

E6-Associated Protein Is Required for Human Papillomavirus Type 16 E6 to Cause Cervical Cancer in Mice

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Abstract

High-risk human papillomaviruses (HPV) cause certain anogenital and head and neck cancers. *E6*, one of three potent HPV oncogenes that contribute to the development of these malignancies, is a multifunctional protein with many biochemical activities. Among these activities are its ability to bind and inactivate the cellular tumor suppressor *p53*, induce expression of telomerase, and bind to various other proteins, including Bak, E6BP1, and E6TP1, and proteins that contain PDZ domains, such as hScrib and hDlg. Many of these activities are thought to contribute to the role of *E6* in carcinogenesis. The interaction of *E6* with many of these cellular proteins, including *p53*, leads to their destabilization. This property is mediated at least in part through the ability of *E6* to recruit the ubiquitin ligase E6-associated protein (E6AP) into complexes with these cellular proteins, resulting in their ubiquitin-mediated degradation by the proteasome. In this study, we address the requirement for E6AP in mediating acute and oncogenic phenotypes of *E6*, including induction of epithelial hyperplasia, abrogation of DNA damage response, and induction of cervical cancer. Loss of E6AP had no discernible effect on the ability of *E6* to induce hyperplasia or abrogate DNA damage responses, akin to what we had earlier observed in the mouse epidermis. Nevertheless, in cervical carcinogenesis studies, there was a complete loss of the oncogenic potential of *E6* in mice nulligenic for E6AP. Thus, E6AP is absolutely required for *E6* to cause cervical cancer. *Cancer Res*; 70(12); 5064–73. ©2010 AACR.

Introduction

Human papillomaviruses (HPV) are etiologically associated with cancers of the head and neck and the anogenital tract, which includes the cervix. Infection with HPVs is attributed with almost all cases of cervical cancer (1, 2). Cervical cancer accounts for ~5% of the world's total tumor burden (3) and is the second leading cause of cancer-related deaths among women (4, 5). Approximately 70% of all cervical cancers are attributed to infection with the "high-risk" HPV types 16 or 18. In cervical cancers, two HPV genes, *E6* and *E7*, are expressed (6, 7). HPV *E6* and *E7* have transforming and oncogenic properties in tissue culture (8, 9) and in animal models (10–12). *E6* and *E7* are primarily known for their abilities to target the tumor suppressors *p53* (13, 14) and *pRb* (15), respectively, for inactivation and ubiquitin-mediated degradation.

High-risk *E6* is known to target *p53* for ubiquitin-mediated degradation through its interaction with the E3 ligase E6-associated protein (E6AP). Similarly, *E6* has been found to induce the degradation of other cellular proteins, and in many cases, it is mediated through interactions with E6AP (16, 17). Although the majority of data support the requirement of E6AP in *E6*-mediated degradation of *p53* (18, 19), our previous studies in the mouse skin indicate that HPV16 *E6* is able to target *p53* for inactivation and degradation in the absence of E6AP (20). Results from other groups using either *in vitro* or *in vivo* systems also suggest the existence of E6AP-independent means for high-risk HPV *E6* to target *p53* for ubiquitin-mediated degradation (21, 22). Therefore, it remained unclear what role *E6AP* would have in *E6*-mediated acute and oncogenic phenotypes in the context of the cervix.

In mice, *E6* and *E7* cooperate to induce cervical carcinogenesis (23, 24). HPV *E6* induces cervical cancer through its ability to inactivate *p53* and other cellular proteins, including PDZ-containing proteins that interact directly with *E6* (24, 25). Similar requirements were needed for *E6* to induce skin cancers in mice (26). Because HPV16 *E6* can target *p53* for inactivation in the skin through E6AP-independent means (20), it was of interest to determine the importance of E6AP in cervical carcinogenesis.

Further interest fueled our pursuits of *E6AP* in the cervix. In addition to its function as an E3 ubiquitin ligase, *E6AP* also functions as a steroid receptor coactivator (27) and may be differentially regulated in cancers of the breast and prostate (28, 29). Recent evidence shows that the nuclear

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hormone receptor estrogen receptor α (*ER α*) is a necessary cofactor in HPV-mediated cervical carcinogenesis (30). Given the involvement of E6AP in the regulation of nuclear hormone receptors, its importance in cervical cancer warranted further investigation.

To determine the role of *E6AP* in the oncogenic effects of *E6*, we generated HPV transgenic mice on an *E6AP*-null (*E6AP*^{-/-}) background. Our results show that in the cervix, loss of E6AP ablates the oncogenic activity of E6. We conclude that *E6AP* is a critical mediator of the oncogenic activities of HPV16 *E6* in the cervix.

Materials and Methods

Mice

The mouse lines *K14E6*^{WT} (31), *K14E7*^{WT} (10), and *E6AP*^{-/-} (20, 32) were maintained on the inbred FVB/n background. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care–approved McArdle Laboratory Animal Care Facility in accordance with an institutionally approved animal protocol.

Estrogen treatment and analysis of reproductive tracts

For acute studies, 5-week-old virgin female mice were treated with 17 β -estradiol for 6 weeks to achieve a state of persistent estrus (24, 25). Mice acutely treated with estrogen were either exposed to 10 Gy of ionizing radiation from a ¹³⁷Cs source or left untreated and sacrificed 24 hours later. One hour before sacrifice, the nucleotide analogue 5-bromo-2'-deoxyuridine (BrdUrd; 12.5 mg/mL in PBS) was injected i.p. at 10 μ L/1 g body weight to label any newly synthesized DNA.

For acute studies that require ovariectomy, 8-week-old virgin female mice were ovariectomized and allowed to finish the remaining cycle and recover for 10 days. The mice were then injected with either estrogen (30 μ g/kg weight) or ethanol vehicle and sacrificed 24 hours later. Animals were perfused with 4% paraformaldehyde, and reproductive tracts were harvested for analysis.

For long-term cancer studies, 5-week-old virgin female mice were either untreated or treated with 17 β -estradiol for a period of 6 to 9 months, after which the reproductive tracts were harvested and fixed according to previously described methods (23) and sectioned. Every tenth, 5- μ m section was stained with H&E and evaluated histopathologically, with the worst lesion scored as the final diagnosis. The severity of disease was evaluated by taking the spectrum of lesions within a given group and given a score of 0 to 5. Specifically, a diagnosis of hyperplasia is given a value of 0; cervical intraepithelial neoplasia 1 (CIN1) is given a value of 1 and increases progressively with each higher lesion grade to a score of 5 for large invasive cancer (LIC).

Quantification of DNA synthesis and DNA damage response

To quantify the levels of DNA synthesis in the epithelium of the cervix, basal and suprabasal BrdUrd-positive cells were counted and divided by the total number of cells and multiplied by 100 to determine the percentage. To quantify the

DNA damage response, the total number of BrdUrd-positive cells was counted and divided by the total number of cells in the epithelium and multiplied by 100. BrdUrd was counted in at least eight, 40 \times microscopic fields per mouse, with a total of at least three or more mice per genotype group.

Statistical analysis

A two-sided Wilcoxon rank sum test was used for determining statistical significance in the quantification of DNA synthesis, tumor multiplicity, tumor burden, and severity of disease. A two-sided Fisher's exact test was used in determining significance for cancer incidence.

Immunohistochemistry and immunofluorescence

Conditions for immunohistochemistry or immunofluorescence for the antibodies BrdUrd (NA20; Calbiochem), p53 (CM5; Novocastra), MCM7 (ab47DC141; NeoMarkers), and ER α (MC20; Santa Cruz Biotechnology) were previously described (20, 24, 25).

Results

E6-induced cervical epithelial hyperplasia is not dependent on E6AP

Previous results indicate that the ubiquitin ligase E6AP is not required for E6-induced epithelial hyperplasia in the skin (20). To determine whether E6AP is required for E6-induced cervical epithelial hyperplasia, we crossed *K14E6*^{WT} transgenic mice onto an *E6AP*^{-/-} background and monitored for proliferation in young female mice treated acutely with estrogen. Acute treatment with estrogen is necessary to avoid variability in the thickness of the cervical epithelium and synchronizes all mice. Animals were injected with the nucleotide analogue BrdUrd 1 hour before sacrifice. Proliferation was subsequently measured by quantifying the incorporation of BrdUrd into nascently synthesized DNA via BrdUrd-specific immunohistochemistry. Similar to previous results, *K14E6*^{WT} transgenic mice were able to induce hyperplasia in the cervical epithelium as indicated by higher levels of suprabasal DNA synthesis relative to nontransgenic (*NTG*) mice (0.9% versus 0.4%; $P = 0.03$; Fig. 1A, inset). Loss of E6AP in *K14E6*^{WT} transgenic mice (*K14E6*^{WT}*E6AP*^{-/-}) did not result in a loss of epithelial hyperplasia (0.9%) and was not different from that of *K14E6*^{WT} mice ($P = 1$). Surprisingly, the loss of E6AP in the absence of HPV E6 (*E6AP*^{-/-}) also resulted in significantly higher levels of suprabasal DNA synthesis relative to *NTG* mice (1.0% versus 0.4%; $P = 0.02$). Thus, E6AP is not the mediator of HPV E6–induced hyperplasia.

A trivial explanation for our observation that E6AP is not required for E6-induced cell proliferation is that E6 is itself causing lower steady-state levels of E6AP. To determine if this is the case, we performed E6AP-specific Western blot analysis. We observed no obvious differences in the levels of E6AP in *NTG* versus *K14E6*^{WT}, *K14E7*^{WT}, or *K14E6*^{WT}*E7*^{WT} transgenic tissues (Supplementary Fig. S1). Thus, in our mouse model, E6 does not alter levels of E6AP.

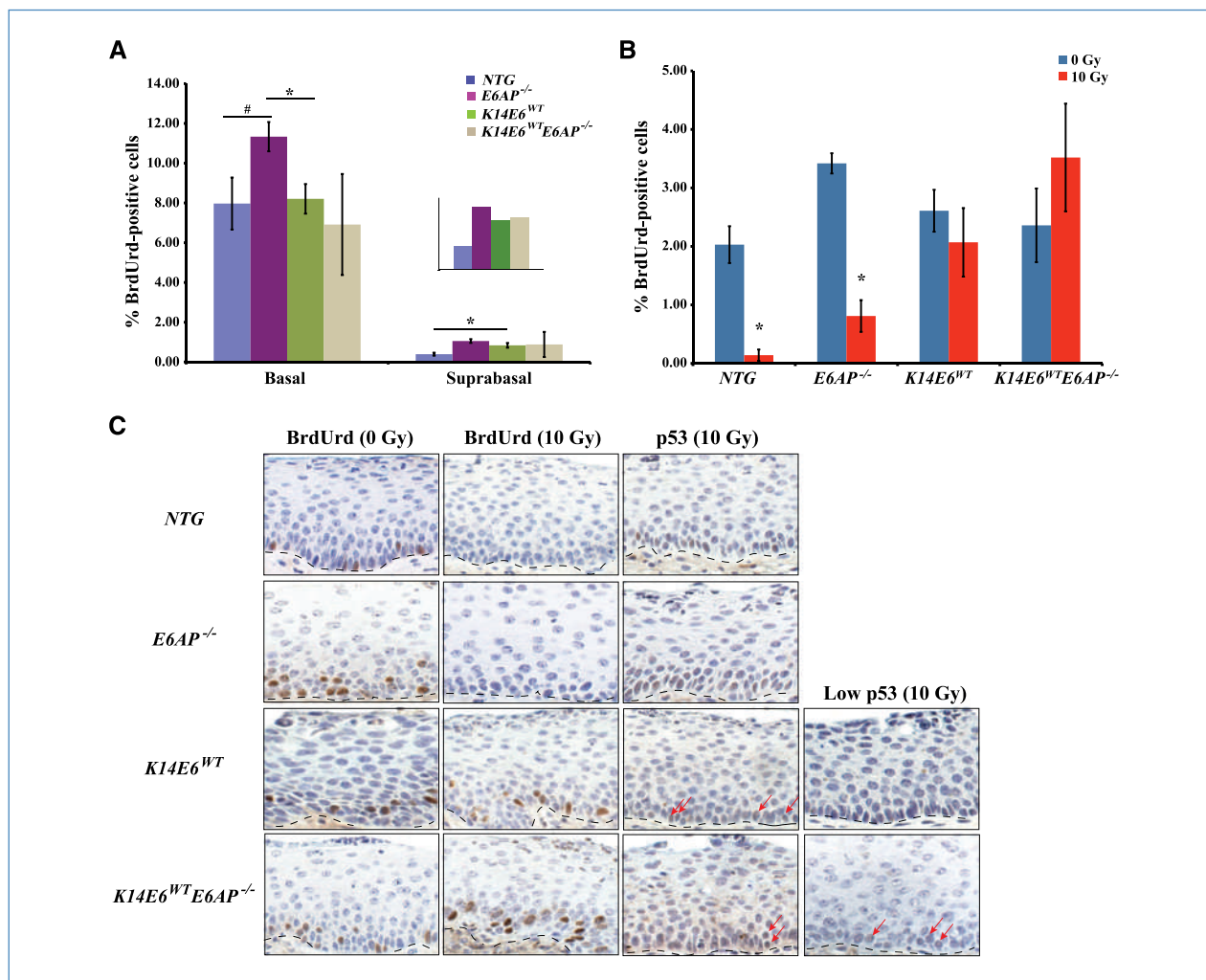


Figure 1. Examination of acute effects in HPV E6 transgenic mice on an E6AP-deficient background in the cervix. A, quantification of DNA synthesis in the cervical epithelium of female mice acutely treated with estrogen to synchronize mice in estrus. Groups of three or more mice per genotype were injected with BrdUrd 1 h before sacrifice. Formalin-fixed, paraffin-embedded tissue was subjected to BrdUrd-specific immunohistochemistry, and the percentage of BrdUrd-positive cells was counted in both the basal and the suprabasal layers in at least eight 40 \times frames/mouse tissue, as a measure of DNA synthesis. Epithelial hyperplasia is defined by an increase in the percentage of suprabasal BrdUrd-positive cells. E6AP^{-/-} mice have a marginal increase in the number of basal BrdUrd-positive cells relative to NTG ($^{\#}$, $P = 0.08$) mice and are significantly different than K14E6^{WT} mice (* , $P = 0.03$). The percentage of suprabasal BrdUrd-positive cells was significantly higher in both E6AP^{-/-} and K14E6^{WT} groups ($P < 0.05$) relative to NTG mice. Loss of E6AP in the presence of HPV E6 did not prevent the induction of epithelial hyperplasia ($P = 1$). Inset, magnification of the number of the BrdUrd-positive cells in the suprabasal layers. B, quantification of DNA synthesis in the cervical epithelium of females acutely treated with estrogen and either given 10 Gy of ionizing radiation or not treated. Groups of three or more mice per genotype were injected with BrdUrd 23 h after irradiation, and 1 h later, tissue was harvested. Like-size groups of age-matched mice not exposed to irradiation served as controls. The percentage of BrdUrd-positive cells was quantified as described in A. Following irradiation, significant reductions in the percentage of cells supporting DNA synthesis were observed in both NTG (* , $P = 0.01$) and E6AP^{-/-} (* , $P = 0.03$) groups. Both K14E6^{WT} and K14E6^{WT}E6AP^{-/-} groups were abrogated in this DNA damage response, maintaining the levels of DNA synthesis seen in the control groups for each genotype (both $P > 0.05$). C, sections of cervical epithelium from mice acutely treated with exogenous estrogen and stained for BrdUrd or p53 by immunohistochemistry. Mice were either unirradiated or irradiated and sacrificed 24 h later. Dotted lines in each picture demarcate the cervical epithelium from the underlying stroma. First and second columns, DNA synthesis, as represented by BrdUrd-positive staining nuclei, is reduced upon irradiation in NTG mice on either an E6AP-sufficient or an E6AP-insufficient background. In contrast, both K14E6^{WT} and K14E6^{WT}E6AP^{-/-} retain the ability to synthesize DNA. Third column, p53 is undetectable in the absence of DNA damage (data not shown); however, it is induced in NTG and E6AP^{-/-} mice upon irradiation. K14E6^{WT} and K14E6^{WT}E6AP^{-/-} mice display variable levels of p53 upon irradiation. Arrows point to examples of p53-positive cells. Fourth column, examples of a strong inhibition of the DNA damage response as exemplified by either a lack of (K14E6^{WT}) or a few (K14E6^{WT}E6AP^{-/-}) p53-positive cells in the epithelium.

Inhibition of the DNA damage response by HPV E6 in the absence of E6AP

Previous results show that HPV E6 transgenic mice retain the ability to inhibit DNA damage responses in response to

ionizing radiation in the epidermis of the mouse skin despite the absence of E6AP (20). Given these results, we wanted to determine whether HPV E6 maintains the ability to inhibit the DNA damage response in the absence of E6AP in the

cervical epithelium. Mice acutely treated with estrogen were either given 10 Gy of ionizing radiation or left untreated and harvested 24 hours later. One hour before sacrifice, all of the mice were injected with BrdUrd to label nascently synthesized DNA. We assessed two different responses to DNA damage: (a) the ability to cease DNA synthesis and (b) the induction and subsequent accumulation of p53. As expected, DNA synthesis was significantly reduced in both *NTG* (2.0 versus 0.3%; $P = 0.01$) and *E6AP*^{-/-} (3.4 versus 0.8%; $P = 0.03$) mice on radiation (Fig. 1B and C). Similar to previous results observed in the skin, DNA synthesis in *K14E6*^{WT} transgenic mice was not significantly changed upon radiation (2.6% versus 2.1%; $P = 0.09$). Interestingly, *K14E6*^{WT}*E6AP*^{-/-} transgenic mice did not show a reduction in DNA synthesis on DNA damage (2.3% versus 3.5%; $P = 0.16$), suggesting that, in the cervical epithelium, E6AP is not required for the targeted inactivation of E6 of p53 in inhibiting the DNA damage response. This result is in kind to that observed previously in the skin (20).

To address whether E6 can prevent the accumulation of p53 after DNA damage in the absence of E6AP, immunohistochemistry for p53 was performed on sections from both unirradiated and irradiated mice. In the absence of any DNA damage, p53 was undetectable in all genotypes (data not shown). As expected, p53 was highly induced in the cervical epithelium of *NTG* and *E6AP*^{-/-} mice upon radiation (Fig. 1C). In contrast to skin where E6 is able to largely prevent induction of detectable p53 following irradiation, p53 levels in the irradiated *K14E6*^{WT} cervix varied from nil to modest levels, with positive cells found predominantly in the basal layer. Thus, in the cervix, E6 partially prevents the accumulation of p53 in response to irradiation, although it efficiently prevents DNA damage-associated growth arrest (Fig. 1B). The cervical epithelia of irradiated *K14E6*^{WT}*E6AP*^{-/-} transgenic mice also displayed low to modest levels of p53 protein, similar to *K14E6*^{WT} mice, indicating that E6AP is not required for the ability of E6 to prevent accumulation of p53 following irradiation. This is similar to what was observed in the skin of E6AP-null mice (20).

The acute response to estrogen is affected in mice without E6AP

In addition to its function as an ubiquitin ligase, E6AP also functions as a steroid receptor coactivator (27). E6AP has

been reported to target various nuclear hormone receptors (29), including ER α , for ubiquitin-mediated degradation (33). Because E6AP is reported to play a role in estrogen signaling, we investigated whether loss of E6AP has any effect in the cervix, where estrogen signaling plays an important role. Effects from estrogen signaling can be observed quickly, and thus, the acute response to estrogen was assessed in groups of *NTG* and *E6AP*^{-/-} mice. Mice were ovariectomized to eliminate endogenous levels of estrogen and treated 10 days later with either vehicle or estrogen for 24 hours, and after which, the reproductive tracts were harvested. Ovariectomy results in a thin epithelium throughout the reproductive tract due to a lack of proliferation signals from ovarian hormones. The normal response to estrogen is an increase in uterine wet weight (34) and the induction of epithelial hyperplasia. The epithelium from *NTG* and *E6AP*^{-/-} groups treated with ethanol vehicle were hypoplastic. On estrogen treatment, the majority of reproductive tracts from *NTG* mice were overtly (data not shown) and histologically thickened (Fig. 2). Acute treatment of *E6AP*^{-/-} mice with estrogen, on the contrary, did not result in either an overt or a histologically thickened epithelium. It is not clear why this is the case because, at the time of sacrifice, the proliferative index in the cervical epithelium of these mice was similar to *NTG* mice as assessed by BrdUrd incorporation over a 1-hour period before sacrifice, and there was no detectable programmed cell death (data not shown). One possible explanation is that there is a slight delay in the response of the E6AP-null tissue to estrogen, given that E6AP-null tissue does undergo hyperplasia when treated long term with estrogen (see below).

E6-induced cervical carcinogenesis is dependent on E6AP

Given that ER α signaling is affected in *E6AP*^{-/-} mice, we investigated whether loss of *E6AP* would result in a reduced ability of HPV E6 to induce cervical cancer. We therefore compared mice nulligenic for E6AP and bred them to *K14E6*^{WT} mice. *K14E6*^{WT} mice develop cervical cancer after treatment with physiologic levels of estrogen for 9 months (24). Cervical lesions that arise in these mice are histopathologically similar to that found in HPV16-infected women. Specifically, we observe a progressive disease that includes the development of CIN grades 1 to 3, followed by the onset

Figure 2. Acute responses to estrogen partially require E6AP. Shown are H&E sections of cervix from mice that were ovariectomized and treated with either ethanol (ETOH) or estrogen (E₂) for 24 h. The first and third columns are sections taken at $\times 1.25$ magnification, whereas the second and fourth columns are sections taken at $\times 40$ magnification. Note the relative lack of epithelial hyperplasia in *E6AP*^{-/-} mice relative to *NTG* mice on treatment with estrogen (far right column).

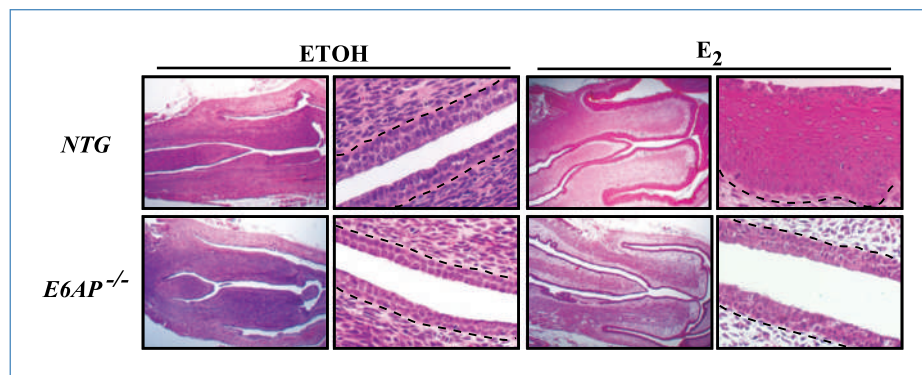


Table 1. Summary of disease in the cervix and vagina of mice after 9 months with estrogen

Mouse genotype		Severity of disease*					
		H	CIN1	CIN2	CIN3	MIC	LIC
Cervix	NTG	17	3	1		2	
	<i>E6AP</i> ^{-/-}	2	11	2	1	3	
	<i>K14E6</i> ^{WT†}	3	9	7	8	5	7
	<i>K14E6</i> ^{WT} <i>E6AP</i> ^{-/-‡}	1	12	2		1	
Vagina	NTG	16	7				
	<i>E6AP</i> ^{-/-}	1	18				
	<i>K14E6</i> ^{WT†}	9	12	15	2	1	
	<i>K14E6</i> ^{WT} <i>E6AP</i> ^{-/-‡}	5	9	2			

Abbreviations: H, hyperplasia; MIC, microinvasive cancer; VAIN, vaginal intraepithelial neoplasia.

*Cohorts of female mice of the indicated genotypes were treated with estrogen for 9 mo, and the reproductive tracts from every mouse were scored for the worst state (severity) of cervical (top half) or vaginal (bottom half of table) disease. The overall severity of disease between different genotypes was compared using a two-sided Wilcoxon rank sum test.

†The shift in the severity of disease in *K14E6*^{WT} transgenic mice is highly significant relative to *NTG* and *E6AP*^{-/-} mice ($P < 0.001$).

‡Loss of *E6AP* results in a highly significant reduction in the severity of disease in *K14E6*^{WT}*E6AP*^{-/-} in the cervix ($P < 0.001$) and a significant reduction in the vagina ($P = 0.05$) relative to *K14E6*^{WT} mice.

of frank cancer of the cervix. We therefore compared the severity of cervical disease by comparing the complete spectrum of lesions observed within any given group (see Materials and Methods for details). Similar to previous findings, *K14E6*^{WT} mice have an increase in the severity of disease, which was significantly different from *NTG* mice ($P < 0.001$). *K14E6*^{WT} transgenic mice have an increase in tumor incidence, multiplicity, and tumor burden (defined as the average area of tumor mass) relative to *NTG* mice (Supplementary Fig. S2; Tables 1 and 2). In *K14E6*^{WT}*E6AP*^{-/-} transgenic mice, however, the severity of disease was significantly reduced ($P < 0.001$) when compared with *K14E6*^{WT}. Specifically, *K14E6*^{WT}*E6AP*^{-/-} mice have reductions in the incidence of cervical cancer (6.3%; $P = 0.04$), tumor multiplicity (0.13; $P = 0.06$), and tumor burden ($P = 0.04$). Indeed, the incidence of cervical disease in the *K14E6*^{WT}*E6AP*^{-/-} mice was not significantly different from that observed in *NTG* mice. These results indicate that the capacity of *E6* to induce cervical cancer was completely abrogated on an *E6AP*-null background.

In human cervical cancers, *E6* is always expressed with *E7*, a second HPV oncogene. HPV16 *E7* is the more potent oncogene in the mouse cervix, as *K14E7*^{WT} mice develop cervical cancers after only treatment with 6 months of 17 β -estradiol (23). In comparison, *K14E6*^{WT} mice only develop cervical cancers when treated for 9 months (24). Double-transgenic mice (*K14E6*^{WT}*E7*^{WT}) expressing both HPV16 *E6* and *E7* develop larger cancers than *K14E7*^{WT} mice when treated for 6 months with 17 β -estradiol (23). To assess the importance of *E6AP* in cervical carcinogenesis in the presence of both *E6* and *E7*, we assessed the severity and incidence of cervical disease in *K14E6*^{WT}*E7*^{WT} mice on an *E6AP*-sufficient or nulligenic back-

ground after treatment with estrogen for 6 months (Supplementary Fig. S2; Tables 2 and 3). Histopathologic diagnosis revealed that in the absence of *E6AP* (*K14E6*^{WT}*E7*^{WT}*E6AP*^{-/-}), the severity of disease is significantly reduced in the cervix of HPV mice ($P = 0.03$). Moreover, *K14E6*^{WT}*E7*^{WT}*E6AP*^{-/-} transgenic mice did not show any incidence of large invasive cancers (LIC) as opposed to an incidence of 41% in *K14E6*^{WT}*E7*^{WT} mice on the *E6AP*-sufficient background. To determine if *E6AP* status had any influence on carcinogenesis when only *E7* is expressed, we compared the severity and incidence of cervical disease in *K14E7*^{WT} mice on an *E6AP*-sufficient or nulligenic background (Table 3). No significant differences in cervical disease were observed. Based on these results, we conclude that *E6AP* is specifically a critical cofactor for HPV16 *E6* in cervical carcinogenesis.

A progressive disease leading to cancer is also observed in the vaginal cavity or lower reproductive tract (LRT) in our HPV transgenic mouse model for cervical carcinogenesis, consistent with the role of high-risk HPVs in vaginal cancers (35, 36). We therefore assessed the influence of *E6AP* on vaginal carcinogenesis. Similar findings were observed as in the cervix. In *K14E6*^{WT} mice treated with estrogen for 9 months, the absence of *E6AP* led to a complete abrogation of *E6*-dependent carcinogenesis in the vagina (Supplementary Fig. S2; Table 1). Likewise, the contribution of *E6* was lost in *K14E6*^{WT}*E7*^{WT} mice treated with estrogen for 6 months in *K14E6*^{WT}*E7*^{WT}*E6AP*^{-/-} mice (Supplementary Fig. S2; Table 3).

It is interesting to note that in our analysis of the influence of *E6AP* status on the acute response to estrogen in the cervical epithelia, we observed a defect in hyperplasia induced by estrogen in the *E6AP*-deficient cervix of ovariectomized

mice. Nevertheless, in our 9-month estrogen-treated *E6AP*^{-/-} mice, we observed hyperplasia, dysplasia, and even cancer at a low frequency, akin to what we observed in E6AP-sufficient mice (Table 1). These results indicate that any effect of E6AP deficiency on the function of estrogen in this tissue is not manifested in the long term and could simply reflect a slight delay in the acute response.

Reduced incidence of cervical cancer is not due to a lack of ER α expression

Given the acute and long-term results observed in mice on the *E6AP*^{-/-} background, we wanted to determine whether the reduced incidence of cancer in *K14E6*^{WT}*E6AP*^{-/-} mice is due to a lack of ER α expression. Previous findings show that ER α is a necessary cofactor in cervical carcinogenesis in our estrogen-dependent HPV model (30). Thus, immunohistochemistry for ER α was performed on long-term-treated mice. There were varying levels of ER α in reproductive tract epithelium across all genotypes (data not shown). No differences were observed in the expression of ER α between *NTG* and *E6AP*^{-/-} cancers. In the presence of either HPV E6 or E7, the loss of E6AP resulted in minor reductions in the expression of ER α ; however, the cancers still expressed appreciable levels of ER α (Fig. 3A).

Reduced incidence of cancer is not a result of reduced proliferation or expression of the HPV biomarker MCM7

To confirm that the reduction in the severity of disease and cancer incidence in HPV transgenic mice on the *E6AP*^{-/-} background is not due to an inability to proliferate, immunohistochemistry for BrdUrd was performed on *NTG*, *E6AP*^{-/-}, *K14E6*^{WT}, *K14E6*^{WT}*E6AP*^{-/-}, *K14E7*^{WT}, *K14E7*^{WT}*E6AP*^{-/-}, *K14E6*^{WT}*E7*^{WT}, and *K14E6*^{WT}*E7*^{WT}*E6AP*^{-/-} mice treated with estrogen for either 6 or 9 months (data not shown). As expected, proliferation was low in *NTG* mice. Similar to acute studies reported above, *E6AP*^{-/-} mice had higher levels of proliferation. No differences in the levels of BrdUrd were observed in comparing HPV transgenic (*K14E6*^{WT} or *K14E6*^{WT}*E7*^{WT}) mice either on an E6AP-sufficient or E6AP-insufficient background.

MCM7 is a biomarker for HPV-positive lesions in both clinical human and murine samples (37). Cancers from the above genotypes were evaluated for the expression of MCM7. Cancers from *NTG* and *E6AP*^{-/-} mice expressed low but detectable levels of MCM7. Mice expressing either HPV E6 or E7 generally had increased levels of MCM7 expression, driven mostly by the inactivation of pRb by E7 (38) or the dysregulation of the p16/pRb pathway by E6 (24, 39), both

Table 2. Summary of tumor characteristics in the cervix and vagina

Genotype	Cancer incidence (%) [*]	LIC incidence (%)	Tumor multiplicity (tumors/mouse)	Tumor burden (area of tumor invasion/mouse; mm ²)
<i>NTG</i> (n = 23)	8.7	0	0.09	5 × 10 ⁻³
<i>E6AP</i> ^{-/-} (n = 19)	15.8	0	0.21	2 × 10 ⁻³
<i>K14E6</i> ^{WT} (n = 39)	30.8	17.9 [†]	0.51 [‡]	0.39 [§]
<i>K14E6</i> ^{WT} <i>E6AP</i> ^{-/-} (n = 16)	6.3	0 [†]	0.13 [¶]	3 × 10 ^{-3**}
<i>NTG</i> (n = 7)	0	0	0	0
<i>K14E7</i> ^{WT} (n = 22)	50	9.1	1.8	0.17
<i>K14E7</i> ^{WT} <i>E6AP</i> ^{-/-} (n = 7)	57.1	14.3 ^{††}	2.3	0.38
<i>K14E6</i> ^{WT} <i>E7</i> ^{WT} (n = 17)	82.4	41	5.6	1.31
<i>K14E6</i> ^{WT} <i>E7</i> ^{WT} <i>E6AP</i> ^{-/-} (n = 6)	50	0	3.2	0.15 ^{‡‡}

NOTE: Animals represented in the top four lines of the table (up to and including *K14E6*^{WT} *E6AP*^{-/-}) were treated with estrogen for 9 mo, whereas the five listed below were treated for 6 mo. All statistical tests were two-sided.

^{*}Cancer incidence was defined as the incidence of cancer that occurred in the cervix and the LRT combined.

[†]Loss of E6AP resulted in a marginally significant reduction in the formation of LIC in *K14E6*^{WT}*E6AP*^{-/-} relative to *K14E6*^{WT} transgenic mice (*P* = 0.09).

[‡]Tumor multiplicity is significantly higher in *K14E6*^{WT} transgenic mice relative to *NTG* mice (*P* < 0.05).

[§]*K14E6*^{WT} transgenic mice have significantly larger tumors than *NTG* mice (*P* < 0.05).

^{||}Loss of E6AP resulted in a reduction in cancer incidence in *K14E6*^{WT}*E6AP*^{-/-} and was significantly different than *K14E6*^{WT} transgenic mice (*P* = 0.04).

[¶]Loss of E6AP results in a reduction in tumor multiplicity in *K14E6*^{WT}*E6AP*^{-/-} relative to *K14E6*^{WT} transgenic mice (*P* = 0.06).

^{**}Loss of E6AP results in a significant reduction in tumor size in *K14E6*^{WT}*E6AP*^{-/-} relative to *K14E6*^{WT} transgenic mice (*P* < 0.05).

^{††}Loss of E6AP did not result in a reduction in the percentage of LIC in *K14E7*^{WT}*E6AP*^{-/-} and was not different than *K14E7*^{WT} transgenic mice (*P* > 0.05).

^{‡‡}Loss of E6AP in *K14E6*^{WT}*E7*^{WT}*E6AP*^{-/-} transgenic mice resulted in smaller tumors relative to *K14E6*^{WT}*E7*^{WT} transgenic mice (*P* = 0.07).

Table 3. Summary of the disease state in the cervix and vagina of mice after 6 months with estrogen

Mouse genotype		Severity of disease*					
		H	CIN1	CIN2	CIN3	MIC	LIC
Cervix	NTG	5	1	1			
	<i>K14E7^{WT}</i> †	1	1	2	7	9	2
	<i>K14E7^{WT}E6AP^{-/-}</i> †‡			2	1	3	1
	<i>K14E6^{WT}E7^{WT}</i> §				3	8	6
	<i>K14E6^{WT}E7^{WT}E6AP^{-/-}</i> ‡		1	2		3	
Vagina	NTG	5	1	1			
	<i>K14E7^{WT}</i> †	2	4	7	1	8	
	<i>K14E7^{WT}E6AP^{-/-}</i> †‡		2	4		1	
	<i>K14E6^{WT}E7^{WT}</i> §	1	2	2		11	1
	<i>K14E6^{WT}E7^{WT}E6AP^{-/-}</i> ‡	1	2	2		1	

*Cohorts of female mice of the indicated genotypes were treated with estrogen for 6 mo, and the reproductive tracts from every mouse were scored for the worst state (severity) of cervical (top half) or vaginal (bottom half of table) disease. The overall severity of disease between different genotypes was compared using a two-sided Wilcoxon rank sum test.

†Regardless of E6AP status, the shift in the severity of disease in transgenic mice expressing HPV E7 in the presence or absence of E6 was all highly significant relative to NTG mice ($P < 0.001$).

‡Loss of E6AP did not result in a reduction in the severity of disease in *K14E7^{WT}E6AP^{-/-}* and was not significantly different than *K14E7^{WT}*.

§The severity of disease is significantly increased in *K14E6^{WT}E7^{WT}* transgenic mice in the cervix ($P = 0.001$) and in the LRT ($P = 0.07$) relative to *K14E7^{WT}* mice.

||Loss of E6AP resulted in a reduction in the severity of disease in *K14E6^{WT}E7^{WT}E6AP^{-/-}* transgenic mice both in the cervix ($P = 0.006$) and in the LRT ($P = 0.01$) relative to *K14E6^{WT}E7^{WT}* mice; the resulting severity of disease is similar to *K14E7^{WT}E6AP^{-/-}* mice ($P > 0.05$).

of which can lead to subsequent activation of E2F target genes. Indeed, there were no significant differences in the expression of MCM7 between HPV transgenic mice on an E6AP-sufficient versus E6AP-insufficient background (Fig. 3B).

Loss of E6AP results in an increased frequency of detectable p53 in the reproductive tract

Due to the reduction in the incidence of cancer and the severity of disease in *K14E6^{WT}E6AP^{-/-}* mice, we wanted to determine whether the inability of E6 to degrade p53 accounted for these observations. To investigate this question, immunohistochemistry for p53 was performed on mice treated with estrogen. Cancers from NTG, *E6AP^{-/-}*, *K14E6^{WT}*, and *K14E6^{WT}E6AP^{-/-}* mice have little to no detectable p53 (Fig. 3B). However, focal expression of p53 is detectable in the reproductive epithelium of *E6AP^{-/-}* and *K14E6^{WT}* mice, with an appreciable increase in *K14E6^{WT}E6AP^{-/-}* mice. Expression of p53 was more likely to be detected in the epithelium of the LRT and thus may explain the rare occurrence of cancers in this region in mice that only express E6 and not the E7 transgene (24, 36).¹ Mice expressing the E7 transgene

have detectable levels of p53 in both the dysplastic epithelium and cancers, likely due to the ability of E7 to cause increases in the levels of p53 (40, 41). Accordingly, *K14E6^{WT}E7^{WT}E6AP^{-/-}* transgenic mice have an appreciable increase in the expression of p53 over that of *K14E6^{WT}E6AP^{-/-}* mice, which is evident both in the epithelium and in the cancers. In sum, the loss of E6AP results in an increased frequency of detectable p53 in the reproductive tract that correlated with decreases in cancer incidence and the severity of disease in E6 transgenic mice.

Discussion

In this study, we found that the loss of *E6AP* had little effect on acute activities of E6 in the cervix, yet had dramatic effects on the ability of E6 to synergize with estrogen to form cancers in the reproductive tract, in the presence or absence of E7. The loss of E6AP resulted in reductions in both cancer incidence and the severity of disease. In sum, this study shows that E6AP plays a critical role in mediating the contributions of HPV16 E6 to cervical cancer.

E6AP is not required for HPV E6-induced acute effects

Similar to previous observations in the skin of HPV E6 transgenic mice (20), E6AP was found to be required neither

¹ Authors' observation.

for the induction of epithelial hyperplasia nor for the inactivation of the DNA damage response in the reproductive tract. E6AP is thought to potentially mediate E6-induced hyperplasia due to its predicted ability to target the PDZ partners of E6, Scribble, and Dlg for degradation. Loss of either of these tumor suppressor genes in *Drosophila* results in the development of epithelial hyperplasia, loss of cell-cell contacts, and cancer development (42-44). In human cervical cancers, hScrib and hDlg become mislocalized and their levels progressively reduced (45, 46). Observations from the skin and

the reproductive tract suggest that E6AP is not the mediator of E6-induced epithelial hyperplasia and/or that in its absence there is alternative ubiquitin ligase that can target these proteins for degradation. Evidence from others suggests that the latter hypothesis may be the case. Work from Massimi and colleagues (22) show that E6 maintains the ability to target both Scribble and Dlg for ubiquitin-mediated degradation in the absence of E6AP, in contrast to reports that argue for a dependence of E6AP (16). Moreover, the ability of E6 to inhibit the DNA damage response in both the skin and reproductive

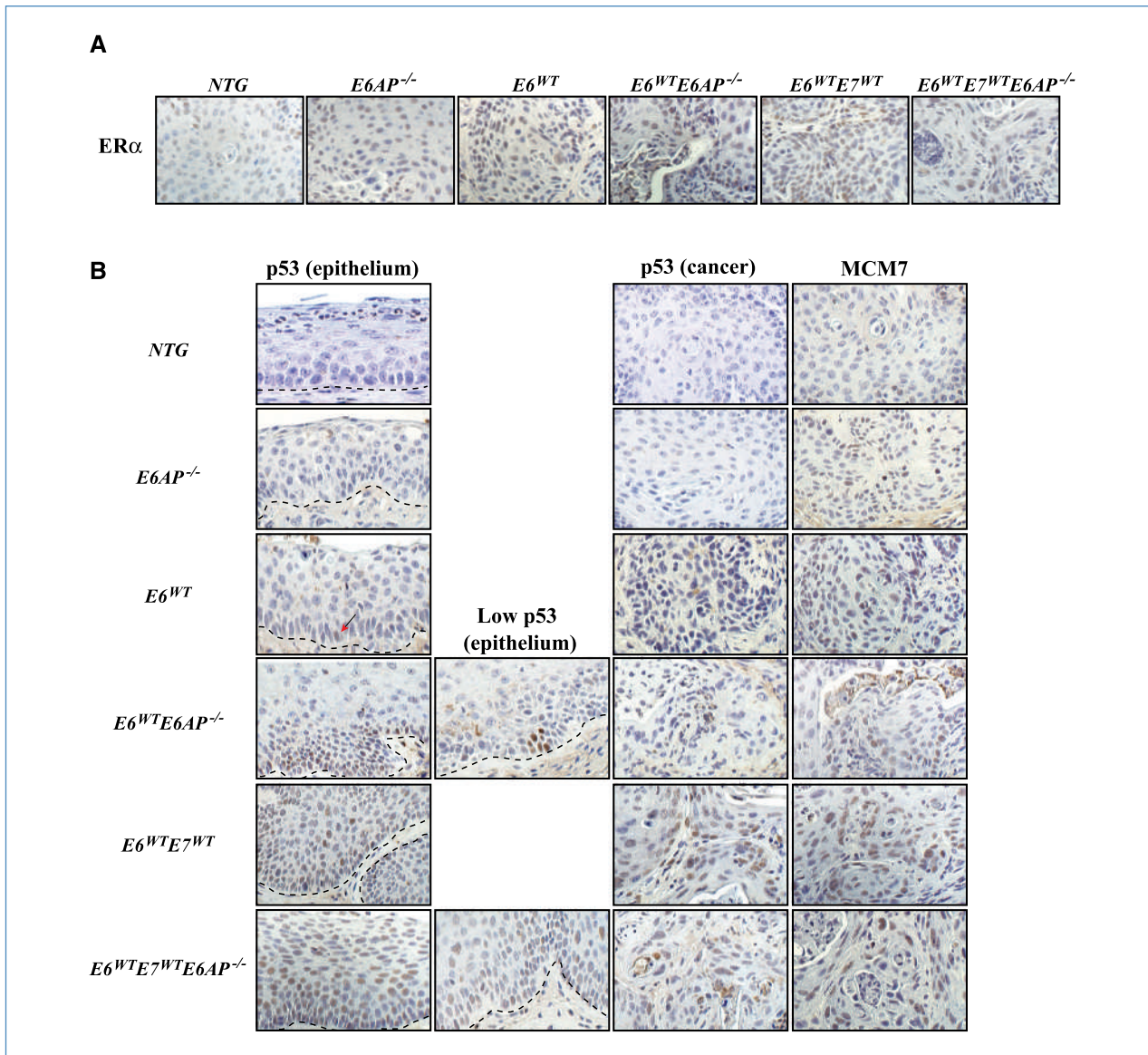


Figure 3. Immunohistochemical characterization of tumors from the reproductive tract. A, sections of cancers taken from the reproductive tract from mice of the indicated genotypes and stained for ERα by immunohistochemistry. All of the cancers from the various genotypes express ERα. Magnification, ×40. B, sections of epithelium (first and second columns) or cancers (third column) taken from the reproductive tract from mice of the indicated genotypes and stained by immunohistochemistry for either p53 or MCM7. Dotted lines in each picture demarcate the cervical epithelium from the underlying stroma. In the epithelium, p53 is not often detected; however, loss of E6AP in *K14E6^{WT}E6AP^{-/-}* mice results in the induction of p53 at either low or higher (second column) levels. p53 is detected both in the epithelium and cancers of *K14E6^{WT}E7^{WT}* and *K14E6^{WT}E7^{WT}E6AP^{-/-}* as p53 is stabilized by HPV E7. MCM7 (fourth column) is expressed at low levels in cancers from *NTG* and *E6AP^{-/-}* mice. Levels of MCM7 are increased in cancers that express HPV oncogenes E6 and/or E7. Magnification, ×40.

tract (this study) in the absence of E6AP similarly suggests that there are alternative means to inactivating p53. Alternatively, proposed mechanisms by which E6 inactivates p53 include repression of transactivation of p53 target genes either by acetylation (47) or by interaction of E6 with CBP/p300 (48), which may be relevant here.

Implications of our findings

Our studies provide clear evidence for an essential role of E6AP in mediating the oncogenic activity of HPV16 E6 but leave unanswered the mechanism underlying its requirement. Specifically, those acute phenotypes of E6 that were measured were retained on the E6AP-deficient background despite the complete loss of neoplastic disease. Our results suggest that the role of E6AP in mediating the activities of E6 has yet to be fully appreciated.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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E6-Associated Protein Is Required for Human Papillomavirus Type 16 E6 to Cause Cervical Cancer in Mice

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