Title
The fanconi anemia pathway limits human papillomavirus replication.

Journal
Journal of Virology, 86(15)

Authors
Hoskins, Elizabeth
Morreale, Richard
Werner, Stephen
et al.

Publication Date
2012-08-01

DOI
10.1128/JVI.00408-12

Peer reviewed

JVI Journals.ASM.org

The Fanconi Anemia Pathway Limits Human Papillomavirus Replication

Elizabeth E. Hoskins,[a] Richard J. Morreale,[a] Stephen P. Werner,[a] Jennifer M. Higginbotham,[a] Laimonis A. Laimins,[b] Paul F. Lambert,[c] Darron R. Brown,[d] Maura L. Gillison,[e] Gerard J. Nuovo,[f] David P. Witte,[g] Mi-Ok Kim,[h] Stella M. Davies,[i] Parinda A. Mehta,[i] Melinda Butsch Kovacic,[j] Kathryn A. Wikenheiser-Brokamp,[k] and Susanne I. Wells[a]

Division of Oncology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA[a]; Department of Microbiology and Immunology, Feinberg School of Medicine, Chicago, Illinois, USA[b]; McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA[c]; Division of Infectious Diseases, Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana, USA[d]; Internal Medicine—Hematology & Oncology, Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, Ohio, USA[e]; Viral Oncology, Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, Ohio, USA[f]; Division of Pathology and Laboratory Medicine, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA[g]; Division of Biostatistics and Epidemiology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA[h]; Division of Bone Marrow Transplantation and Immune Deficiency, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA[i]; Division of Asthma Research, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA[j]; and Division of Pathology and Laboratory Medicine and Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA[k]

High-risk human papillomaviruses (HPVs) deregulate epidermal differentiation and cause anogenital and head and neck squamous cell carcinomas (SCCs). The E7 gene is considered the predominant viral oncogene and drives proliferation and genome instability. While the implementation of routine screens has greatly reduced the incidence of cervical cancers which are almost exclusively HPV positive, the proportion of HPV-positive head and neck SCCs is on the rise. High levels of HPV oncogene expression and genome load are linked to disease progression, but genetic risk factors that regulate oncogene abundance and/or genome amplification remain poorly understood. Fanconi anemia (FA) is a genome instability syndrome characterized at least in part by extreme susceptibility to SCCs. FA results from mutations in one of 15 genes in the FA pathway, whose protein products assemble in the nucleus and play important roles in DNA damage repair. We report here that loss of FA pathway components FANCA and FANCD2 stimulates E7 protein accumulation in human keratinocytes and causes increased epithelial proliferation and basal cell layer expansion in the HPV-positive epidermis. Additionally, FANCD2 loss stimulates HPV genome amplification in differentiating cells, demonstrating that the intact FA pathway functions to restrict the HPV life cycle. These findings raise the possibility that FA genes suppress HPV infection and disease and suggest possible mechanism(s) for reported associations of HPV with an FA cohort in Brazil and for allelic variation of FA genes with HPV persistence in the general population.

Human papillomaviruses (HPVs) are double-stranded DNA viruses comprised of more than 100 subtypes, some of which, designated high risk, cause cervical and approximately one quarter of head and neck squamous cell carcinomas (HNSCCs) (1, 20, 21). HPV is associated primarily with HNSCCs that occur in the oropharynx, which includes the back of the tongue, walls of the throat, the soft palate, and the tonsils. Approximately 60% of oropharyngeal SCCs are caused by one of the high-risk types of HPV, and the vast majority of those are positive for HPV type 16 (HPV16) (10). Importantly, these HNSCCs are a distinct type distinguishable from HPV-negative tumors which have alcohol and tobacco use as the predominant risk factors (1).

HPV is the causative agent of nearly all cervical cancers and is best studied in that disease. Although HPV infections are common, with up to 90% of women estimated to be infected in their lifetime, most infections are cleared by the immune system and do not progress to cancer. A small fraction of HPV infections, however, lead to low-grade and high-grade squamous intraepithelial lesions and cervical cancer. Persistent infection is the most significant risk factor for developing cervical cancer. While the exact progression of events is still not entirely understood, replication, persistence, integration of the viral genome, and increased expression of the viral E6 and E7 oncogenes are important steps in malignant progression. The tumor suppressors p53 and Rb are frequently inactivated in human SCC either by mutation or by the E6 and E7 oncogenes following high-risk HPV infection. HPV E6 causes the degradation of p53 and the induction of hTERT. HPV E7 is considered the predominant oncogene and inhibits Rb, resulting in the activation of E2F transcription factors which stimulate proliferation and inhibit differentiation. While there are many genes whose expressions are altered by E7, the most critical downstream targets and pathways are still being elucidated. One downstream effector of E7 is ΔNp63, a transcription factor which is required both to maintain the pool of epithelial progenitor cells and to initiate skin stratification (30). Less is known about upstream signaling pathways which modify E7 abundance and their effects on the HPV life cycle and SCC development.

Fanconi anemia (FA) is a rare inherited autosomal recessive disease characterized by genome instability, bone marrow failure, leukemia, and an extreme susceptibility to anogenital and head

Received 17 February 2012 **Accepted** 14 May 2012
Published ahead of print 23 May 2012
Address correspondence to Susanne I. Wells, Susanne.Wells@cchmc.org.

doi:10.1128/JVI.00408-12

and neck SCCs (2, 3, 22). Detection of HPV in primary HNSCCs from individuals with FA has found both increased prevalence of HPV in the United States (19) and decreased prevalence in the Netherlands (31) relative to that of the general population; thus, the role of HPV in FA HNSCC remains controversial. A recent report from Brazil has demonstrated increased HPV prevalence in oral rinses from an FA cohort compared to that in controls (8). Furthermore, allelic variation of FA pathway components is a reported risk factor for HPV persistence in cervical cancers in the general population (32). FA is caused by mutations in one of 15 genes in the FA pathway (including FANCA and FANCD2). Their respective protein products assemble in the nucleus and play important roles in DNA repair. In response to DNA damage or replication stress, eight FA proteins, including FANCA, form the nuclear core complex. Complex formation leads to monoubiquitination of the FANCD2/FANCI (ID) complex, followed by colocalization with DNA repair proteins, including BRCA2/FANCD1 and BRCA1 (16, 17, 29). The bone marrow failure that characterizes FA may be associated with immune defects (9). However, successful bone marrow transplantation does not protect from early and aggressive SCC development in individuals with FA, suggesting that the FA pathway prevents epithelial cell transformation through cell-autonomous mechanisms.

Previous molecular studies have demonstrated that the HPV E7 oncogene upregulates the transcription and function of these FA proteins (13, 27, 28). Furthermore, FANCA loss in human keratinocytes caused increased cellular proliferation and hyperplasia in high-risk HPV-positive organotypic epithelial rafts (14). Additionally, FANCD2 knockout stimulated HPV16 E7-driven head and neck cancer in mice (26). While it is therefore evident that FA loss supports SCC phenotypes in epidermal models, it is unknown whether this is specific to the HPV-positive environment, and relevant molecular links and consequences for the HPV life cycle have not yet been reported. Here, we show that FA loss in keratinocytes and organotypic rafts leads to elevated levels of ΔNp63 and that both elevated ΔNp63 levels and cellular hyperplasia are specific to HPV-positive epidermis and not observed in HPV-negative epidermis. Furthermore, FANCA and FANCD2 knockdown stimulated the accumulation of E7 protein levels in the absence of any other HPV gene products and through a posttranscriptional mechanism. Finally, FA loss increased HPV genome amplification in organotypic rafts and differentiated cells, thus demonstrating that the HPV life cycle is attenuated by the intact FA pathway.

MATERIALS AND METHODS

Cell culture. Primary human foreskin keratinocytes (HFKs) (34), near diploid immortalized keratinocytes that form skin (NIKS), HPV16$^+$ NIKS, and CIN 612 cells were carried as previously described (4, 35). Irradiated J2-3T3 mouse fibroblasts were used as feeder cells.

Retroviral and lentiviral transduction. Nontargeting and FANCD2- and FANCA-specific short hairpin RNA (shRNA)-expressing lentiviral vectors were obtained through the Sigma MISSION shRNA program (Sigma-Aldrich) as previously described (14). LXSN 16E7, LXSN 16E7^{DLYC}, and pBABE 16E7 vectors were previously described (12, 15). Cells were transduced at 30 to 50% confluence for a total of 4 h for retroviruses or 8 h for lentiviruses in a final concentration of 8 μg/ml Polybrene. For the LXSN vectors, cells were selected for 2 days in 200 μg/ml G418. For the pBABE and lentiviral vectors, cells were selected and carried in 1 μg/ml puromycin.

Organotypic epithelial raft culture. Organotypic rafts were generated as previously described (25). After 16 days of growth, the resulting tissue was fixed in 2% paraformaldehyde, embedded in paraffin, sectioned, and morphologically examined by hematoxylin and eosin (H&E) staining.

Ventana *in situ* HPV DNA detection. A chromogenic *in situ* hybridization test for the detection of several high-risk HPV genomes, including HPV16 and HPV31(Ventana Medical Systems), was carried out by the CCHMC Division of Pathology. Sections were incubated with a fluorescein-tagged DNA probe and counterstained using the fully automated Ventana BenchMark instrument (6).

Immunofluorescence. Immunofluorescence was performed as previously described (14). Antibodies used were mouse monoclonal BrdU (1:100; Invitrogen), mouse monoclonal ΔNp63 (1:200; Santa Cruz), mouse monoclonal K10 (1:200), and rabbit polyclonal K14 (1:2,000) (both from Covance Research Products). Primary antibodies were detected with either a mouse or rabbit secondary antibody conjugated to rhodamine (1:200; Jackson ImmunoResearch). The sections were mounted in Vectashield/DAPI (4′,6-diamidino-2-phenylindole; Vector Labs) mounting medium and analyzed using a Leica DMI6000 microscope (Leica) and Openlab5 software (Improvision).

Immunohistochemistry. Tissue sections were deparaffinized with xylene and rehydrated. Following antigen retrieval in 10 mM sodium citrate, sections were blocked in M.O.M. mouse IgG (Vector Laboratories) in a humidity chamber for 1 h. Sections were then incubated for 1 h with a mouse monoclonal BrdU antibody (1:100; Invitrogen). After washes in phosphate-buffered saline (PBS), sections were incubated with biotinylated secondary antibody for 30 min. Staining was developed in DAB (Vector Laboratories) and counterstained with nuclear fast red (Biomeda; M209). Sections were then dehydrated in ethanol followed by xylene and mounted in Permount (Fisher Scientific).

Keratinocyte differentiation in methylcellulose. Cells were suspended in F medium containing 1.5% methylcellulose, allowed to differentiate for 24 or 48 h, and harvested by stringent washes with cold PBS (33). DNA or protein was extracted for analysis.

Calcium differentiation. A total of 4×10^5 cells were seeded in 60-mm dishes on J2-3T3 feeder fibroblasts. When 80% confluence was reached, feeders were removed by 0.05% trypsin/1× EDTA treatment, and cells were calcium starved overnight with Epilife medium plus HKGS (Cascade Biologics). Epilife medium containing 20% fetal bovine serum (FBS) and 5 mM $CaCl_2$ was added, and cells were allowed to differentiate for 24 or 48 h. Media and cells were collected, and DNA or protein was extracted for analysis.

Quantitative RT-PCR. RNA was reverse transcribed into cDNA using the Quantitech reverse transcription (RT) kit (Qiagen). Primers used were HPV16 E7 (5′-GAG ATA CAC CTA CAT TGC ATG-3′ and 5′-TGC TTT GTA CGC ACA ACC GAA-3′) and c-abl (5′-TGC CCA GAG AAG GTC TAT GAA CT-3′ and 5′-AAC ATT GTT TCA AAG GCT TGG TG-3′). cDNA was run in duplicate for each sample in 2× SYBR green master mix (Applied Biosystems) using an ABI 7300 real-time PCR system. A serial dilution of cDNA was used to generate a standard curve for HPV16 E7 and c-abl. Threshold cycle (C_T) values were compared back to the standard curve, and HPV16 E7 was normalized to the c-abl control.

Quantitative PCR. Primers used were HPV31 E7 (5′-AAT TAC CCG ACA GCT CAG ATG-3′ and 5′-GGC ACA CGA TTC CAA ATG AG) and GAPDH (5′-CCG CAG AGG TGT GGT GGC TG-3′ and 5′-CAG CCT GGC CTT TGG GGT CG-3′). DNA was run in duplicate for each sample in 2× SYBR green master mix (Applied Biosystems) using an ABI 7300 real-time PCR system. A serial dilution of CIN 612 DNA was used to generate a standard curve for HPV31 E7 and GAPDH. C_T values were compared back to the standard curve and HPV31 E7 was normalized to the GAPDH control.

Western blot analyses. Whole-cell protein extracts were harvested with lysis buffer (0.1% Nonidet P-40, 250 mM NaCl, 50 mM Tris [pH 7.4], and 5 mM EGTA) and supplemented with protease inhibitor cocktail

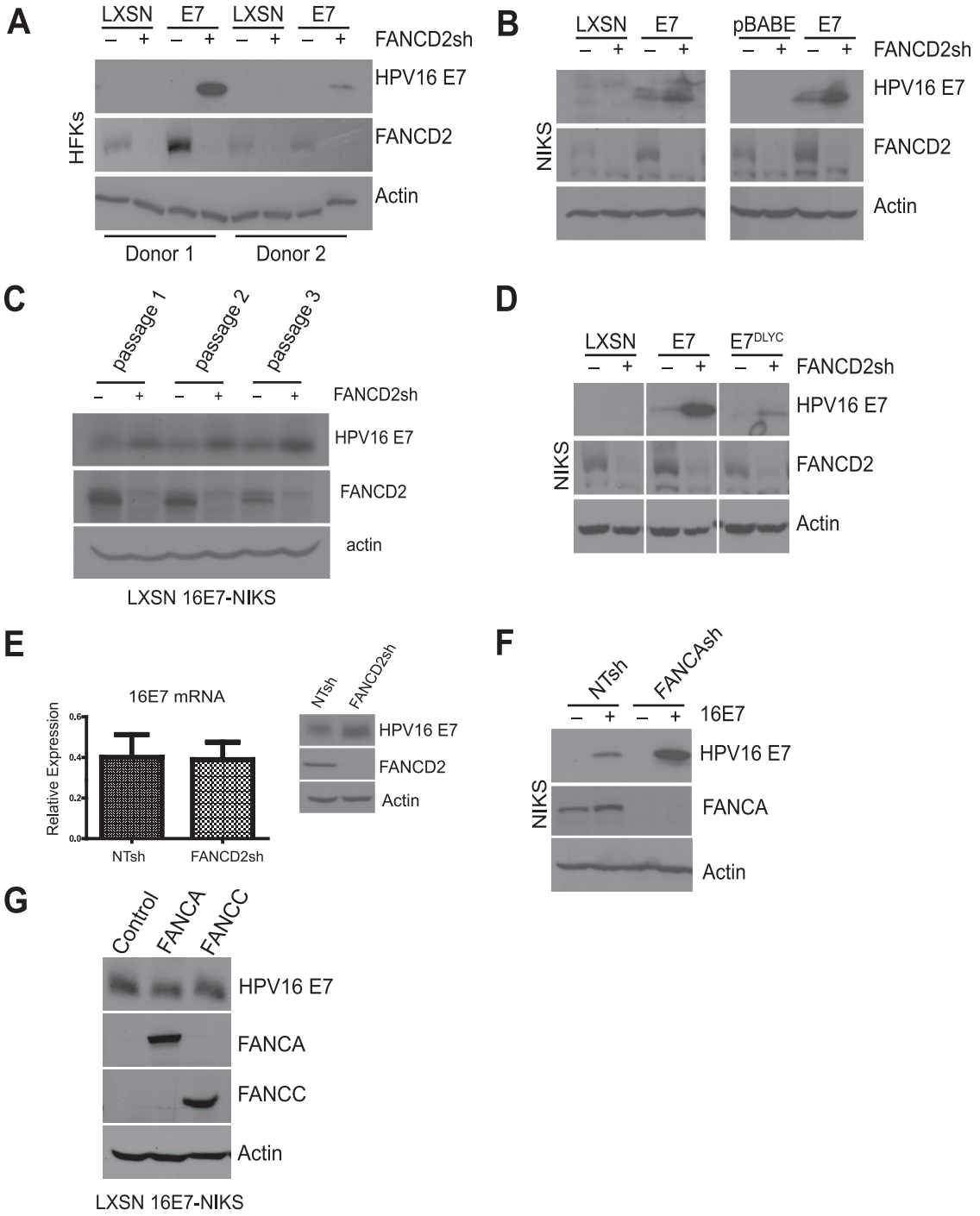

FIG 1 FA gene knockdown increases HPV16 E7 protein levels in keratinocytes, though E7 transcript levels are unchanged. (A) HPV16 E7 protein levels are increased in FANCD2-depleted LXSN-E7-expressing HFKs from 2 different donors (−, nontargeting short hairpin control; +, FANCD2 knockdown) (B) and with LXSN-E7- or pBABE-E7-transduced NIKS (C). Elevated E7 protein levels are maintained for at least 3 cell passages in LXSN-E7-transduced NIKS (D) and are still observed with a mutant unable to bind and degrade Rb ($E7^{DLYC}$). (E) Although E7 protein levels are consistently higher when FANCD2 is knocked down, E7 mRNA levels remain unchanged ($n = 3$; $P = 0.94$) (means ± SEM). (F) Knockdown of FANCA produces a similar increase in E7 protein (G), but overexpression of FANCA or FANCC does not alter E7 levels (G).

(Pharmingen), 10 mM NaF, 5 mM $NaVO_3$, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Blotting was performed as previously described (34). Antibodies used were mouse monoclonal ΔNp63 (Santa Cruz), rabbit polyclonal FANCD2 (Novus), rabbit polyclonal FANCA (Cascade Bioscience), or mouse pan-actin-specific antiserum (gift from J. Lessard, CCHMC). For Western blots in which HPV16 E7 was also probed, whole-cell extracts were harvested in supplemented radioimmunoprecipitation assay (RIPA) buffer. A primary antibody mix of mouse monoclonal anti-16E7 antibody (1:150, 8C9 [Invitrogen]; 1:200, ED17 [Santa Cruz]) and SuperSignal West Femto substrate (Pierce) was used for detection.

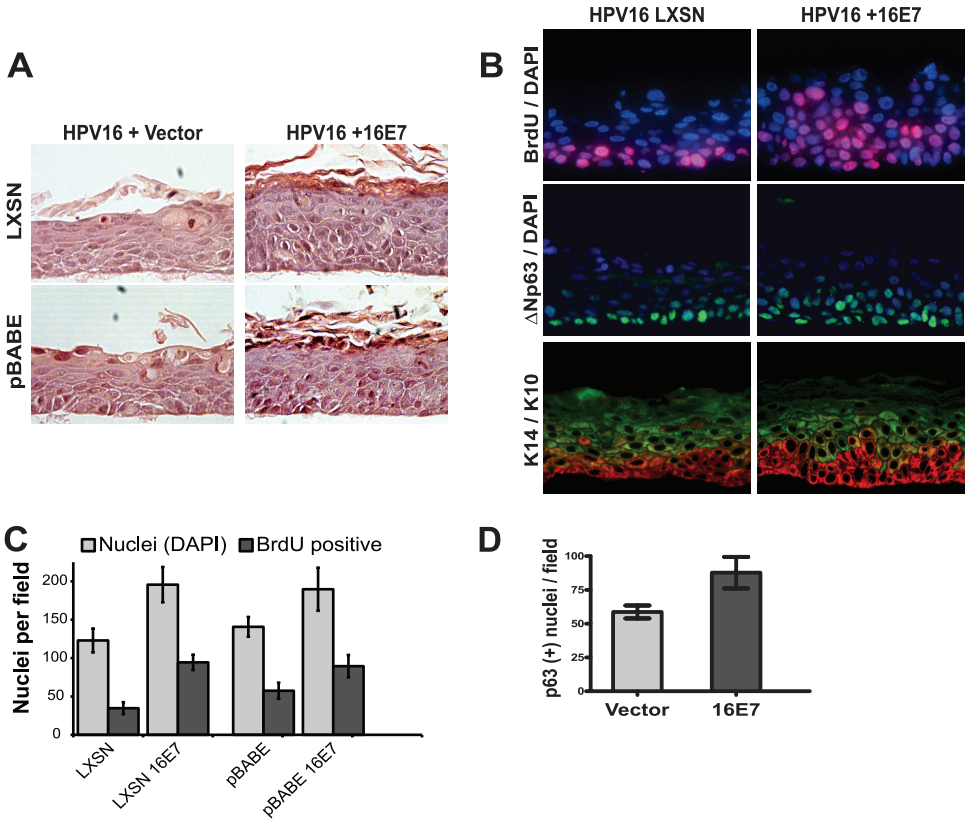

FIG 2 Exogenous E7 oncoprotein expression exacerbates HPV-driven hyperplasia. (A) Retroviral LXSN-E7 or pBABE-E7 expression in NIKS already harboring HPV16 causes hyperplasia in organotypic rafts (shown by H&E). (B to D) Increased proliferation (BrdU), increased number of ΔNp63-positive cells, and expansion of the basal cell compartment (K14/K10) (means ± SD).

Statistics. Time course experiments measuring HPV genome copy number in cells transduced with FANCD2 shRNA or nontargeting shRNA were performed in triplicate ($n = 3$ independent experiments). Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests using an α value of 0.05. All other significance was determined using a Student *t* test. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS

FA gene knockdown stimulates the accumulation of E7 in primary and immortalized keratinocytes. Our previous data showed that HPV-immortalized keratinocytes derived from individuals with FA were more proliferative than their isogenic, gene-corrected counterparts. Furthermore, FANCA and FANCD2 knockdown in HPV-positive keratinocytes stimulated proliferation. Since loss of FA pathway function in HPV-positive cells exacerbated epidermal proliferation and hyperplasia (14), and since these are phenotypes closely associated with HPV E7 protein activities, we tested directly whether FA knockdown could regulate E7. Levels of E7 oncoprotein were examined by Western blot analysis in E7-transduced, FANCD2-knockdown versus control keratinocytes. FANCD2 depletion in E7-expressing primary human foreskin keratinocytes (HFKs) was sufficient to stimulate E7 protein accumulation compared to cells transduced with the nontargeting short hairpin control (NTsh) (Fig. 1A). Transduction of near-diploid immortalized keratinocytes that form skin (NIKS) with two different retroviral E7 expression vectors (LXSN or pBABE) yielded similar results (Fig. 1B). The elevated E7 protein level in NIKS was maintained for at least 3 cell passages (Fig. 1C). Furthermore, the observed E7 accumulation was not dependent upon the ability of E7 to bind and degrade the tumor suppressor Rb, as shown by transduction with a mutant E7 construct defective for binding RB and p21 (DLYC E7) (Fig. 1D). Detection of E7 message levels in FANCD2sh versus NTsh keratinocytes did not reveal significant differences; thus, we conclude that E7 regulation occurs posttranscriptionally (Fig. 1E). E7 protein levels also increased following knockdown of FANCA, a pathway component upstream of FANCD2 (Fig. 1F). Finally, when either FANCA or FANCC were overexpressed in E7-NIKS, protein levels of E7 remained unchanged compared to those of vector control-transduced cells (Fig. 1G). Taken together, these data show that FA deficiency stimulates E7 accumulation in the absence of other HPV proteins. The finding that FA gene overexpression does not repress E7 protein levels suggests that E7 regulation by FA is not a straightforward epistatic relationship.

Previous research indicates that HPV-driven hyperplasia is enhanced in FA-deficient cells (14). One possibility is that elevated E7 levels mediate the phenotype observed upon FA pathway loss. We therefore determined whether additional E7 expression in cells stably harboring episomal HPV was sufficient to exacerbate hyperplasia and to further disrupt differentiation. NIKS containing 30 to 50 copies of HPV16 genomic DNA was transduced with a retroviral vector expressing HPV16 E7 (either LXSN or pBABE)

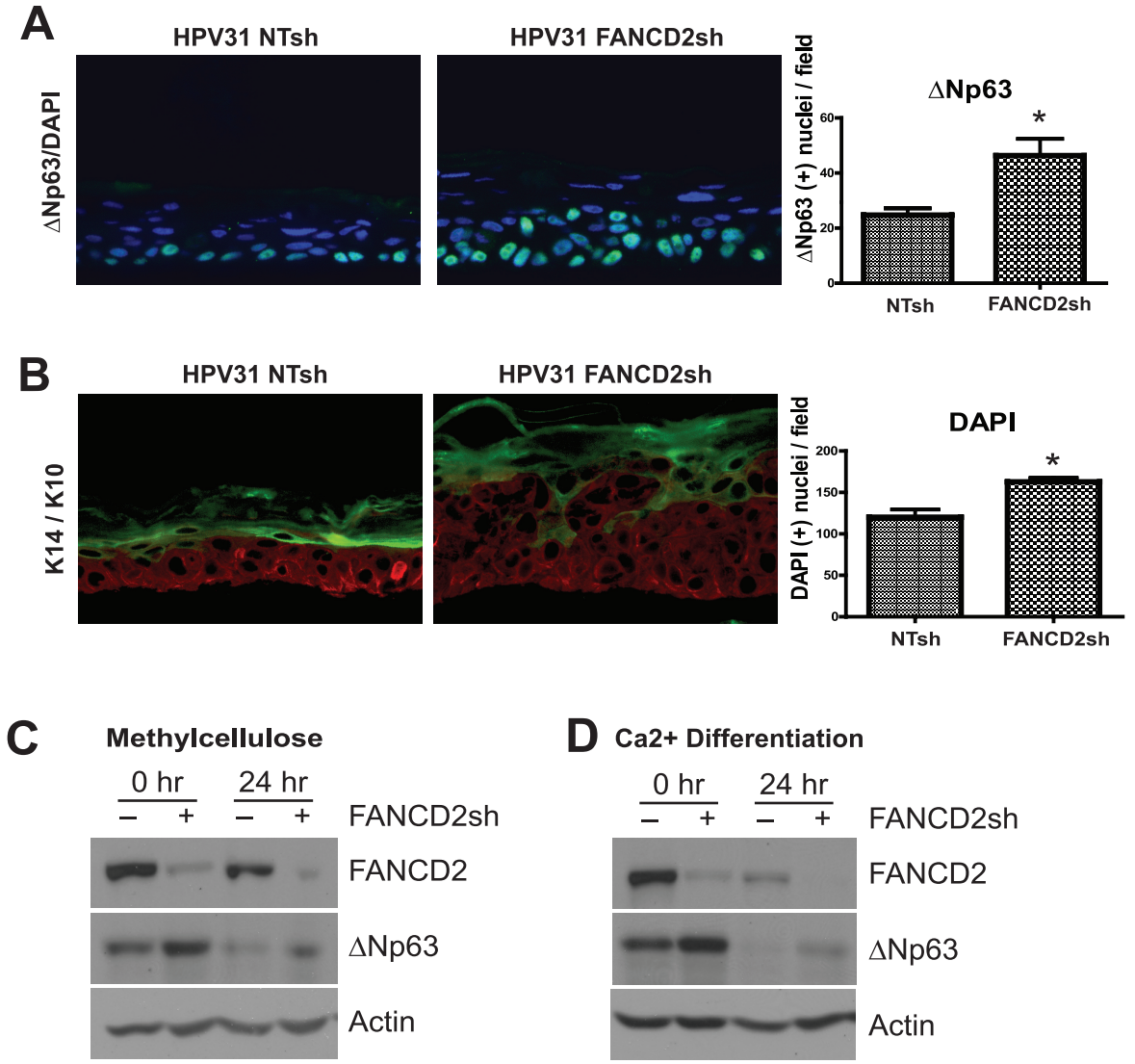

FIG 3 FANCD2 knockdown mimics E7 overexpression by stimulating ΔNp63 expression and expanding the basal cell compartment. (A) ΔNp63-positive cells are increased in FANCD2-knockdown HPV31$^+$ CIN 612 cells (*, $n = 3$; $P < 0.05$) (means ± SEM). (B) K14/K10-positive cell layers are skewed toward the K14-positive basal cell compartment. Total nuclei are also increased as quantified by DAPI counts (*, $n = 3$; $P < 0.05$) (means ± SEM). ΔNp63 protein levels are elevated under nondifferentiating conditions and following 24 h of differentiation in methylcellulose (C) or in calcium in FANCD2sh CIN 612 cells (D).

and subsequently cultured in organotypic rafts, a three-dimensional system that permits keratinocyte differentiation and formation of fully stratified epithelium. E7-transduced compared to control rafts exhibited increased proliferation in excess of that induced by HPV alone, as shown by exaggerated hyperplasia (H&E), BrdU incorporation, and nuclear counts (means ± standard deviations [SD]) (Fig. 2A to C). Squamous cell carcinoma is thought to originate in the stem and/or progenitor cell compartment. Proliferation correlated with an expansion of this basal cell compartment, marked by increased numbers of ΔNp63- and K14-positive cells (means ± SD) (Fig. 2B and D).

Differentiation abnormalities in response to FA loss are specific to HPV-positive epidermis. E7 expression gains within HPV16-positive epidermis caused abnormal differentiation and augmented hyperplasia reminiscent of FA-deficient HPV16-positive epidermis (14) (Fig. 2B). Viral replication is tightly dependent upon host cell differentiation, and the consequences of deficient FA pathway function on HPV replication are unknown. The HPV31 CIN 612 cell line is known to productively replicate HPV genomes under multiple differentiation conditions (4), and we therefore first characterized FANCD2sh-induced differentiation abnormalities in these cells. FANCD2 loss increased the proportion of ΔNp63 ($P < 0.05$) (means ± standard errors of the means [SEM]) and K14-positive cells compared to those of NTsh control raft cultures (Fig. 3A and B). In all cases, control NTsh rafts had a continuous and clearly defined K10 layer, while the K10 layers of the FANCD2sh rafts were irregular and often absent (Fig. 3B). The loss of K10-positive cell layers in the FANCD2sh rafts correlated with increasing numbers of K14-positive cells in the basal compartment, often extending as clusters into suprabasal areas, and an overall increase in total nuclei as quantified by DAPI counts ($P < 0.05$) (means ± SEM) (Fig. 3B). Elevated ΔNp63 expression in

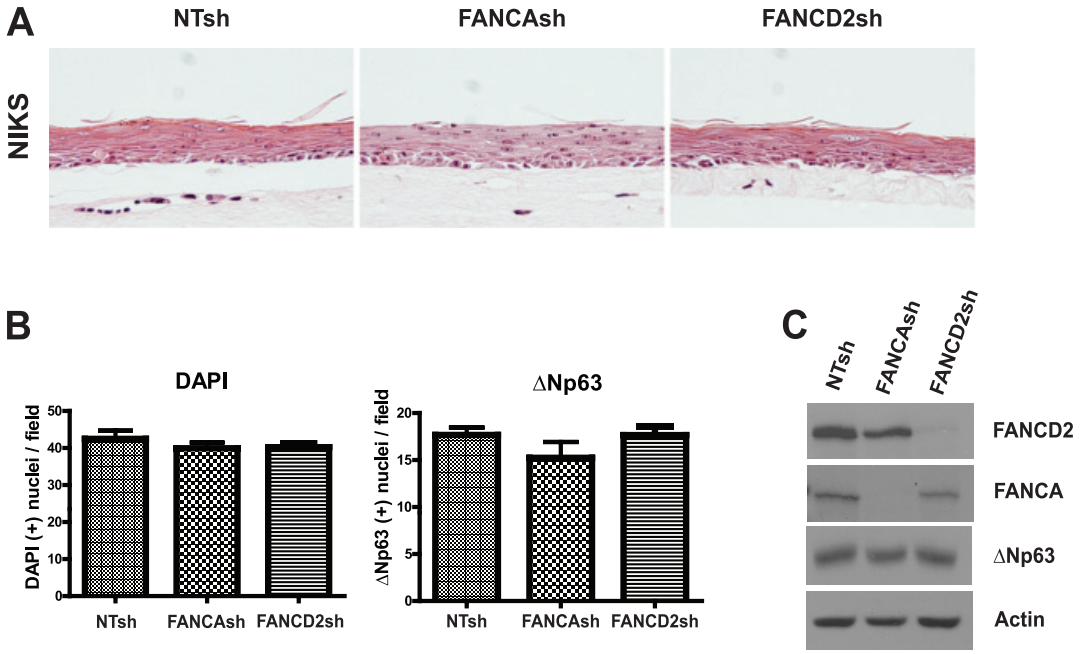

FIG 4 Tumor phenotypes are not observed in FA-deficient, HPV-negative keratinocytes. (A) Hyperplasia (H&E) does not occur following knockdown of FANCA or FANCD2 in HPV-negative NIKS, nor are DAPI counts or ΔNp63-positive cell numbers (B) (means ± SEM) or ΔNp63 protein levels significantly different by Western blot analysis (C).

response to FANCD2 knockdown was also observed by Western blot analysis under nondifferentiating conditions or following differentiation either by methylcellulose (Fig. 3C) or calcium (Fig. 3D). Importantly, FANCD2 depletion in HPV-negative NIKS did not result in hyperplasia (Fig. 4A), increased ΔNp63 expression, or the number of DAPI-positive cells in organotypic rafts (Fig. 4B and C), thus demonstrating for the first time that at least some of the hyperplastic phenotypes caused by FA loss are specific to the HPV-positive environment.

FA loss increases HPV DNA replication. To determine the effects of a deficient FA pathway on HPV replication, we performed *in situ* hybridization assays for viral DNA detection in organotypic raft cultures generated with cells containing HPV16 or HPV31. These were stably transduced either with NTsh control vector or FANCD2 shRNA expression vector. Assay sensitivity was below the 30 to 50 genomes present per cell in each case; thus, a positive signal denotes productive replication. As previously described (14), hyperplasia and cellular proliferation were increased following FANCD2 knockdown in HPV16$^+$ NIKS (Fig. 5A). Interestingly, the number of cells that productively replicated HPV16 viral genomes was also increased (means ± SD) (Fig. 5B), suggesting that productive HPV replication is more abundant following perturbation of FA pathway function. Increased replication was observed immediately after FANCD2 knockdown and at passage 28 (Fig. 5B). To confirm these findings, we used three independent methods to assay the replication of the closely related high-risk HPV31 in FANCD2-deficient CIN 612 cells. Similar to HPV16, replication of HPV31 was increased in organotypic rafts as measured by *in situ* hybridization (Fig. 6A). HPV31 replication was also increased in FANCD2 knockdown CIN 612 cells relative to NTsh controls when embedded in methylcellulose (Fig. 6B). Finally, when cells were differentiated in high-

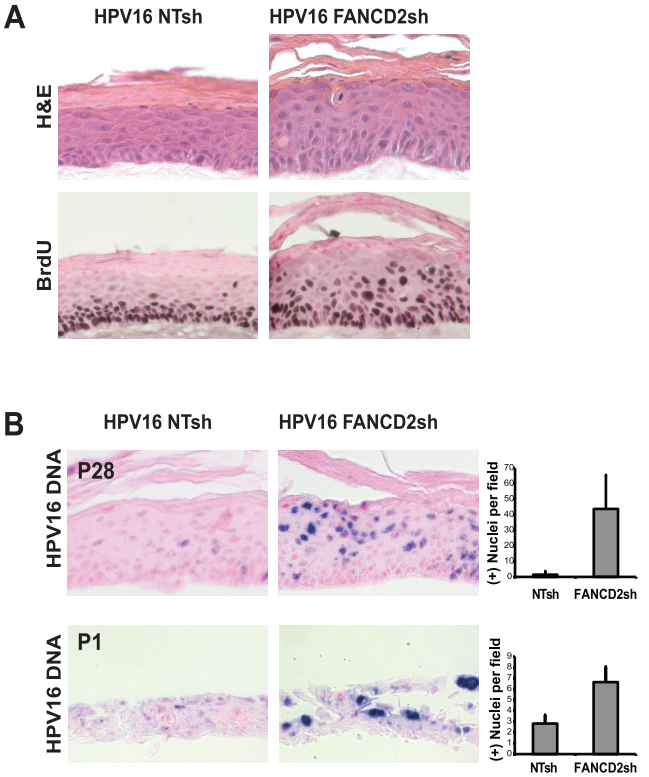

FIG 5 FANCD2 knockdown stimulates HPV16 replication. Organotypic raft culture hyperplasia (H&E) and cellular proliferation (BrdU) (A) and viral replication (*in situ* hybridization for HPV genomes) (B) are increased in FANCD2-deficient HPV16$^+$ NIKS (means ± SD). P28, passage 28; P1, passage 1.

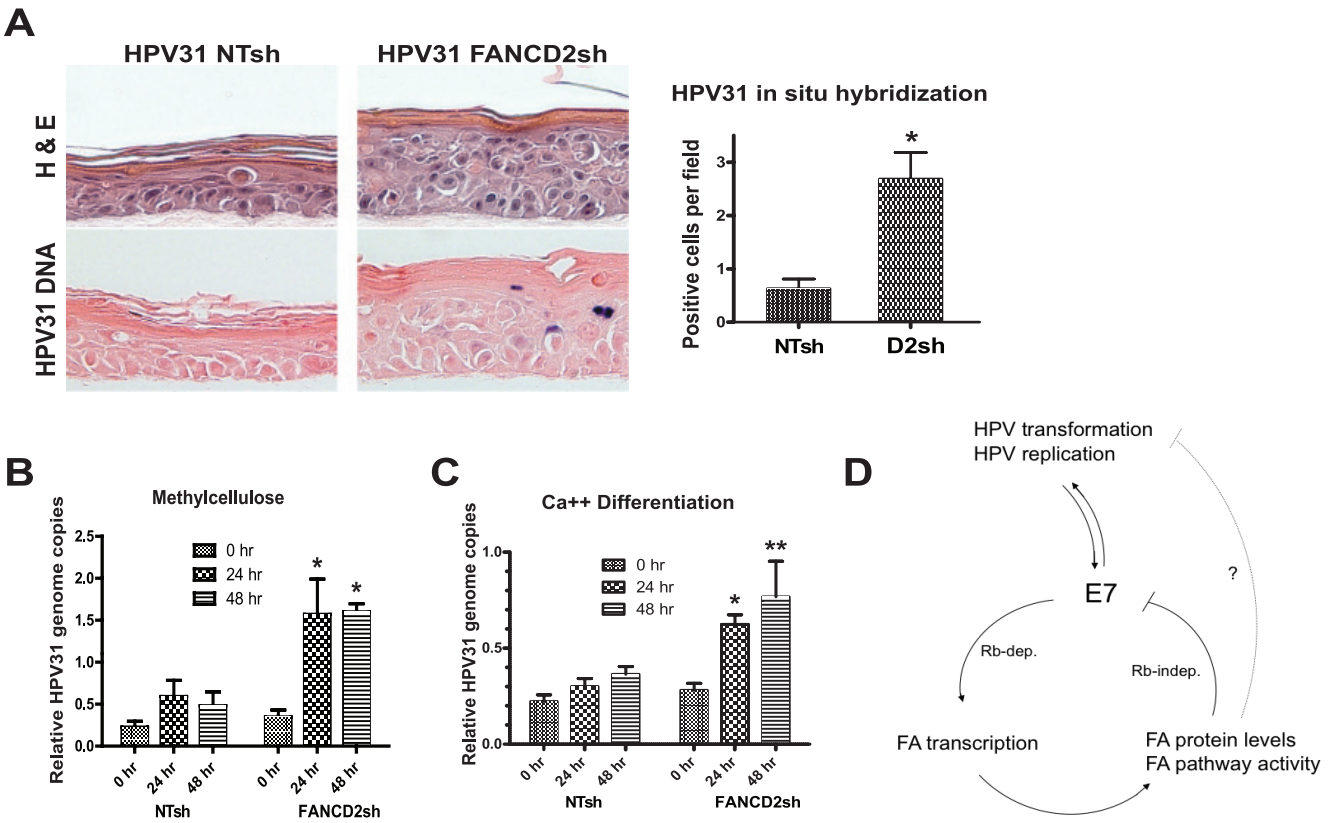

FIG 6 FANCD2 knockdown stimulates HPV31 replication. (A) Organotypic raft hyperplasia (H&E) and viral replication (*in situ* hybridization for HPV genomes) are upregulated in FANCD2-deficient HPV31$^+$ CIN 612 cells. Increased numbers of cells that productively replicate HPV are observed in FANCD2-deficient CIN 612 rafts (*, $n = 3$; $P < 0.05$) (means ± SEM). Increased genome copy number is observed during methylcellulose differentiation (DNA quantified by qPCR) (*, $n = 3$; $P < 0.05$) (means ± SEM) (B) or calcium-induced differentiation (DNA quantified by qPCR) (**, $n = 3$; $P < 0.01$; *, $P < 0.05$) (means ± SEM) (C). (D) Model depicting FA and E7 cross talk. A negative feedback loop is depicted wherein the FA pathway limits HPV replication and transformation through E7-dependent mechanisms. Arrows indicate activation, and lines indicate repression. Under normal circumstances, HPV infection and E7 production upregulate FA gene expression through Rb inhibition and lead to FANCD2 monoubiquitination and pathway activation. The intact FA pathway in turn limits E7 accumulation through unknown but Rb-independent mechanisms. Loss of FA function and consequent release of E7 from the negative feedback loop may hyperstimulate HPV replication and transformation. The dotted line indicates the possibility of E7-independent effects on viral phenotypes.

calcium medium, FANCD2 knockdown cells showed significantly greater HPV31 replication compared to NTsh controls (Fig. 6C). HPV31 replication also increased when the FA pathway was perturbed via FANCA knockdown (data not shown). Taken together, these data show that FA loss stimulates the HPV replicative life cycle.

DISCUSSION

We demonstrate here that the FA pathway, widely known for its important role in DNA repair, controls the replication of a major human pathogen, HPV. In following up on this finding, we uncovered a new and unexpected role for FA in regulating the accumulation of the viral E7 oncoprotein. HPV infection is the cause of a subset of anogenital and head and neck cancers and is responsible for 4% of all cancers worldwide. In the year 2000 alone, the total direct medical costs attributable to sexually transmitted HPV infection in the United States were an estimated $2.9 billion, with an overwhelming majority of the funds spent on monitoring and treating abnormal pap results (7). Blanket therapy has thus achieved tremendous reductions in the incidence of the HPV-associated cancer but has come at a cost to society and distress to affected individuals who are often treated unnecessarily. Importantly, decades of HPV research have not identified specific genetic modifiers of high-risk HPV infection and transformation, thus limiting our ability to identify and focus on the population at greatest risk for malignant conversion. Since HPV infection is linked to human cancer, the finding that FA loss stimulates HPV oncogene production and the viral life cycle has implications for our understanding of HPV-associated squamous cell carcinoma. The observed HPV hyperreplication in FA-deficient epidermis may be relevant for individuals with FA: recent data from Brazil suggest increased HPV prevalence in oral rinses from an FA cohort compared to controls. However, whether this prevalence translates into viral persistence and ultimately disease remains to be determined, particularly since HPV genome presence in FA SCCs is controversial.

FANCD2 or FANCA knockdown in E7-transduced keratinocytes increased the accumulation of E7 protein but did not affect message levels. It is likely that E7 stabilization is responsible for the observed protein accumulation, but we emphasize that these data do not rule out other posttranscriptional regulatory mechanisms. Experiments to define the relevant molecular pathways and processes which link the FA pathway with E7 production are now critical. The E7 protein results were extremely reproducible in E7-

expressing cells and particularly when E7 expression was low. However, it is important to note that a similar induction by FA loss was only sometimes, and not consistently, observed in cells coexpressing E6 and E7, either for technical or biological reasons. In contrast, upregulated ΔNp63 protein levels are observed in E6/E7-positive cells upon FA loss (Fig. 3C and D), together with an expansion of the ΔNp63-associated stem and progenitor cell compartment (Fig. 3A). Published data have defined ΔNp63 as an induced target of E7, and E7 expression was sufficient for stimulating ΔNp63 expression through inhibition of miR-203 microRNA production (23). Moreover, ΔNp63 expression promoted productive viral DNA amplification (24). It is possible that E7 stimulates ΔNp63 expression to support FA phenotypes and viral replication. Alternatively, these events may be separate from each other. Regardless, increased copy numbers of transcriptionally active HPV genome templates in infected FA cells would be expected to stimulate E6/E7 oncogene expression and thus cancer risk. In the cervix, high viral load and persistent viral infection are strongly associated with cytological abnormalities and disease severity, specifically for HPV16 (11, 18). HPV E7 is known to promote transformation in cells where the life cycle has been interrupted, which often occurs through spontaneous integration of the viral genome (5). Loss of the FA pathway may therefore increase the risk of viral integration not only through associated genome instability but potentially also through stimulation of viral replication and thus increased genome load. In conclusion, our data demonstrate that the FA pathway restricts HPV E7 protein levels and viral genome amplification in differentiated epidermis and should encourage epidemiological studies of HPV prevalence in FA populations and in sporadic SCCs which are deficient for FA gene functions.

ACKNOWLEDGMENTS

We thank E. Krumpelbeck for tissue processing and sectioning, M. Taylor for the Ventana HPV *in situ* detection, J. Lessard for the pan-actin antibody, and D. Galloway for E7 retroviral vectors.

Funding was provided by R01 CA102357 and by a grant from the Fanconi Anemia Research Fund.

S.I.W., E.E.H., and R.J.M. conceived the study and experimental design, performed and analyzed experiments, and cowrote the manuscript. S.P.W. and J.M.H. performed experiments and provided experimental advice. D.R.B., M.B.K., M.L.G., and G.J.N. provided intellectual and experimental support with HPV detection and diagnostics. P.F.L. and L.A.L. provided reagents and critical technical advice. S.M.D. and P.A.M. provided intellectual advice on FA clinical phenotypes and data interpretation. K.A.W.-B. analyzed tissue pathology, D.P.W. performed Ventana *in situ* hybridization, and M.-O.K. supported statistical analyses.

REFERENCES

1. **Ang KK, et al.** 2010. Human papillomavirus and survival of patients with oropharyngeal cancer. N. Engl. J. Med. **363**:24–35.
2. **Auerbach AD.** 2009. Fanconi anemia and diagnosis. Mutat. Res. **668**:4–10.
3. **Bagby GC, Alter BP.** 2006. Fanconi anemia. Semin. Hematol. **43**:147–156.
4. **Bedell MA, et al.** 1991. Amplification of human papillomavirus genomes *in vitro* is dependent on epithelial differentiation. J. Virol. **65**:2254–2260.
5. **Bodily J, Laimins LA.** 2011. Presistence of human papillomavirus infection: keys to malignant progression. Trends Microbiol. **19**:33–39.
6. **Bolick DR, et al.** 2003. Laboratory implementation of human papillomavirus testing. Arch. Pathol. Lab Med. **127**:984–990.
7. **Chesson HW, Blandford JM, Gift TL, Tao G, Irwin KL.** 2004. The estimated direct medical cost of sexually transmitted diseases among American youth, 2000. Persp. Sex. Reprod. Health **36**:11–19.
8. **de Araujo MR, et al.** 2010. High prevalence of oral human papillomavirus infection in Fanconi's anemia patients. Oral Dis. **17**:572–576.
9. **Fagerlie SR, Bagby GC.** 2006. Immune defects in Fanconi anemia. Crit. Rev. Immunol. **26**:81–96.
10. **Gillison M.** 2010. HPV and its effect on head and neck cancer prognosis. Clin. Adv. Hematol. Oncol. **8**:680–682.
11. **Gravitt PE, et al.** 2007. High load for most high risk human papillomavirus genotypes is associated with prevalent cervical cancer precursors but only HPV16 load predicts the development of incident disease. Int. J. Cancer **121**:2787–2793.
12. **Halbert CL, Demers GW, Galloway DA.** 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J. Virol. **65**:473–478.
13. **Hoskins EE, et al.** 2008. Coordinate regulation of Fanconi anemia gene expression occurs through the Rb/E2F pathway. Oncogene **27**:4798–4808.
14. **Hoskins EE, et al.** 2009. Fanconi anemia deficiency stimulates HPV-associated hyperplastic growth in organotypic epithelial raft culture. Oncogene **28**:674–685.
15. **Jones DL, Thompson DA, Munger K.** 1997. Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to HPV type 16 E7-induced apoptosis. Virology **239**:97–107.
16. **Kennedy RD, D'Andrea AD.** 2005. The Fanconi anemia/BRCA pathway: new faces in the crowd. Genes Dev. **19**:2925–2940.
17. **Kim Y, et al.** 2011. Mutations of the SLX4 gene in Fanconi anemia. Nat. Genet. **43**:142–146.
18. **Kovacic MB, et al.** 2006. Relationships of human papillomavirus type, qualitative viral load, and age with cytologic abnormality. Cancer Res. **66**:10112–10119.
19. **Kutler DI, et al.** 2003. Human papillomavirus DNA and p53 polymorphisms in squamous cell carcinomas from Fanconi anemia patients. J. Natl. Cancer Inst. **95**:1718–1721.
20. **Leemans CR, Braakhuis BJM, Brakenhoff RH.** 2011. The molecular biology of head and neck cancer. Nat. Rev. **11**:9–22.
21. **Lowy DR, Munger K.** 2010. Prognostic implications of HPV in oropharyngeal cancer. N. Engl. J. Med. **363**:82–84.
22. **Mathew CG.** 2006. Fanconi anaemia genes and susceptibility to cancer. Oncogene **25**:5875–5884.
23. **Melar-New M, Laimins LA.** 2010. Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J. Virol. **84**:5212–5221.
24. **Mighty KK, Laimins LA.** 2011. p63 is necessary for the activation of human papillomavirus late viral functions upon epithelial differentiation. J. Virol. **85**:8863–8869.
25. **Nakahara T, Peh WL, Doorbar J, Lee D, Lambert PF.** 2005. Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle. J. Virol. **79**:13150–13165.
26. **Park JW, et al.** 2010. Deficiencies in the Fanconi anemia DNA damage response pathway increase sensitivity to HPV-associated head and neck cancer. Cancer Res. **70**:9959–9968.
27. **Spardy N, et al.** 2007. The human papillomavirus type 16 E7 oncoprotein activates the Fanconi anemia (FA) pathway and causes accelerated chromosomal instability in FA cells. J. Virol. **81**:13265–13270.
28. **Spardy N, Duensing A, Hoskins EE, Wells SI, Duensing S.** 2008. 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. **68**:9954–9963.
29. **Stoepker C, et al.** 2011. SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. Nat. Genet. **43**:138–141.
30. **Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA.** 2006. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev. **20**:3185–3197.
31. **van Zeeburg HJ, et al.** 2008. Clinical and molecular characteristics of squamous cell carcinomas from Fanconi anemia patients. J. Natl. Cancer Inst. **100**:1649–1653.
32. **Wang SS, et al.** 2009. Common variants in immune and DNA repair genes and risk for human papillomavirus persistence and progression to cervical cancer. J. Infect. Dis. **199**:20–30.
33. **Wilson R, Laimins LA.** 2005. Differentiation of HPV-containing cells using organotypic "raft" culture or methylcellulose. Methods Mol. Med. **119**:157–169.
34. **Wise-Draper TM, et al.** 2006. Apoptosis inhibition by the human DEK oncoprotein involves interference with p53 functions. Mol. Cell. Biol. **26**:7506–7519.
35. **Wise-Draper TM, et al.** 2009. DEK proto-oncogene expression interferes with the normal epithelial differentiation program. Am. J. Pathol. **174**:71–81.