THE HUMAN PAPILLOMAVIRUS TYPE 16 E7 (HPV-16 E7) ONCOPROTEIN AND THE HOST CELL DNA DAMAGE RESPONSE

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High-risk human papillomaviruses (HPVs), such as HPV-16, are the etiological agents of squamous cell carcinomas (SCCs) of the anogenital tract and a subset of oropharyngeal cancers. High-risk HPVs encode two oncoproteins, E6 and E7, which promote unscheduled host cell proliferation by targeting the p53 and pRB tumor suppressor proteins, respectively. HPV-16 E7 has been shown to stimulate structural chromosomal instability and DNA breakage. These findings raise several important questions. First, how does HPV-16 E7 induce DNA damage? Second, what are the precise consequences of HPV-16 E7-induced DNA damage for host cell genomic integrity, and lastly, how do HPV-16 E7-expressing cells maintain proliferation despite activated DNA damage checkpoints? Here, we show that HPV-16 E7 activates the Fanconi Anemia (FA) pathway, a branch of the host cell DNA damage response that primarily responds to stalled DNA replication forks. Importantly, we show that HPV-16 E7 expression in FA-deficient cells accelerates the formation of structural chromosomal alterations, which may help to explain the heightened susceptibility of FA patients to HPV-associated tumors. However, we also provide evidence that HPV-16 E7-induced FA pathway activation in FA-proficient cells may contribute to evasion of anti-proliferative host cell barriers by promoting alternative lengthening of telomeres (ALT). Finally, we demonstrate that HPV-16 E7 circumvents DNA damage checkpoint control and promotes aberrant mitotic entry by increasing the proteolytic

turnover of claspin, which plays a role in the ATR/CHK1-mediated replication stress response. Collectively, our results underscore that HPV-16 E7 interferes with host cell genome integrity by inducing DNA replication stress. The detrimental effects of HPV-16 E7 on the genomic integrity of host cells with a deficient FA pathway support the notion that this DNA damage response pathway is crucial to prevent HPV-16 E7-induced genomic instability and malignant progression. However, we also provide evidence that HPV-16 E7 can exploit the FA pathway to promote cellular immortalization. Future experiments to explore these events for cancer therapy and/or prevention are warranted.

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1.0 GENERAL INTRODUCTION

1.1 HUMAN PAPILLOMAVIRUSES (HPVS) AND CANCER

Human papillomaviruses (HPVs) are circular double-stranded DNA tumor viruses that infect cutaneous and mucosal epithelial surfaces (1). Over 200 different genotypes of the virus have been identified and those that infect mucosal epithelial surfaces can be further categorized into “high” and “low” risk types. High-risk HPV types, such as HPV-16, -18, -31 and -33, are intimately associated with squamous cell carcinomas (SCCs) of the anogenital tract, including the cervix, vulva, anus, and penis, as well as a subset of cancers in the oropharyngeal region. Low-risk HPV types, such as HPV-6 and -11, cause benign tumor formation (condyloma acuminata) with little propensity for malignant progression (2, 3). Other HPVs that have been linked with cancer include HPV-5 and HPV-8, which are associated with the rare skin disease epidermodysplasia verruciformis (EV). Patients with EV develop wart-like, macular skin lesions and display a high incidence of non-melanoma skin cancer following ultraviolet (UV) exposure (4).

The major route of viral transmission for mucosa-associated HPVs is through sexual contact (5). It has been estimated that approximately two thirds of adults are infected with HPV within two years of becoming sexually active (6). However, in most cases the virus is eliminated by the host immune system (7, 8). Risk factors for HPV-associated tumor formation include persistence, the presence of multiple HPV types, a compromised immune system (such as in the case of HIV-affected individuals), cigarette smoke and inherited gene mutations that are linked with genetic cancer susceptibility syndromes, such as Fanconi anemia (FA) (9-11).

Greater than 90% of all cervical cancers are associated with chronic high-risk HPV infection (12, 13). Precancerous lesions, also referred to as cervical intraepithelial neoplasias

(CINs) are graded on a histopathological scale (I to III) based on the degree of disruption of the epithelial tissue architecture. It can take many years for pre-cancerous lesions to progress to invasive cancer, which underscores that HPV-associated carcinogenesis is a multi-step process that requires additional genomic insults. Cervical cancer remains the 3rd leading cause of cancer-related deaths in women worldwide (2, 13). Recently, a prophylactic vaccine has been developed, which prevents infection of HPV types, -6, -11, -16, and -18 (14). However, additional preventive and therapeutic options are required for previously infected persons and for those with little access to the vaccine.

1.2 THE BIOLOGY OF HPVS

The HPV genome consists of circular double-stranded DNA of approximately eight kilobases in size. HPV genomes contain roughly eight open reading frames (ORFs) that encode for "early" (E) and "late" (L) genes, which are transcribed as polycistronic mRNAs (Fig. 1) (6). The "early" gene products include the E1 and E2 genes, which regulate viral replication and gene transcription. Other early genes include the E4 and E5 genes, which play roles in virion assembly/release or epidermal growth factor (EGFR) signaling respectively, and the E6 and E7 oncogenes, which are required to disrupt host cell cycle control in order to promote viral propagation. The "late" genes, L1 and L2, encode for the structural capsid proteins (15).

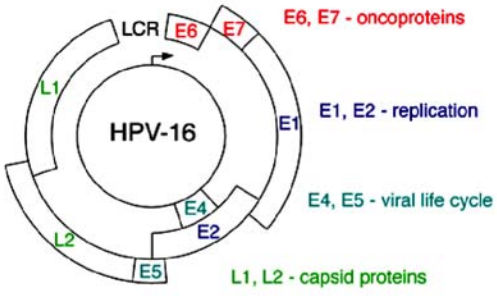

Figure 1. Map of the HPV-16 genome.

HPV-16 encodes for 8 open reading frames, including early genes (E) involved in various aspects of the viral life cycle and late genes (L) encoding for the structural capsid proteins. Viral transcription and replication is controlled by the long control region (LCR).

Viral transcripts are processed by alternative splicing and are thought to be translated by leaky ribosomal scanning, a mechanism in which a proportion of translation complexes may scan the region upstream of the ORF until a proper start codon is identified (16). High-risk HPVs contain two promoters, an early promoter (p97) and late promoter (p742). The early promoter is found upstream of the E6 ORF and is primarily active early in infection, while the late promoter is found within the E7 ORF and is activated upon differentiation during the productive phase of the virus (6, 17).

Early viral gene transcription is regulated by a non-coding region found upstream of the E6 ORF, known as the long control region (LCR) or upstream regulatory region (URR). The LCR contains binding sites for various host cellular transcription factors, including Sp-1, Oct-1 and YY1, which serve to regulate HPV gene transcription (17). The LCR also harbors binding sites for the HPV viral E1 and E2 proteins which are involved in initiating viral replication. E1 is

responsible for melting the DNA near the viral origin of replication and possess helicase activity, while E2 increases the binding specificity of E1 to viral origins (18-20).

The life cycle of the virus is intimately connected to the differentiation process of the epithelium (Fig. 2). Squamous epithelium consists of stratified cell layers with a proliferating basal keratinocyte layer. Normally, as the daughter cells migrate upwards within the epithelium they undergo differentiation, which is associated with permanent exit from the cell cycle (6).

The virus gains access to the proliferating basal cell layer through microlesions to the epithelial surface and enters the keratinocyte through mechanisms that are not fully understood in detail (21). Following infection, early viral genes are expressed and viral replication is kept at 50 to 100 extrachromosomal HPV genomes per cell. During the early phase of viral infection, HPV E2 transcriptionally regulates the HPV E6 and E7 oncogenes and limits their expression (15, 22).

Due to its small size, the HPV genome does not encode for any viral replication enzymes and therefore, must commandeer that of its host cell in order to establish viral replication. Since differentiation normally leads to irreversible cell cycle exit, the virus needs to override differentiation-associated cell cycle withdrawal in order to maintain a milieu that is competent for replication (23). Through the concerted efforts of the high-risk HPV E6 and E7 oncoproteins, which target the p53 and retinoblastoma (pRB) tumor suppressor proteins, respectively, the virus is able to maintain an S phase-like milieu that is conducive for viral replication in terminally differentiated keratinocytes (6, 24, 25). Within the suprabasal levels, the differentiation-dependent HPV promoter is activated and late viral genes are expressed. High viral genome amplification occurs, thereby increasing the chance for successful viral transmission. The virus is assembled into virions and sloughed off with the uppermost layer of the epithelium (26).

Throughout a productive infection, the HPV genome is maintained episomally (22). Approximately 99% of HPV-associated cancers however, harbor viral DNA that has become integrated into the host cell genome. While the precise mechanism of HPV integration is not understood in detail, it usually occurs at a position that results in disruption of the HPV-16 E2 gene and all others HPV-16 genes besides HPV-16 E6 and E7. Loss of HPV-16 E2 consequently leads to deregulation of the HPV-16 E6 and E7 oncogenes, and accordingly, the majority of HPV-associated tumors are characterized with high levels of HPV-16 E6 and E7. It is important to mention, however, that pre-cancerous lesions have been found to contain episomal and integrated HPV genomes (27-29). This finding highlights the notion that integration of the virus occurs during multistep malignant progression and, although it terminates the viral life cycle, may ultimately provide a growth advantage that results in the development of cancer.

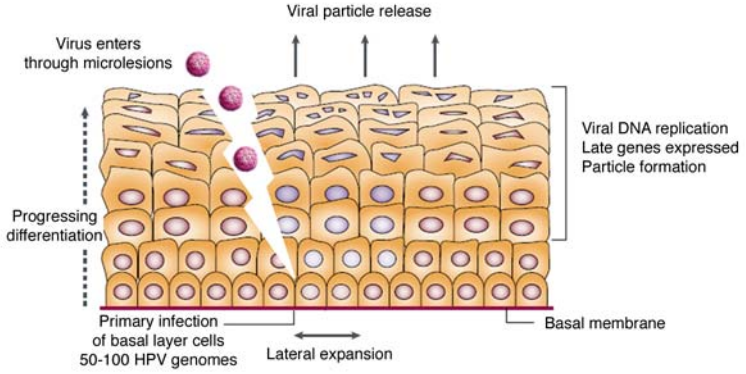

Figure 2. The HPV life cycle.

The virus enters through microlesions on the epithelial surface and infects the proliferating basal cell layer. Early viral genes are expressed and viral copy number is strictly regulated. The HPV oncoproteins attenuate G1/S checkpoint control and create an S phase-like milieu that is permissive for viral genome replication in terminally differentiated keratinocytes. Legend continued on following page.

Within the suprabasal layers, the late viral genes are expressed, high viral genome amplification occurs and the virus is packaged into virions. HPV particles are then sloughed off with the uppermost layer of the epithelium. Adapted by permission from Macmillian Publishers Ltd: [Nature Reviews Cancer] 2(5); 342-350, copyright 2002.

1.3 MOLECULAR TARGETS OF THE HIGH-RISK HPV ONCOPROTEINS

The high-risk HPV E6 and E7 oncoproteins disrupt cell cycle regulation and promote proliferation by interacting with various host cell proteins. Manipulation of cell cycle proteins by high-risk HPV oncoproteins has also been linked to their ability to stimulate the immortalization and transformation of various human and rodent cell lines (25).

1.3.1 The HPV-16 E7 oncoprotein

The high-risk HPV E7 oncoprotein is a small, short-lived phosphoprotein consisting of 98 amino acids that is localized mainly in the nucleus. HPV-16 E7 contains two copies of the Cys-X-X-Cys zinc binding domain in its C-terminus and is phosphorylated by casein kinase II (CKII) (Fig. 3) (30). HPV-16 E7 harbors sequences that share homology with the adenovirus (Ad) E1A protein and SV40 large tumor antigen (T Ag), commonly referred to as conserved regions 1 and 2 (CR1 and CR2) (31). The HPV-16 E7 oncoprotein is known for its ability to bind and induce the degradation of the retinoblastoma (pRB) tumor suppressor protein and the related "pocket proteins" p107 and p130 through the LXCXE motif found in CR2 and amino acids in CR1 (32-36). HPV-16 E7 destabilizes the pRB protein by binding and redirecting the cullin 2 ubiquitin ligase complex (37). The pocket proteins play a critical role in regulating the G1/S

phase transition of the cell cycle and are required to inhibit E2F-dependent gene transcription prior to S phase (38). HPV-16 E7 further disrupts the pRB/E2F-regulatory axis by inhibiting the cyclin dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (39-41). Consequently, HPV-16 E7-expressing cells are characterized by deregulated levels of E2F, as well as E2F targets, including cyclin E, cyclin A, and CDC25A (42-44).

HPV-16 E7 interacts with various other host cell targets in order to stimulate proliferation (45). These include chromatin remodeling factors such as the histone acetyl transferase (HAT) p300 (46), the DNA methyltransferase Dnmt1 (47), and histone deacetylases (HDACs) 1 and 2 through an interaction with Mi2β, a component of the NURD histone deacetylase complex (48). HPV-16 E7 has also been hypothesized to inhibit anchorage-independent apoptosis (anoikis) by binding p600 and can directly bind E2F, cyclin E/CDK2 and cyclinA/CDK2 complexes in order to deregulate their activity (47, 49-51).

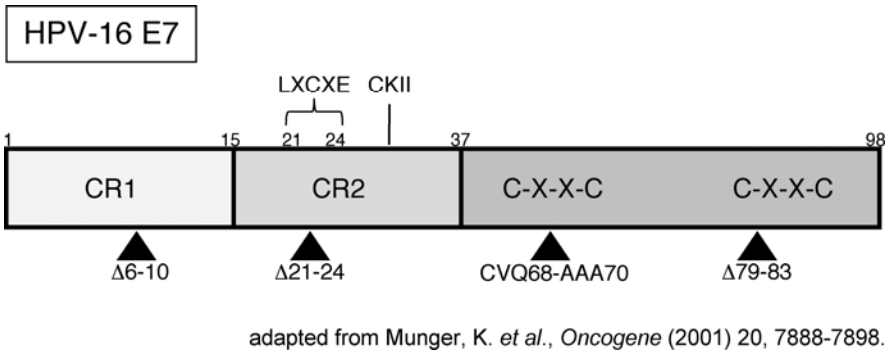

Figure 3. Schematic representation of the HPV-16 E7 oncoprotein.

HPV-16 E7 contains two conserved regions (CR1 and CR2) that are highly homologous to that of the adenovirus E1A and SV40 large T Ag proteins. CR2 contains the LXCXE pRB binding motif and a casein kinase II phosphorylation site. Several important mutations are depicted with black triangles. HPV-16 E7 Δ6-10 binds but not does not degrade pRB. Legend continued on following page.

HPV-16 E7 Δ21-24 does not bind or degrade pRB. C-terminal mutants (CVQ68-AAA70 and Δ79-83) are unable to overcome a $p21^{Cip1}$-associated growth arrest. Adapted by permission from Macmillian Publishers Ltd: [Oncogene] 54(20); 7888-7898, copyright 2001.

1.3.2 The HPV-16 E6 oncoprotein

The E2F family of transcription factors however, also controls anti-proliferative targets, such as $p14^{Arf}$, which if deregulated can lead to the stabilization of the p53 transcription factor (52-54). p53 is a crucial mediator of the cellular stress response and is involved in stimulating the transcription of pro-apoptotic factors and/or checkpoint proteins following negative growth conditions (55). Therefore, the cooperating HPV-16 E6 oncoprotein has evolved to target the p53 protein for degradation by redirecting E6-AP, a member of the HECT domain family of host cell ubiquitin ligases (56-59). Further disruption of the p53 tumor suppressor protein is achieved through an interaction between the high-risk HPV E6 oncoprotein and p300, a co-transcriptional activator of p53 (60, 61).

High-risk HPV E6 oncoprotein also drives proliferation by activating the promoter region of hTERT, the catalytic subunit of telomerase that is involved in maintaining telomere length (62). High-risk HPV E6 has been shown to deregulate hTERT activity through multiple mechanisms, including the binding of the transcription factor c-myc to the hTERT promoter region (63). hTERT activity is further enhanced by HPV-16 E6-induced histone aceytlyation of the hTERT promoter and degradation of NFX1-91, a transcriptional repressor of hTERT. Both of these activities have been shown to occur in an E6AP-dependent manner (64-66).

Other binding partners of high-risk HPV E6 include paxillin and proteins that contain a PDZ binding domain, including hDlg (the human homologue of the *Drosophila melanogaster* tumor suppressor disc large), MUPP1 and hScrib (the human homologue of *D. melanogaster* tumor suppressor scribble). Interactions between high-risk HPV E6 oncoproteins and PDZ proteins contribute to regulation of cell-cell contact, cell polarity and signaling (67-70).

Due to subtle changes in amino acid sequence, the low-risk HPV E6 and E7 oncoproteins are unable to inactivate p53 and pRB, respectively, or do so with greatly reduced efficiency when compared to their high-risk counterparts (71). Low-risk HPV oncoproteins are also unable to promote immortalization of cells and exhibit little to no transformation abilities (25). These results suggest that the ability of high-risk HPV oncoproteins to effectively target the pRB and p53 tumor suppressor proteins is linked with their oncogenecity. It is important to note however, that more recent work has illustrated that low-risk HPV-6 E7 can destabilize the p130 protein, but not pRB or p107, similar to HPV-16 E7 (72). These results indicate that disruption of p130 by the low-risk HPV E7 oncoprotein may be required to relax the differentiation programming in order to facilitate later stages of the viral life cycle, while complete disruption of pRB and pRB-like proteins by the high-risk HPV E7 oncoprotein is linked with its ability to promote tumorigenesis.

By disrupting the p53 and pRB tumor suppressor pathways, the HPV-16 E6 and E7 oncoproteins are able to relax G1/S cell cycle checkpoint control and induce unscheduled entry into S phase (Fig. 4). Unscheduled proliferation, associated with deregulated E2F, cyclin E or CDC25A levels, has previously been shown to stimulate aberrant replication dynamics and DNA replication stress (73-75). Whether the high-risk HPV oncoproteins trigger a host cell DNA replication stress response has not been investigated in detail.

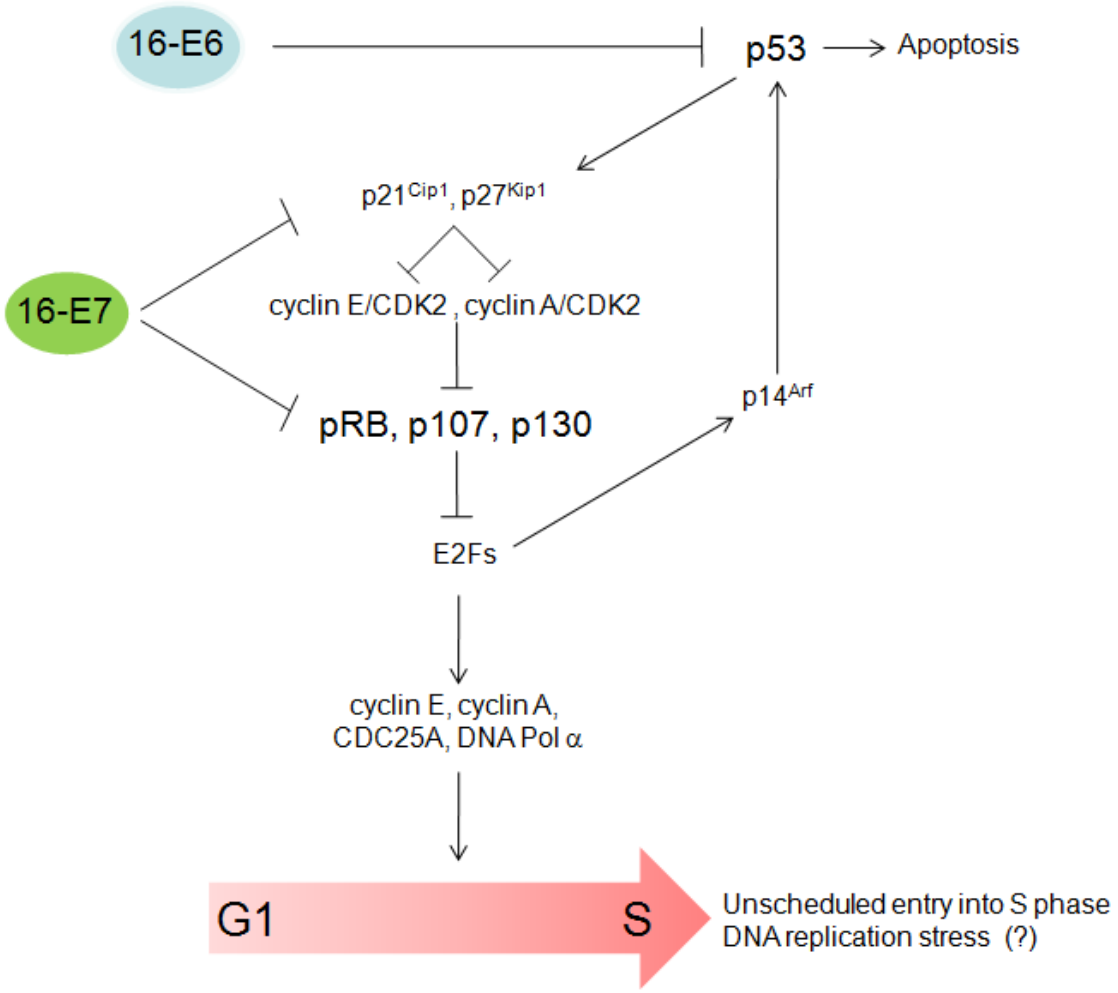

Figure 4. Disruption of G1/S checkpoint control by the high-risk HPV oncoproteins.

HPV-16 E7 (16-E7) disrupts the pRB-regulatory axis and promotes deregulated E2F-mediated gene transcription by binding and inducing the degradation of the pRB family of proteins, as well as inhibiting the cyclin dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. In order to evade anti-proliferative E2F-mediated signals, such as $p14^{Arf}$, HPV-16 E6 (16-E6) targets p53 for degradation. Together, the HPV oncoproteins relax G1/S checkpoint control and induce unscheduled entry into S phase, an activity that has been hypothesized to stimulate oncogenic stress associated with aberrant replication dynamics.

1.4 HIGH-RISK HPV ONCOPROTEINS, DNA DAMAGE AND CHROMOSOMAL INSTABILITY

1.4.1 DNA damage, chromosomal instability and cancer

Genomic instability is a hallmark of cancer, including HPV-associated cervical neoplasms (76). Loss of genomic integrity is a major factor for malignant progression and important for HPV oncoprotein-associated malignant transformation of cells (77-81).

Chromosomal instability has been defined as "the accelerated rate of gains and losses of whole or portions of chromosomes in the context of continuous growth" (82, 83). While changes in chromosome number (aneuploidy) have been linked with mitotic defects, such as misaligned chromosomes or failed cytokinesis, altered chromosome structure is typically associated with DNA breaks (82). Exposed DNA ends can promote chromosome translocations or gene amplification/deletions as a result of aberrant chromosome fusions or repeated breakage-bridge cycles (84).

During the evolution of a tumor, certain structural aberrations are selected for if they provide a growth advantage for the cell ie. gain of oncogenes or deletion of tumor suppressors. The idea that DNA breakage-induced chromosomal instability can drive malignant progression is supported by the observation that patients suffering from mutations in certain DNA repair enzymes suffer from enhanced cancer susceptibility (85).

DNA breaks can occur from exogenous insults, eg. ultraviolet rays (UV) or gamma irradiation (86). However, it is conceivable that the most common endogenous source of DNA breaks are stalled replication forks that have collapsed (87). Stalled replication forks can form for

a variety of reasons, including physical barriers to replication such as interstrand crosslinks or single-strand breaks, replication enzyme deficiencies, dNTP pool imbalance or as a result of deregulated replication origin firing (73-75, 88). Other sources of DNA DSBs include chemotherapeutic agents, ionizing radiation, free radicals and DNA repair intermediates (86, 89).

1.4.2 Evidence of DNA breakage in HPV-positive tumor cells

Cytogenetic analysis of HPV lesions has revealed that, in addition to changes in ploidy, the majority of HPV tumors (up to 100%) harbor structural chromosomal changes (90, 91). Reoccurring patterns of chromosome gains and losses have been found in a broad spectrum of HPV tumors, suggesting that certain structural chromosomal aberrations are preferentially retained throughout HPV carcinogenesis (92). In line with this, gain of chromosome 3q has been correlated with the transition from pre-invasive to invasive cervical carcinomas (90). Other structural alterations commonly observed in HPV-associated neoplasms, include gains of genetic material on 1q, 5p, 6p and 20 and losses mapped to 2q, 3p, 4, 8p and 13q (92-94).

1.4.3 High-risk HPV oncoproteins induce DNA damage and DNA damage checkpoints

The finding that HPV tumors display structural chromosomal instability raises the important question whether high-risk HPV oncoproteins can induce DNA damage. Work from several labs suggests that this is the case and that high-risk HPV oncoproteins are in fact able to promote DNA breakage in host cells. First, HPV-16 E6 and/or E7 expression has been reported to act as mutagenic agents in primary human keratinocytes (95-97). Second, the HPV-16 E6 or E7 oncoproteins enhance the integration of foreign DNA, an event that requires DNA DSB

formation (98). Third, comet assay analyses, a technique which visualizes DNA breaks on a cell by cell basis, has revealed that stable expression of HPV-16 E6 or E7 in primary human keratinocytes stimulates increased DNA breakage (99). Fourth, HPV-16 E6 and/or E7 have been shown to induce structural chromosomal instability as evidenced by anaphase bridge formation (Fig. 5) and micronuclei, in the absence of telomere attrition (99, 100). Anaphase bridges form when exposed DNA ends fuse together to produce a dicentric chromosome and can ultimately lead to repeated cycles of breakage-bridge-fusion events (84). Lastly, HPV-16 E7 expression was found to stimulate nuclear foci containing γ–H2AX, a marker of DNA DSBs and an early signaling event in the cellular stress response (refer to section 1.5 for a complete overview of the host cell DNA damage response).

Other viral oncoproteins such as Ad E1A and SV40 large T Ag, which share similar functions as HPV-16 E7, have also been shown to activate DNA damage checkpoints, trigger DNA breakage and promote structural chromosomal instability (Table 1) (101-104). Previous work suggests that expression of E1A and large T Ag promotes chromosome breaks independent of viral replication and it has been hypothesized that host cells in S phase are particularly susceptible to viral oncoprotein-induced DNA breakage (101-104).

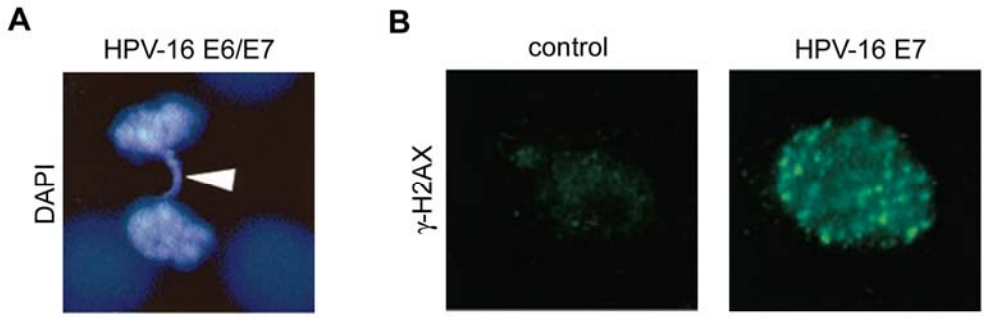

Illustration adapted from Duensing and Munger, *Cancer Res.* (2002) 62, 7075-82.

Figure 5. The high-risk HPV oncoproteins stimulate structural chromosomal instability and activate the host cell DNA damage response.

(A) Primary human keratinocytes engineered to stably express the HPV-16 E6 and E7 oncoproteins were analyzed microscopically for structural abnormalities following a nuclear DAPI stain. The white arrow points to an anaphase bridge. (B) Primary human keratinocytes stably expressing HPV-16 E7 or empty vector control (control) were processed for immunofluorescence microscopic analysis of γ–H2AX. Note the appearance of γ–H2AX foci in HPV-16 E7-expressing cells. Adapted by permission from Copyright Clearance Center: [Cancer Research] 62(23); 7075-7082, copyright 2002.

How do HPV-16 oncoproteins stimulate DNA damage? While it is thought that HPV-16 E6-induced degradation of p53 permits the accumulation of DNA damage (96), the mechanisms behind HPV-16 E7-induced DNA breaks are less clear. Importantly, expression of low-risk HPV-6 E7 in human keratinocytes has not been found to stimulate mutagenesis (95). Since low-risk HPV-6 E7 oncoprotein is unable to efficiently target the pRB protein for degradation, this result suggests that deregulation of the pRB/E2F axis by HPV-16 E7 may play a role in stimulating host cell DNA damage. Therefore, the goals of this thesis was to investigate 1) the molecular mechanisms behind HPV-16 E7-induced host cell DNA damage, 2) the consequences of HPV-16 E7-associated DNA damage on host cell genomic integrity and 3) to determine how

HPV-16 E7-expressing cells continue to proliferate in a DNA damage response-activated host cell environment.

Virus	Viral Protein	Evidence of DNA breaks	Refs
Adenovirus	E1A	Structural chromosomal instability	[103, 104]
HPV	HPV-16 E6	Structural chromosomal instability, mutagenesis, foreign DNA integration, DNA comet assay	[95-100]
HPV	HPV-16 E7	Structural chromosomal instability, mutagenesis, foreign DNA integration, DNA comet assay, γ–H2AX foci	[95-100]
SV40	Large T Ag	Structural chromosomal instability, ATM/ATR activation	[101, 102]

Table 1. Summary of viral oncoproteins that induce host cell DNA damage.

1.5 THE HOST CELLULAR DNA DAMAGE RESPONSE

1.5.1 ATM and ATR signaling cascades

Cells has evolved a highly complex signaling network, known as the DNA damage response, which is required to detect and facilitate the repair of damaged DNA as well as coordinate cell cycle arrest or apoptosis (Fig. 6) (105-107). The DNA damage response is orchestrated mainly by the phospho-inositide kinase (PIK)-related ataxia telangiestacia mutated (ATM) and ATR (ATM and Rad 3-related) protein kinases, which become activated in response to specific types of DNA lesions and phosphorylate multiple downstream targets that lead to the

assembly of DNA repair proteins on chromatin and activation of checkpoint proteins (105). ATM is primarily activated in response to DNA DSBs, while ATR is generally linked with replication stress and stalled replication forks (106). However, there is a significant amount of cross talk as evidenced by the fact that ATR can also be activated in response to DNA DSBs, albeit with slower kinetics (108-112).

In response to DNA DSBs, the MRN (Mre11-Rad50-Nbs1) complex is required to recruit ATM to the chromatin, where ATM is then activated through an intermolecular autophosphorylation at serine 1981 (pATM S1981) (113-115). Activated ATM phosphorylates histone variant H2AX at serine 139 (γ–H2AX), which serves as a platform for the adaptor protein, MDC1 (116). Through multiple interactions between MDC1, γ–H2AX and the MRN complex, the DNA damage signal is spread to surrounding chromatin and facilitates the rapid recruitment of DNA repair factors into what are termed nuclear foci (117, 118). The detection of nuclear foci is a common method used to characterize DNA damage response activation. Additional ATM substrates found to localize to broken DNA include BRCA1 and the p53 binding partner, 53BP1 among others (119).

In contrast, ATR is activated by stretches of RPA-coated single stranded DNA that form at replication blocks as a result of continued DNA unwinding (120). Activation of the ATR kinase is facilitated in part by ATRIP (ATR-interacting protein), which regulates the localization of ATR to sites of stalled replication, and the TOPBP1 protein (121-125). ATR requires the mediator protein, claspin, in order to phosphorylate the downstream checkpoint kinase, Chk1, which signals to various targets in order to arrest the cell cycle to allow time for DNA repair (126-128).

ATR and ATM signal primarily through the downstream effector checkpoint kinases, Chk1 and Chk2, respectively (129). Activated Chk1 and Chk2 phosphorylate multiple substrates, including p53, $p21^{Cip1}$ and the CDC25 family of phosphatases, which play a more direct role in imposing a cell cycle arrest by inhibiting CDK activity. Many of these targets can also be directly phosphorylated by ATM and ATR as well (130).

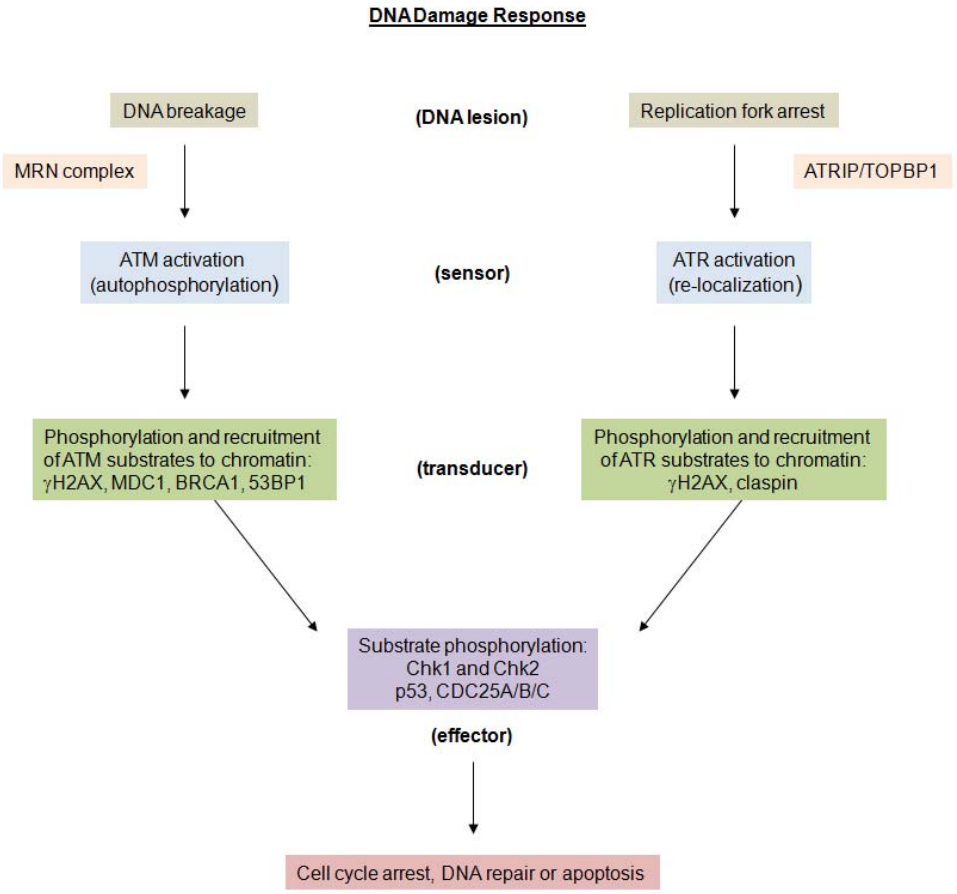

Figure 6. The DNA damage response signaling cascade.

In response to specific types of DNA lesions the ATM or ATR kinases are activated by either autophosphorylation or re-localization. The ATM and ATR kinases phosphorylate various substrates which are required to transduce/spread the DNA damage signal throughout the cell. Phosphorylation of the effector checkpoint kinases facilitates cell cycle arrest, DNA repair or apoptosis.

1.5.2 The Fanconi Anemia (FA) pathway

The Fanconi Anemia pathway (FA) is a signaling cascade that participates in the DNA damage response and is specifically activated following DNA replication stress and stalled DNA replication forks (131). Much of what is known today about the FA pathway stems from research done using patient-derived cell lines that harbor inherited inactivating mutations in one of the many FA genes (132). FA is an extremely rare, recessive or X-linked, genetic cancer susceptibility syndrome that affects 1 in every 350,000 live births. FA patients display a variety of abnormalities, including bone marrow failure, developmental defects and a high incidence of solid tumors, specifically SCCs (133). FA-deficient cells are characterized by high levels of spontaneous chromosomal instability and exhibit hypersensitivity to interstrand cross linking agents, such as Mitomycin C (MMC), which causes chromosome breaks and abnormal radial structures (134).

The FA pathway consists of at least thirteen proteins, which assemble into multi-protein core complexes that are thought to play a role in the detection, stabilization and restart of stalled replication forks (135). Following genotoxic stress, eight of the FA proteins (FANCA, B, C, E, F, G, L, M) and the interacting proteins FAAP24 and FAAP100 form a high molecular weight nuclear core complex that serves to monoubiquitinate the FANCI and FANCD2 proteins (FA-ID) at conserved lysine residues (Fig. 7A) (136). FANCL has been shown to harbor E3 ligase activity and therefore, is likely to be involved in ubiquitin moiety transfer (137, 138). However, each of the indicated FA proteins is required for core complex stability and to facilitate the monoubiquitination of the FA-ID proteins (139). Monoubiquitination of the FA-ID proteins leads to their translocation to the chromatin where it then interacts with FANCD1/BRCA2,

FANCJ/BRIP1, FANCN/PALB2 and several DNA damage proteins including RAD51 (140) (Fig. 7B). These proteins have various enzymatic functions and have been implicated in replication fork restart and DNA repair through mechanisms that are thought to involve homologous recombination (HR) and DNA translesion synthesis (141, 142). In line with this, FA-deficient cells are characterized by hypersensitivity to MMC-induced interstrand crosslinks and display high levels of spontaneous chromosomal instability. The pathway is then switched off through the deubiquitination of the ID complex by the USP1 enzyme (143).

FANCD2 monoubiquitination occurs in an ATR-dependent manner and is required for the formation of FANCD2 nuclear foci in response to genotoxic stress (144). Therefore, the appearance of FANCD2 foci formation is commonly used as a surrogate marker for FA pathway activation.

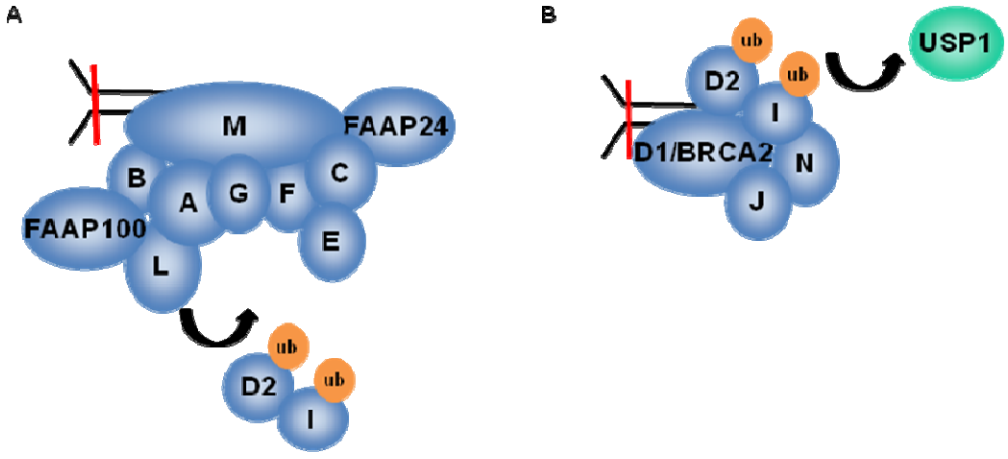

Figure 7. The FA DNA damage pathway.

(A) In response to stalled replication forks, eight of the FA-associated proteins and several FA-interacting proteins form a core complex that serves to monoubiquitinate the FANCD2 and FANCI proteins. Legend continued on following page.

(B) Monoubiquitinated FANCD2 and FANCI are recruited to the chromatin and assembled into nuclear foci where they interact with FANCD1/BRCA2, FANCJ, FANCN and other DNA damage proteins (not depicted) in order to stabilize and restart stalled replication forks. The pathway is turned off by the deubiquitinating enzyme USP1.

1.6 FANCONI ANEMIA (FA) AND HPV

A recent epidemiological study revealed that FA patients are 50-times more likely to develop solid tumors (145) and have a 500-700 fold higher incidence of head and neck squamous cell carcinomas (HNSCCs) than the normal population (146). Greater than 50% of all solid tumors found in FA patients form at organ sites with a predilection for infection with high-risk human papillomaviruses (HPVs), including the anogenital and oropharynx regions (145-152). The precise contribution of high-risk HPV infection with respect to FA tumor burden however, remains unclear due to conflicting results in the literature. An analysis of HNSCCs from FA patients found that approximately 80% were positive for high-risk HPV DNA, type 16 (HPV-16) being the most prevalent (9). However, contradictory reports have been unable to detect HPV DNA in a separate subset of FA HNSCCs and tumor-derived cell lines (153, 154). Despite these differences, FA genes have been found to be epigenetic silencing in HPV-associated tumors (155). The reason for the increased susceptibility of FA patients to develop HPV tumors at a significantly younger age than the normal population remains unknown.

1.7 THESIS OUTLINE

Chapter 1 consists of a general introduction to the biology of human papillomaviruses (HPVs) and how they are associated with cancer. The role of the high-risk HPV oncoproteins in the viral life cycle and in stimulating host cell DNA damage and genomic instability will then be discussed. This chapter also provides a review of the DNA damage response and presents clinical findings that support a potential link between the genetic cancer susceptibility syndrome Fanconi Anemia (FA) and HPV-associated carcinogenesis.

Chapter 2 describes activation of the FA pathway by the HPV-16 E7 oncoprotein. Hallmarks of FA pathway activation were observed in HPV-associated squamous cell carcinoma (SCC) tissue as well as organotypic raft cultures harboring full length HPV-16 episomes. Further experiments using an *in vitro* cell culture model revealed that the HPV-16 E7 oncoprotein is involved in stimulating FA pathway activation. Finally, we show that high-risk HPV E6 and E7-positive raft cultures generated from patient-derived FANCA-deficient primary cells display increased DNA DSBs and that expression of the HPV-16 E7 oncoprotein in a FANCD2-deficient cell line accelerates structural chromosomal instability. These results suggest that HPV-16 E7 triggers a host cell DNA replication stress response, which is reflected by FA pathway activation, and indicates that certain DNA repair pathways are essential to prevent HPV-16 E7-associated DNA damage and genomic instability.

Chapter 3 describes a functional consequence of HPV-16 E7-associated replication stress for host cell genomic integrity in FA-proficient cells. HPV-16 E7-induced replication stress and

associated FA pathway activation was found to play a role in the telomerase-independent alternative lengthening of telomeres (ALT). We show that the HPV-16 E7 oncoprotein stimulates the appearance of ALT-associated promyelocytic leukemia bodies (APBs), a surrogate marker of ALT activity, which contain the FANCD2 protein. Further characterization of these nuclear structures revealed that their formation is linked with replication stress at telomeres and points to a role for the FA pathway in ALT-mediated telomere homeostasis. Finally, the HPV-16 E7 oncoprotein was shown to stimulate the formation of FANCD2-containing APBs in primary human keratinocytes. Collectively, these findings may help to explain how the HPV-16 E7 oncoprotein contributes to the immortalization of cells in a telomerase-independent manner and furthermore, suggests that HPV-16 E7-associated activation of the DNA damage response may aid in the circumvention of anti-proliferative host cell barriers.

Chapter 4 describes a novel mechanism through which the HPV-16 E7 oncoprotein promotes cell cycle progression despite anti-proliferative stimuli. HPV-16 E7 was found to attenuate DNA damage checkpoint control and stimulate aberrant mitotic entry in the presence of DNA damage by accelerating the degradation of claspin. Claspin is a DDR protein that plays a role in the ATR/CHK1 signaling pathway and its degradation has recently been implicated in DNA damage checkpoint recovery. Several E2F-responsive components of the $SCF^{\beta\text{-}TrCP}$-based claspin degradation machinery were found deregulated in the presence of the HPV-16 E7 oncoprotein. These findings suggest that manipulation of the pRB/E2F-axis by HPV-16 E7 may contribute to altered claspin protein stability and thus relax DNA damage checkpoint control in order to maintain mitotic entry in the presence of anti-proliferative stimuli.

Chapter 5 summarizes findings from each of the previous sections and develops a comprehensive model of how the HPV-16 E7 oncoprotein activates and exploits the host cell DNA damage response. This chapter will highlight results which illustrate how interplay between HPV-16 E7 and the host cell DNA damage response contribute to genomic instability, malignant progression and the viral life cycle. Future directions will also be discussed.

2.0 THE HPV-16 E7 ONCOPROTEIN ACTIVATES THE FANCONI ANEMIA (FA) PATHWAY AND CAUSES ACCELERATED CHROMOSOMAL INSTABILITY IN FA CELLS

Work described in this section was published in the Journal of Virology (JVI 2007, 81:13265-13270) with authors Nicole Spardy, Anette Duensing, Domonique Charles, Nathan Haines, Tomomi Nakahara, Paul F. Lambert and Stefan Duensing.

D. Charles and N. Haines performed experiments using the organotypical raft cultures. T. Nakahara and P.F. Lambert provided the organotypical raft cultures. N. Spardy performed all other experiments described in this section. A. Duensing helped with the experimental design and data analysis. N. Spardy and S. Duensing conceived the project, analyzed the results and wrote the manuscript.

Work described in this section was also done in collaboration with Susanne Wells (Cincinnati Children's Hospital) and was published in Oncogene 2009, (28:674-685) with authors EE Hoskins, TA Morris, JM Higginbotham, N Spardy, E Cha, P Kelly, DA Williams, KA Wikenheiser-Brokamp, S Duensing and SI Wells.

2.1 INTRODUCTION

As reviewed in Chapter 1, the HPV-16 E7 oncoprotein plays a critical role in the viral life cycle by disrupting the pRB/E2F-axis in order to create a replication-competent milieu conducive for viral replication in growth-arrested keratinocytes. However, unscheduled S phase entry and deregulated cyclin expression has been hypothesized to trigger aberrant replication dynamics and DNA replication stress (73-75). The FA pathway is part of the host cell DNA damage response that is specifically activated in response to stalled replication forks (136). Therefore, we tested whether HPV-16 oncoprotein expression triggers host cell replication stress, using induction of the FA pathway as a molecular readout. Since replication stress can ultimately lead to DNA breakage (87), we also examined whether a functional FA pathway is required to prevent high-risk HPV oncoprotein-associated DNA breakage. Lastly, based on epidemiological studies which suggest that FA patients are increasingly susceptible to HPV tumors (9, 148), we tested whether FA-deficient cells are more prone to HPV-16 oncoprotein-induced structural chromosomal instability.

We show that hallmarks of FA pathway activation are observed in HPV-associated SCCs as well as organotypic raft cultures harboring full length HPV-16 episomes. Further experiments revealed that the HPV-16 E7 oncoprotein is involved in activating the FA pathway, as assessed by FANCD2 foci formation and recruitment of monobiquitinated FANCD2 and FANCD1/BRCA2 to chromatin. Importantly, FA gene deficiency was found to enhance high-risk HPV oncoprotein-associated DNA breakage and furthermore, that HPV-16 E7 expression in

patient-derived FANCD2-deficient cells accelerates the accumulation of structural chromosomal instability. Collectively, our results suggest that FA pathway activation represents an early host cell response to HPV infection and that HPV-16 E7 oncoprotein activity is involved in promoting FA pathway induction. Our finding that HPV-16 E7 expression exerts deleterious effects for the genomic integrity of FA-deficient cells supports the notion that certain DNA repair pathways are crucial to prevent malignancy and may help to explain the increased susceptibility of FA patients to HPV tumors.

2.2 MATERIALS AND METHODS

Cell culture and transfection

(hTERT)-immortalized normal human oral keratinocytes (NOKs), kindly provided by Karl Münger (Channing Laboratory, Brigham & Women's Hospital, Boston, MA) were maintained in serum-free keratinocyte growth media (KGS, Invitrogen, Carlsbad, CA) supplemented with 50 U/ml penicillin (Cambrex, Walkersville, MD), 50 μg/ml streptomycin (Cambrex) and fungizone (Gibco/Invitrogen). FA complementation group D2 (GM16633; Coriell Cell Repositories, Camden, NJ) immortalized fibroblasts were grown in Dulbecco Modified Eagle's medium (DMEM; Biowhittaker/Lonza Inc., Allendale, NJ) supplemented with 10% FBS and antibiotics as described. Stable populations of NOKs expressing the high-risk HPV-16 E6, HPV-16 E7, HPV-16E6/E7, or low-risk HPV-6 E6 or HPV-6 E7 oncoproteins were created by transfecting 2 μg of plasmid using nucleofection (Lonza Inc.), followed by antibiotic selection in G418 and/or puromycin. Stable populations of FANCD2-deficient fibroblasts

expressing the various HPV oncoproteins were generated using 2μg of plasmid and fugene lipofection (Invitrogen), followed by antibiotic selection. pCMV-based plasmids containing HPV-16 E6 or HPV-16 E7 were kindly provided by Karl Münger. LXSN-based low-risk HPV-6 E6 and HPV-6 E7 constructs were kindly provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) and subcloned into pCMV-based vectors. Correct plasmid expression was verified using PCR (data not shown).

Immunological methods

Whole cell lysates were prepared by scraping cells into lysis buffer (1% NP-40, 50 mM Tris-Hcl pH 8.0, 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Lysates were incubated for 1 hr with rocking at 4°C and then cleared by centrifugation for 30 min at 13,000 rpm at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA).

Chromatin fractionation was done according to Mendez and Stillman (156). Briefly, cells of even confluency were spun down at 1,100 rpm for 10 min at room temperature (RT) and washed once in PBS. The pellet was resuspended in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM NaF, 340 mM sucrose, 10% glycerol, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1% Triton X-100, 5 μg/ml leupeptin, 10 μg/ml aprotinin) and incubated on ice for 5 min. The nuclear pellet (P1) was collected by centrifugation at 3,500 rpm for 5 min at 4°C, and the supernatant containing the cytoplamic fraction of soluble proteins (S2) was further clarified by high-speed centrifugation at 13,000 rpm for 15 min to remove cell debris and insoluble aggregates. The nuclear pellet was

washed once in Buffer A, and then lysed in Buffer B (3 mM EDTA, 0.2 mM EGTA, 1mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin) on ice for 10 min. The soluble nuclear proteins (S3) and insoluble chromatin fraction (P3) were collected by centrifugation at 4,000 rpm for 5 min at 4°C. The pellet was washed once in Buffer B and then resuspended in SDS-sample buffer containing 40% β-mercaptoethanol and sheared for 15 sec in a 550 Sonic Dismembrator (Fischer Scientific, Pittsburgh, PA) at 15% amplitude.

Equal amounts of protein were then loaded onto a 3-8% Tris-Acetate gel (Invitrogen) or a 4-12% Bis-Tris gel (Invitrogen) and blotted onto a nitrocellulose membrane.

For immunofluorescence analysis of organotypical raft cultures and paraffin embedded tissue samples, slides were deparaffinized by baking overnight, followed by xylene treatment and dehydration in 100% ethanol. After rehydration in a graded ethanol series (90%, 70%, 50%), slides were washed twice in dH_2O and microwave treated in 0.01 M Citrate buffer, pH 6.0 for 30 min. Slides were allowed to cool, washed once in dH_2O and twice in PBS, followed by digested using pepsin solution (Digest-all 3; Zymed Laboratories, San Francisco, CA) for 30 sec at 37°C. Slides were rinsed in PBS and blocked in 10% normal donkey serum in distilled water for 30 min at room temperature. Primary antibodies were diluted in PBS and slides were incubated for at least 2 nights at 4°C, followed by several hours at 37°C. Slides were washed in PBS and a FITC-conjugated secondary antibody was applied and incubated overnight at 4°C, followed by 37°C for several hours. Slides were washed in PBS and counterstained with 4',6'-diamidino-2-phenylinodole (DAPI, Vector Laboratories, Burlingame, CA). Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

For immunofluorescence analysis of cells grown on coverslips, cells were washed briefly in PBS and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Following a brief wash in PBS, cells were permeabilized using 1% Triton-X 100 in PBS for 30 min at room temperature, washed in PBS and blocked in 10% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in distilled water for 15 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C, followed by several hours at 37°C in a humidified chamber. Cells were washed in PBS, incubated with a FITC-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch) for at least 2 h at 37°C, washed in PBS and counterstained with DAPI

Primary antibodies used for immunoblotting, and immunofluorescence were FANCD1/BRCA2 (Calbiochem, San Diego, CA), FANCD2 (Genetex, Irvine, CA) and ORC2 (BD Biosciences PharMingen, San Jose, CA). Ponceau stain (Pon S) was used to demonstrate equal protein loading.

Metaphase spread analysis

Cells were treated 2 h prior to harvesting with 0.1 μg/ml colcemid solution (Sigma, St. Louis, MO). Cells were then spun down at 1,100 rpm for 10 min at RT. The pellet was resuspended in 50 μl of the supernatant and 1 ml of prewarmed 75 mM KCl and then incubated for 10 min at 37°C. The cells were collected by centrifugation at 1,100 rpm for 10 min at RT. The supernatant was discarded and the pellet was resuspended drop by drop with Carnoy's fixative (3:1, methanol: glacial acetic acid). The cells were collected by centrifugation at 1,100 rpm for 10 min at RT and resuspended in Carnoy's fixative. The cell suspension was then

dropped from an approximate height of 10 cm onto glass slides. Chromosomes were visualized with DAPI stain.

Densitometric analysis

Quantification of band intensities was performed using NIH Image J software, http://rsbweb.nih.gov/ij/.

Statistical significance

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

2.3 RESULTS

The FA pathway is activated in the presence of high-risk HPV oncoproteins.

We first determined the activation status of the FA pathway, using FANCD2 foci formation as a surrogate marker of FA pathway activation, in two different tissue arrays (Cybridi, Bethesda, MD and Biomax USA, Rockville, MD) that contained a total of 82 cervical squamous cell carcinomas (SCCs) and 39 control tissue specimens. Immunofluorescence microscopic analysis for FANCD2 revealed different staining patterns in cancer tissue versus that in normal cervical tissue. In 17 out of the 39 control samples (43.6%) we observed small punctate FANCD2 foci that was restricted to the supra-basal/basal strata of the epithelium and therefore, likely represents S phase-associated FA pathway activation. In contrast, a proportion

of SCC samples displayed cells with large FANCD2 foci in addition to the smaller foci, with some of the larger FANCD2 foci displaying a ring-like morphology (Fig. 8). Out of a total of 82 cervical cancers, 50 tumor samples (61%) were found to contain large FANCD2 foci. The number of tumor cells positive for large FANCD2 foci ranged from a few positive cells (1 per high-power field (HPF); 27 tumors), to a moderate number (1 to 5 per HPF; 15 tumors), to a high number (>5 per HPF; 8 tumors). Large FANCD2 foci were also observed in high-risk HPV-associated anal SCCs (data not shown). Since large FANCD2 foci were not detected any of the normal tissue samples this suggests that their formation represents FA pathway activation that is associated with oncogenic stress.

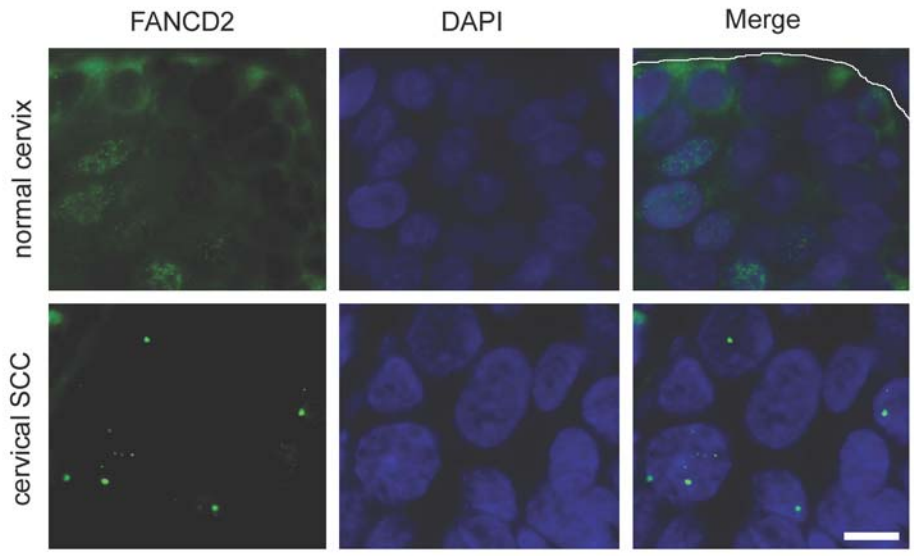

Figure 8. FANCD2 foci in normal cervical tissue and cervical SCCs.

Immunofluorescence analysis of FANCD2 revealed small punctate FANCD2 foci in normal tissue (upper panel) in contrast to large FANCD2 foci observed in SCC samples (lower panel). Nuclei were stained with DAPI. The white line in the top right panel indicates the basement membrane. Scale bar indicates 10 μm.

Activation of the FA pathway involves the HPV-16 E7 oncoprotein and occurs independently of viral integration.

The majority of HPV-associated tumors contain HPV DNA that has been integrated within the host cell genome, which leads to increased HPV oncoprotein activity. Precancerous lesions however, maintain the virus in an episomal state (157). Therefore, we were interested in determining whether episomal expression of the high-risk HPV oncoprotein is sufficient to activate the FA pathway. An immunofluorescence microscopic analysis of FANCD2 on three independently generated organotypical raft cultures from spontaneously immortalized human foreskin keratinocytes that stably express full length HPV-16 episomes or control was performed (Fig. 9A). We found that a significant proportion of cells harboring HPV-16 episomes contained large FANCD2 foci (1.7%) in comparison to control raft cultures, in which no such staining was observed (0%, $p \leq 0.05$, Fig. 9B). Inactivation of HPV-16 E7 by a translational termination linker (TTL) mutant in two independently generated raft cultures was found to significantly reduce the formation of large FANCD2 foci to 0.08% ($p \leq 0.05$, Fig. 9B). Raft cultures expressing a mutant HPV-16 E7 oncoprotein in which the pRB binding motif had been deleted was also found to decrease the percentage of cells with large FANCD2 foci (0.8%, data not shown). These findings suggest that the HPV-16 E7 oncoprotein is involved in stimulating the FA pathway and that FA activation occurs independently of viral integration.

In order to further explore the contributing roles of the high-risk HPV oncoproteins with respect to FA pathway induction, we performed immunofluorescence microscopic analysis of FANCD2 on hTERT-immortalized normal oral keratinocytes (kindly provided by K. Munger, Channing Laboratory, Brigham and Women's Hospital, Boston, MA) that have been engineered to stably express the high-risk HPV-16 E6 and/or HPV-16 E7 or low-risk HPV-6 E6 or E7

oncoproteins (Fig. 9C). We found that HPV-16 E7 expression lead to a significant 5.6-fold increase in the percentage of cells that contain large FANCD2 foci, from 1.5% in controls to 8.4% ($p \leq 0.0005$, Fig. 9D). Expression of the HPV-16 E6 oncoprotein did not lead to a significant increase of large FANCD2 foci as compared to the corresponding controls (4.6% versus 5.3%, $p > 0.05$, Fig. 9D). Co-expression of HPV-16 E6 and E7 lead to a moderate further increase of cells with large FANCD2 foci, from 12.1% in cells expressing HPV-16 E7 (HPV-16 E7/neo) to 14.9% in cells expressing both HPV-16 E6 and E7 (Fig. 9E). No significant change in the percentage of cells positive for large FANCD2 foci was observed in cell populations expressing the low-risk HPV-6 E6 or E7 oncoproteins (3.5% and 5.5%, respectively) compared to controls (4.8%, $p > 0.05$; Fig. 9F). The differences in the base line percentage of control cells positive for large FANCD2 foci are likely due to the fact that cells observed in Figs. 9E and F as well as HPV-16 E6-expressing cells and the corresponding controls (Fig. 9D) underwent two rounds of selection, whereas the HPV-16 E7-expressing cells and corresponding controls were only selected once. Nevertheless, the HPV-16 E7 oncoprotein was able to stimulate a significant increase in the percentage of cells that contain large FANCD2 foci regardless of the background levels in the respective controls.

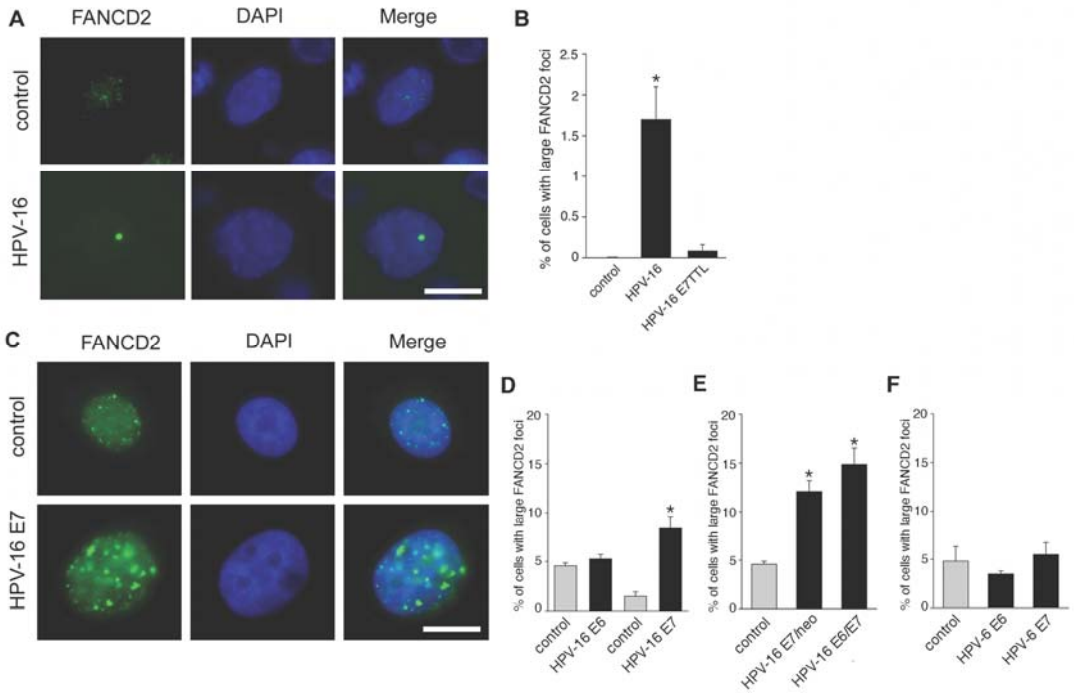

Figure 9. Induction of large FANCD2 foci involves HPV-16 E7 and occurs independently of viral integration.

(A) Immunofluorescence microscopic analysis of FANCD2 in organotypic raft cultures generated from primary human keratinocytes stably transduced with either HPV-16 episomes or control. Note the small punctate FANCD2 foci in a control cell in contrast to the large nuclear FANCD2 focus in a cell with HPV-16. Nuclei were stained with DAPI. Scale bar indicates 10 μm. (B) Quantification of the percentage of cells with large FANCD2 foci observed in organotypic rafts cultures transduced with control, full length HPV-16 episomes or HPV-16 episomes with a TTL inserted at nucleotide 711 of the E7 gene (E7TTL). Each bar indicates the mean and standard error for at least two independent immunofluorescence experiments, with at least 100 cells counted per experiment. Asterisk indicates statistical significance difference in comparison to controls. (C) Immunofluorescence microscopic analysis of FANCD2 in hTERT-immortalized normal oral keratinocytes stably transfected with either HPV-16 E7 or control. Note the different FANCD2 foci pattern in control versus HPV-16 E7-expressing cells. Nuclei stained with DAPI. Scale bar indicates 10 μm. (D to F) Quantification of the percentage of cells with large FANCD2 foci in cells expressing HPV-16 E6 or HPV-16 E7 (D), co-expression of HPV-16 E6 and E7 (E), or low-risk HPV-6 E6 or E7 (F) and their corresponding controls. Each bar represents the mean and standard error for triplicate quantifications of at least 100 cells. Asterisks indicate statistically significant differences in comparison to controls.

In order to provide further biochemical evidence that the HPV-16 E7 oncoprotein activates the FA pathway, we performed a chromatin fractionation analysis of cell lines stably expressing the various high- and low-risk HPV oncoproteins (Fig. 10). Chromatin fractions (P3) and soluble nuclear fractions (S3) were collected from stable cell populations and analyzed by western blot analysis for FANCD2 and FANCD1/BRCA2 levels. As expected, the chromatin fractions contained mostly the monoubiquitinated form of FANCD2 (also referred to as "long form"; L), while the soluble fraction consisted primarily of the faster migrating "short form; S" of FANCD2. HPV-16 E7-expressing cells were found to contain a 2.3-fold increase of monoubiquitinated FANCD2 present in the chromatin fraction compared to control cells (densitometric analysis of band intensities was quantified using the NIH image J program), indicative of FA pathway activation (Fig. 10A). Furthermore, HPV-16 E7-expressing cells displayed an increase of FANCD1/BRCA2 at the chromatin as well. Levels of the short form of FANCD2 and FANCD1/BRCA2 were also increased in the soluble fraction of HPV-16 E7-expressing cells compared to controls.

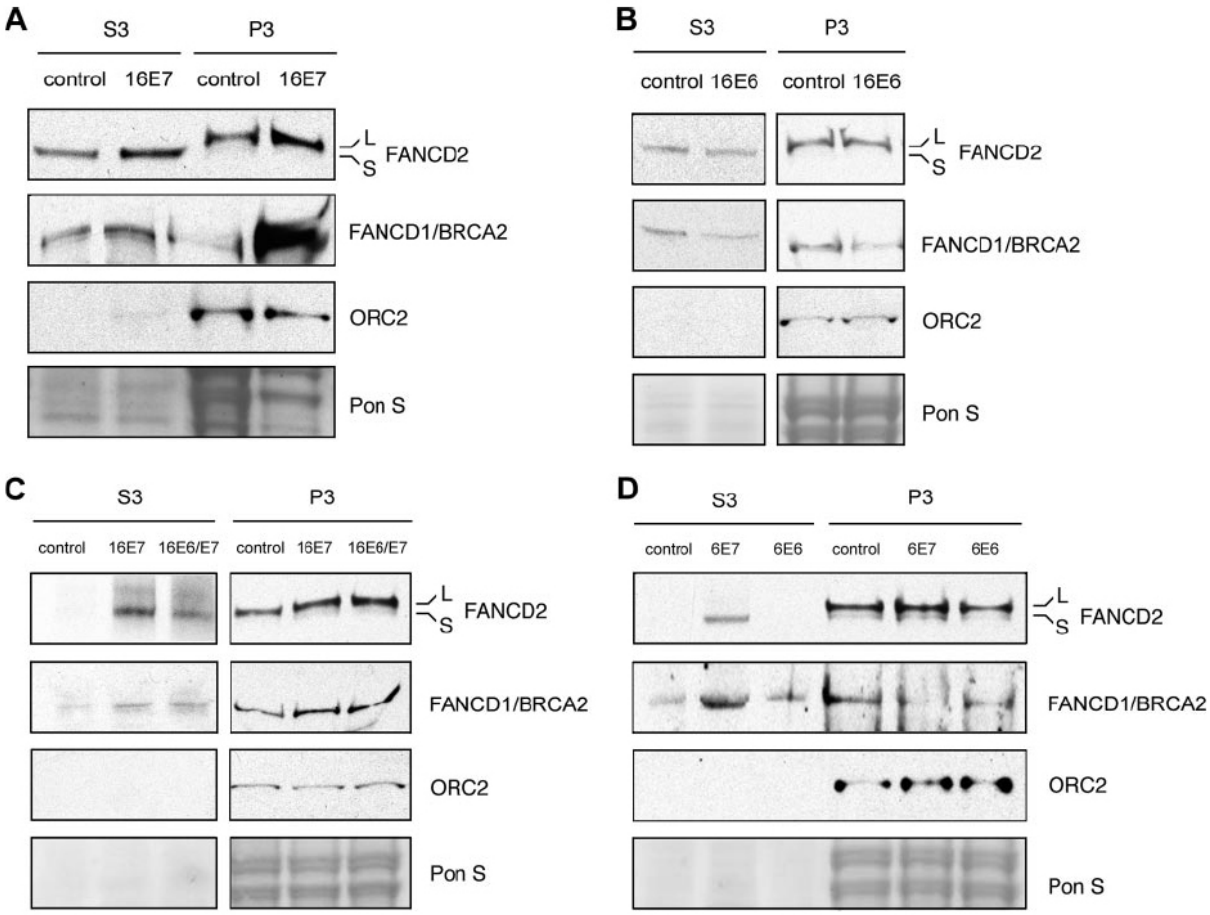

Figure 10. HPV-16 E7 stimulates the recruitment of monoubiquitinated FANCD2 and FANCD1/BRCA2 to chromatin.

(A-D) Chromatin fractionation analysis of hTERT-immortalized keratinocytes stably expressing HPV-16 E7 (A), HPV-16 E6 (B), HPV-16 E6/E7 (C) or low-risk HPV-6 E6 or E7 (D) and their corresponding controls. Immunoblot analysis was used to assess the amount of FANCD2 and FANCD1/BRCA2 protein found in the soluble nuclear (S3) and chromatin-bound (P3) fractions. Note the molecular shift of FANCD2 corresponding to the monoubiquitinated form of FANCD2 (long form, L) or non-ubiquitinated (short form, S). Immunoblot for ORC2 is shown to demonstrate chromatin enrichment of P3 fractions. Ponceau S (Pon S) staining is shown to visualize total protein loading.

Expression of the HPV-16 E6 oncoprotein was not found to increase the recruitment of monoubiquitinated FANCD2 or FANCD1/BRCA2 to the chromatin compared to controls (Fig. 10B). Co-expression of the HPV-16 E6 and E7 oncoprotein lead to a slight further increase in the amount of monoubiquitinated FANCD2 recruited to the chromatin compared to cells expressing the HPV-16 E7 alone (2.4-fold increase versus the 2.2-fold increase, respectively, Fig. 10C). No further increase of FANCD1/BRCA2 at the chromatin was observed in cells co-expressing HPV-16 E6 and E7 compared to HPV-16 E7-expressing cells. Co-expression of the high-risk HPV oncoproteins did not result in a further increase of FANCD2 or FANCD1/BRCA2 found in the soluble fractions compared to cells expressing HPV-16 E7 alone.

Ectopic expression of the low-risk HPV E7 oncoprotein was found to stimulate only a minor increase of monoubiquitinated FANCD2 (1.3-fold) recruited to chromatin compared to controls (Fig. 10D). Interestingly however, cells expressing HPV-6 E7 displayed an increase of both FANCD2 and FANCD1/BRCA1 found in the soluble fraction. No difference in the amount of FANCD2 or FANCD1/BRCA2 was observed in either of the cellular fractions between cells expressing the HPV-6 E6 oncoprotein and controls.

These findings further corroborate that the HPV-16 E7 oncoprotein activates the FA DNA damage response.

FA-deficiency enhances high-risk HPV oncoprotein-associated DNA damage.

Based on these results and the fact that persistently stalled replication forks can collapse to form DNA DSBs, we hypothesized that deficiencies in the FA pathway would lead to an increase of DNA breakage in high-risk HPV E6/E7-positive cells. In order to test this, we performed immunofluorescence microscopic analysis for the p53 binding protein 53BP1, a

marker of DNA DSBs, in organotypic raft cultures that had been generated from patient-derived FANCA-deficient primary keratinocytes that had been immortalized by retroviral transduction with an HPV-18 E6/E7 expression vector, and then either complemented with a FANCA-expressing retroviral vector (FANCA complemented) or transduced with an empty vector control (denoted FANCA$^{-/-}$) (Fig. 11A). We observed that HPV-18 E6/E7-positive, FANCA$^{-/-}$ raft cultures displayed a significant increase in the percentage of cells that contain 53BP1 foci when compared to HPV-18 E6/E7-positive FANCA-complemented cells. Furthermore, HPV-18 E6/E7-expressing FANCA deficient cells contained significantly more 53BP1 foci per nucleus as compared to HPV-18 E6/E7-positive FANCA-complemented cells (Fig. 11B). These results suggest that FA-deficient cells are increasingly susceptible to high-risk HPV-induced DNA breakage and furthermore, that the FA pathway plays a role in preventing or repairing host cell DNA damage induced by high-risk HPV oncoprotein expression.

The HPV-16 E7 oncoprotein accelerates chromosomal instability in FA-deficient cells.

We next sought to determine whether the increase of HPV-associated DNA breakage in FA-deficient cells has a negative impact for host cell genomic integrity. In order to test this, we performed metaphase spread analysis on immortalized patient-derived FANCD2-deficient fibroblasts that had been engineered to stably express HPV-16 E6 and/or E7, HPV-6 E6 or E7 or empty vector control. hTERT-immortalized NOKs stably expressing HPV-16 E7 or empty vector control were used as FA-proficient control cells. Cells were stained with DAPI and microscopically analyzed for the appearance of chromosomal abnormalities. We observed that a proportion of HPV-16 E7-expressing FANCD2-deficient cells displayed structural genomic alterations, including chromosome breaks and fusions (Fig. 12A). In line with previous findings,

HPV-16 E7 was shown to stimulate an increase of chromosomal abnormalities in FA-proficient keratinocytes, from 1.6% in controls to 6.7%, albeit without reaching statistical significance (Fig. 12B). In FANCD2-deficient cells however, HPV-16 E7 expression stimulated a statistically significant 2.9-fold increase of chromosomal abnormalities, from 3.3% in controls to 9.7% ($p \leq 0.001$, Fig. 12C). Co-expression of HPV-16 E6 and E7 lead to only a minor further increase in the percentage of abnormal metaphases, from 9.8% in cells expressing HPV-16 E7 alone (HPV-16 E7/neo) to 12.3% (1.3-fold). Expression of HPV-16 E6 or low-risk HPV-6 E6 or E7 did not lead to a significant increase in the percentage of chromosome alterations compared to controls. These results underscore that HPV-16 E7 expression accelerates the accumulation of structural chromosomal instability in FA deficient cells.

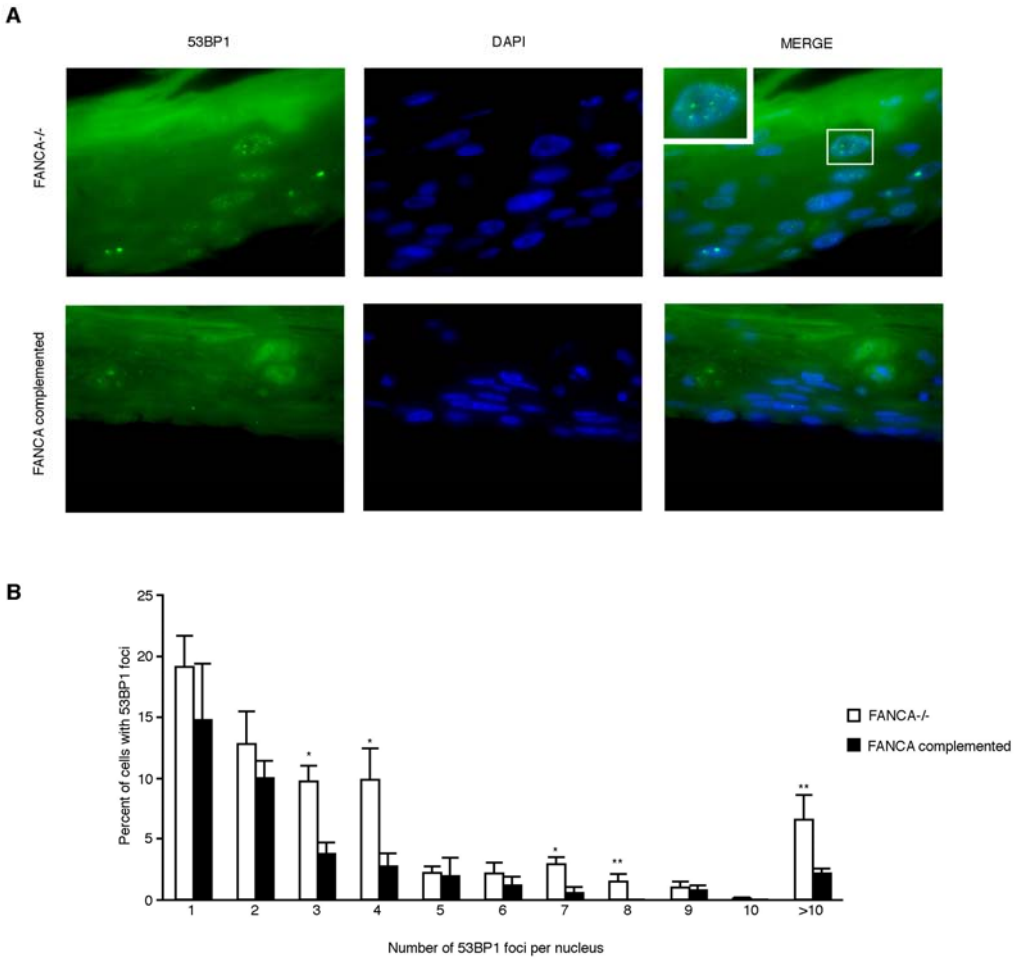

Figure 11. FANCA deficiency augments high-risk HPV oncoprotein-associated DNA damage.

(A) Immunofluorescence microscopic analysis of 53BP1 in organotypic raft cultures generated from HPV-18 E6/E7-immortalized FANCA-deficient keratinocytes complemented with either control vector ($FANCA^{-/-}$, upper panel) or FANCA (FANCA complemented, bottom panel). White box indicates the 53BP1-positive FANCA-deficient cell shown at higher magnification in insert. Nuclei stained with DAPI. Scale bar indicates 10 μm. (B) Quantification of the proportion of FANCA-deficient or complemented cells that contain the indicated number of 53BP1 foci per cell. Each bar represents the mean and standard error of two independent experiments with at least triplicate quantification of a minimum of 100 cells. Asterisks indicate statistically significant differences in comparison to controls (*$p \leq 0.05$, **$p \leq 0.01$). Adapted by permission from Macmillan Publishers Ltd: [Oncogene] (28:674-685), copyright (2009).

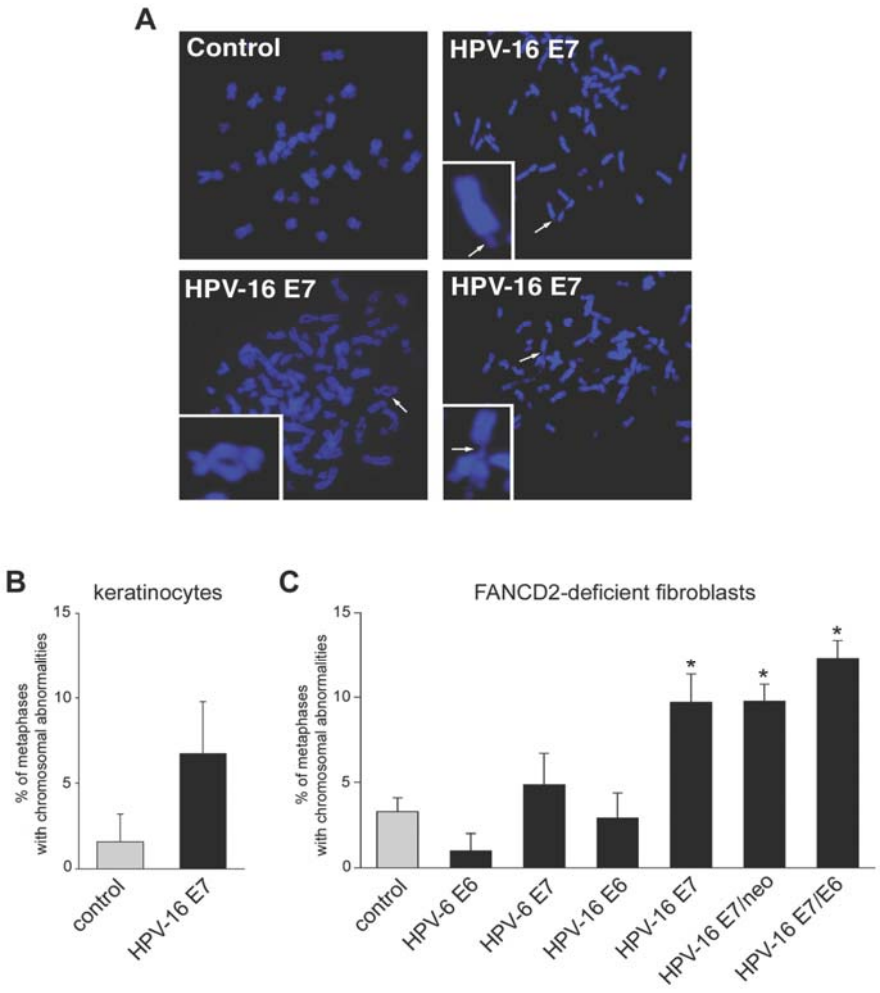

Figure 12. HPV-16 E7 accelerates structural chromosomal instability in patient-derived FANC2-deficient cells. (A) FANCD2-deficient immortalized fibroblasts were stably transfected with HPV-16 E7 or control and subjected to metaphase spread analysis. Chromosomes were stained with DAPI. Inserts depict chromatid breaks (right panels) and fusion events (bottom left panel) observed in HPV-16 E7-expressing FA-deficient cells at higher magnification. (B and C) Quantification of the percentage of metaphase spreads with chromosome aberrations from stably transfected empty vector (control) or HPV-16 E7-expressing immortalized human keratinocytes (FA-proficient) (B) or immortalized FANCD2-deficient fibroblasts stably expressing low-risk HPV-6 E6 or E7 or high-risk HPV-16 E6 and/or E7 (C). Each bar represents the mean and standard error for at least triplicate quantifications of a minimum of 25 metaphases. Asterisks indicate statistically significant differences in comparison to controls.

2.4 DISCUSSION

Here, we show that high-risk HPV oncoproteins activate the FA pathway in a manner which involves the HPV-16 E7 oncoprotein and occurs independently of viral integration. We provide evidence that a FA-deficient host cell environment enhances high-risk HPV oncoprotein-induced DNA breakage and moreover, that HPV-16 E7 accelerates the accumulation of structural chromosomal instability in FANCD2-deficient cells. Collectively, our findings suggest that activation of the FA pathway represents an early host cell response to HPV infection and that a functional FA pathway is crucial to prevent high-risk HPV oncoprotein-associated DNA breakage and structural chromosomal instability.

Although our results clearly point to a role for the HPV-16 E7 oncoprotein in activating the FA pathway, the molecular mechanism through which this occurs remains unclear. Since the FA pathway responds primarily to DNA replication stress (132), it is plausible that HPV-16 E7-associated unscheduled entry into S phase stimulates aberrant host cell replication dynamics, which ultimately cause DNA replication forks to stall. In line with this, deregulated cyclin E, CDC25A and E2F levels, important characteristics of HPV-16 E7-expressing cells, has been found to trigger DNA replication stress (73-75). Further evidence that HPV-16 E7-associated disruption of cell cycle control plays a role in activating the FA pathway is supported by our finding that deletion of the core pRB binding motif (Δ21-24), which is required to promote unscheduled entry into S phase, lead to a reduction in the percentage of cells with large FANCD2 foci (data not shown). It is important to note however, that the decrease of cells with large FANCD2 foci in HPV-16 E7 Δ21-24 mutant rafts was not as pronounced as in HPV-16 E7TTL rafts, which indicates that pRB-independent activities of HPV-16 E7 may also contribute

to FA pathway activation. These results suggest that the HPV-16 E7 oncoprotein activates the FA pathway; however, further experiments to directly show how HPV-16 E7 expression affects replication fork dynamics are warranted.

A similar frequency of large FANCD2 foci was detected in both HPV-16 episome-containing raft cultures and HPV-associated neoplastic tissue, which underscores that episomal expression of HPV-16 oncoproteins is sufficient to activate the FA pathway. Since the majority of precancerous lesions harbor episomal HPV DNA, these findings support the notion that HPV-16 oncoprotein-induced replication stress and subsequent FA pathway activation may play a role in promoting early steps of carcinogenesis.

FA deficiency was found to increase high-risk HPV oncoprotein-induced DNA breakage, as evidenced by the increase of 53BP1 foci observed in FANCA-deficient, HPV-18 E6/E7-positive organotypic raft cultures. Since a functional FA pathway is required to maintain replication fork stability (158), these results suggest that the collapse of stalled replication forks may drive DNA DSB formation and structural chromosomal instability in cells expressing the HPV-16 E7 oncoprotein.

Our results support the notion that a FA-deficient host cell environment facilitates the acquisition of genomic alterations in HPV-positive cells that are required for malignant progression. In line with this, certain FA genes have been found to be epigenetically silenced in a subset of HPV cancers (155). Interestingly, the FA pathway has been implicated in maintaining the stability of chromosomal fragile sites (CFS) (159), which are specific regions with the host cell genome that are hypothesized to be hot spots for HPV integration (160-163). While the molecular mechanisms behind HPV integration are unknown, it has been hypothesized to involve host and viral genome breakage. Based on our results, we suggest that inherited or

epigenetic silencing of the FA pathway may enhance viral integration. Since viral integration is thought to drive malignant progression in HPV tumors, the idea that HPV integration may be accelerated in FA-deficient host cells may help to explain the rapid progression of HPV tumors in FA patients.

FA-deficient cells have previously been shown to be readily transformed by viral oncoproteins, including the SV40 large T Ag (164), which shares key functions with the HPV-16 E7 oncoprotein. SV40-transformed cells, like HPV-positive malignant cells (165), have been characterized with structural chromosomal instability (166, 167). Hence, our model that HPV-16 E7-induced replication stress contributes to host cell genomic instability may also be applicable to other small DNA tumor viruses that disrupt cell cycle progression in a manner similar to HPV.

Our results suggest that FA-deficient cells are increasingly susceptible to HPV-16 E7-induced chromosomal instability. However, it must be emphasized that the precise role of HPV infection with respect to malignancies in FA patients is currently unclear. Alternatively, it may be that FA deficiency, in the absence of HPV infection, facilitates genomic instability at these anatomical sites in a manner that is similar to that observed during HPV infection in normal populations. Furthermore, despite our inability to detect a significant increase of chromosomal changes in FA-deficient cells expressing HPV-16 E6, it is likely that HPV-16 E6-mediated p53 degradation also contributes to genomic instability in these cells. In support of this, p53-deficient FANCD2 knockout mice have been found to develop an increase of epithelial cancers (168).

Collectively, our findings provide evidence that the HPV-16 E7 oncoprotein triggers activation of the host cell FA pathway and that FA deficiency promotes the rapid accumulation of structural chromosomal instability in HPV-16 E7-expressing cells. Future experiments are

required to assess how induction of the FA pathway by HPV-16 E7 contributes to the viral life cycle and malignant progression in FA-proficient host cells.

3.0 HPV-16 E7 REVEALS A LINK BETWEEN DNA REPLICATION STRESS, FANCONI ANEMIA D2 PROTEIN AND ALTERNATIVE LENGTHENING OF TELOMERE-ASSOCIATED PROMYELOCYTIC LEUKEMIA BODIES

Work described in this section was published in Cancer Research (Cancer Res. 2008, 68: (23); 9954-9962) with authors Nicole Spardy, Anette Duensing, Elizabeth E. Hoskins, Susanne I. Wells and Stefan Duensing

E.E. Hoskins and S.I. Wells provided the primary human foreskin keratinocytes stably expressing the HPV-16 E7 oncoprotein or corresponding control. N. Spardy performed all the experiments described in this section. A. Duensing contributed to experimental designs and data interpretation. N. Spardy and S. Duensing conceived the project, analyzed and interpreted the results and wrote the manuscript.

3.1 INTRODUCTION

The high-risk HPV oncoproteins profoundly disrupt cell cycle control in order to promote host cell proliferation (reviewed in Chapter 1). However, unscheduled proliferation is accompanied by gradual telomere loss, which ultimately leads to anti-proliferative responses including replicative senescence or apoptosis. The acquisition of mechanisms by which to maintain telomere length contributes to the immortalization of cells and represents a critical step in tumor evolution. The human papillomavirus (HPV) represents a useful tool through which to study activation of telomere maintenance mechanisms, as HPV-associated oncoproteins have been found to promote proliferation by interfering with telomere homeostasis through multiple mechanisms in order to maintain telomere length and extend the lifespan of primary human cells (62, 169, 170).

Telomeres are structures found at the end of chromosomes, which are required to protect DNA ends from being recognized as DNA double strand breaks. Structurally, telomeres consist of 10 to 15 kb of tandem TTAGGG repeats with a 3' single strand overhang of the G-rich strand. Erosion of telomeric DNA occurs with each successful round of cell division due to the inability of the DNA polymerase to completely replicate the ends of chromosomes during lagging strand synthesis. Critically short telomere length leads to aberrant fusions of chromosomes and negative growth signaling, which results in apoptosis or replicative senescence (171). Self-renewing cells, such as male germ cells, are able to maintain telomere length as a result of activated telomerase. Telomerase is a specialized enzyme that encodes a RNA component that allows the addition of

telomeric repeats to the 3' end of telomeres. Telomerase activation is also observed in a large proportion of tumor cells and is thought to be a major mechanism involved in the immortalization of cells (172).

Expression of the high-risk HPV oncoproteins E6 and E7 is able to independently extend the life span of primary human keratinocytes by interfering directly with telomere maintenance mechanisms (62). The HPV-16 E6 oncoprotein contributes to cellular immortalization by upregulating hTERT, the catalytic subunit of telomerase, through mechanisms described in Chapter 1. Despite high levels of telomerase however, late passages of HPV-16 E6-expressing primary human keratinocytes do not maintain telomere length (62). This finding supports the notion that additional activities besides telomerase may contribute to the complete immortalization of cells by high-risk HPV oncoproteins. In contrast, the HPV-16 E7 oncoprotein has been found to avoid replicative senescence associated with critical telomere shortening through a mechanism that is independent of the HPV-16 E6 oncoprotein and telomerase. Late passages of HPV-16 E7-expressing primary human keratinocytes are able to maintain heterogenous telomere length in the absence of detectable telomerase activity (169, 170). While the precise telomere maintenance mechanism utilized by the HPV-16 E7 oncoprotein remains unknown, it has been suggested that the alternative lengthening of telomeres (ALT) may be involved (169, 170).

ALT was originally identified in lower organisms and is believed to extend chromosome ends through a mechanism that involves the recombination of homologous DNA sequences found at telomeres (173, 174). Further evidence that ALT is a recombination-based mechanism is supported by the fact that various HR proteins, including MRE11 and RAD51, are required for ALT-associated activity (175). Signs of ALT have since been observed in approximately 10% of

human cancers, frequently detected in tumors of mesenchymal origin, and in various tumor-derived cell lines (176). Cells with activated ALT are characterized by heterogenous telomere length, extrachromosomal telomeric DNA circles and the appearance of subnuclear promyelocytic leukemia bodies (PML) (177). While the precise function of ALT-associated PML bodies (APBs) remains unclear, they are thought to represent sites of recombining telomeric DNA, as they have been found to contain telomeric DNA, telomere binding proteins and various proteins involved in HR. APBs appear at the onset of ALT immortalization and therefore are often used as a surrogate marker of ALT activity (177, 178).

Telomeric DNA is replicated using normal DNA replication machinery. However, recent reports suggest that the TTAGGG repeats found at chromosome ends can be particularly difficult to replicate and prone to replication fork stalling (179-181). Since stalled replication forks are excellent substrates for HR, it is not surprising that patients suffering from HR gene deficiencies, such as Fanconi Anemia (FA), display telomere defects. FA patient cells have been characterized by accelerated telomere erosion and breakage, as well as the presence of extrachromosomal telomeric DNA, indicative of disrupted telomere homeostasis (182, 183).

We have previously described how the HPV-16 E7 oncoprotein triggers a host cell DNA replication stress response, as reflected by FA pathway activation (Chapter 2, (184). The HPV-16 E7 oncoprotein was found to accelerate chromosomal aberrations, including those which frequently occurred at chromosome ends. Whether activation of the FA pathway by HPV-16 E7 plays a role in preserving telomere integrity in FA-proficient cells currently remains unknown.

Here, we provide evidence that the HPV-16 E7 oncoprotein stimulates the appearance of APBs that contain the FA-associated D2 (FANCD2) protein. We provide several lines of evidence that HPV-16 E7-induced FANCD2-positive APBs form as part of a replication stress

response at telomeres. First, FANCD2-containing APBs co-localize with proteins commonly found at stalled replication forks, most notably BRCA2 and MUS81, as well as single-stranded DNA. Second, treatment of cells with replication stress agents lead to a similar increase in the frequency of FANCD2-containing APBs and finally, their formation was found to depend on the ATR replication stress kinase. We show that depletion of FANCD2 using a small hairpin construct lead to an increase of dysfunctional telomeres, suggesting that the FA pathway is required to maintain telomere homeostasis in ALT-positive cells. Importantly, HPV-16 E7 was able to stimulate FANCD2-positive APB formation in early passage primary human keratinocytes, which may help to explain how the HPV-16 E7 oncoprotein contributes to cellular immortalization in a telomerase-independent manner. Collectively, our results suggest that HPV-16 E7-associated replication stress and FA pathway activation at telomeres promotes recombination-based ALT activity. These findings underscore that viral oncoproteins may be able to manipulate the DNA damage response in order to bypass anti-proliferative host cell barriers.

3.2 MATERIALS AND METHODS

Cell culture, treatment and transfection

U-2 OS, MCF-7, HeLa cells (ATCC, Manassas, VA) and GM00847 fibroblasts (Coriell, Camden, NJ) were maintained in DMEM (Biowhittaker/Lonza, Inc.) as described earlier. Primary human keratinocytes were maintained in serum-free keratinocyte growth medium

(Epilife, Cascade Biologics/Invitrogen). Cells were treated with 100 ng/ml mitomycin C (MMC; Sigma), 1 mM hydroxyurea (HU; Calbiochem) or dH_2O for the indicated time intervals.

U-2 OS cells were stably transfected using Fugene 6 (Invitrogen) transfection reagent and 2 μg of plasmid DNA followed by selection in DMEM supplemented with 3 mg/ml of G418 (Cellgro, Herndon, VA) for 48 h. pCMV-based plasmids containing HPV-16 E6, HPV-16 E7 or mutant HPV-16 E7 Δ21-24 were kindly provided by K. Münger (Channing Laboratory, Brigham & Women's Hospital, Boston, MA). LXSN-based low-risk HPV-6 E7 construct and mutant HPV-16 E7 CVQ68-70AAA and E7 Δ79-83 constructs were kindly provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) and subcloned into pCMV-based vectors. The pCDNA3-3HA-Mus81 plasmid was kindly provided by Clare H. McGowan (The Scripps Research Institute, La Jolla, CA). Empty vector controls were included in all experiments. All stable cell lines were passaged at least five times after antibiotic selection.

Primary human foreskin keratinocytes (HFKs) were transduced with an LXSN-based high-risk HPV-16 E7 construct or LXSN empty vector followed by selection in G418-supplemented medium.

Immunological methods

Preparation of cell lysates and immunofluorescence staining were performed as described earlier, with the exception that cells processed for immunofluorescence of PML were first treated with a soluble protein extraction buffer (10 mM PIPES, 300 mM sucrose, 3 mM $MgCl_2$, 20 mM NaCl, 0.5% Triton-X 100, [pH 6.8]) as previously described (185).

Primary antibodies used for immunoblotting, and immunofluorescence were 53BP1 (Novus Biologicals, Littleton, CO), actin (Sigma), ATM (Genetex), phosphorylated ATM at

serine 1981 (Novus Biologicals), ATR (Genetex), BLM (Bethyl, Montgomery, TX), BRCA2 (Calbiochem), 5'-bromo-2'deoxyuridine (BrdU; Roche Diagnostics, Indianapolis, IN), FANCD2 (Genetex), HA (Santa Cruz Biotechnology, Santa Cruz, CA), HPV-16 E7 (Santa Cruz Biotechnology), γ-H2AX (Trevigen, Gaithersburg, MD), MRE11 (Genetex), PML (Santa Cruz Biotechnology), RAD51 (Genetex), RPA32 (Lab Vision, Fremont, CA), and TRF2 (Imgenex, San Diego, CA).

Detection of single-stranded DNA (ssDNA)

To detect ssDNA, cells were labeled for 24 h in DMEM supplemented with 10 μmol/L of BrdU (Roche Diagnostics). All cells were washed once with fresh DMEM and processed immediately for BrdU immunofluorescence as described above without any prior DNA denaturation step. Cells were then incubated with a mouse monoclonal BrdU antibody (Roche Diagnostics), followed by incubation with a Rhodamine Red-conjugated anti-mouse secondary antibody (Jackson Immunoresearch). This technique utilizes the property that the monoclonal BrdU antibody only recognizes the incorporated base analog when the DNA is found in the single-stranded form (186).

Small interfering RNA (siRNA) and small hairpin RNA (shRNA)

Synthetic RNA duplexes were used to reduce ATM or ATR expression. Oligonucleotides targeting ATM, ATR, or control were obtained commercially (siGENOME SMARTpool; Dharmacon, Lafayette, CO). For each experiment cells were grown on coverslips in 2 ml DMEM free of antibiotics. Cells were transfected with 12 μl of 20 μM annealed RNA duplexes using

Oligofectamine (Invitrogen) transfection reagent and 0.5 μg of DsRED (Clontech). Cells were processed for further analyses at 72 h after transfection.

Small hairpin RNA (shRNA) constructs against FANCD2 or control (GFP) were obtained commercially (HuSh, Origene, Rockville, MD). U2-OS were transiently transfected using nucleofection (Lonza, Inc.) with either pooled shFANCD2 contructs (2 μg of each of the four constructs provided) or 2 μg of shGFP plus 6 μg of herring sperm. Cells were processed for further analyses at 72 h after transfection.

Telomere fluorescence in situ hybridization (FISH)

Telomere FISH was performed using a Cy3-conjugated telomere PNA probe (Telomere PNA FISH kit/Cy3; Dako, Glostrup, Denmark) according to the manufacturer's protocol. Briefly, cells grown on coverslips were rinsed in PBS and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then washed in PBS, permeabilized with 1% Triton-X 100 in PBS for 30 min at room temperature, and washed in PBS. Cells were then rinsed in tris buffered saline (TBS), incubated in 3.7% formaldehyde in TBS for 2 min, and washed 2 times 5 min in TBS. Cells were then incubated in a proteinase K pre-treatment solution for 10 min, followed by 2 times 5 min in TBS. Cells were then dehydrated in an ice cold ethanol series (70%, 85%, 90%) and allowed to air dry. Cells were incubated with the Telomere PNA probe/Cy3 for 5 min at 80°C, followed by hybridization at room temperature for 1.5 h in the dark. The cells were then rinsed, and washed for 5 min at 65°C. Cells were then blocked with 10% normal donkey serum for 15 min at room temperature and processed for immunofluorescence analyses as described above.

Statistical methods

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

3.3 RESULTS

HPV-16 E7 overexpression stimulates the formation of FANCD2 nuclear foci that show key characteristics of APBs.

In Chapter 2 we show that HPV-16 E7 expression activates the FA pathway, as evidenced by the appearance of large nuclear FANCD2 foci (184). Unexpectedly, a small proportion of FANCD2 foci observed in squamous cell carcinoma (SCC) tissue and organotypic raft cultures containing HPV-16 episomes displayed a ring-like structure. Ring-shaped foci have previously been found in cells which maintain their telomeres by the alternative lengthening of telomeres (ALT) (177). Therefore, in order to test whether HPV-associated oncoproteins are able to stimulate ring-shaped FANCD2 foci, we performed immunofluorescence microscopic analysis of FANCD2 in U-2 OS cells that have been engineered to stably express high-risk HPV-16 E7 or HPV-16 E6, low-risk HPV-6 E7 or empty vector control. We observed that a proportion of HPV-16 E7-expressing cells harbored ring-shaped FANCD2 foci (Fig. 13A). The HPV-16 E7 oncoprotein was found to stimulate a significant 2.0-fold increase in the percentage of cells that contain ring-shaped FANCD2 foci from 7.2% in controls to 14.3% in HPV-16 E7-expressing cells ($p \leq 0.005$; Fig. 13B). In contrast, stable expression of HPV-16 E6 (Fig. 13B) or low-risk

HPV-6 E7 (Fig. 13C) was unable to stimulate an increase of ring-shaped FANCD2 foci when compared to control cells.

A mutational analysis was performed in order to test which sequences of HPV-16 E7 are required to induce ring-shaped FANCD2 foci. We observed that expression of the HPV-16 E7 mutant Δ21-24, which is deficient in pRB binding and degradation, was unable to stimulate a significant increase of ring-shaped FANCD2 foci compared to control cells (Fig.13D). Expression of C-terminal HPV-16 E7 mutants (CVQ68-AAA70 or Δ79-83; kindly provided by D. Galloway), which can degrade pRB but are deficient in overcoming a $p21^{Cip1}$-associated growth arrest were found to stimulate only a minor increase of ring-shaped FANCD2 foci compared to control cells (Fig. 13D). These results suggest the pRB-dependent and pRB-independent activities of the HPV-16 E7 oncoprotein are involved in stimulating these specific FANCD2-positive structures.

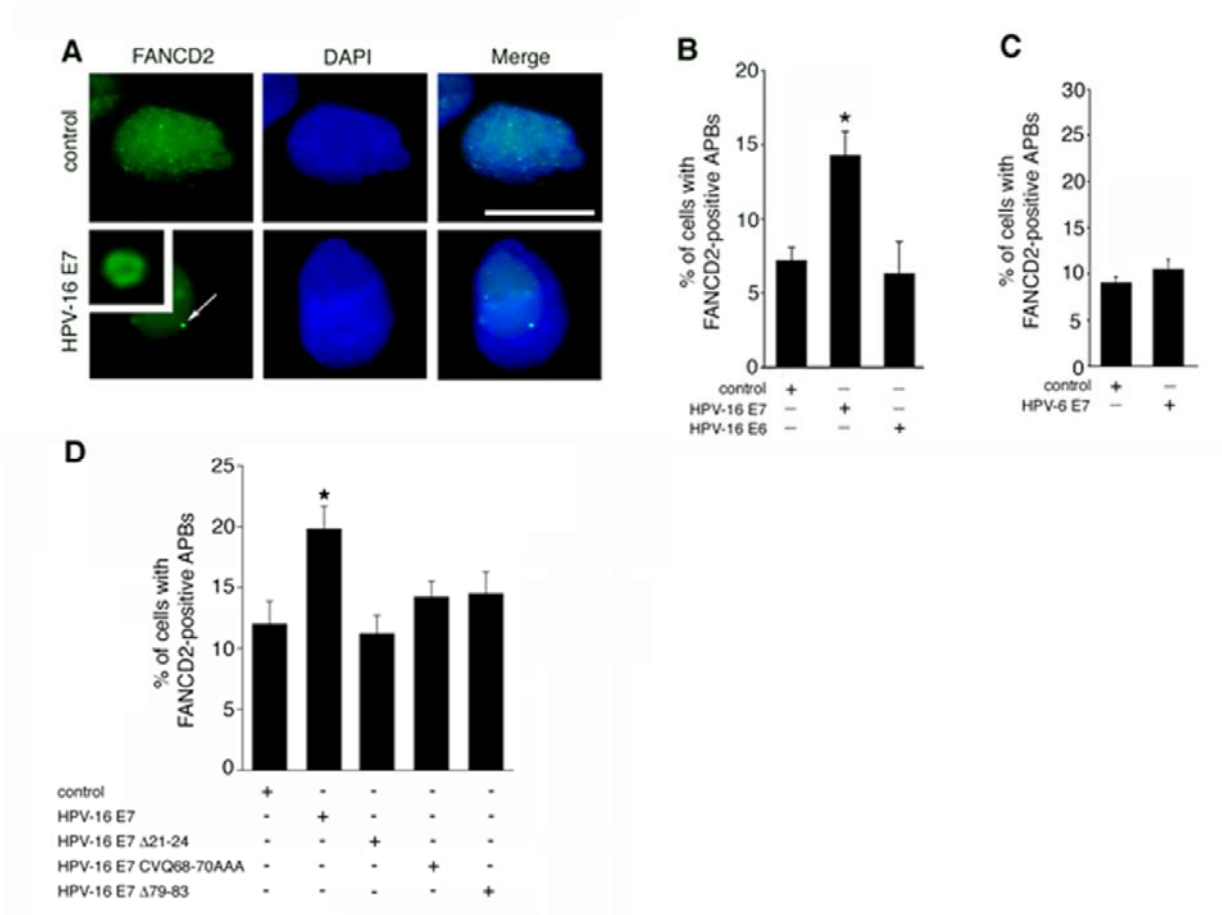

Figure 13. HPV-16 E7 stimulates the appearance of ring-shaped FANCD2 foci.

(A) Immunofluorescence microscopic analysis of FANCD2 in U-2 OS cells stably expressing HPV-16 E7 or control. Note the ring-shaped FANCD2 foci (arrow, insert) in contrast to the smaller FANCD2 foci observed in the control cell. Nuclei stained with DAPI. Scale bar indicates 10 μm. (B-D) Quantification of the percentage of cells with ring-shaped FANCD2 foci in U-2 OS cells stably expressing high-risk HPV-16 E7 or E6 (B), low-risk HPV-6 E7 (C) or various HPV-16 E7 mutants (D) and their corresponding controls (empty vector). Bars represent mean and standard error from three independent experiments with at least 100 cells counted (B) or mean and standard error from a representative experiment with at least 100 cells counted in triplicate (C,D). Asterisks indicate statistically significant differences in comparison to controls.

ALT cells are characterized by ring-shaped nuclear foci, commonly referred to as ALT-associated PML bodies or APBs, which contain PML, telomere binding proteins and telomeric DNA (177). In order to test whether FANCD2 is localized to APB structures, we performed co-immunofluorescence microscopic analysis of FANCD2 and known ABP components in HPV-16 E7-expressing U-2 OS cells (Fig. 14A). Ring-shaped FANCD2 foci were found to co-localize with PML (72.5%) and TRF2 (80.4%). By combining immunofluorescence analysis of FANCD2 and *in situ* hybridization using a telomere DNA probe we show that ring-shaped FANCD2 structures harbor telomeric TTAGGG repeats (94.1%; Fig 14A). FANCD2 was also found to co-localize with PML and TRF2 in a second ALT positive cell line, GM00847, but not in telomerase-positive Hela cells (data not shown). Collectively, these results underscore that FANCD2 is a novel component of APBs.

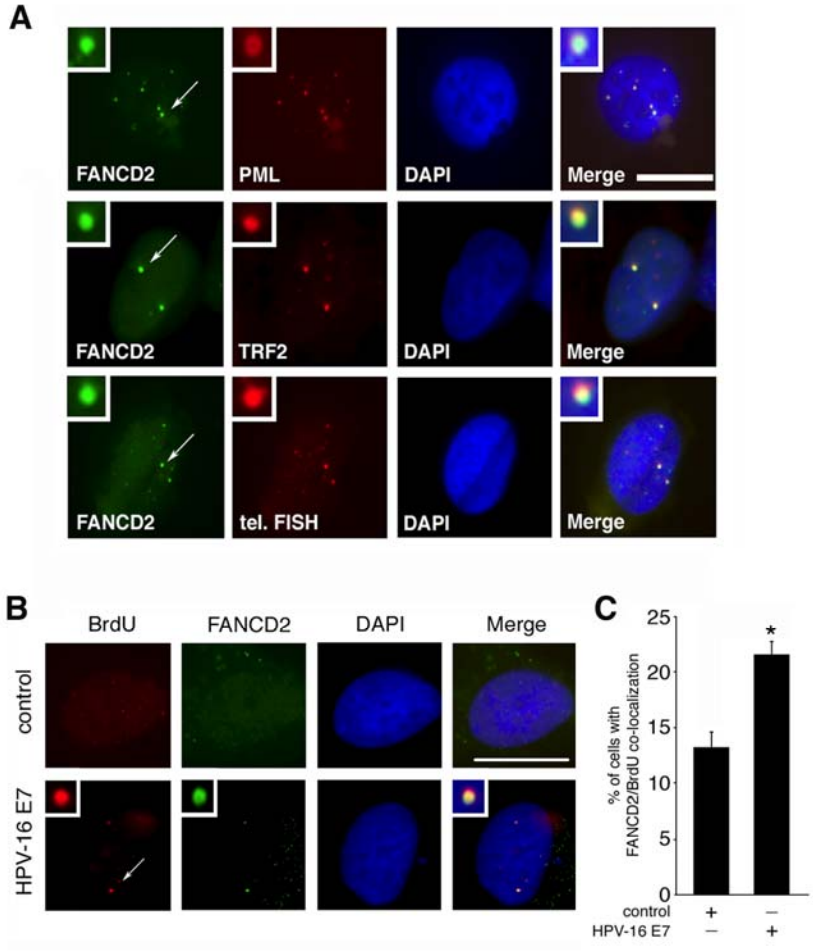

Figure 14. Ring-shaped FANCD2 foci co-localize with known APB components and ssDNA.

(A) Co-immunofluorescence microscopic analysis of HPV-16 E7-expressing U-2 OS cells for FANCD2 and PML (top) or TRF2 (middle). Immunofluorescence microscopic analysis of FANCD2 combined with FISH using a Cy3-conjugated telomeric DNA probe (bottom) in HPV-16 E7-expressing cells. Arrows indicate foci shown at higher magnification in the insert. Nuclei stained with DAPI. Scale bar indicated 10 μm. (B) Co-immunofluorescence microscopic analysis of FANCD2 and BrdU in HPV-16 E7-expressing U-2 OS cells. Arrow indicates foci shown at higher magnification in inserts. Nuclei stained with DAPI. Scale bar indicates 10 μm. (C) Quantification of the proportion of cells with BrdU and FANCD2-positive APBs in U-2 OS cell populations stably expressing empty vector (control) or HPV-16 E7. Each bar represents the mean and standard error for two independent experiments with at least triplicate quantification of a minimum of 100 cells. Asterisks indicate statistically significant differences in comparison to controls.

HPV-16 E7-induced FANCD2-positive APBs co-localize with ssDNA and DNA damage proteins involved in the processing and restart of stalled replication forks.

FANCD2 is recruited to chromatin in response to replication stress, as evidenced by chromatin fractionation analyses performed in Chapter 2. Therefore, we were interested in testing whether FANCD2-positive APBs contain DNA structures or DNA damage proteins that are commonly found at stalled replication forks. We show, using a modified BrdU technique, that a proportion of HPV-16 E7-induced FANCD2-positive APBs contain single-stranded DNA (ssDNA; Fig. 14B). ssDNA frequently forms at stalled replication forks as a result of continued DNA unwinding. HPV-16 E7 expression was found to stimulate a significant increase of FANCD2-positive APBs that harbor ssDNA (1.6-fold; 21.5%) when compared to control cells (13.2%, $p \leq 0.001$; Fig. 14C).

Next, we performed co-immunofluorescence microscopic analyses of FANCD2 and a variety of DNA damage proteins that localize to sites of stalled replication. We found that several DNA damage proteins, which have previously been identified as APB components, co-localize with FANCD2-positive APB structures in HPV-16 E7-expressing cells. These proteins include RPA32, BLM, RAD51 and MRE11 (Fig. 15). The percentages of co-localization between FANCD2-APBs and the respective proteins were as follows, 100% for RPA32, 86% for BLM, 27.3% for RAD51 and 86.6% for MRE11 in HPV-16 E7-expressing cells. We also identified BRCA2 and MUS81 as novel components of APB structures (Fig. 15). The percentage of co-localization between FANCD2-positive APBs and BRCA2 and MUS81 in HPV-16 E7-expressing cells was 33% and 38.4%, respectively. BRCA2 (also known as FANCD1) has been linked with the loading of FANCD2 to chromatin in response to genotoxic stress and is required for replication fork stability (187, 188). MUS81 possesses endonuclease activity that is involved

in processing abnormal DNA structures, such as Holliday junctions that form during HR-mediated replication fork restart (189). DNA double strand breaks (DSBs) have also been suggested to form as HR intermediates. In line with this, a small subset of ring-shaped FANCD2 were found to co-localize with phosphorylated ATM at serine 1981 and γ–H2AX, two markers of DNA breaks (data not shown). Collectively, our findings that FANCD2-APBs contain ssDNA and DNA damage proteins, in particular BRCA1 and MUS81, suggests that the processing of stalled replication forks may be a trigger of ALT activity in HPV-16 E7-expressing cells.

Replication stress-inducing agents stimulate FANCD2-positive APB formation.

In order to further corroborate these results, we next examined whether treatment of U-2 OS cells with replication stress-inducing agents was able to phenocopy the HPV-16 E7-associated increase of FANCD2-positive APBs. U-2 OS cells treated with the cross-linking agent, Mitomycin C (MMC) for 24 h was found to stimulate a significant 2.3-fold increase in the percentage of cells harboring FANCD2-containing APBs (23.2%) when compared to control treated cells (10.1%, $p \leq 0.001$; Fig. 16A, left panel). MMC is a classical activator of the FA pathway, and thus verifies that the localization of FANCD2 to APBs represents a bona fide activation of the FA pathway. MMC treatment however, may also lead to DNA DSBs due to replication fork breakdown. Therefore, to directly test whether FANCD2-APBs form as a result of stalled replication forks, we treated cells with hydroxyurea (HU), a ribonucleotide reductase inhibitor that causes forks to stall by depleting the deoxynucelotide pool. We observed a significant 2.3-fold increase of FANCD2-positive APBs in cells treated with HU for 72 h, from 15.3% in controls to 34.8% ($p \leq 0.005$; Fig. 16A, right panel). These results further support the notion that replication stress is a trigger for FANCD2-positive APB formation.

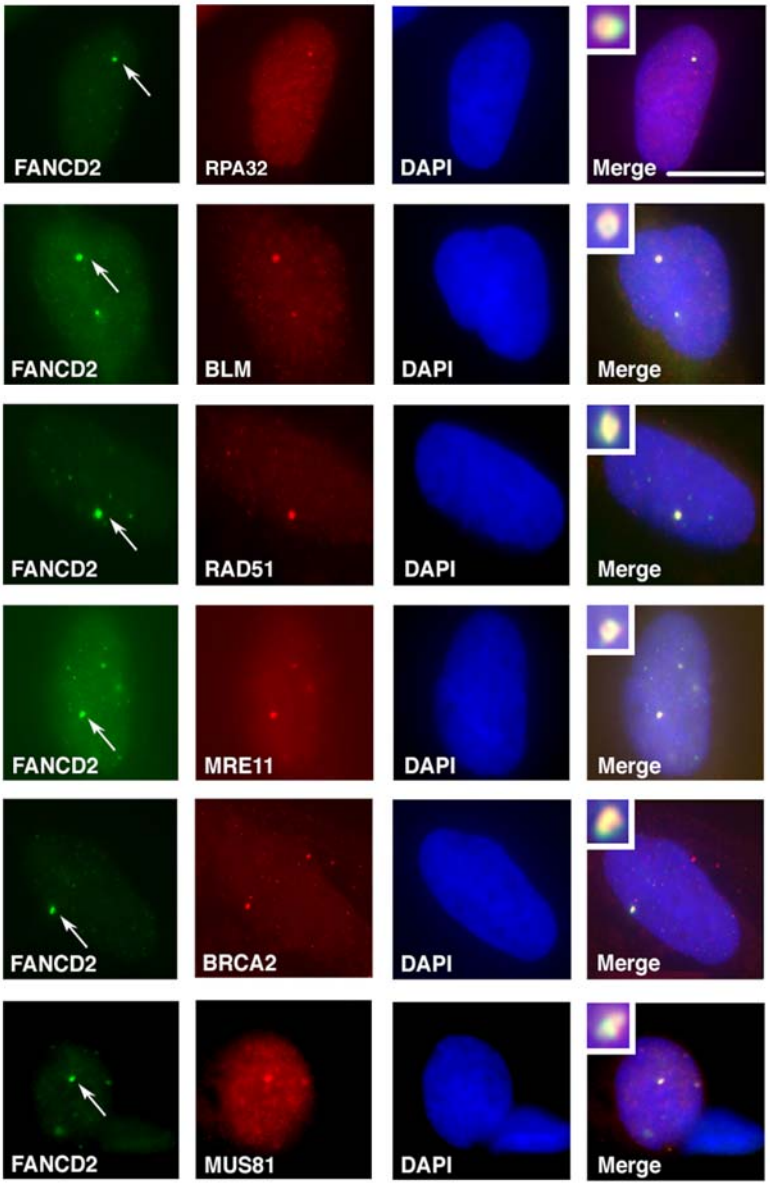

Figure 15. FANCD2-APBs co-localize with DNA damage proteins commonly found at stalled replication forks. Co-immunofluorescence microscopic analysis of HPV-16 E7-expressing U-2 OS cells for FANCD2 in combination with RPA32, BLM, RAD51, MRE11, BRCA1 or MUS81. MUS81 was detected by transiently transfecting cells with a plasmid encoding for MUS81-HA, followed by immunofluorescence analysis for HA. Arrows indicate the FANCD2-APBs shown at higher magnification in inserts. Nuclei stained with DAPI. Scare bar indicates 10 μm.

HPV-16 E7-associated FANCD2-positive APB formation requires the ATR DNA damage kinase.

Since replication stress is known to activate the FA pathway via the ATR-CHK1-signaling axis (144), we next sought to determine whether FANCD2-positive APB formation is dependent on the ATR DNA damage kinase. HPV-16 E7-expressing U-2 OS cells were transiently transfected with small interfering RNA (siRNA) duplexes targeting ATM, ATR or control. Immunoblot analysis showed a significant reduction in the respective protein levels at 72 hours (Fig. 16A). Immunofluorescence microscopic analysis of FANCD2 revealed that cells depleted for ATR lead to a significant 2.8-fold reduction in the percentage of cells harboring FANCD2-positive APBs (4.8%) when compared to cells treated with control siRNA for 72 h (13.7%, $p \leq 0.0005$; Fig. 16C&D). Depletion of ATM had no significant affect on the frequency of HPV-16 E7-expressing U-2 OS cells with FANCD2-positive APBs (15%, $p > 0.05$; Fig. 16D) when compared to control cells. Notably, previous reports have not detected a requirement for ATM or ATR in conventional APB formation (190). Our finding that the HPV-16 E7 oncoprotein stimulates FANCD2-positive APBs in an ATR-dependent manner further underscores that their formation is part of a replication stress response.

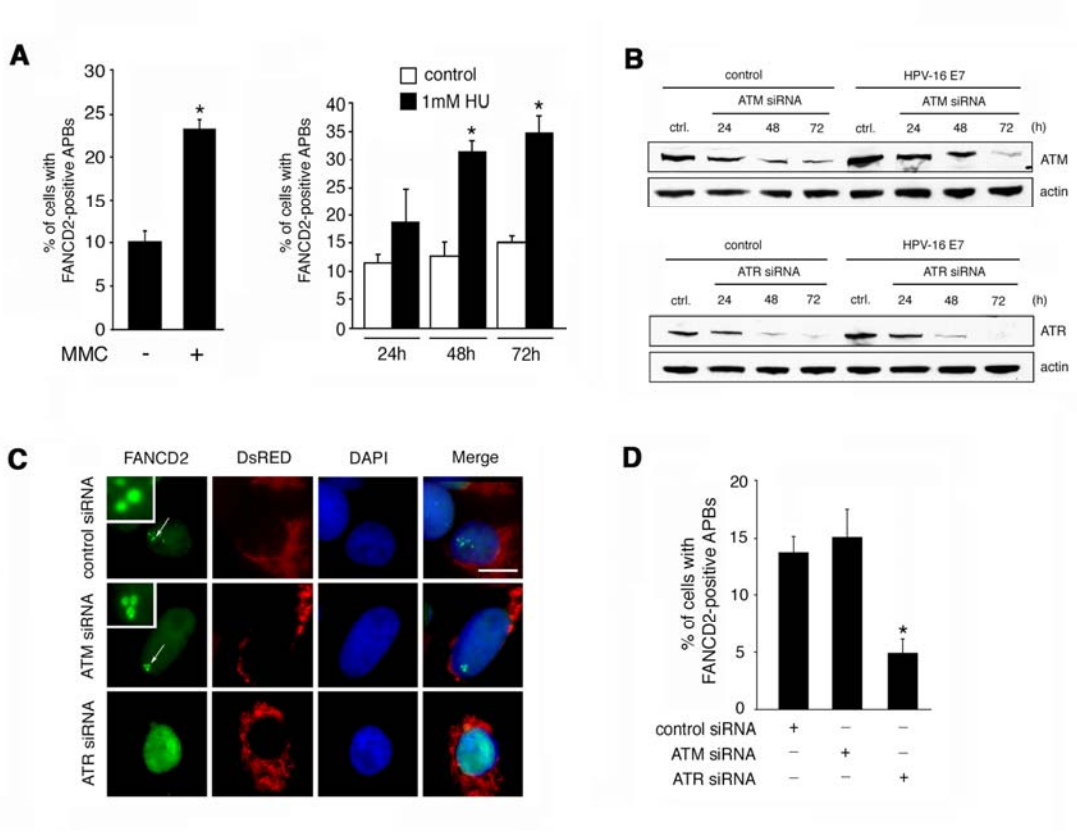

Figure 16. FANCD2-APBs form in response to exogenous replication stress and their formation requires the ATR DNA damage kinase in HPV-16 E7-expressing cells.

(A) Quantification of the proportion of U-2 OS cells harboring FANCD2-APBs following treatment with 100 ng/ml MMC or dH2O for 24 h (left) or treated with 1 mM HU or dH_2O (control) for the indicated time intervals (right). Each bar represents the mean and standard error for two independent experiments with at least triplicate quantification of a minimum of 50 cells. Asterisks indicate statistically significant differences in comparison to controls. (B) Immunoblot analysis of U-2 OS cell expressing HPV-16 E7 or control treated with either control (ctrl.), ATM or ATR siRNA duplexes for the indicated time intervals. Immunblot for actin demonstrates total protein loading. (C) Immunofluorescence analysis for FANCD2 in HPV-16 E7-expressing cells treated with ctrl., ATM or ATR duplexes for 72 h, using dsRED as a transfection control. Note the disappearance of FANCD2-APBs in HPV-16 E7-expressing cells treated with ATR siRNA. Legend continued on next page.

Arrows indicate the FANCD2-APBs shown at higher magnification in inserts. Nuclei were stained with DAPI. Scale bar indicates 10 μm. (D) Quantification of the proportion of HPV-16 E7-expressing cells with FANCD2-APBs following treatment with respective siRNA duplexes. Bars indicate the mean and standard deviation of two independent experiments with at least triplicate quantification of a minimum of 100 cells. Asterisk indicates statistically significant differences in comparison to controls.

FANCD2 depletion leads to telomere dysfunction in ALT-positive cells.

To directly test the role of FANCD2 in maintaining telomere homeostasis in ALT-immortalized cells, we depleted FANCD2 protein by transfecting U-2 OS cells with small hairpin RNA (shRNA) constructs targeting FANCD2 or control (GFP; Fig. 17A). Previous reports have suggested that critically short telomere length can lead to the binding of DNA damage proteins to chromosome ends (also termed telomere dysfunction-induced foci, TIF) (191). By combining *in situ* hybridization using a telomeric DNA probe with immunofluorescence analysis of the DNA damage marker, 53BP1 we found that depletion of FANCD2 in U-2 OS cells leads to dysfunctional telomeres, as evidence by an increase of telomeric signals that co-localize with 53BP1 (Fig. 17B). A significant 6.9-fold increase of cells that harbored at least 5 TIFs per nucleus was observed in FANCD2-depleted cells (17.9%) when compared to control treated cells (2.6%, $p \leq 0.0001$; Fig. 17C). These results suggest that the FANCD2 protein plays a role in maintaining telomere integrity in ALT-immortalized cells.

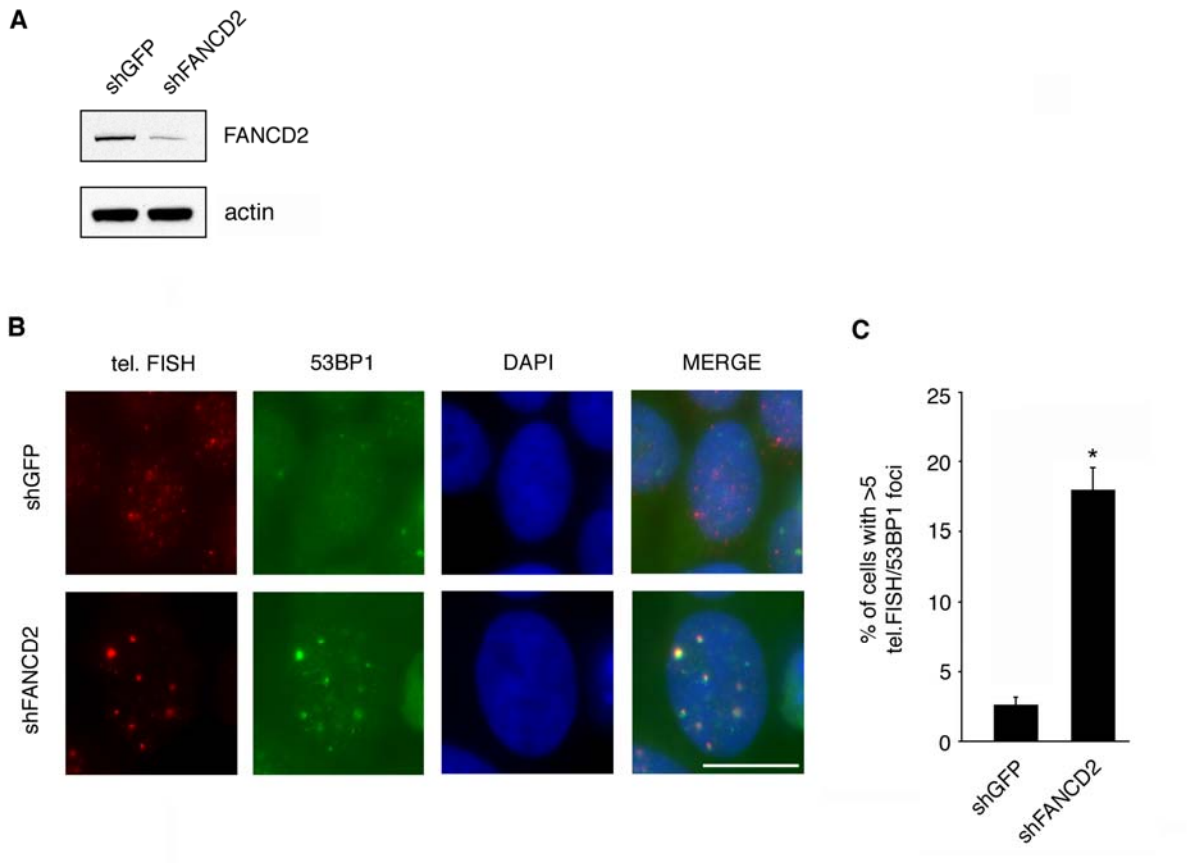

Figure 17. FANCD2 depletion in U-2 OS cells leads to telomere dysfunction.

(A) Immunoblot analysis of U-2 OS cells 72 h after transient transfection with shRNA targeting FANCD2 (shFANCD2) or control (shGFP). Immunoblot for actin demonstrates total protein loading. (B) Immunofluorescence analysis of 53BP1 in combination with FISH using a Cy3-conjugated telomere probe in U-2 OS cells 72 h after transfection with shFANCD2 or shGFP. Nuclei were stained with DAPI. Scale bar indicates 10 μm. (C) Quantification of the proportion of U-2 OS cells transiently transfected with shFANCD2 or shGFP that display >5 telomeric signals that co-localize with 53BP1. Bars indicate mean and standard error of two independent experiments with at least duplicate quantification of a minimum of 50 cells. Asterisk indicates statistically significant differences in comparison to controls.

The HPV-16 E7 oncoprotein stimulates APB formation in early passage primary human keratinocytes.

Lastly, we were interested in determining whether HPV-16 E7 expression stimulates FANCD2-positive APBs in non-immortalized primary human foreskin keratinocytes. Early passage primary human keratinocytes that had been stably transduced with a LXSN-based construct encoding HPV-16 E7 or control were analyzed by immunofluorescence microscopic analysis of FANCD2 and TRF2 for the appearance of FANCD2-positive APBs (Fig. 18A). Remarkably, FANCD2-positive APBs were observed in a small percentage of HPV-16 E7-expressing primary keratinocytes (0.05%) as early as passage 2 (Fig. 18B). The frequency of HPV-16 E7-expressing cells positive for FANCD2-APB foci gradually increased with passage number, reaching 1.05% in cells at passage 9 ($p \leq 0.05$ when compared to control; Fig. 18B). Later passages of HPV-16 E7-expressing cells showed a fluctuation in the percentage of cells positive for FANCD2-containing APBs ranging between 0.2% and 1.7% (passage 18). Importantly, FANCD2-positive APBs were not detected in control cells from passage 2 to passage 9 when cells ceased to proliferate. These results underscore that the HPV-16 E7 oncoprotein is capable of stimulating FANCD2-positive APBs in primary human keratinocytes and furthermore, suggest that cells harboring FANCD2-positive APBs may exhibit a growth advantage.

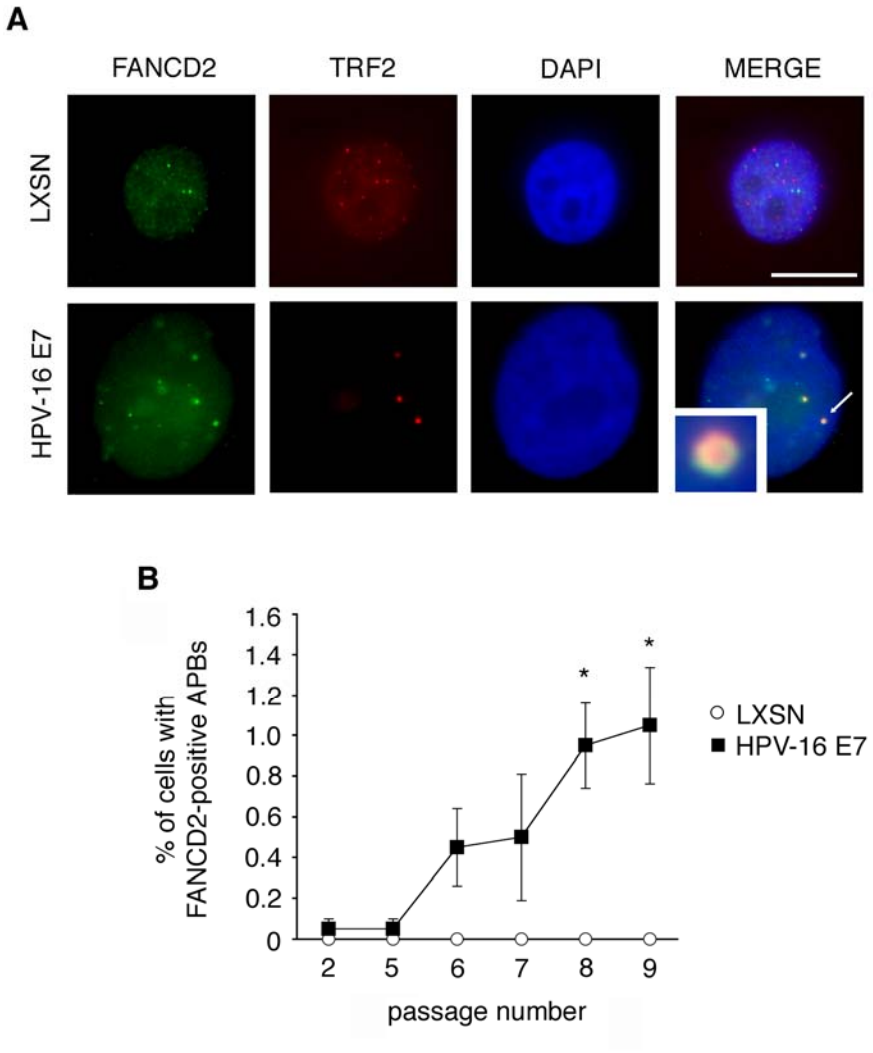

Figure 18. HPV-16 E7 stimulates FANCD2-APBs in early passage primary human foreskin keratinocytes.

(A) Co-immunofluorescence microscopic analysis of FANCD2 and TRF2 in primary human foreskin keratinocytes stably transduced with an LXSN-based HPV-16 E7 construct or empty vector control (LXSN). Note the appearance of FANCD2-APB foci in HPV-16 E7-expressing primary cells. Arrow points to the FANCD2-APB depicted at higher magnification in insert. Nuclei were stained with DAPI. Scale bar indicates 10 μm. (B) Quantification of the proportion of primary human keratinocytes stably expressing HPV-16 E7 (closed square) or control (open circle) that contains FANCD2-APBs. Each point corresponds to the mean and standard deviation of at least triplicate quantification of 500 cells at the passage number indicated. Asterisks indicate statistically significant differences in comparison to controls.

3.4 DISCUSSION

The HPV-16 E7 oncoprotein has previously been found to extend the lifespan of primary human keratinocytes and promote telomere lengthening through a mechanism independent of telomerase (169, 170). The precise molecular process through which this occurs remains unclear; however, late passages of HPV-16 E7-expressing cells share key characteristics with cells that maintain their telomeres through the ALT pathway (169, 170).

Here, we show that the HPV-16 E7 oncoprotein increases the formation of APBs that contain FANCD2, a critical protein within the FA pathway, which is activated and recruited to the chromatin at stalled replication forks (132). We also identify BRCA2/FANCD1 and MUS81 as novel components of FANCD2-containing APBs. This is the first report that these DNA damage proteins are localized to APBs; however, work from multiple labs has since recapitulated our findings that FANCD2 and MUS81 are localized to APBs (192, 193). These results lend support to the notion that aberrant replication structures, such as stalled replication forks, are linked with APB formation in HPV-16 E7-expressing cells. In line with these results, we show that treatment of U-2 OS cells with replication stress-inducing agents increases the frequency of cells that harbor FANCD2-positive APB foci. Furthermore, HPV-16 E7-induced FANCD2-containing APBs were found to form in an ATR-dependent manner. A previous study failed to detect a change in APB frequency following HU treatment, which may be explained by cell-type specific differences between the U-2 OS cells used in our study and the primary cells used by Fasching and co-workers (194).

Remarkably, we found that depletion of FANCD2 in ALT-positive U-2 OS cells rapidly leads to telomere dysfunction. The FANCD2 protein has been shown to localize to meiotic

telomeres (195) and has previously been implicated in protecting telomere integrity, as evidenced by the fact that patient-derived FA-deficient cells display telomere defects, including telomere breakage and increased extrachromosomal telomeric DNA. Despite the normal telomere length observed in FANCG-deficient mice (196), FANCD2-deficient mice have been reported to form epithelial cancers which are typically associated with telomere erosion (168). Whether the telomere dysfunction observed in FANCD2-depleted ALT cells reflects a general role for FANCD2 in maintaining telomere homeostasis in all cells however, requires further experiments.

ALT is believed to result from deregulated HR at telomeres (197) and the FA pathway has been implicated in HR-mediated replication fork restart. Hence, it is possible that deregulated FANCD2-associated activity, potentially involving aspects of HR, at telomeres is a source for extrachromosomal telomeric circles or telomeric sister chromatid exchanges, both hallmarks of ALT.

Our mutational analysis suggests that HPV-16 E7-associated inactivation of pRB, pRB family members and the $p21^{Cip1}$ CDK inhibitor are involved in promoting FANCD2-positive APB formation. These functions are essential for HPV-16 E7-induced deregulation of cyclin/CDK activity and accelerated entry into S phase (39, 41). Work presented in Chapter 2 suggests that HPV-16 E7-induced unscheduled entry into S phase is likely to lead to replication stress, as evidenced by FA pathway activation (Chapter 2, (184). Therefore, it is plausible that aberrant host cell DNA replication dynamics, which stem from HPV-16 E7-associated cell cycle disruption, are likely to affect processive replication through telomeric DNA, as the TTAGGG repeats have been shown to be particularly difficult to replication and prone to replication fork stalling (179-181). Since stalled replication forks are excellent substrates for HR, our results

may help to provide an explanation for how replication stress at telomeres may provide a stimulus for ALT activity. This idea is supported by the finding that cells deficient for pRB and the related pocket proteins display key characteristics of ALT-positive cells (198). Furthermore, the majority of ALT cell lines have been immortalized using SV40, which expresses the large T Ag, which shares key functions with HPV-16 E7, including its ability to inactivate pRB (173). An intriguing possibility, based on previous studies and work presented here, is that disruption of the pRB/E2F-axis by viral oncoproteins such as SV40 large T Ag or HPV-16 E7 promotes ALT by stimulating replication stress at telomeric DNA. However, whether HPV-16 E7-associated replication stress is sufficient to promote ALT or if HPV-16 E7 directly targets regulators of ALT/HR awaits further clarification.

A recent study has identified that DNA methyltransferase (DNMTs) play a role in maintaining telomere length and that DNMT-deficient cells display deregulated telomere recombination and APB formation (199). HPV-16 E7 has been found to interact with DNMT1 (47). However, this interaction leads to an upregulation of DNMT1's DNA methyltransferase activity and therefore, is unlikely to contribute to the increased APB frequency observed in HPV-16 E7-expressing U-2 OS cells.

HPV-16 E7 and other high-risk and low-risk HPV proteins (E1, E2, E6, E1^E4, and L2) have been shown to co-localize and/or interact with PML or PML-containing oncogenic domains (PODs) (200). This study by Nakahara and Lambert (200) revealed that viral E2-dependent transcription and viral replication occurs independently of the PML protein and that the increase of PML-positive foci detected in HPV-positive cells was linked with the presence of extrachromosomally replicating viral DNA rather than E6 and/or E7 expression. Therefore, we

believe the results presented here do not disagree with these findings, as PML has been reported to localize to many different subsets of nuclear foci.

While we have not directly tested whether a physical interaction between HPV-16 E7 and PML affects APB frequency, several lines of evidence suggest that this mechanism is unlikely to play a role. In previous studies, both high- and low- risk HPV E7 oncoproteins were found to interact with PML and equally abrogate PML IV-induced senescence (201). In contrast, our results show a clear difference between the ability of high-risk and low-risk HPV E7 oncoproteins to stimulate FANCD2-positive APB foci. Furthermore, wild type HPV-16 E7 and mutant HPV-16 E7 C24G have been found to display similar affinities to PML (201), and yet differed in the ability to promote FANCD2-positive APB formation (our results, data not shown). These inconsistencies suggest that direct interactions between HPV-16 E7 and PML may not play a major role in promoting APB formation. In line with this, previous reports show that ectopically expressed HPV-16 E7 does not co-localize with PML in the ALT-positive U2-OS cell line (202).

Despite the fact that most advanced cervical neoplasias and HPV-positive cells lines display high levels of telomerase activity, some precancerous cervical lesions lack detectable telomerase expression and display telomere attrition (203). How a minimum telomere length necessary to evade anti-proliferative responses is maintained in these lesions, which can persist for many years, remains unknown. Our finding that HPV-16 E7 can stimulate APB formation in a small but significant proportion of early passage non-immortalized primary human foreskin keratinocytes suggests that this mechanism may contribute to telomere length maintenance in a subset of cells. Importantly, APBs were never detected in control keratinocytes which ceased to proliferate past passage 9. Upregulation of telomerase has clearly been found to be the more

efficient mechanism of telomere maintenance (204) and therefore, clonal evolution will undoubtedly lead to the predominance of cells with sufficient telomerase activity as evidenced by the high frequency of telomerase expression observed in cervical malignancies (205, 206). Nonetheless, the correlation between APB formation and extended cell life span may suggest that APB-positive cells may exhibit a growth advantage at early stages of immortalization.

In conclusion, our results point to a novel link between DNA replication stress, FANCD2 and ALT activity, as reflected by APB formation, in HPV-16 E7-expressing cells. These findings highlight the exploitation of certain aspects of the DNA damage response by HPV-16 E7 to circumvent anti-proliferative barriers in infected host cells. Our results suggest a model in which HPV-16 E7-induced replication stress leads to FA pathway activation, which if it occurs at telomeres, may be beneficial to the virus as it contributes to ALT-mediated extension of host cell lifespan and ultimately immortalization.

4.0 HPV-16 E7 ATTENUATES DNA DAMAGE CHECKPOINT CONTROL BY INCREASING THE PROTEOLYTIC TURNOVER OF CLASPIN

Work described in this section is currently under review at *Cancer Research* with authors Nicole Spardy, Anette Duensing, Katherine Covella, Elliot Cha, Elizabeth E. Hoskins, Susanne I. Wells and Stefan Duensing

K. Covella generated the human fibroblasts stably expressing mutant HPV-16 E7 Δ21-24-HA. E. Cha performed preliminary immunofluorescence analysis experiments in human fibroblasts stably expressing HPV-16 E7 or control. E.E. Hoskins and S.I. Wells provided the primary human foreskin keratinocytes stably expressing the HPV-16 E7 oncoprotein or corresponding control. A. Duensing contributed to experimental designs and data interpretation. N. Spardy and S. Duensing conceived the project, analyzed and interpreted the results and wrote the manuscript.

4.1 INTRODUCTION

The HPV-16 E7 oncoprotein has been shown to induce DNA breakage, most likely as a result of host cell DNA replication stress (Chapters 2 and 3; (184, 207, 208). Despite signs of DNA damage and checkpoint activation, HPV-16 E7-expressing cells continue to proliferate through mechanisms that are not understood in detail. While several reports suggest that HPV-16 E7 promotes proliferation in the presence of negative growth signaling by deregulating cell cycle proteins (209, 210), it has not been determined whether HPV-16 E7 can interfere with the DNA damage response in order to evade DNA damaged-induced cell cycle arrest.

Here, we show that HPV-16 E7-expressing cells enter mitosis despite DNA damage. We provide evidence that this occurs as a result of HPV-16 E7's ability to attenuate DNA damage checkpoint control by accelerating the proteolytic destruction of the host cell DNA damage response protein claspin. Claspin is a part of the ATR/CHK1-axis treatment (127) and its degradation by the

$SCF^{\beta\text{-}TrCP}$ pathway has recently been found to be required prior to mitotic entry in cells with activated DNA damage checkpoints (211-213). Further experiments revealed that claspin accumulation inhibits mitosis in HPV-16 E7-expressing cells and that disruption of the pRB/E2F-axis by HPV-16 E7 contributes to deregulated claspin levels and aberrant entry into mitosis. Collectively, our findings highlight a novel mechanism through which the HPV-16 E7 oncoprotein relaxes DNA damage checkpoint control in order to maintain cell cycle progression in a DNA damage response-activated, anti-proliferative host cell environment.

4.2 MATERIALS AND METHODS

Cell culture, treatments and transfections

Primary human foreskin keratinocytes (HFKs) were harvested from foreskins and maintained as described earlier. Human foreskin fibrobasts (BJ; ATCC) and C33A cells (ATCC) were maintained in DMEM (Biowhittaker, Lonza, Inc.) as described earlier. CaSki cells (ATCC) were maintained in RPMI media (Biowhittaker/Lonza, Inc.) supplemented with 1% L-glutamine (Invitrogen) and antibiotics as described earlier. To assess protein stability cells were treated with 30 μg/ml cycloheximide (Calbiochem) or dH_2O for the indicated times. The proteasome was inhibited by treating cells with 1 μM Z-Leu-Leu-Leu-Vinyl Sulfone ($Z\text{-}L_3VS$; BioMol International/Enzo Life Sciences, Inc., Plymouth Meeting, PA) or DMSO for 24 h. Stalled replication forks were induced by treating cells with 1 mM HU (Calbiochem) or dH_2O for 24 h.

Primary HFKs were transduced with an LXSN-based high-risk HPV-16 E7 construct or LXSN empty vector followed by selection in G418-supplemented media. Expression was confirmed through immunoblot analysis. Transient transfections of primary HFKs stably expressing HPV-16 E7 was done using nucleofection (Lonza, Inc.) with 2 μg of plasmids encoding a HA-tagged mutant claspin S30/34A (kindly provided by Michele Pagano, New York University School of Medicine, New York, NY) or empty vector control (neo-HA) in combination with 0.5 μg of dsRED as transfection marker. Tissue culture media was replaced 24 h after transfection and cells were fixed and mitotic index was assessed 48 h after transfection.

Immunological Methods

Whole cell lysates were prepared and immunoblot analysis was performed as described earlier. Quantification of band intensities was performed using NIH Image J software, http://rsbweb.nih.gov/ij/.

Immunofluorescence analysis of cells grown on coverslips was performed as described earlier, with the exception that cells were permeabilized using 1% Triton-X 100 in PBS for 15 min.

Paraffin-embedded tissue samples were retrieved from the archives of the Department of Pathology, University of Pittsburgh School of Medicine (IRB # 0505181) and HPV typed using the Rembrandt/PanPath in situ hybridization kit (Invitrogen). Immunofluorescence microscopic analysis of paraffin-embedded tissue samples was performed as described earlier, with the exception that slides were not subjected to pepsin digest.

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, Inc., Danvers, MA), Claspin (antibody kindly provided by Raimundo Freire, Unidad de Investigación, Hospital Universitario de Canarias, Tenerife, Spain), CUL-1 (Santa Cruz Biotechnology, Inc.), cyclin A (Novocastra Laboratories Ltd, Newcastle-upon-Tyne, England), phosphorylated H2AX at serine 139 (Upstate, Temecula, CA), HPV-16 E7 (Santa Cruz Biotechnology, Inc), phosphorylated PLK1 at threonine 210 (BD PharMingen), PLK1 (Santa Cruz Biotechnology, Inc.) and β-TrCP (Zymed/Invitrogen).

Statistical Analysis

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

4.3 RESULTS

HPV-16 E7-expressing cells enter mitosis despite DNA damage.

To determine the frequency with which HPV-positive cells enter mitosis with DNA damage, an immunofluorescence microscopic analysis for the DNA damage marker γ–H2AX was performed in tissue biopsies obtained from high-risk HPV-positive (types 16/18 and/or 31/33) anal neoplasms or non-malignant tissue (Fig. 19A, left panel). A total of six carcinomas in situ (CIS) samples, seven squamous cell carcinomas (SCCs) and six non-malignant controls (hemorrhoids) were analyzed for the presence of mitotic cells with γ–H2AX foci. Microscopic analysis revealed that HPV-positive biopsies displayed a statistically significant increase in the percentage of γ–H2AX-positive mitotic cells from 24.5% in non-malignant tissue to 52.6% in CIS and 62.4% in SCC tissue ($p \leq 0.01$ and $p \leq 0.0001$, respectively; Fig. 19A, right panel). Since DNA damage checkpoints are crucial to prevent mitotic entry in the presence of unrepaired DNA breaks (214), these findings suggest that high-risk HPV oncoproteins may attenuate DNA damage checkpoint control that normally is involved in arresting cells at the G2/M phase of the division cycle.

The HPV-16 E7 oncoprotein activates DNA damage checkpoints associated with DNA replication stress (Chapters 2 and 3; (184, 208) and stimulates DNA damage in a cell cycle-

dependent manner (Fig. 20). Since HPV-16 E7-expressing cell populations continue to proliferate and enter mitosis, we next asked whether HPV-16 E7 is involved in circumventing DNA damage checkpoint control. We performed an immunofluorescence microscopic analysis for γ–H2AX in primary human foreskin keratinocytes (HFKs) stably transduced with HPV-16 E7 or empty vector control (LXSN) and tested whether HPV-16 E7 expression, independent of HPV-16 E6, is sufficient to permit mitotic entry in the presence of DNA damage. We found that a significant proportion of HPV-16 E7-expressing cells found in pro-/metaphase and later stages of mitosis (ana-/telophase) harbor γ–H2AX foci (Fig. 19B, left panel). A statistically significant 1.6-fold increase in the percentage of total γ–H2AX-positive mitotic cells was observed in HPV-16 E7-expressing cells (72.3%) compared to controls (44%, Fig. 19B, right panel/top position; $p \leq 0.005$). Furthermore, we show that the presence of HPV-16 E7 leads to a significant 1.5-increase of γ–H2AX-positive pro-/metaphase (75.2%) compared to controls (49.5%, Fig. 1B, right panel/middle position; $p \leq 0.005$) and a significant 2.0-fold increase of γ–H2AX-positive ana-/telophases in HPV-16 E7-expressing cells (68%) compared to controls (33.7%, Fig. 19B, right panel/bottom position; $p \leq 0.005$). Collectively, these results underscore that HPV-16 E7 expression inflicts host cell DNA damage and furthermore, suggest that HPV-16 E7-expressing cells are able to enter mitosis in the presence of DNA damage.

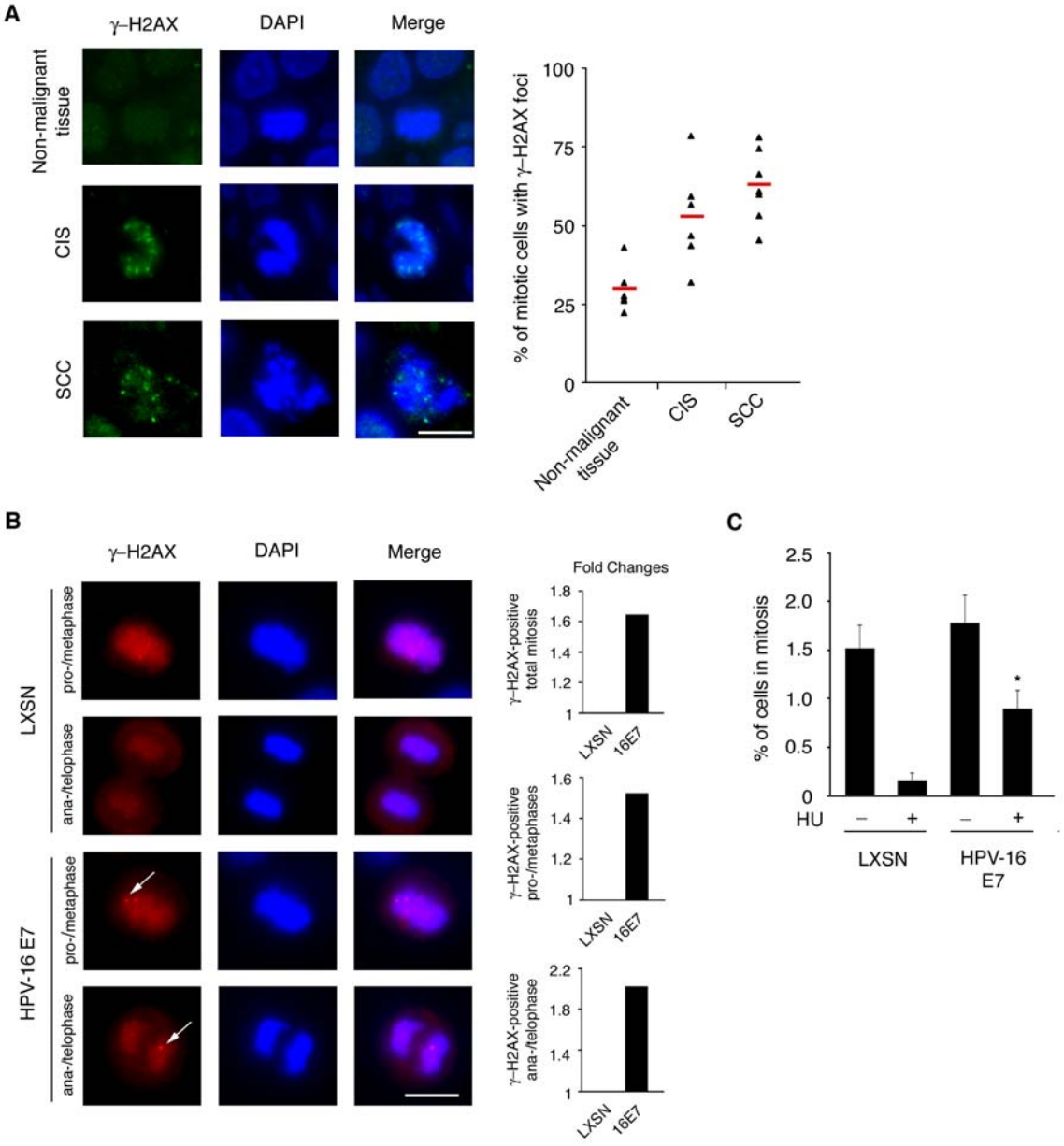

Figure 19. The HPV-16 E7 oncoprotein promotes mitotic entry despite the presence of DNA damage.

(A) Immunofluorescence microscopic analysis of γ–H2AX in high-risk HPV-positive anal carcinomas in situ (CIS), squamous cell carcinomas (SCCs) or non-malignant tissue samples (hemorrhoids). Nuclei stained with DAPI. Scale bar indicates 10 μm (left panel). Legend continued on following page.

Quantification of the proportion of mitotic cells with γ–H2AX foci in CIS, SCC or non-malignant tissue samples. Bar indicates the mean percentage of γ–H2AX-postitive mitotic cells observed in at least six cases with at least 350 mitotic cells analyzed for each category (right panel). (B) Immunofluorescence microscopic analysis of γ–H2AX in primary HFKs stably expressing HPV-16 E7 or control (LXSN). Nuclei stained with DAPI. Note the appearance of γ–H2AX foci (arrows) in HPV-16 E7-expressing cells at various stages of mitosis. Scale bar indicates 10 μm (left panel). Bars indicate the fold changes of the percentage of total mitotic cells (top), pro-/metaphase cells (middle) or ana-/telophase cells (bottom) that contain γ–H2AX foci. Five counts of at least 50 cells were analyzed from a representative experiment (right panel). (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 mM HU or dH_2O for 24 h. Bars indicate mean and standard error of two independent experiments with triplicate quantification of a minimum of 50 cells. Asterisk indicates statistically significant differences in comparison to controls.

HPV-16 E7 attenuates DNA damage checkpoint control and promotes mitotic entry in the presence of replication stress.

We next sought to rule out that the increase of mitotic cells with γ–H2AX foci in HPV-16 E7 cell populations is not simply as a result of an overall increase of DNA damage. In order to directly test whether HPV-16 E7 relaxes DNA damage checkpoints required to prevent aberrant mitotic entry, HPV-16 E7-expressing or control primary HFKs were treated for 24 hours with hydroxyurea (HU), 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. HU treatment was chosen as it mimics certain aspects of the DNA damage response observed in HPV-16 E7-expressing cells, such as activation of the FA pathway. No significant difference in the mitotic index of HPV-16 E7-expressing cells (1.8%) compared to control cells (1.5%, $p>0.05$) was observed following control treatment (Fig. 19C). In contrast, a statistically significant 4.5-fold increase of HPV-16 E7-expressing cells were found in mitosis

(0.9%) following HU treatment compared to control cells (0.2%, $p \leq 0.001$; Fig. 19C). These results underscore that a proportion of HPV-16 E7-expressing cells enter mitosis despite exogenous replication stress, which suggests that the HPV-16 E7 oncoprotein can attenuate DNA damage checkpoint responses that would normally arrest cells following DNA replication stress.

HPV-positive cells lines show altered claspin protein stability.

Claspin facilitates ATR-mediated phosphorylation of the CHK1 kinase in response to DNA replication stress (126, 127). Its degradation however, has recently been implicated in the recovery from DNA damage checkpoint arrest (211-213). Therefore, we next monitored whether claspin expression is altered in the presence of high-risk HPV-16 oncoproteins. Claspin protein stability was assessed by treating HPV-16-positive cancer cells (Caski) or HPV-negative cancer cells (C33A) with cycloheximide (CHX) for up to 8 hours, followed by immunoblot analysis for claspin protein levels (Fig. 21A, upper panel). Densitometric analyses revealed a 6.2-fold drop in claspin protein found in Caski cells following an 8 hour CHX block, as compared to a 2.1-fold decrease in C33A cells (Fig. 21A, lower panel). These results suggest that claspin protein stability is negatively affected in cells expressing HPV-16 oncoproteins.

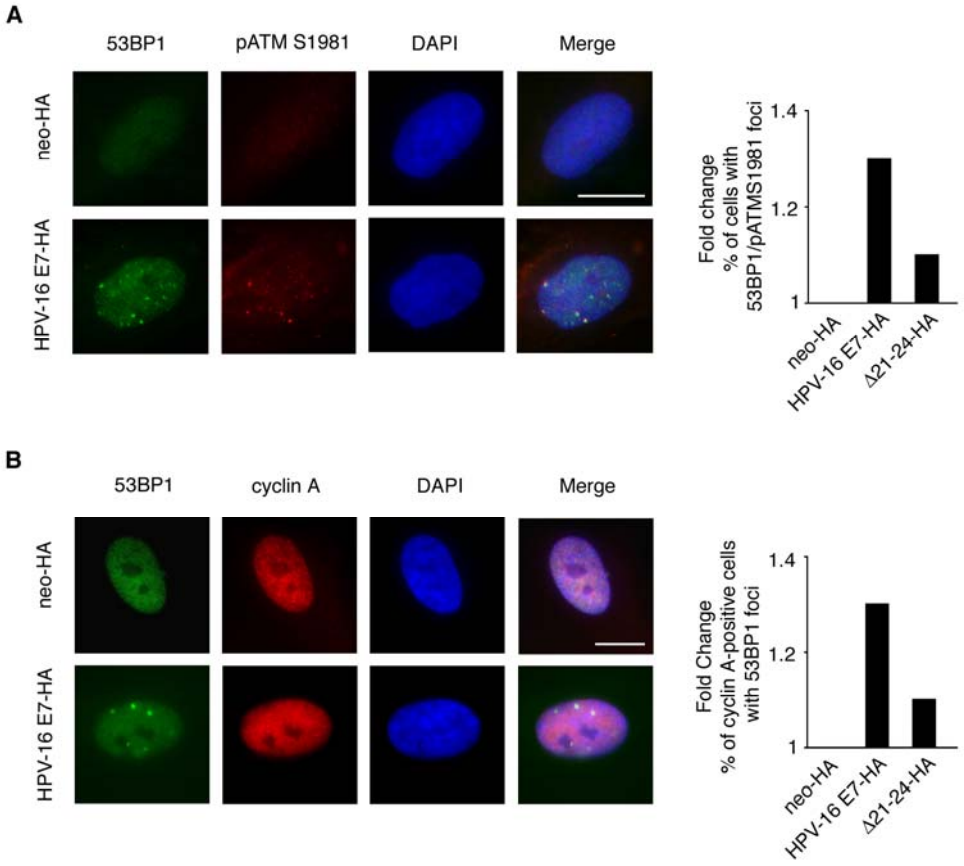

Figure 20. The HPV-16 E7 stimulates DNA breaks in a cell cycle dependent manner.

(A) Co-immunofluorescence microscopic analysis of 53BP1 and pATM in BJ fibroblasts stably expressing HPV-16 E7-HA or control (neo-HA). Nuclei stained with DAPI. Scale bar indicates 10 μm (left panel). Quantification of the percentage of BJ cells stably expressing control (neo-HA), HPV-16 E7-HA or mutant HPV-16 E7 Δ21-24-HA that contain 53BP1/pATM S1981 foci. Bars indicate the mean value of fold changes generated from two independent experiments with triplicate counts of a minimum of 50 cells (right panel). (B) Co-immunofluorescence microscopic analysis of 53BP1 and cyclin A in BJ fibroblasts stably expressing HPV-16 E7-HA or control (neo-HA). Nuclei stained with DAPI. Scale bar indicates 10 μm (left panel). Quantification of the percentage of BJ cells stably expressing control (neo-HA), HPV-16 E7-HA or mutant HPV-16 E7 Δ21-24-HA that contain cyclin A–positive cells with 53BP1 foci. Bars indicate the mean value of fold changes generated from two independent experiments with triplicate counts of a minimum of 50 cells (right panel).

The HPV-16 E7 oncoprotein accelerates the proteolytic turnover of claspin.

To test whether HPV-16 E7 plays a role in regulating claspin protein stability, we treated primary HFKs stably expressing HPV-16 E7 or control cells with CHX and monitored claspin protein levels (Fig. 21B, upper panel). Immunoblot analysis revealed an increase in the base-line level of claspin in HPV-16 E7-expressing cells in comparison to control cells. Importantly, however, densitometric analyses revealed a 3.3-fold decrease in claspin protein levels in HPV-16 E7-expressing cells following CHX treatment for 2 hours as compared to the 2-fold decrease in control cells (Fig. 21B, lower panel). These results support the notion 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.

To further corroborate the role of HPV-16 E7 in claspin proteolysis, we next tested whether inhibition of the proteasome lead to claspin protein stabilization. Treatment of primary HFKs stably expressing HPV-16 E7 or control cells with the proteasome inhibitor Z-L_3VS for 24 hours led to a greater fold increase in claspin protein levels in HPV-16 E7-expressing cells (2.8-fold) compared to control cells (1.8-fold; Fig. 21C). These results underscore that the ubiquitin-proteasome pathway plays an important role in HPV-16 E7-associated claspin protein destabilization.

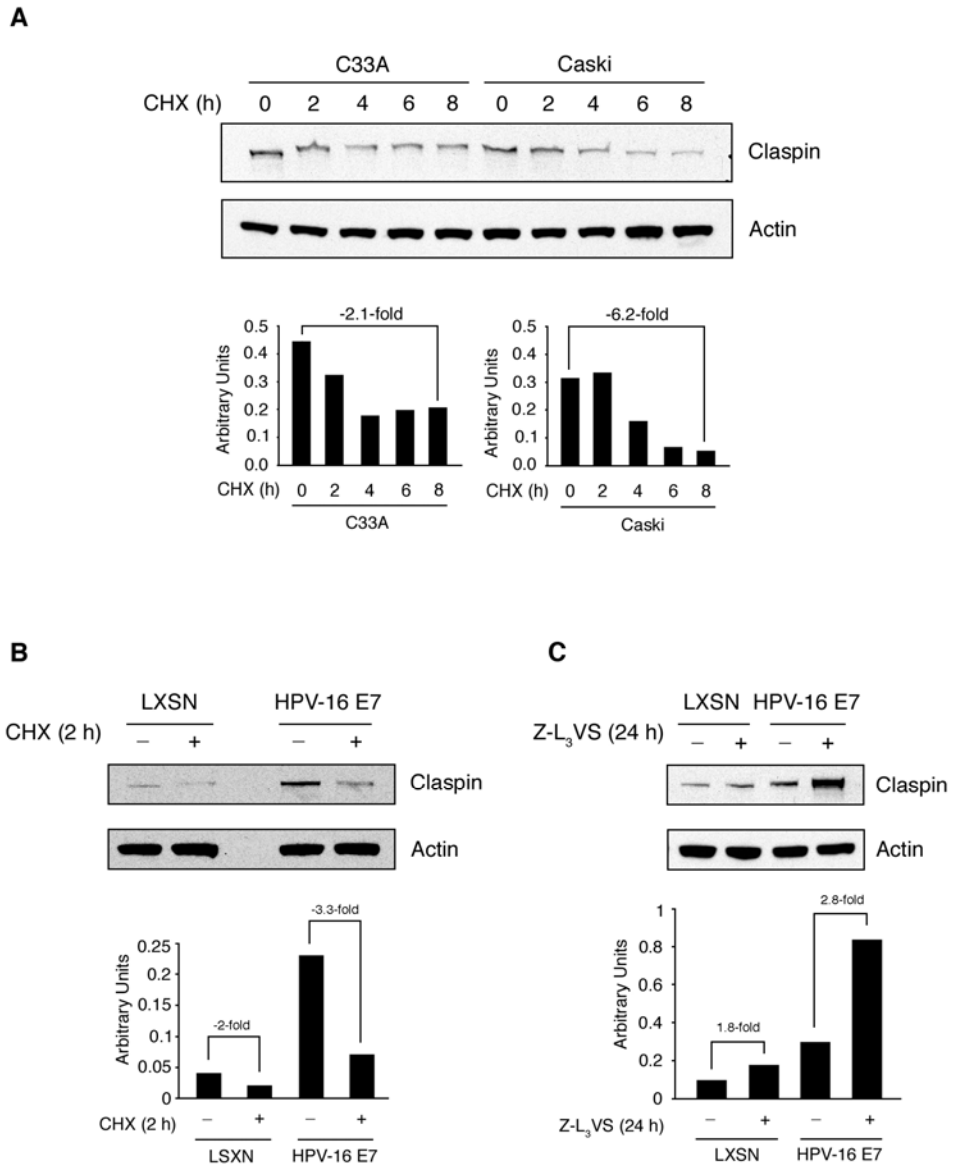

Figure 21. The proteolytic turnover of claspin is accelerated in HPV-positive cells and involves the HPV-16 E7 oncoprotein.

(A) Immunoblot analysis of claspin in HPV-negative (C33A) and HPV-positive (Caski) cells treated with cycloheximide (CHX) for the indicated times. Immunoblot for actin was used to demonstrate total protein loading. Densitometric analyses was used to quantify claspin levels and the fold changes were plotted using arbitrary units.

Legend continued on following page.

(B) Immunoblot analysis of claspin in primary HFKs stably expressing HPV-16 E7 or control (LXSN) following CHX treatment. Immunoblot for actin was used to demonstrate total protein loading. Densitometric analyses was used to quantify claspin levels and the fold changes were plotted using arbitrary units. (C) Immunoblot analysis of claspin in primary HFKs stably expressing HPV-16 E7 or control (LXSN) treated with Z-L_3VS or dH_2O for 24 h. Immunoblot for actin was used to demonstrate total protein loading. Densitometric analyses was used to quantify claspin levels and the fold changes were plotted using arbitrary units.

Claspin degradation is required for mitotic entry in HPV-16 E7-expressing cells.

Mitotic entry following DNA damage checkpoint responses requires the destruction of claspin, as it relieves further ATR-mediated CHK1-associated cell cycle arrest. To directly test whether claspin degradation by the G2/M-specific claspin proteolysis machinery facilitates entry into mitosis in HPV-16 E7-expressing cells, we transiently transfected primary HFKs stably expressing HPV-16 E7 or LXSN with a non-degradable mutant of claspin, in which two conserved serine residues (30 and 34) within the phospho-degron motif of claspin have been mutated to alanines (S30/34A; kindly provided by M. Pagano, New York University School of Medicine, New York, NY) or empty vector control and monitored the mitotic index 48 hours following transfection. Transfection of control cells (LXSN) with the phospho-degron mutant claspin S30/34A did not change the mitotic index (1.35%) as compared to cells transfected with empty vector control (1.32%, $p>0.05$). In contrast, transfection of HPV-16 E7-expressing primary cells with the phospho-degron mutant claspin S30/34A lead to a significant 1.8-fold decrease in the percentage of cells in mitosis (2.2%) compared to cells transfected with empty vector control (1.2%, $p\leq0.001$; Fig. 22). These findings underscore that acceleration of claspin degradation by HPV-16 E7 promotes entry into mitosis and suggests that accumulation of claspin in HPV-16 E7-expressing cells reinforces G2/M checkpoint control.

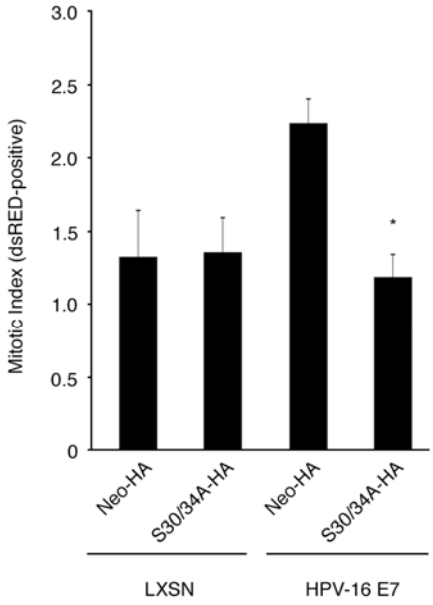

Figure 22. A non-degradable mutant of claspin inhibits mitotic entry in HPV-16 E7-expressing cells.

The mitotic index was assessed in HPV-16 E7-expressing or control (LXSN) primary human keratinocytes 48 h after transient transfection with either a HA-tagged phospho-degron mutant of claspin (S30/34A-HA) or empty vector control (Neo-HA) using dsRED as a transfection marker. Mean values and standard error was generated from a representative experiment for LXSN cells and three independent experiments for HPV-16 E7-expressing cells, with at least 100 cells counted in triplicate per experiment. Asterisk indicates statistically significant differences in comparison to controls.

HPV-16 E7-induced claspin degradation involves deregulation of multiple components of the $SCF^{\beta\text{-}TrCP}$-based claspin degradation machinery.

DNA damage checkpoint recovery is facilitated, in part, by the mitotic kinase Polo-like kinase 1 (PLK1), which promotes $SCF^{\beta\text{-}TrCP}$-mediated claspin proteolysis in the G2/M phase of the cell cycle (211-213). Therefore, we next sought to test whether HPV-16 E7 affects claspin

protein stability by deregulating components of the $SCF^{\beta\text{-}TrCP}$-based claspin degradation machinery. Immunoblot analysis of primary HFKs expressing HPV-16 E7 or control revealed that multiple proteins implicated in $SCF^{\beta\text{-}TrCP}$-mediated claspin degradation were upregulated in the presence of the HPV-16 E7 oncoprotein, including cullin 1 (CUL1), β-TrCP, Aurora A and PLK1 (Fig. 23A). However, since claspin protein stability is also regulated by the deubiquitinating enzymes (DUBs), USP7 and USP28 (215-217), we were also interested in assessing their expression level in HPV-16 E7-expressing and control primary HFKs. No significant difference in the USP7 expression level, which primarily counteracts $SCF^{\beta\text{-}TrCP}$-based claspin ubiquitination in G2/M (215), was detected in HPV-16 E7-expressing cells compared to control cells (Fig. 23B). A slight increase of USP28 protein levels, which opposes APC/C^{Cdh1}-based claspin degradation in G1 (215, 216), was observed in HPV-16 E7-expressing cells compared to controls. Collectively, these findings suggest that HPV-16 E7-associated claspin proteolysis may involve the deregulation of the $SCF^{\beta\text{-}TrCP}$-based claspin degradation machinery, but not the deregulation of the DUB that thwarts $SCF^{\beta\text{-}TrCP}$-mediated claspin degradation.

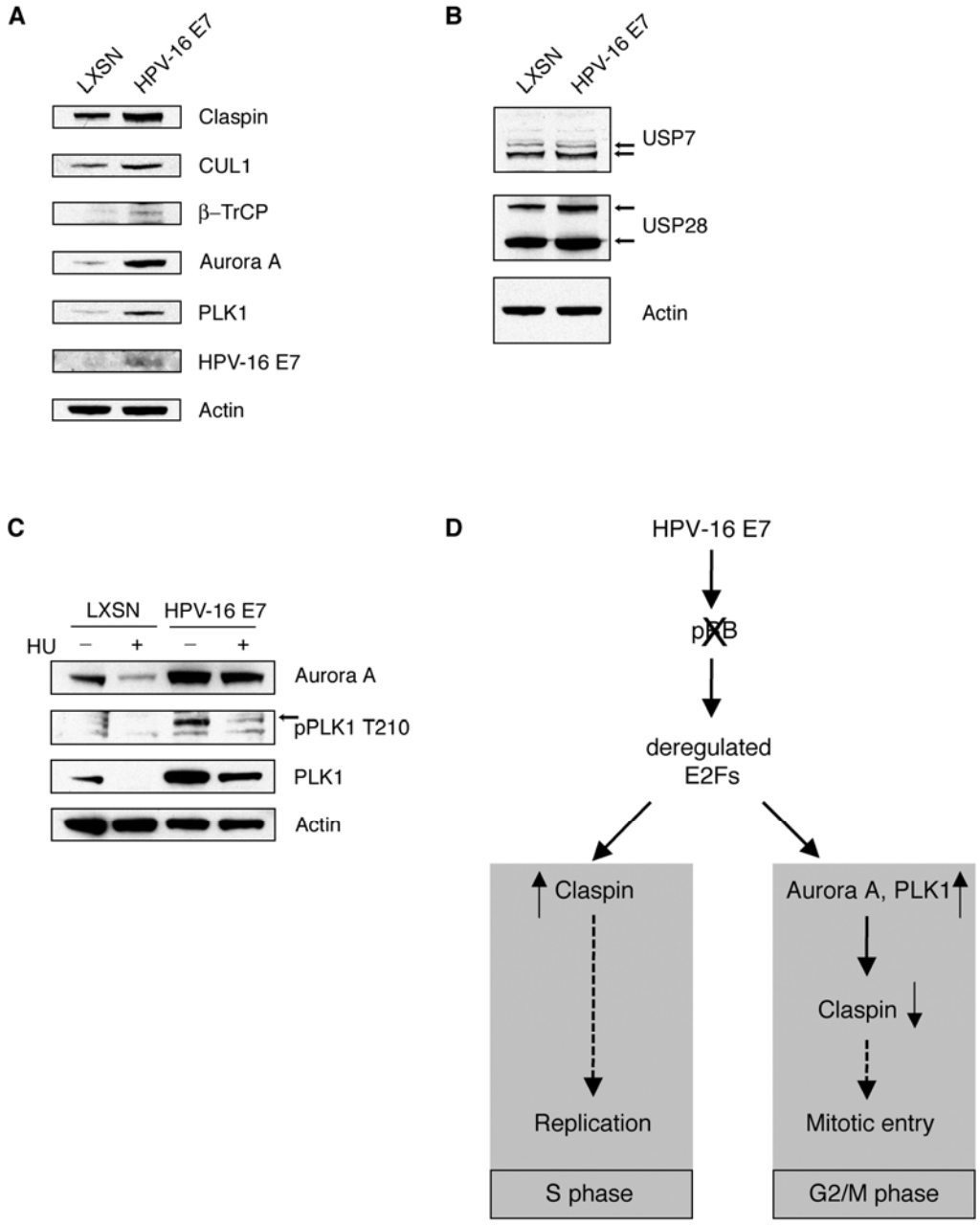

Figure 23. The HPV-16 E7 oncoprotein deregulates multiple components of the claspin degradation machinery. (A) Immunoblot analysis of various claspin degradation machinery in primary HFKs stably expressing HPV-16 E7 or control (LXSN). Immunblot for HPV-16 E7 was used to verify correct expression. Immunblot for actin was used to demonstrate total protein loading. Legend continued on following page.

(B) Immunoblot analysis of claspin deubiquitinating enzymes in primary HFKs stably expressing HPV-16 E7 or control (LXSN). Immunblot for actin was used to demonstrate total protein loading. (C) Immunoblot analysis of claspin degradation machinery in primary HFKs stably expressing HPV-16 E7 or control (LXSN) following treatment with HU or dH_2O for 24 hours. Immunblot for actin was used to demonstrate total protein loading. Arrow points to band corresponding to pPLK1 T210. (D) Proposed model of HPV-16 E7-induced deregulation of claspin protein stability. HPV-16 E7-associated pRB/E2F disruption leads to increased levels of claspin in S-phase in order to promote DNA replication. However, deregulated E2F gene transcription in G2/M may stimulate an increase of PLK1 and Aurora A expression levels, which accelerates $SCF^{\beta\text{-}TrCP}$-mediated claspin proteolysis, thereby facilitating aberrant entry into mitosis in the presence of DNA damage.

Activation of PLK1 prior to DNA damage checkpoint recovery is regulated by the Aurora A kinase (218). Therefore, we next analyzed Aurora A and PLK1 expression as well as PLK1 activation in primary HFKs stably expressing HPV-16 E7 or control cells exposed to exogenous DNA replication stress. We found that HPV-16 E7-expressing cells maintain high levels of Aurora A in response to HU-induced replication stress compared to control cells (Fig. 23C). In addition, HPV-16 E7-expressing cells maintained detectable levels of total PLK1 protein as well as Aurora A-mediated phosphorylation of PLK1 at threonine 210 (T210). Phosphorylation at this residue is a prerequisite for PLK1 to promote mitotic entry after DNA damage checkpoint arrest (218). Together, these results show that HPV-16 E7 disrupts multiple layers of control involved in DNA damage checkpoint recovery.

4.4 DISCUSSION

Here, we show that HPV-16 E7-expressing cells enter mitosis despite DNA damage. We provide evidence that HPV-16 E7 circumvents DNA damage checkpoints required to prevent aberrant mitotic entry by accelerating the proteolysis of claspin, a critical mediator of the ATR/CHK1 signaling axis. Previous reports have linked $SCF^{\beta\text{-}TrCP}$-based claspin degradation in G2/M with recovery from DNA damage checkpoint activation (211-213). We found that HPV-16 E7 deregulates the expression and activity of several key proteins involved in this process, thus creating a cellular environment that permits mitotic entry despite the presence of anti-proliferative stimuli.

Although our results clearly point to a role for HPV-16 E7 in attenuation of DNA damage checkpoint control (Fig. 19C), it is noteworthy that we discovered γ–H2AX-positive mitotic cells in non-malignant tissue samples and control keratinocyte populations. It is possible that genotoxic stress associated with unfavorable growth conditions or oxidative damage contributes to the appearance of γ–H2AX foci in these cells. Recent studies suggest that cells with un-repairable DNA damage escape prolonged cell cycle arrest and enter mitosis through a process known as checkpoint adaptation before being eliminated by mitotic catastrophe or apoptosis (219-223).

We show that HPV-16 E7 accelerates claspin proteolysis, which may provide an explanation for how HPV-16 E7-expressing cells are able to overcome replication-stress associated DNA damage checkpoint responses and aberrantly enter mitosis. While we assume that HPV-16 E7 accelerates claspin degradation at the G2/M-transition of the cell division cycle, the inability to efficiently synchronize HPV-16 E7-expressing keratinocyte populations

prevented us from directly testing this hypothesis. We do, however, show that the HPV-16 E7-associated decrease in claspin protein stability is not simply due to a higher percentage of cells in mitosis, as no significant difference was detected in the mitotic indexes of HPV-16 E7-expressing and control cell populations.

Since claspin is required for ATR-mediated activation of CHK1, 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/CHK1-signaling axis in HPV-16 E7-expressing cells, but that it is more efficiently degraded as cells nears the G2/M transition in order to alleviate DNA damage checkpoint responses and promote mitotic entry. This line of thinking is supported by our finding that a non-degradable mutant of claspin inhibits mitotic entry in HPV-16 E7-expressing cells, thus underscoring that claspin accumulation reinforces G2/M checkpoint control.

Several components of the $SCF^{\beta\text{-}TrCP}$-based claspin degradation machinery were found upregulated in HPV-16 E7-expressing cells, including CUL1, β-TrCP, PLK1 and Aurora A. Recently, however, claspin protein stability has also been shown to be regulated by the deubiquitinating enzymes (DUBs) USP7 and USP28, which counteract $SCF^{\beta\text{-}TrCP}$- and APC/C^{Cdh1}-mediated claspin degradation, respectively, in order to re-establish claspin-associated DNA damage checkpoint activation (215-217). Despite the slight upregulation of USP28 levels, no such increase in USP7 protein expression was detected in HPV-16 E7 keratinocyte populations compared to controls.

Prior to DNA damage checkpoint recovery, claspin must be targeted for degradation by PLK1-mediated phosphorylation which creates a recognition motif for the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase (211-213). Recent studies have implicated Aurora A-mediated phosphorylation of PLK1 at

T210 as a pre-requisitive for PLK1 activation and DNA damage checkpoint recovery (218). Here, we show that HPV-16 E7-expressing cells maintain elevated levels of Aurora A and PLK1 in the presence of HU-induced replication stress when compared to control cells. Furthermore, we found that phosphorylation of PLK1 at T210 also remains detectable in HPV-16 E7-expressing cells treated with HU. Our finding that HU-treatment in control keratinocytes lead to significantly less Aurora A and PLK1 protein levels could be explained by the fact that these cells are more efficiently arrested by HU treatment in early S phase, when PLK1 and Aurora A levels are kept low (224-226). In contrast, our result that HPV-16 E7-expressing cells display higher levels of Aurora A and PLK1 despite HU treatment may suggest that HPV-16 E7-expressing cells do not arrest properly following HU and/or that HPV-16 E7 disrupts the regulation of these two mitotic kinases.

Collectively, these findings indicate that the HPV-16 E7 oncoprotein accelerates claspin proteolysis by disrupting the balance between positive and negative regulators involved in $SCF^{\beta\text{-}TrCP}$-mediated claspin protein stability during DNA damage checkpoint recovery in the G2/M phase of the cell division cycle. At this time however, we are unable to rule out the possibility that HPV-16 E7 may accelerate claspin degradation in a more direct manner.

Interestingly, claspin, PLK1 and Aurora A genes have each been shown to have E2F-responsive promoter elements (227-229). Therefore, it is likely that HPV-16 E7-associated disruption of the pRB/E2F-axis contributes to claspin expression levels in both a positive and negative manner. Deregulation of E2F-mediated gene transcription by HPV-16 E7 is likely to stimulate higher levels of claspin in S phase in order to promote more efficient host cell DNA replication. This is in line with our result that HPV-16 E7-expressing keratinocytes display increased baseline levels of claspin when compared to control cells. However, enhanced E2F-

mediated transcription would also deregulate expression and/or activity of components of the $SCF^{\beta\text{-}TrCP}$ degradation machinery in cells nearing the G2/M transition, thereby leading to accelerated and/or premature claspin degradation and hence, attenuated DNA damage checkpoint control and aberrant entry into mitosis. Our finding that inhibition of claspin degradation prevents mitotic entry in HPV-16 E7-expressing cells suggests that Aurora A or PLK1 inhibitors may be potentially therapeutic in treating high-risk HPV-associated malignancies and in cancers that display deregulated claspin degradation.

5.0 GENERAL DISCUSSION

The high-risk HPV-16 E7 oncoprotein has been shown to stimulate host cell DNA breakage and structural chromosomal instability through mechanisms that are not understood in detail (95, 99, 100). Therefore, the goal of this report was to answer several key questions: (1) How does HPV-16 E7 induce DNA damage? (2) What are the precise consequences of HPV-16 E7-associated DNA damage for host cell genomic integrity? and (3) How do HPV-16 E7-expressing cells continue to proliferate in the presence of DNA damage and activated DNA damage checkpoints? This chapter will discuss our findings and highlight how the disruption of host cell cycle regulation by HPV-16 E7 triggers DNA damage checkpoint activation and DNA DSBs. Furthermore, we will discuss how the interplay between HPV-16 E7 and the host cell DNA damage response contributes to host cell genome instability, malignant progression and the viral life cycle.

5.1 HOW DOES THE HPV-16 E7 ONCOPROTEIN INDUCE HOST CELL DNA DAMAGE?

HPV-16 E7-associated replication stress contributes to DNA breakage

Stalled replication forks are a frequent source of DNA DSBs and several lines of evidence suggest that replication stress contributes to DNA breakage in HPV-16 E7-expressing cells. First, we show that HPV-16 E7 expression activates the FA pathway, as evidenced by increased FANCD2 foci and recruitment of FANCD2 to chromatin (Chapter 2; (184). Activation of the FA pathway was used as a surrogate marker of replication stress, since techniques currently used to monitor the progression of replication forks are difficult and error-prone.

Second, we demonstrate that FANCA gene deficiency enhances high-risk HPV oncoprotein-induced DNA breakage (Chapter 2, (207). Further experiments revealed that the HPV-16 E7 oncoprotein is responsible for causing an accelerated accumulation of structural chromosomal instability in FA-deficient host cells (Chapter 2; (184). Third, we show that HPV-16 E7 expression in non-immortalized human foreskin fibroblasts stimulates an increase of 53BP1 foci, a marker of DNA breaks, in cells that at the same time express S phase markers (cyclin A) (Chapter 4). Finally, we report that HPV-16 E7 activates the FA pathway and induces DNA DSBs in a cell cycle dependent manner. This was evidenced by the fact that expression of the HPV-16 E7 Δ21-24 mutant, which is deficient in pRB binding/degradation and accelerating S phase entry, was unable to stimulate an increase of cells with FANCD2 foci (Chapter 2; (184) or cyclin A-positive cells that harbor 53BP1 foci (Chapter 4). These results underscore that HPV-16 E7 is likely to drive DNA DSB formation by triggering host cell replication stress.

It is important to note however, that attenuation of G1/S checkpoint control by HPV-16 E7 may also create an environment that permits cells to progress from G1 to S phase in the presence of DNA DSBs (ie. prior to DNA repair) or other lesions that subsequently lead to DNA DSBs in S phase. Therefore, further experiments, which combine the labeling of replicated DNA with DSB identification, are required to directly determine the proportion of DNA DSBs that are generated during replication in HPV-16 E7-expressing cell populations.

How does the HPV-16 E7 oncoprotein stimulate replication stress?

Replication stress can be caused by a variety of stimuli including physical barriers that impede replication fork progression (88). One example of a physical replication block are interstrand DNA crosslinks (ICLs). ICLs are caused by the chemotherapeutic agent Mitomycin C

(MMC) and are considered the classical activator of the FA pathway (134). Currently, there is little evidence to suggest that HPV-16 E7 can directly interact with DNA in order to stimulate ICL formation or other types of DNA adducts. However, certain metabolic processes have been shown to produce reactive oxygen species (ROS), which can chemically alter DNA base structure as well as block replication fork progression (230). Since HPV-16 E7 can disrupt host cell homeostasis on multiple levels (45), we cannot rule out the possibility that increased ROS production may lead to chemically altered DNA structures or DNA breaks, which ultimately cause replication forks to stall.

DSBs and altered DNA structures are also commonly formed as DNA repair intermediates. Many viral proteins, including HPV-16 E6, have been shown to interact with host cell DNA repair proteins (231-233). Whether HPV-16 E7 can similarly deregulate certain types of DNA repair is not known. However, manipulation of DNA repair pathways, resulting in too much or too little repair activity, could cause DNA DSBs to accumulate, which would ultimately lead to activation of a replication stress response.

Currently, the most plausible explanation is that HPV-16 E7-induced replication stress arises from defects in replication initiation (74, 75). Normally, cells ensure that replication occurs once and only once during the cell cycle by strictly regulating two key events 1) the licensing of replication origins by pre-replication complex (pre-RC) assembly in G1 and 2) the firing of replication forks during S phase (234). Since HPV-16 E7 is well known for its ability to profoundly disrupt host cell cycle control, it likely that replication stress may occur in HPV-16 E7-expressing cells as a result of deregulated origin activity. Extensive research has revealed that pre-RC components (ORC, CDC6, CDT1 and MCM2-7) are recruited to origins in G1 when cyclin levels are kept low. Additional pre-RC assembly (and re-replication) is then prevented, as

cyclin levels rise at the G1/S transition and stay elevated throughout the remainder of the cell cycle (234). HPV-16 E7-expressing cells however, are characterized by deregulated expression of cyclin E and A (43), which may cause the window of time within the cell cycle that is permissive for pre-RC assembly to shorten, thus leading to a reduction in the number of properly licensed origins. Since multiple origins are typically fired in close proximity of one another to ensure that stalled replication forks can be "rescued" by surrounding forks (234); decreased pre-RC assembly (and subsequent fork firing) could increase the chance of replication fork collapse and DNA DSB formation following MUS81-mediated cleavage (235). In support of this notion, deregulated expression of E2F and cyclin E has previously been shown to inhibit the recruitment of certain pre-RC components to replication origins (74, 75). Furthermore, we show here that sequences of HPV-16 E7 that are involved in disrupting the pRB/E2F-axis are required to stimulate replication stress, as evidenced by FA pathway activation (Chapter 2; (184). Further experiments however, are required to analyze whether pre-RC recruitment is altered in HPV-16 E7-expressing cells.

It is also possible that HPV-16 E7 may manipulate origin activity by directly binding pre-RC components. In line with this, the c-myc proto-oncogene, which is functionally related to HPV-16 E7, has been shown to directly bind pre-RC components and manipulate the activity of replication origins (236). We feel that this scenario is unlikely based on our inability to detect a physical interaction between HPV-16 E7 and various pre-RC components (data not shown); however, at this time we cannot rule out the possibility that HPV-16 E7 may cause replication stress by interfering more directly with host cell replication enzymes.

5.2 WHAT ARE THE PRECISE CONSEQUENCES OF HPV-16 E7-INDUCED DNA DAMAGE FOR HOST CELL GENOMIC INTEGRITY?

Host cell genetic background affects the consequences of HPV-16 E7-induced DNA damage for genomic integrity

The results presented in this thesis support a model in which HPV-16 E7-associated replication stress and FA pathway activation elicit specific consequences for chromosomal integrity depending on the genetic background of the host cell (Fig. 24). We provide evidence that in FA-deficient host cells, stalled replication forks induced by HPV-16 E7 grow increasingly unstable and are prone to collapse, which ultimately leads to the accelerated accumulation of DNA damage and chromosome breaks (Chapter 2; (184). However, in FA-proficient host cells, when HPV-16 E7-induced replication stress occurs within telomeric DNA it can trigger ALT-associated recombination activity (Chapter 3; (208), thereby preserving the integrity of host cell telomeres. We hypothesize that in FA-proficient cells, HPV-16 E7-associated replication stress that occurs outside of telomeric DNA, such as at chromosomal fragile sites, may also lead to replication fork collapse and DNA breaks, despite activation of the FA pathway. This notion is supported by our finding that HPV-16 E7 expression in BJ fibroblasts, which have a functional FA pathway, stimulates an increase of 53BP1 foci in cells that are also expressing cyclin A (Fig. 20).

Together, these results underscore that replication stress caused by HPV-16 E7 can exert positive or negative effects on genome integrity of the host cell. Ultimately however, each of these consequences contributes to malignant progression, either by facilitating cellular

immortalization or by promoting the acquisition of genomic changes that may provide a growth advantage.

Role of the FA pathway during HPV carcinogenesis

In line with our result that FA deficiency accelerates HPV 16 E7-induced structural chromosomal instability, certain FA genes have been found to be epigenetically silenced in a subset of HPV tumors (155). These findings suggest that inactivation of the FA pathway may be selected for at some point during HPV tumor evolution, and raise the question what the requirement of the FA pathway during early and late stages of HPV tumorigenesis in FA-proficient cells may be.

We hypothesize that the FA pathway is likely to serve several key functions early in HPV tumorigenesis. First, our results indicate that a functional FA pathway may be required to prevent excessive and hence, lethal levels of chromosomal breakage in the host cell. We show that high-risk HPV oncoproteins induce greater levels of DNA breakage in FA-deficient cells (Chapter 2; (207), which is likely to lead to anti-proliferative host cell responses. In line with this, HPV-16 E7 expression in FANCD2-deficient cells was found to stimulate signs of apoptosis (data not shown). These results suggest that in early stages of cancer, the FA pathway may be necessary to prevent levels of chromosomal breakage that could be toxic to the cell, despite the ability of HPV-16 E6 to degrade p53.

A second potential requirement for the FA pathway in early stages of cancer involves its role in the ALT mechanism of telomere lengthening (Chapter 3; (208). While the majority of malignant HPV cancers show activation of telomerase, precancerous lesions display little to no telomerase activity, and yet can remain present for decades (205, 237). The mechanisms through

which precancerous cells maintain a telomere length sufficient to circumvent anti-proliferative host cell barriers are unknown. We hypothesize that replication stress-induced FA-mediated ALT activity may promote the early stabilization of telomeres. This idea is supported by several lines of evidence. First, we show that episomal expression of HPV-16 oncoproteins (commonly observed in precancerous lesions) is sufficient to activate the FA pathway (Chapter 2; (184). Second, HPV-16 E7 expression was found to stimulate the formation of FANCD2-positive ALT-associated PML bodies (APBs) in a small proportion of primary human keratinocytes as early as passage 2. Moreover, HPV-16 E7-expressing keratinocytes harboring FANCD2-APBs were shown to exhibit a slight growth advantage (Chapter 3; (208). These results suggest that activation of ALT by HPV-16 E7 may represent an early host cell event that occurs prior to viral integration.

Why then are the majority of HPV tumors telomerase positive? It may be that precancerous lesions contain a mixed population of cells, which maintain their telomeres via either telomerase or ALT. Since telomerase has been shown to be the dominant mechanism of telomere maintenance (204), cells that acquire higher levels of HPV-16 E6 after viral integration or additional mutations leading to elevated levels of telomerase, will ultimately exhibit a growth advantage and clonally expand.

Collectively, our results suggest that a functional FA pathway may be beneficial early in HPV tumorigenesis by promoting ALT and protecting cells from anti-proliferative responses associated with gross chromosomal breakage. However, at later stages of malignancy, when the cancer cells have gained higher levels of telomerase and additional genomic changes, FA gene silencing may be selected for as it would further promote host cell proliferation and create an

environment that allows the acquisition of additional chromosomal alterations that are growth promoting.

5.3 HOW DO HPV-16 E7-EXPRESSING CELLS CONTINUE TO PROLIFERATE DESPITE ACTIVATED DNA DAMAGE CHECKPOINTS?

Claspin: the "Achilles heel" of the ATR signaling cascade?

Since DNA damage checkpoint responses pose a threat to viral propagation, it is not surprising that the HPV-16 E7 oncoprotein has evolved a mechanism to circumvent ATR-mediated responses. Here, we provide evidence that HPV-16 E7 attenuates DNA damage checkpoint control and promotes aberrant mitotic entry by accelerating the proteolytic turnover of claspin, a mediator of ATR activity (Chapter 4).

Claspin represents an excellent host cell target for viral oncoproteins to exploit, as it can first be "used" (231) during S phase to promote host cell replication and then "abused" (231) by accelerating its degradation at the G2/M transition in order to aid aberrant mitotic entry. Based on our results that deregulated E2F transcriptional control may be linked to premature/accelerated claspin degradation (Chapter 4), it will be interesting to see whether other viral oncoproteins that can similarly disrupt the pRB/E2F axis (Ad E1A and SV40 large T Ag) are also able to attenuate DNA damage checkpoint control by manipulating claspin protein stability. Normal human fibroblasts immortalized with SV40 large T Ag have previously been shown to efficiently degrade claspin prior to mitotic entry (211); however, it has not been

determined in detail whether this contributes to attenuation of DNA damage checkpoint responses.

Collectively, our results suggest that HPV-16 E7-expressing cells may rely heavily on branches of the ATR pathway that are required to restart stalled replication forks (Chapter 2 and 3), but to also dampen other aspects of the ATR signaling axis that are involved in eliciting a cell cycle arrest (Chapter 4). The host cell DNA damage response however, is a highly complex signaling network with a significant level of cross-talk, especially between the ATM and ATR kinases (105). These findings raise the question whether HPV-16 E7 also manipulates other branches of the DNA damage response, specifically the ATM kinase, in order to maintain host cell proliferation. Based on our finding that HPV-16 E7-expressing cells display nuclear foci containing pATM S1981, γ–H2AX and 53BP1 (Chapter 4), which are targets of ATM that are phosphorylated quickly in response to DNA breakage (119), we hypothesize that HPV-16 E7 may also be able to induce a disconnect in the ATM axis, where early signaling is maintained and more downstream events that are required to induce cell cycle arrest are attenuated.

It is important to emphasize that HPV-16 E6-mediated degradation of p53 has also been shown to contribute to relaxation of G2/M checkpoint control in HPV-positive cells (238). In response to DNA damage, activated ATM and ATR signal through p53 in order to upregulate $p21^{Cip1}$ activity and/or promote nuclear exclusion of 14-3-3σ, which leaves cyclin B/CDK1 in an inactivated state and facilitates a G2/M arrest (239). These findings underscore that the HPV-16 oncoproteins can profoundly disrupt at least two branches of the DNA damage response (p53 and claspin) that feed into activation of the G2/M checkpoint. Ultimately, these mechanisms promote aberrant mitotic entry, which facilitates viral propagation and host cell genomic instability. Future experiments will provide interesting insights on whether HPV-16 oncoproteins can

exploit additional aspects of the DNA damage response in order to effectively evade anti-proliferative host cell responses.

Does exploitation of the DNA damage response by HPV oncoproteins contribute to the viral life cycle?

In a recent review, Weitzman and co-workers have outlined how manipulation of the host cell DNA damage response by viral proteins can facilitate the viral life cycle (231). For example, certain adenoviral proteins have been shown to evade the DNA damage response by promoting the degradation and mislocalization of DNA repair proteins in order to evade viral genome concatemerization (240). In contrast however, efficient SV40 replication was shown to depend on ATM signaling (241, 242).

Remarkably, many DNA damage proteins are also required for host cell DNA replication (158). Therefore, it is plausible that the HPV virus may benefit from a partially activated DNA damage response. Our finding that the HPV-16 E7 oncoprotein activates the FA pathway (Chapter 2 and 3) may suggest that certain pathways within the DNA damage response that are involved in maintaining host cell replication fork progression during S phase may be preferentially retained in HPV-positive cells, in order to promote viral replication and the ALT pathway.

On the other hand, aberrant host cell replication dynamics induced by HPV-16 oncoproteins may also lead to replication fork stalling within viral DNA. Whether this results in a selection pressure to silence the FA pathway in order to evade slowing of viral replication is unknown. The finding that FA genes can be silenced during HPV tumor evolution suggests that

this may be a clear possibility. Further experiments are required to determine whether a functional FA pathway helps or hinders HPV replication.

5.4 MODEL: INTERPLAY BETWEEN THE HOST CELL DNA DAMAGE RESPONSE AND HPV-16 ONCOPROTEINS CONTRIBUTES TO CARCINOGENESIS

Based on the work presented here, we provide a comprehensive model (Fig. 24), which describes how the interplay between HPV-16 oncoproteins and the DNA damage response contributes to host cell genomic instability and malignant progression.

HPV-16 E7 promotes unscheduled entry into S phase by binding and inducing the degradation of the pRB family of proteins and inhibiting $p21^{Cip1}$ and $p27^{Kip1}$. These activities lead to deregulated expression of E2F and E2F-targets, including claspin, which helps to promote efficient host cell replication. Accelerated entry into S phase however, causes aberrant host cell dynamics, DNA replication stress, and activation of the FA pathway (Chapter 2; (184). HPV-16 E7-induced replication stress and FA pathway activation can exert positive (ALT activity) or negative (structural chromosomal changes) consequences for genomic integrity (184, 208), depending on the genetic and/or epigenetic background of the host cell.

Importantly, enhanced E2F-mediated transcription also plays a role in circumventing DNA damage checkpoint responses (associated with replication stress-associated claspin accumulation) by upregulating components of the $SCF^{\beta\text{-}TrCP}$-based claspin degradation pathway. HPV-16 E7-induced deregulation of Aurora A and PLK1 expression promotes the accelerated

and/or premature degradation of claspin and hence, stimulates aberrant entry into mitosis (Chapter 4).

Ultimately, HPV-16 E7-induced deregulated E2F activity and DNA breakage can lead to the stabilization of p53 via $p14^{Arf}$ and ATM/ATR signaling. Therefore, the HPV-16 E6 oncoprotein is required to target p53 for degradation, thereby circumventing p53-mediated DNA damage checkpoint responses and growth arrest. These results underscore the intricate interactions between viral oncoproteins and the DNA damage response of the host during viral replication as well as malignant progression.

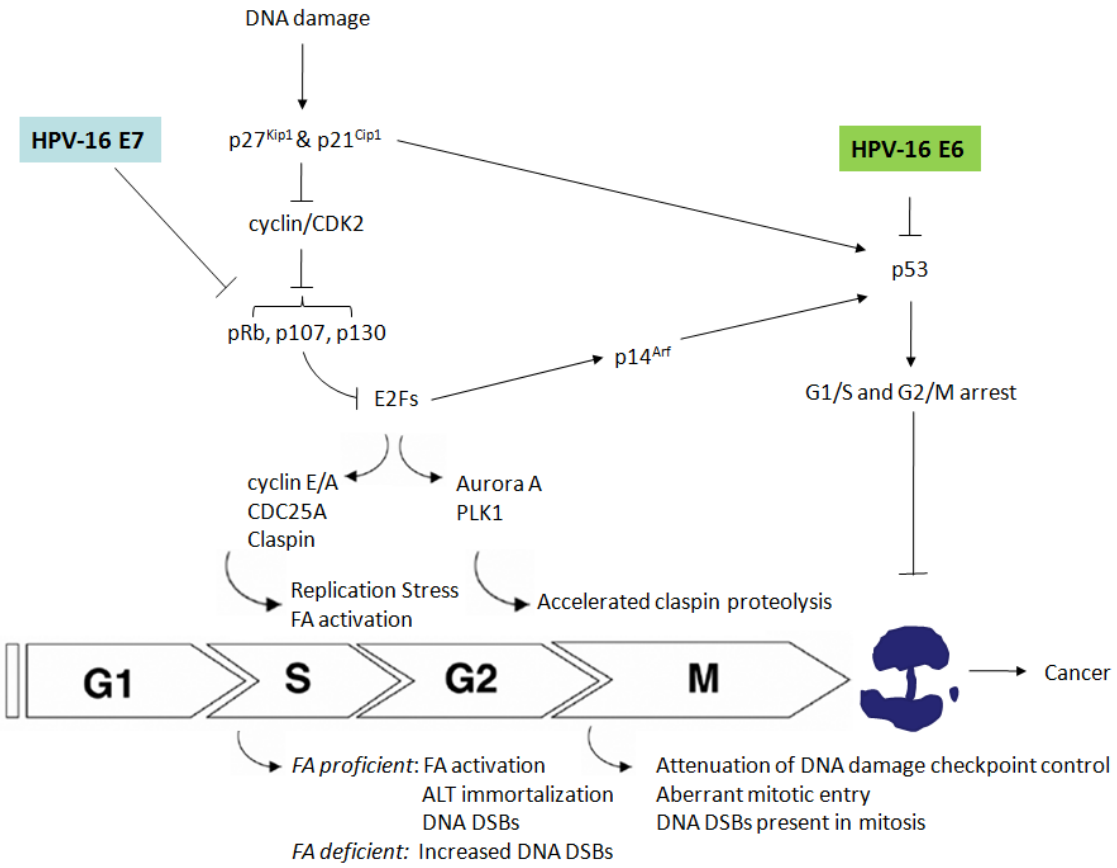

Figure 24. Interplay between HPV-16 oncoproteins and the host cell DNA damage response contributes to chromosomal instability.

HPV-16 E7 accelerates S phase entry by disrupting the pRB family of proteins and the $p21^{Cip1}$ and $p27^{Kip1}$ CDK inhibitors. Deregulated E2F activity upregulates S phase genes (claspin), triggers replication stress and activates the FA pathway. In FA-proficient cells HPV-16 E7-associated stress can lead to ALT activity and/or DNA DSBs as a result of replication fork collapse. In FA-deficient cells, HPV-16 E7 expression leads to enhanced DNA DSBs and structural chromosomal instability due to reduced replication fork stability. Deregulated E2F-transcription of Aurora A and PLK1 by HPV-16 E7 promote the accelerated degradation of claspin and aberrant entry into mitosis. HPV-16 E6-mediated degradation of p53 contributes to relaxation of G1/S and G2/M checkpoint control. By exploiting multiple avenues of the host cell DNA damage response, HPV-16 oncoproteins promote structural chromosomal instability, which can ultimately contribute to cancer.

5.5 FUTURE DIRECTIONS

Work presented in this thesis describes how exploitation of the host cell DNA damage response by the HPV-16 E7 oncoprotein contributes to various aspects of carcinogenesis. Our findings raise several key issues that warrant further investigation in the future.

While we have shown that HPV-16 E7 accelerates chromosomal instability in FA-deficient host cells, several key questions remain unanswered with respect to the role of HPV in FA tumor burden. First, why do HPV-associated tumors progress at a significantly faster rate in FA patients as compared to that in the normal population? Second, what role does immunosuppression of host cells (commonly observed in FA patients following bone marrow transplant) play in persistency of HPV infection and/or HPV carcinogenesis in FA patients? Based on the conflicting reports found within the literature (9, 153, 154), it is apparent that additional studies, using larger sample sizes with more precise methods for HPV detection, will be required to definitively answer these questions.

Little is known about the molecular mechanisms behind HPV integration, but it has been hypothesized to involve DNA breakage within host and viral genomes. Based on our finding that FA gene deficiency increases DNA breakage in HPV-positive cells, we suggest that inherited or epigenetic silencing of FA genes may enhance viral integration. Interestingly, HPV has been shown to preferentially integrate at chromosomal fragile sites (160, 161), which are known to be increasingly unstable in FA-deficient cells (159). Experiments designed to assess the precise manner in which FA gene deficiency influences HPV integration are currently underway in the Duensing laboratory.

Despite the development of a prophylactic vaccine, additional therapeutic options are required for patients with pre-existing infections or those with little access to the vaccine. Our finding that the HPV-16 E7 oncoprotein preferentially targets the ATR axis suggests that small molecular inhibitors of specific branches within this pathway or inhibitors of ALT may be potentially useful in treating or preventing HPV-associated neoplasms.

Finally, it is important to note that small DNA tumor viruses have been instrumental in deciphering how normal host cell processes become deregulated in cancer. The findings presented in this thesis underscore that viral oncoproteins have also evolved mechanisms by which to circumvent anti-proliferative host cell barriers governed by the DNA damage response. By continuing to utilize HPV as a molecular tool, we will undoubtedly learn even more about how deregulation of the DNA damage response contributes to various aspects of carcinogenesis.

Dedication

This thesis is dedicated to my family and to Stefan and Anette Duensing. Each of you has been instrumental in helping me to become the person that I am today. Thank you for your support, encouragement and friendship.

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To my grandparents, I appreciate the sacrifices that each of you has made to ensure that hard work and education was, and is, a main priority in our family. My grandfather (Albert Spardy, Sr.) has a saying, “Tenacity conquers mediocrity”, and this has been my mantra throughout my graduate career.

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6.0 BIBLIOGRAPHY

1. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002; 2: 342-50.
2. zur Hausen H. Papillomavirus infections--a major cause of human cancers. Biochim Biophys Acta 1996; 1288: F55-78.
3. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000; 92: 709-20.
4. de Villiers EM. Human papillomavirus infections in skin cancers. Biomed Pharmacother 1998; 52: 26-33.
5. Koutsky L. Epidemiology of genital human papillomavirus infection. Am J Med 1997; 102: 3-8.
6. Longworth MS, Laimins LA. Pathogenesis of human papillomaviruses in differentiating epithelia. Microbiol Mol Biol Rev 2004; 68: 362-72.
7. Hopfl R, Heim K, Christensen N, et al. Spontaneous regression of CIN and delayed-type hypersensitivity to HPV-16 oncoprotein E7. Lancet 2000; 356: 1985-6.
8. Jenson AB, Kurman RJ, Lancaster WD. Tissue effects of and host response to human papillomavirus infection. Dermatol Clin 1991; 9: 203-9.
9. Kutler DI, Wreesmann VB, Goberdhan A, et al. Human papillomavirus DNA and p53 polymorphisms in squamous cell carcinomas from Fanconi anemia patients. J Natl Cancer Inst 2003; 95: 1718-21.
10. Wang SS, Bratti MC, Rodriguez AC, et al. Common Variants in Immune and DNA Repair Genes and Risk for Human Papillomavirus Persistence and Progression to Cervical Cancer. J Infect Dis 2009; 199: 20-30.
11. Kjellberg L, Hallmans G, Ahren AM, et al. Smoking, diet, pregnancy and oral contraceptive use as risk factors for cervical intra-epithelial neoplasia in relation to human papillomavirus infection. Br J Cancer 2000; 82: 1332-8.
12. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999; 189: 12-9.
13. Bosch FX, Burchell AN, Schiffman M, et al. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine 2008; 26 Suppl 10: K1-16.
14. Hausen HZ. Papillomaviruses--to vaccination and beyond. Biochemistry (Mosc) 2008; 73: 498-503.
15. Psyrri A, DiMaio D. Human papillomavirus in cervical and head-and-neck cancer. Nat Clin Pract Oncol 2008; 5: 24-31.

16. Stacey SN, Jordan D, Williamson AJ, Brown M, Coote JH, Arrand JR. Leaky scanning is the predominant mechanism for translation of human papillomavirus type 16 E7 oncoprotein from E6/E7 bicistronic mRNA. J Virol 2000; 74: 7284-97.
17. Thierry F. Transcriptional regulation of the papillomavirus oncogenes by cellular and viral transcription factors in cervical carcinoma. Virology 2009; 384: 375-9.
18. Yang L, Mohr I, Fouts E, Lim DA, Nohaile M, Botchan M. The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. Proc Natl Acad Sci U S A 1993; 90: 5086-90.
19. Sedman J, Stenlund A. The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities. J Virol 1998; 72: 6893-7.
20. Sedman J, Stenlund A. Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus in vivo and in vitro. EMBO J 1995; 14: 6218-28.
21. Smith JL, Campos SK, Ozbun MA. Human papillomavirus type 31 uses a caveolin 1- and dynamin 2-mediated entry pathway for infection of human keratinocytes. J Virol 2007; 81: 9922-31.
22. Stubenrauch F, Laimins LA. Human papillomavirus life cycle: active and latent phases. Semin Cancer Biol 1999; 9: 379-86.
23. Hebner CM, Laimins LA. Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity. Rev Med Virol 2006; 16: 83-97.
24. DiMaio D, Liao JB. Human papillomaviruses and cervical cancer. Adv Virus Res 2006; 66: 125-59.
25. Munger K, Howley PM. Human papillomavirus immortalization and transformation functions. Virus Res 2002; 89: 213-28.
26. Hummel M, Hudson JB, Laimins LA. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J Virol 1992; 66: 6070-80.
27. Corden SA, Sant-Cassia LJ, Easton AJ, Morris AG. The integration of HPV-18 DNA in cervical carcinoma. Mol Pathol 1999; 52: 275-82.
28. Jeon S, Allen-Hoffmann BL, Lambert PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J Virol 1995; 69: 2989-97.
29. Melsheimer P, Vinokurova S, Wentzensen N, Bastert G, von Knebel Doeberitz M. DNA aneuploidy and integration of human papillomavirus type 16 e6/e7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri. Clin Cancer Res 2004; 10: 3059-63.
30. Munger K, Basile JR, Duensing S, et al. Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. Oncogene 2001; 20: 7888-98.
31. Phelps WC, Yee CL, Munger K, Howley PM. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell 1988; 53: 539-47.
32. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res 1996; 56: 4620-4.

33. Davies R, Hicks R, Crook T, Morris J, Vousden K. Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. J Virol 1993; 67: 2521-8.
34. Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 1989; 243: 934-7.
35. Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J 1989; 8: 4099-105.
36. Jones DL, Munger K. Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. J Virol 1997; 71: 2905-12.
37. Huh K, Zhou X, Hayakawa H, et al. Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor. J Virol 2007; 81: 9737-47.
38. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell 1995; 81: 323-30.
39. Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes Dev 1997; 11: 2090-100.
40. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P. Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. Oncogene 1996; 13: 2323-30.
41. Jones DL, Alani RM, Munger K. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. Genes Dev 1997; 11: 2101-11.
42. Katich SC, Zerfass-Thome K, Hoffmann I. Regulation of the Cdc25A gene by the human papillomavirus Type 16 E7 oncogene. Oncogene 2001; 20: 543-50.
43. Martin LG, Demers GW, Galloway DA. Disruption of the G1/S transition in human papillomavirus type 16 E7-expressing human cells is associated with altered regulation of cyclin E. J Virol 1998; 72: 975-85.
44. Zerfass K, Schulze A, Spitkovsky D, Friedman V, Henglein B, Jansen-Durr P. Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. J Virol 1995; 69: 6389-99.
45. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. Virology 2008.
46. Bernat A, Avvakumov N, Mymryk JS, Banks L. Interaction between the HPV E7 oncoprotein and the transcriptional coactivator p300. Oncogene 2003; 22: 7871-81.
47. Burgers WA, Blanchon L, Pradhan S, de Launoit Y, Kouzarides T, Fuks F. Viral oncoproteins target the DNA methyltransferases. Oncogene 2007; 26: 1650-5.
48. Brehm A, Nielsen SJ, Miska EA, et al. The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. EMBO J 1999; 18: 2449-58.
49. Huh KW, DeMasi J, Ogawa H, Nakatani Y, Howley PM, Munger K. Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. Proc Natl Acad Sci U S A 2005; 102: 11492-7.
50. McIntyre MC, Ruesch MN, Laimins LA. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. Virology 1996; 215: 73-82.
51. Nguyen CL, Munger K. Direct association of the HPV16 E7 oncoprotein with cyclin A/CDK2 and cyclin E/CDK2 complexes. Virology 2008; 380: 21-5.

52. Bates S, Phillips AC, Clark PA, et al. p14ARF links the tumour suppressors RB and p53. Nature 1998; 395: 124-5.
53. Qin XQ, Livingston DM, Kaelin WG, Jr., Adams PD. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A 1994; 91: 10918-22.
54. Wu X, Levine AJ. p53 and E2F-1 cooperate to mediate apoptosis. Proc Natl Acad Sci U S A 1994; 91: 3602-6.
55. Helton ES, Chen X. p53 modulation of the DNA damage response. J Cell Biochem 2007; 100: 883-96.
56. Huibregtse JM, Scheffner M, Howley PM. Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. Mol Cell Biol 1993; 13: 4918-27.
57. Huibregtse JM, Scheffner M, Howley PM. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol Cell Biol 1993; 13: 775-84.
58. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 1993; 75: 495-505.
59. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990; 63: 1129-36.
60. Patel D, Huang SM, Baglia LA, McCance DJ. The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. EMBO J 1999; 18: 5061-72.
61. Zimmermann H, Degenkolbe R, Bernard HU, O'Connor MJ. The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300. J Virol 1999; 73: 6209-19.
62. Klingelhutz AJ, Foster SA, McDougall JK. Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature 1996; 380: 79-82.
63. Veldman T, Liu X, Yuan H, Schlegel R. Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter. Proc Natl Acad Sci U S A 2003; 100: 8211-6.
64. Gewin L, Myers H, Kiyono T, Galloway DA. Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. Genes Dev 2004; 18: 2269-82.
65. Xu M, Luo W, Elzi DJ, Grandori C, Galloway DA. NFX1 interacts with mSin3A/histone deacetylase to repress hTERT transcription in keratinocytes. Mol Cell Biol 2008; 28: 4819-28.
66. James MA, Lee JH, Klingelhutz AJ. HPV16-E6 associated hTERT promoter acetylation is E6AP dependent, increased in later passage cells and enhanced by loss of p300. Int J Cancer 2006; 119: 1878-85.
67. Nakagawa S, Huibregtse JM. Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. Mol Cell Biol 2000; 20: 8244-53.
68. Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T, Ishibashi M. Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A 1997; 94: 11612-6.

69. Lee SS, Glaunsinger B, Mantovani F, Banks L, Javier RT. Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. J Virol 2000; 74: 9680-93.
70. Lee SS, Weiss RS, Javier RT. Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A 1997; 94: 6670-5.
71. Scheffner M, Romanczuk H, Munger K, Huibregtse JM, Mietz JA, Howley PM. Functions of human papillomavirus proteins. Curr Top Microbiol Immunol 1994; 186: 83-99.
72. Zhang B, Chen W, Roman A. The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. Proc Natl Acad Sci U S A 2006; 103: 437-42.
73. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005; 434: 864-70.
74. Tanaka S, Diffley JF. Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. Genes Dev 2002; 16: 2639-49.
75. Ekholm-Reed S, Mendez J, Tedesco D, Zetterberg A, Stillman B, Reed SI. Deregulation of cyclin E in human cells interferes with prereplication complex assembly. J Cell Biol 2004; 165: 789-800.
76. zur Hausen H. Viruses in human cancers. Science 1991; 254: 1167-73.
77. Matlashewski G, Schneider J, Banks L, Jones N, Murray A, Crawford L. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. EMBO J 1987; 6: 1741-6.
78. Noda T, Yajima H, Ito Y. Progression of the phenotype of transformed cells after growth stimulation of cells by a human papillomavirus type 16 gene function. J Virol 1988; 62: 313-24.
79. Pirisi L, Yasumoto S, Feller M, Doniger J, DiPaolo JA. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J Virol 1987; 61: 1061-6.
80. Yasumoto S, Burkhardt AL, Doniger J, DiPaolo JA. Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J Virol 1986; 57: 572-7.
81. Yasumoto S, Doniger J, DiPaolo JA. Differential early viral gene expression in two stages of human papillomavirus type 16 DNA-induced malignant transformation. Mol Cell Biol 1987; 7: 2165-72.
82. Gagos S, Irminger-Finger I. Chromosome instability in neoplasia: chaotic roots to continuous growth. Int J Biochem Cell Biol 2005; 37: 1014-33.
83. Rajagopalan H, Jallepalli PV, Rago C, et al. Inactivation of hCDC4 can cause chromosomal instability. Nature 2004; 428: 77-81.
84. McClintock B. The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics 1941; 26: 234-82.
85. Levitus M, Joenje H, de Winter JP. The Fanconi anemia pathway of genomic maintenance. Cell Oncol 2006; 28: 3-29.
86. Kastan MB. Our cells get stressed too! Implications for human disease. Blood Cells Mol Dis 2007; 39: 148-50.
87. Budzowska M, Kanaar R. Mechanisms of dealing with DNA damage-induced replication problems. Cell Biochem Biophys 2009; 53: 17-31.
88. Osborn AJ, Elledge SJ, Zou L. Checking on the fork: the DNA-replication stress-response pathway. Trends Cell Biol 2002; 12: 509-16.

89. Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993; 362: 709-15.
90. Heselmeyer K, Schrock E, du Manoir S, et al. Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. Proc Natl Acad Sci U S A 1996; 93: 479-84.
91. Atkin NB. Cytogenetics of carcinoma of the cervix uteri: a review. Cancer Genet Cytogenet 1997; 95: 33-9.
92. Heselmeyer K, Macville M, Schrock E, et al. Advanced-stage cervical carcinomas are defined by a recurrent pattern of chromosomal aberrations revealing high genetic instability and a consistent gain of chromosome arm 3q. Genes Chromosomes Cancer 1997; 19: 233-40.
93. Rader JS, Gerhard DS, O'Sullivan MJ, et al. Cervical intraepithelial neoplasia III shows frequent allelic loss in 3p and 6p. Genes Chromosomes Cancer 1998; 22: 57-65.
94. Wistuba, II, Montellano FD, Milchgrub S, et al. Deletions of chromosome 3p are frequent and early events in the pathogenesis of uterine cervical carcinoma. Cancer Res 1997; 57: 3154-8.
95. Liu X, Han S, Baluda MA, Park NH. HPV-16 oncogenes E6 and E7 are mutagenic in normal human oral keratinocytes. Oncogene 1997; 14: 2347-53.
96. Havre PA, Yuan J, Hedrick L, Cho KR, Glazer PM. p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res 1995; 55: 4420-4.
97. Kim HJ, Guo W, Park NH. HPV-16 E6 oncoprotein induces mutations via p53-dependent and -independent pathways. Oncol Rep 2000; 7: 707-12.
98. Kessis TD, Connolly DC, Hedrick L, Cho KR. Expression of HPV16 E6 or E7 increases integration of foreign DNA. Oncogene 1996; 13: 427-31.
99. Duensing S, Munger K. The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. Cancer Res 2002; 62: 7075-82.
100. Hashida T, Yasumoto S. Induction of chromosome abnormalities in mouse and human epidermal keratinocytes by the human papillomavirus type 16 E7 oncogene. J Gen Virol 1991; 72 (Pt 7): 1569-77.
101. Hein J, Boichuk S, Wu J, et al. Simian virus 40 large T antigen disrupts genome integrity and activates a DNA damage response via Bub1 binding. J Virol 2009; 83: 117-27.
102. Chang PL, Gunby JL, Tomkins DJ, Mak I, Rosa NE, Mak S. Transformation of human cultured fibroblasts with plasmids carrying dominant selection markers and immortalizing potential. Exp Cell Res 1986; 167: 407-16.
103. Braithwaite AW, Cheetham BF, Li P, Parish CR, Waldron-Stevens LK, Bellett AJ. Adenovirus-induced alterations of the cell growth cycle: a requirement for expression of E1A but not of E1B. J Virol 1983; 45: 192-9.
104. Caporossi D, Bacchetti S. Definition of adenovirus type 5 functions involved in the induction of chromosomal aberrations in human cells. J Gen Virol 1990; 71 (Pt 4): 801-8.
105. Harper JW, Elledge SJ. The DNA damage response: ten years after. Mol Cell 2007; 28: 739-45.
106. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature 2004; 432: 316-23.
107. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 2004; 73: 39-85.
108. Stiff T, Walker SA, Cerosaletti K, et al. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. EMBO J 2006; 25: 5775-82.

109. Hall-Jackson CA, Cross DA, Morrice N, Smythe C. ATR is a caffeine-sensitive, DNA-activated protein kinase with a substrate specificity distinct from DNA-PK. Oncogene 1999; 18: 6707-13.
110. Hurley PJ, Bunz F. ATM and ATR: components of an integrated circuit. Cell Cycle 2007; 6: 414-7.
111. Lakin ND, Hann BC, Jackson SP. The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53. Oncogene 1999; 18: 3989-95.
112. Tibbetts RS, Brumbaugh KM, Williams JM, et al. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev 1999; 13: 152-7.
113. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003; 421: 499-506.
114. Kim YC, Gerlitz G, Furusawa T, et al. Activation of ATM depends on chromatin interactions occurring before induction of DNA damage. Nat Cell Biol 2009; 11: 92-6.
115. Kitagawa R, Kastan MB. The ATM-dependent DNA damage signaling pathway. Cold Spring Harb Symp Quant Biol 2005; 70: 99-109.
116. Stucki M, Jackson SP. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA Repair (Amst) 2006; 5: 534-43.
117. Kim JE, Minter-Dykhouse K, Chen J. Signaling networks controlled by the MRN complex and MDC1 during early DNA damage responses. Mol Carcinog 2006; 45: 403-8.
118. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000; 10: 886-95.
119. Bekker-Jensen S, Lukas C, Kitagawa R, et al. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol 2006; 173: 195-206.
120. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 2003; 300: 1542-8.
121. Kumagai A, Lee J, Yoo HY, Dunphy WG. TopBP1 activates the ATR-ATRIP complex. Cell 2006; 124: 943-55.
122. Ball HL, Cortez D. ATRIP oligomerization is required for ATR-dependent checkpoint signaling. J Biol Chem 2005; 280: 31390-6.
123. Ball HL, Myers JS, Cortez D. ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation. Mol Biol Cell 2005; 16: 2372-81.
124. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: partners in checkpoint signaling. Science 2001; 294: 1713-6.
125. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol 2008; 9: 616-27.
126. Kumagai A, Kim SM, Dunphy WG. Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1. J Biol Chem 2004; 279: 49599-608.
127. Chini CC, Chen J. Claspin, a regulator of Chk1 in DNA replication stress pathway. DNA Repair (Amst) 2004; 3: 1033-7.
128. Petermann E, Helleday T, Caldecott KW. Claspin promotes normal replication fork rates in human cells. Mol Biol Cell 2008; 19: 2373-8.
129. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 2003; 3: 421-9.

130. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 2001; 15: 2177-96.
131. Wang X, D'Andrea AD. The interplay of Fanconi anemia proteins in the DNA damage response. DNA Repair (Amst) 2004; 3: 1063-9.
132. Cohn MA, D'Andrea AD. Chromatin recruitment of DNA repair proteins: lessons from the fanconi anemia and double-strand break repair pathways. Mol Cell 2008; 32: 306-12.
133. Kennedy RD, D'Andrea AD. The Fanconi Anemia/BRCA pathway: new faces in the crowd. Genes Dev 2005; 19: 2925-40.
134. Cervenka J, Arthur D, Yasis C. Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and Fanconi anemia. Pediatrics 1981; 67: 119-27.
135. Green AM, Kupfer GM. Fanconi anemia. Hematol Oncol Clin North Am 2009; 23: 193-214.
136. Wang W. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet 2007; 8: 735-48.
137. Meetei AR, de Winter JP, Medhurst AL, et al. A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet 2003; 35: 165-70.
138. Meetei AR, Yan Z, Wang W. FANCL replaces BRCA1 as the likely ubiquitin ligase responsible for FANCD2 monoubiquitination. Cell Cycle 2004; 3: 179-81.
139. Alpi AF, Patel KJ. Monoubiquitylation in the Fanconi anemia DNA damage response pathway. DNA Repair (Amst) 2009; 8: 430-5.
140. Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M, D'Andrea AD. S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 2002; 100: 2414-20.
141. Nakanishi K, Yang YG, Pierce AJ, et al. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A 2005; 102: 1110-5.
142. Seki S, Ohzeki M, Uchida A, et al. A requirement of FancL and FancD2 monoubiquitination in DNA repair. Genes Cells 2007; 12: 299-310.
143. Nijman SM, Huang TT, Dirac AM, et al. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell 2005; 17: 331-9.
144. Andreassen PR, D'Andrea AD, Taniguchi T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev 2004; 18: 1958-63.
145. Rosenberg PS, Greene MH, Alter BP. Cancer incidence in persons with Fanconi anemia. Blood 2003; 101: 822-6.
146. Kutler DI, Auerbach AD, Satagopan J, et al. High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Arch Otolaryngol Head Neck Surg 2003; 129: 106-12.
147. Kennedy AW, Hart WR. Multiple squamous-cell carcinomas in Fanconi's anemia. Cancer 1982; 50: 811-4.
148. Alter BP. Cancer in Fanconi anemia, 1927-2001. Cancer 2003; 97: 425-40.
149. Alter BP, Joenje H, Oostra AB, Pals G. Fanconi anemia: adult head and neck cancer and hematopoietic mosaicism. Arch Otolaryngol Head Neck Surg 2005; 131: 635-9.
150. Mathew CG. Fanconi anaemia genes and susceptibility to cancer. Oncogene 2006; 25: 5875-84.
151. Tremblay S, Pintor Dos Reis P, Bradley G, et al. Young patients with oral squamous cell carcinoma: study of the involvement of GSTP1 and deregulation of the Fanconi anemia genes. Arch Otolaryngol Head Neck Surg 2006; 132: 958-66.

152. Wilkinson EJ, Morgan LS, Friedrich EG, Jr. Association of Fanconi's anemia and squamous-cell carcinoma of the lower female genital tract with condyloma acuminatum. A report of two cases. J Reprod Med 1984; 29: 447-53.
153. van Zeeburg HJ, Snijders PJ, Pals G, et al. Generation and molecular characterization of head and neck squamous cell lines of fanconi anemia patients. Cancer Res 2005; 65: 1271-6.
154. van Zeeburg HJ, Snijders PJ, Wu T, et al. Clinical and molecular characteristics of squamous cell carcinomas from Fanconi anemia patients. J Natl Cancer Inst 2008; 100: 1649-53.
155. Narayan G, Arias-Pulido H, Nandula SV, et al. Promoter hypermethylation of FANCF: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. Cancer Res 2004; 64: 2994-7.
156. Mendez J, Stillman B. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol 2000; 20: 8602-12.
157. zur Hausen H. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology 1991; 184: 9-13.
158. Thompson LH, Hinz JM, Yamada NA, Jones NJ. How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 2005; 45: 128-42.
159. Howlett NG, Taniguchi T, Durkin SG, D'Andrea AD, Glover TW. The Fanconi anemia pathway is required for the DNA replication stress response and for the regulation of common fragile site stability. Hum Mol Genet 2005; 14: 693-701.
160. Cannizzaro LA, Durst M, Mendez MJ, Hecht BK, Hecht F. Regional chromosome localization of human papillomavirus integration sites near fragile sites, oncogenes, and cancer chromosome breakpoints. Cancer Genet Cytogenet 1988; 33: 93-8.
161. Thorland EC, Myers SL, Gostout BS, Smith DI. Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene 2003; 22: 1225-37.
162. Wentzensen N, Vinokurova S, von Knebel Doeberitz M. Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. Cancer Res 2004; 64: 3878-84.
163. Dall KL, Scarpini CG, Roberts I, et al. Characterization of naturally occurring HPV16 integration sites isolated from cervical keratinocytes under noncompetitive conditions. Cancer Res 2008; 68: 8249-59.
164. Todaro GJ, Green H, Swift MR. Susceptibility of human diploid fibroblast strains to transformation by SV40 virus. Science 1966; 153: 1252-4.
165. Solinas-Toldo S, Durst M, Lichter P. Specific chromosomal imbalances in human papillomavirus-transfected cells during progression toward immortality. Proc Natl Acad Sci U S A 1997; 94: 3854-9.
166. Moorhead PS, Saksela E. The sequence of chromosome aberrations during SV 40 transformation of a human diploid cell strain. Hereditas 1965; 52: 271-84.
167. Walen KH. Chromosome instability in cell lineages of amniocyte clones morphologically transformed by Simian virus 40. Cancer Genet Cytogenet 1987; 25: 149-59.
168. Houghtaling S, Granville L, Akkari Y, et al. Heterozygosity for p53 (Trp53+/-) accelerates epithelial tumor formation in fanconi anemia complementation group D2 (Fancd2) knockout mice. Cancer Res 2005; 65: 85-91.
169. Yamamoto A, Kumakura S, Uchida M, Barrett JC, Tsutsui T. Immortalization of normal human embryonic fibroblasts by introduction of either the human papillomavirus type 16 E6 or E7 gene alone. Int J Cancer 2003; 106: 301-9.

170. Stoppler H, Hartmann DP, Sherman L, Schlegel R. The human papillomavirus type 16 E6 and E7 oncoproteins dissociate cellular telomerase activity from the maintenance of telomere length. J Biol Chem 1997; 272: 13332-7.
171. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 2005; 19: 2100-10.
172. De Lange T. Telomere-related genome instability in cancer. Cold Spring Harb Symp Quant Biol 2005; 70: 197-204.
173. Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. Oncogene 2002; 21: 598-610.
174. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J 1995; 14: 4240-8.
175. Jiang WQ, Zhong ZH, Henson JD, Neumann AA, Chang AC, Reddel RR. Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex. Mol Cell Biol 2005; 25: 2708-21.
176. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 1997; 3: 1271-4.
177. Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res 1999; 59: 4175-9.
178. Henson JD, Hannay JA, McCarthy SW, et al. A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 2005; 11: 217-25.
179. Verdun RE, Karlseder J. The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres. Cell 2006; 127: 709-20.
180. Fouche N, Ozgur S, Roy D, Griffith JD. Replication fork regression in repetitive DNAs. Nucleic Acids Res 2006; 34: 6044-50.
181. Ohki R, Ishikawa F. Telomere-bound TRF1 and TRF2 stall the replication fork at telomeric repeats. Nucleic Acids Res 2004; 32: 1627-37.
182. Leteurtre F, Li X, Guardiola P, et al. Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. Br J Haematol 1999; 105: 883-93.
183. Callen E, Samper E, Ramirez MJ, et al. Breaks at telomeres and TRF2-independent end fusions in Fanconi anemia. Hum Mol Genet 2002; 11: 439-44.
184. Spardy N, Duensing A, Charles D, et al. The human papillomavirus type 16 E7 oncoprotein activates the Fanconi anemia (FA) pathway and causes accelerated chromosomal instability in FA cells. J Virol 2007; 81: 13265-70.
185. Davies SL, North PS, Dart A, Lakin ND, Hickson ID. Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. Mol Cell Biol 2004; 24: 1279-91.
186. Dolbeare F, Beisker W, Pallavicini MG, Vanderlaan M, Gray JW. Cytochemistry for bromodeoxyuridine/DNA analysis: stoichiometry and sensitivity. Cytometry 1985; 6: 521-30.
187. Lomonosov M, Anand S, Sangrithi M, Davies R, Venkitaraman AR. Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. Genes Dev 2003; 17: 3017-22.
188. Wang X, Andreassen PR, D'Andrea AD. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol 2004; 24: 5850-62.

189. Osman F, Whitby MC. Exploring the roles of Mus81-Eme1/Mms4 at perturbed replication forks. DNA Repair (Amst) 2007; 6: 1004-17.
190. Nabetani A, Yokoyama O, Ishikawa F. Localization of hRad9, hHus1, hRad1, and hRad17 and caffeine-sensitive DNA replication at the alternative lengthening of telomeres-associated promyelocytic leukemia body. J Biol Chem 2004; 279: 25849-57.
191. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. Curr Biol 2003; 13: 1549-56.
192. Fan Q, Zhang F, Barrett B, Ren K, Andreassen PR. A role for monoubiquitinated FANCD2 at telomeres in ALT cells. Nucleic Acids Res 2009.
193. Zeng S, Xiang T, Pandita TK, et al. Telomere recombination requires the MUS81 endonuclease. Nat Cell Biol 2009.
194. Fasching CL, Neumann AA, Muntoni A, Yeager TR, Reddel RR. DNA damage induces alternative lengthening of telomeres (ALT) associated promyelocytic leukemia bodies that preferentially associate with linear telomeric DNA. Cancer Res 2007; 67: 7072-7.
195. Garcia-Higuera I, Taniguchi T, Ganesan S, et al. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 2001; 7: 249-62.
196. Franco S, van de Vrugt HJ, Fernandez P, Aracil M, Arwert F, Blasco MA. Telomere dynamics in Fancg-deficient mouse and human cells. Blood 2004; 104: 3927-35.
197. Bechter OE, Shay JW, Wright WE. The frequency of homologous recombination in human ALT cells. Cell Cycle 2004; 3: 547-9.
198. Garcia-Cao M, Gonzalo S, Dean D, Blasco MA. A role for the Rb family of proteins in controlling telomere length. Nat Genet 2002; 32: 415-9.
199. Gonzalo S, Jaco I, Fraga MF, et al. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 2006; 8: 416-24.
200. Nakahara T, Lambert PF. Induction of promyelocytic leukemia (PML) oncogenic domains (PODs) by papillomavirus. Virology 2007; 366: 316-29.
201. Bischof O, Nacerddine K, Dejean A. Human papillomavirus oncoprotein E7 targets the promyelocytic leukemia protein and circumvents cellular senescence via the Rb and p53 tumor suppressor pathways. Mol Cell Biol 2005; 25: 1013-24.
202. Guccione E, Massimi P, Bernat A, Banks L. Comparative analysis of the intracellular location of the high- and low-risk human papillomavirus oncoproteins. Virology 2002; 293: 20-5.
203. Zhang A, Wang J, Zheng B, et al. Telomere attrition predominantly occurs in precursor lesions during in vivo carcinogenic process of the uterine cervix. Oncogene 2004; 23: 7441-7.
204. Wen J, Cong YS, Bacchetti S. Reconstitution of wild-type or mutant telomerase activity in telomerase-negative immortal human cells. Hum Mol Genet 1998; 7: 1137-41.
205. Jiao Y, Zhang W, Liu J, et al. Telomere attrition and chromosome instability via downregulation of TRF2 contributes to arsenic trioxide-induced apoptosis of human T-Cell leukemia cell line molt-4 cells. Cancer Biol Ther 2007; 6: 1186-92.
206. Ito H, Kyo S, Kanaya T, Takakura M, Inoue M, Namiki M. Expression of human telomerase subunits and correlation with telomerase activity in urothelial cancer. Clin Cancer Res 1998; 4: 1603-8.
207. Hoskins EE, Morris TA, Higginbotham JM, et al. Fanconi anemia deficiency stimulates HPV-associated hyperplastic growth in organotypic epithelial raft culture. Oncogene 2008.

208. Spardy N, Duensing A, Hoskins EE, Wells SI, Duensing S. HPV-16 E7 reveals a link between DNA replication stress, fanconi anemia D2 protein, and alternative lengthening of telomere-associated promyelocytic leukemia bodies. Cancer Res 2008; 68: 9954-63.
209. Song S, Gulliver GA, Lambert PF. Human papillomavirus type 16 E6 and E7 oncogenes abrogate radiation-induced DNA damage responses in vivo through p53-dependent and p53-independent pathways. Proc Natl Acad Sci U S A 1998; 95: 2290-5.
210. Helt AM, Galloway DA. Destabilization of the retinoblastoma tumor suppressor by human papillomavirus type 16 E7 is not sufficient to overcome cell cycle arrest in human keratinocytes. J Virol 2001; 75: 6737-47.
211. Peschiaroli A, Dorrello NV, Guardavaccaro D, et al. SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. Mol Cell 2006; 23: 319-29.
212. Mailand N, Bekker-Jensen S, Bartek J, Lukas J. Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. Mol Cell 2006; 23: 307-18.
213. Mamely I, van Vugt MA, Smits VA, et al. Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery. Curr Biol 2006; 16: 1950-5.
214. Bartek J, Lukas J. DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol 2007; 19: 238-45.
215. Faustrup H, Bekker-Jensen S, Bartek J, Lukas J, Mailand N. USP7 counteracts SCF{beta}TrCP- but not APCCdh1-mediated proteolysis of Claspin. J Cell Biol 2009.
216. Bassermann F, Frescas D, Guardavaccaro D, Busino L, Peschiaroli A, Pagano M. The Cdc14B-Cdh1-Plk1 axis controls the G2 DNA-damage-response checkpoint. Cell 2008; 134: 256-67.
217. Zhang D, Zaugg K, Mak TW, Elledge SJ. A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response. Cell 2006; 126: 529-42.
218. Macurek L, Lindqvist A, Lim D, et al. Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature 2008; 455: 119-23.
219. Toczyski DP, Galgoczy DJ, Hartwell LH. CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. Cell 1997; 90: 1097-106.
220. Lupardus PJ, Cimprich KA. Checkpoint adaptation; molecular mechanisms uncovered. Cell 2004; 117: 555-6.
221. Yoo HY, Kumagai A, Shevchenko A, Dunphy WG. Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. Cell 2004; 117: 575-88.
222. Sandell LL, Zakian VA. Loss of a yeast telomere: arrest, recovery, and chromosome loss. Cell 1993; 75: 729-39.
223. Syljuasen RG. Checkpoint adaptation in human cells. Oncogene 2007; 26: 5833-9.
224. Kimura M, Kotani S, Hattori T, et al. Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. J Biol Chem 1997; 272: 13766-71.
225. Lindon C, Pines J. Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. J Cell Biol 2004; 164: 233-41.
226. Uchiumi T, Longo DL, Ferris DK. Cell cycle regulation of the human polo-like kinase (PLK) promoter. J Biol Chem 1997; 272: 9166-74.

227. Iwanaga R, Komori H, Ishida S, et al. Identification of novel E2F1 target genes regulated in cell cycle-dependent and independent manners. Oncogene 2006; 25: 1786-98.
228. He L, Yang H, Ma Y, Pledger WJ, Cress WD, Cheng JQ. Identification of Aurora-A as a direct target of E2F3 during G2/M cell cycle progression. J Biol Chem 2008; 283: 31012-20.
229. Ishida S, Huang E, Zuzan H, et al. Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol Cell Biol 2001; 21: 4684-99.
230. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 2004; 19: 169-85.
231. Lilley CE, Schwartz RA, Weitzman MD. Using or abusing: viruses and the cellular DNA damage response. Trends Microbiol 2007; 15: 119-26.
232. Weitzman MD, Carson CT, Schwartz RA, Lilley CE. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) 2004; 3: 1165-73.
233. Iftner T, Elbel M, Schopp B, et al. Interference of papillomavirus E6 protein with single-strand break repair by interaction with XRCC1. EMBO J 2002; 21: 4741-8.
234. Diffley JF. Regulation of early events in chromosome replication. Curr Biol 2004; 14: R778-86.
235. Gao H, Chen XB, McGowan CH. Mus81 endonuclease localizes to nucleoli and to regions of DNA damage in human S-phase cells. Mol Biol Cell 2003; 14: 4826-34.
236. Dominguez-Sola D, Ying CY, Grandori C, et al. Non-transcriptional control of DNA replication by c-Myc. Nature 2007; 448: 445-51.
237. Takakura M, Kyo S, Kanaya T, Tanaka M, Inoue M. Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. Cancer Res 1998; 58: 1558-61.
238. Thompson DA, Belinsky G, Chang TH, Jones DL, Schlegel R, Munger K. The human papillomavirus-16 E6 oncoprotein decreases the vigilance of mitotic checkpoints. Oncogene 1997; 15: 3025-35.
239. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene 2001; 20: 1803-15.
240. Stracker TH, Carson CT, Weitzman MD. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature 2002; 418: 348-52.
241. Shi Y, Dodson GE, Shaikh S, Rundell K, Tibbetts RS. Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo. J Biol Chem 2005; 280: 40195-200.
242. Zhao X, Madden-Fuentes RJ, Lou BX, et al. Ataxia telangiectasia-mutated damage-signaling kinase- and proteasome-dependent destruction of Mre11-Rad50-Nbs1 subunits in Simian virus 40-infected primate cells. J Virol 2008; 82: 5316-28.