Pocket Proteins Suppress Head and Neck Cancer

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Abstract

Head and neck squamous cell carcinomas (HNSCC) is a common cancer in humans long known to be caused by tobacco and alcohol use, but now an increasing percentage of HNSCC is recognized to be caused by the same human papillomaviruses (HPVs) that cause cervical and other anogenital cancers. HPV-positive HNSCCs differ remarkably from HPV-negative HNSCCs in their clinical response and molecular properties. From studies in mice, we know that E7 is the dominant HPV oncoprotein in head and neck cancer. E7 is best known for its ability to inactivate pRb, the product of the retinoblastoma tumor susceptibility gene. However loss of pRb function does not fully account for E7’s potency in causing head and neck cancer. In this study, we characterized the cancer susceptibility of mice deficient in the expression of pRb and either of two related “pocket” proteins, p107 and p130, that are also inactivated by E7. pRb/p107 deficient mice developed head and neck cancer as frequently as do HPV16 E7 transgenic mice. The head and neck epithelia of the pRb/p107 deficient mice also displayed the same acute phenotypes and biomarker readouts as observed in the epithelia of E7 transgenic mice. Mice deficient for pRb and p130 in their head and neck epithelia showed intermediate acute and tumor phenotypes. We conclude that pRb and p107 act together to efficiently suppress head and neck cancer, and are therefore highly relevant targets of HPV16 E7 in its contribution to HPV-positive HNSCC.
Introduction

Head and neck squamous cell carcinomas (HNSCC) is the sixth most common human cancer. Tobacco use and alcohol consumption have long been known to contribute to increased risk of HNSCC. In the past decade, there has been a growing appreciation that Human Papillomaviruses (HPVs) cause approximately 20% of all HNSCCs and up to 60% of HNSCC arising in the oropharynx including the tonsils (1-2). In the US and Europe, the incidence of HPV-positive (HPV+)HNSCC is increasing while incidence of HPV-negative (HPV-)HNSCC is declining, the latter correlating with reduced use of tobacco in these countries (3). HPV type 16, a high-risk human papillomavirus (HR-HPVs), is associated with the vast majority of HPV+HNSCC (1, 4).

HPV+HNSCC differ from HPV-HNSCC at multiple levels including patient demographics, tumor histopathology, mutation and gene expression profiles, and clinical prognosis. Incidence of HPV+HNSCC is higher in young individuals and less tightly linked with alcohol consumption and smoking (5-6). Patients with HPV+HNSCC have higher survival rates compared to those with HPV-HNSCC when provided standard of care treatment, despite the fact that HPV+HNSCC tend to be more poorly differentiated cancers. On a molecular basis, HPV+HNSCC differ from HPV-HNSCC remarkably in their mutational profiles, with greatly reduced frequencies of mutations in cellular genes, and the absence of mutations in genes such as p53, which is commonly mutated in HPV-HNSCC (6). This difference holds true even for HPV+HNSCC arising in smokers, which supports the premise that HPV is the major driver of the cancer phenotype in HNSCCs positive for this tumor virus. Consistent with this premise, knock down of
expression of HPV oncogenes in HPV+HNSCC cell lines leads to cell death (7).

HPV+HNSCC also have gene expression profiles very distinct from HPV-HNSCC and more similar to that of HPV-positive cervical cancer (8). These molecular differences have been largely attributed to the activities of the HPV-encoded oncogenes.

HR-HPVs such as HPV16 encode for three oncogenes; E5, E6, and E7. Of these, E6 and E7 are commonly found to be expressed in HPV+ HNSCC (9), much like in cervical cancer (10). HR-HPV E6 binds to and induces to the degradation of p53, while HR-HPV E7 binds to and induces degradation of pRb and its related pocket protein family members, p107 and p130 (11-12). Previously, we reported that HPV-16 E7 is the dominant viral oncogene in HPV-associated HNSCC, based on studies in which HPV16 transgenic mice were treated with the chemical carcinogen, 4-nitroquinoine 1-oxide (4-NQO) (13-14). We have also learned that pRb inactivation by E7 is not sufficient to account for the oncogenic potential of E7 in HNSCC: conditional deletion of pRb in head and neck epithelia only partially recapitulated the acute and tumorigenic phenotypes bestowed on mice by E7 (13). These results led us ask what other cellular proteins targeted by E7 contribute to HNSCC.

Here, we report that the combinational loss of pRb along with either p107 or p130 with pRb increased greatly the susceptibility of mice to head and neck tumors over that seen in mice deficient for any one pocket protein. Mice deficient for pRb and p107 in their epithelia had head and neck cancer incidence and severity of disease indistinguishable from that seen in E7 transgenic mice. The oncogenic phenotype in mice deficient for both p107 and pRb was more severe than in mice deficient for both p130 and pRb. Biomarker expression profiles in p107/pRb deficient mice approximated that in
E7 transgenic mice. Compensatory increases in the expression of the remaining pocket protein was observed in mice deficient for two pocket proteins, which in the case of the p130/pRb deficient mice might account for the reduced overall tumor susceptibility. The high degree of susceptibility of p107/pRb deficient mice to head and neck cancer is consistent with the hypothesis that E7 inactivation of these two pocket proteins primarily drives E7 oncogenic properties in HPV+HNSCC.
Materials and Methods

Mice: K14E7 mice have been described previously (13-14). Rb$^{ff}$p107$^{-/-}$ and Rb$^{ff}$p130$^{-/-}$ mice were kindly provided by Dr. Julian Sage (15-17). KRT14-cre/Esr1 (K14CreER) was obtained from Jackson Laboratory. K14CreRb$^{ff}$ has been described previously (13). To generate mice inactivated both pRb and p107, KRT14-cre/Esr1 (i.e., K14CreER$^{tam}$$^{+/-}$ on inbred CD1 genetic background, The Jackson Laboratory) were crossed to Rb$^{ff}$p107$^{-/-}$ mice (on a mixed 129/C57 genetic background) and K14CreER$^{tam}$$^{+/-}$ Rb$^{ff}$p107$^{+/+}$ offspring then crossed to Rb$^{ff}$p107$^{-/-}$ mice to generate K14CreERRb$^{ff}$p107$^{-/-}$ and Rb$^{ff}$p107$^{-/-}$ mice on a CD1/129/C57 mixed genetic background. Control nontransgenic and K14E7 transgenic mice were generated on the same mixed genetic background for all comparisons made in this study.

To generate mice inactivated both pRb and p130, K14CreRb$^{ff}$ (maintained on the inbred FVB/N genetic background), which have been described previously (13), were crossed to Rb$^{ff}$p130$^{-/-}$ mice (on the inbred 129/C57 genetic background) and K14Cre$^{+/-}$ Rb$^{ff}$p130$^{+/-}$ offspring then crossed to Rb$^{ff}$p130$^{-/-}$ mice to generate Rb$^{ff}$p130$^{-/-}$, K14CreRb$^{ff}$, and K14CreRb$^{ff}$p130$^{-/-}$ mice on a FVB/129/C57 mixed genetic background. Control nontransgenic and K14E7 transgenic mice were generated on the same mixed genetic background for all comparisons made in this study.

For the irradiation studies, mice were exposed to 0 or 12 Gy ionizing radiation from a 137Cs source 24 hours before mice were injected IP with BrdU (10µl per g body weight of 12.5mg/ml solution). These mice were then sacrificed 1 hour later and tissues harvested. All mice were bred and maintained in the American Association for Cancer Research.
Accreditation of Laboratory Animal Care–approved McArdle Laboratory Cancer Center Animal Care Facility and were managed in accordance with an approved animal protocol.

**Inducible Cre-mediated recombination.** To generate the Rb-nulligenic state in the head and neck epithelia of K14CreERRb/f p107−/− mice, 6 weeks old K14CreERRb/f p107−/− mice were intraperitoneally injected with tamoxifen (4mg of tamoxifen dissolved in corn oil (Fisher Scientific Inc.) /day) for 5 consecutive days.

**4-nitroquinoline-N-oxide (4-NQO)–induced head and neck carcinogenesis study and histological analysis.** For the head and neck carcinogenesis studies, we started to supply 4-nitroquinoline-N-oxide dissolved water 7 days following completion of treatment with tamoxifen. The treatment and guidelines for histologic analysis were previously described (13). To evaluate the tumor in head and neck, the tongue and esophagus were fixed in 4% (vol/vol) paraformaldehyde solution, embedded in paraffin, and thin sectioned (5μm). Approximately 20 sections were collected at 100 μm intervals for H&E staining and examined for the presence of either papillomas or carcinomas. Based upon detailed assessment of the grade of disease in each section, a diagnosis of the worst grade of disease for each mouse was assigned. Initial histological analysis to identify location of lesions was made by M.K.S. Final diagnoses were made by H.C.P., a mouse pathologist, who was blinded to the mouse genotype. Quantification of DNA synthesis were performed as previously described (18-20).

**Immunohistochemistry.** All of immunohistochemistry were performed as as previously described (18-20). Briefly, the primary antibodies were used in different condition as follows: anti-pRb (1:25, in 5% horse serum, BD Biosciences), anti-p107 (1:125 in 5% non-fat milk/5% horse serum, Santa Cruz Biotechnology), anti-p130 (1:100
in 5% non-fat milk/5% horse serum, BD Biosciences), anti-BrdUrd (1:50 in 5% horse serum, Calbiochem), anti-Mcm7 (1:200 in 5% non-fat milk/5% horse serum, LabVision Neomarkers), anti-p16 (1:50 in 5% non-fat milk/5% horse serum, Santa Cruz Biotechnology), anti-E2F1 (1:150 in 5% non-fat milk/5% horse serum, Abcam), and anti-EZH2 (1:50 in 5% non-fat milk/5% horse serum, BD Biosciences) were used for immunohistochemistry.

Statistical analysis. A two-sided Fisher’s exact test was used to determine the significance of differences in the incidence of overt tumor between each mice group. To determine the significance of differences in the severity of disease and DNA synthesis level between each mouse group, a two-sided Wilcoxon rank sum test was used.
Results

Conditional inactivation of both Rb and p107, or Rb and p130 in head and neck epithelia. We have previously shown that pRb inactivation by E7 is not sufficient to account for HPV-16 E7-mediated oncogenic potential in head and neck cancer (13). HPV-16 E7 has been reported to bind and degrade other pocket protein family members, p107 and p130 (21). To determine if combinatorial loss of two pocket proteins by E7 contributes to increased susceptibility to HNSCC in mice, we generated mice that were inactivated for both pRb and p107, or pRb and p130 in their stratified squamous epithelia. To generate mice inactivated for both pRb and p107 in this tissue, K14CreERtm transgenic mice expressing the tamoxifen inducible CreER from the Keratin 14 (K14) promoter were bred to Rb<sup>fl</sup>p107<sup>-/-</sup> mice, carrying a conditionally homozygous null alleles of Rb in which exon 3 is flanked with loxP sites (22). The use of an inducible Cre was necessitated by the fact that K14CreRb<sup>fl</sup>p107<sup>-/-</sup> mice, in which Cre is constitutively active in stratified epithelia, die before day 10 due to hyperplasia in the oral epithelia precluding them from drinking their mother’s milk (our unpublished observations and (23)). Young adult K14CreERtmRb<sup>fl</sup>p107<sup>-/-</sup> mice were treated with tamoxifen as described in the Materials and Methods section to induce recombination of the floxed Rb allele. When these young adult mice were inactivated for pRb, we did not observe any mortality. We assume this reflects the fact that the oral cavity has grown in size in adult mice and therefore any hyperplasia does not lead to complete occlusion of the cavity. To generate conditional mutant mice inactivated both pRb and p130 in their epithelium, we crossed
Rb\textsuperscript{\textit{\textgamma}}p130\textsuperscript{\textgamma} mice to K14Cre mice. Mortality/high morbidity issues were not observed with the mice. To verify the expression level of each pocket protein in K14CreER\textsuperscript{\textit{\textgamma}}Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma}, K14CreRb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma}, we performed pRb, p107 and p130 immunohistochemical staining on the lingual and esophageal epithelium from these transgenic mice as well as nontransgenic, Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma}, Rb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma}, K14CreRb\textsuperscript{\textgamma}, K14E7 transgenic mice (Fig. 1, data not shown in esophageal epithelium). As expected, pRb staining was infrequently detected in the tamoxifen-treated K14CreER\textsuperscript{\textit{\textgamma}}Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma} mice as well as the K14CreRb\textsuperscript{\textgamma} and K14CreRb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma} indicating that the floxed Rb allele had undergone recombination. In contrast, pRb staining was detected in Cre-negative Rb\textsuperscript{\textgamma} mice, such as Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma}, Rb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma} mice, as well as in nontransgenic mice (Fig. 1, left panel). As expected, we could not detect p107 in lingual epithelium from Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma} and K14CreER\textsuperscript{\textit{\textgamma}}Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma} mice, but it was detected in lingual epithelium from Rb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma}, K14CreRb\textsuperscript{\textgamma}, K14CreRb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma} and nontransgenic mice (Fig. 1, middle panel).

Interestingly, levels of p107 were elevated in Rb-deficient tissues from K14CreRb\textsuperscript{\textgamma}, and K14CreRb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma} mice. Moreover, p107 staining was more strongly and evenly detected in K14CreRb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma} mice compared to K14CreRb\textsuperscript{\textgamma} mice. p130 staining was not detectable in lingual epithelium nulligenic for p130, such as Rb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma}, K14CreRb\textsuperscript{\textgamma}p130\textsuperscript{\textgamma} mice (Fig. 1, right panel). We also observed an increased expression of p130 in K14CreRb\textsuperscript{\textgamma}, K14CreER\textsuperscript{\textit{\textgamma}}Rb\textsuperscript{\textgamma}p107\textsuperscript{\textgamma} tissues. These data indicate that the K14 driven Cre transgenes did drive expression of Cre and recombination of the floxed Rb allele in the desired epithelia within the oral cavity/esophagus. They also demonstrate that compensatory increases in expression of functional pocket proteins can arise in these tissues when deficient for other pocket proteins.
Suprabasal DNA synthesis in head and neck epithelia deficient in pocket proteins

A hallmark of E7 is its ability to induce DNA synthesis in the normally quiescent suprabasal compartment of the stratified epithelia (24-26) including head and neck epithelia (13). Loss of pRb in head and neck epithelia recapitulated some but not all E7-mediated effects on DNA synthesis (13). To determine if combined loss of pRb and p107, or pRb and p130 in head and neck epithelia was able to more completely account for this acute phenotype of E7, we analyzed the proliferative index in the lingual and esophageal epithelium by quantifying the frequency of bromo-deoxyuridine (BrdUrd)-positive suprabasal cells in sections of tissue, from mice injected with this nucleoside analog one hour prior to sacrifice, that were subjected to BrdUrd-specific immunohistochemistry. In tamoxifen-treated $K14\text{CreER}^{tm}\text{Rb}^{fl}\text{p107}^{-/-}$ mice, we observed a significant increase in suprabasal DNA synthesis compared to either nontransgenic or $\text{Rb}^{fl}\text{p107}^{-/-}$ mice but this induction of suprabasal DNA synthesis was significantly lower than that observed in $K14\text{E7}$ mice (Fig. 2A). In $K14\text{CreRb}^{fl}$ mice, pRb inactivation alone induced a significant increase in suprabasal DNA synthesis compared to either nontransgenic or $\text{Rb}^{fl}\text{p130}^{-/-}$ mice but this induction was less than that quantified in either $K14\text{CreRb}^{fl}\text{p130}^{-/-}$ mice or $K14\text{E7}$ mice (Fig. 2B), and is consistent with our prior study (13). Similarly, with tamoxifen-treated $K14\text{CreER}^{tm}\text{Rb}^{fl}\text{p107}^{-/-}$ mice, we observed an increase of suprabasal DNA synthesis in $K14\text{CreRb}^{fl}\text{p130}^{-/-}$ mice compared to mice inactivated for one of these pocket proteins, but this induction again was less than that caused by E7 expression in the same epithelia (Fig. 2B). These observations demonstrate that combinational loss of pRb and either p107 or p130 is not sufficient to account fully
for E7’s ability to induce suprabasal DNA synthesis in head and neck epithelia.

Inhibition of DNA damage response in irradiated head and neck epithelia deficient for pRb and p107, or pRb and p130. HR-HPV E7 is able to disrupt DNA damage responses including those that arise in epithelia of mice exposed to ionizing radiation (27-29). In mouse epithelia, this DNA damage response is reflected in the reduction in frequency cells supporting DNA synthesis 24 hours following exposure to radiation. pRb inactivation alone was necessary and sufficient to inhibit DNA damage response in the epidermis of mouse skin, but not in the murine cervix (19, 30-31). To determine the influence of pocket proteins on DNA-damage response in head and neck epithelia, we quantified the frequency of cells undergoing DNA synthesis in the lingual and esophageal epithelia of mice deficient for pocket proteins that were exposed to 12 Gy ionizing radiation, a dose we had determined is sufficient to cause an arrest in DNA synthesis in these tissues in syngeneic (pocket protein sufficient) mice. DNA synthesis was scored by monitoring incorporation of BrdUrd into newly synthesized DNA in basal cells by immunohistochemistry (mice injected with BrdUrd one hour prior to sacrifice). For comparison, we monitored DNA damage responses in the head and neck epithelia of K14E7 transgenic mice. Radiation-induced arrest of DNA synthesis was observed in nontransgenic, pocket protein sufficient mice but abrogated in K14E7 mice (Fig. 2C&D). In K14CreRb<sup>fl</sup>, no effect of pRb inactivation alone was seen in blocking DNA-damage induced arrest of DNA synthesis (Fig. 2D). Similarly inactivation of either p107 (Fig. 2C) or p130 (Fig. 2D) failed to influence the DNA damage response. However, this response was as completely inhibited in tamoxifen-treated K14CreERtmRb<sup>fl</sup>p107<sup>−/−</sup> mice (Fig. 2C).
and in $K14\text{Cre}Rb^{f/f}p130^{-/-}$ mice (Fig. 2D) as seen in E7 transgenic mice. Thus radiation-induced arrest of DNA synthesis in head and neck epithelia requires the function of multiple pocket proteins. Knocking out just one is not sufficient to abrogate the DNA damage response in this tissue.

As an aside, we saw a reduction in baseline levels of DNA synthesis (i.e. in the absence of radiation) in the basal cells deficient for either p107 (Fig 2C) or p130 (Fig 2D) alone compared to nontransgenic (p107 and p130 sufficient) mice, but this reduction was not seen in tissues deficient for pRb alone (Fig 2D). This reduction in DNA synthesis in the basal cells of p107 or p130 deficient tissue was not due to an increase in pRb levels (see Fig. 1).

**Incidence of overt tumors in the head and neck of 4-NQO–treated mice conditionally inactivated for either pRb and p107, or pRb and p130.** To assess the susceptibility of pocket protein deficient mice to HNSCC, we made use of a previously established protocol for inducing head and neck tumors in mice that employs the chemical carcinogen, 4-nitroquinoline 1-oxide (4-NQO) as a co-carcinogen and scores for tumors in the tongue and esophagus, two head and neck tissues that can be easily retrieved upon necropsy (32). This protocol was applied to groups of mice with the following genotypes: nontransgenic, $Rb^{f/f}p107^{-/-}$, $Rb^{f/f}p130^{-/-}$, $K14\text{Cre}Rb^{f/f}$, $K14\text{CreERTm}Rb^{f/f}p107^{-/-}$, $K14\text{Cre}Rb^{f/f}p130^{-/-}$, and $K14E7$. Consistent with our prior studies (13), $K14E7$ mice developed a high incidence of overt tumors at greater than 90% while the nontransgenic mice had less than 10% incidence of overt tumors (Fig. 3A, 3B). $K14\text{Cre}Rb^{f/f}$ mice had a significant increase in the incidence of overt tumors compared
with that observed in the nontransgenic mice (Fig. 3B; p<0.05), but significantly lower incidence than that developed observed in the \textit{K14E7} mice (Fig. 3B, p<0.05). \textit{Rb}^{\text{ff}}p130^+/− mice had no significant increase in the incidence of overt tumors compared with that observed in the nontransgenic mice (Fig 3B; p=0.26). \textit{Rb}^{\text{ff}}p107^+/− mice had a marginally significant increase in the incidence of overt tumors compared with that observed in the nontransgenic mice (Fig. 3A; p=0.064). In contrast, \textit{K14CreRb}^{\text{ff}}p130^+/− mice had a significant increase in the incidence of overt tumors compared to that observed in \textit{K14CreRb}^{\text{ff}} mice (Fig 3B; p<0.05), but was still significantly lower than the incidence observed in \textit{K14E7} mice (Fig. 3B, p<0.05). \textit{K14CreERtmRb}^{\text{ff}}p107^+/− mice were highly susceptible to head and neck tumors. Out of a group of 34 animals, 29 had overt tumors at the end point of study. This tumor incidence was not significantly different from that observed in the \textit{K14E7} mice (Fig. 3A; p=0.69). These observations indicate that combined loss of pRb and p107 or pRb and p130 leads to increasing susceptibility to head and neck cancers than that observed by inactivation of any single pocket protein.

**Severity of disease in the head and neck of 4-NQO–treated mice conditionally inactivated for either pRb and p107, or pRb and p130.** To determine if combinational inactivation of pRb and p107, or pRb and p130 causes an increased severity of neoplastic disease in the head and neck region, we scored the histopathological grade of disease in the tongue and esophagus of 4-NQO–treated mice. To do so, the tissues from a subset of mice of each genotype were randomly selected, embedded in paraffin, sectioned, stained with H&E and subjected to detailed histopathological analysis. Similar to what we have previously observed (13), half or
more of the K14E7 mice developed invasive carcinomas (50% on CD1/129/C57 mixed background; Table 1, 66.7% on FVB/129/C57 mixed background; Table 2) and they also developed more severe disease overall compared to nontransgenic mice (Tables 1 and 2). Mice inactivated for either pRb or p107 did not develop any invasive cancers (Tables 1 & 2); they only developed benign papillomas. Furthermore, there was no statistically significant difference in their severity of disease in tongue and esophagus compared to that in nontransgenic mice. Interestingly, Rb\textsuperscript{fl}p130\textsuperscript{−/−} mice had a couple of invasive carcinomas and also the severity of disease was significantly worse than nontransgenic mice, but not statistically different compared to K14CreRb\textsuperscript{fl} mice (Table 2). In K14CreRb\textsuperscript{fl}p130\textsuperscript{−/−} mice, 13 out of 15 mice had either no tumor or just a benign papilloma, and the other two mice had invasive carcinoma (Table 2). The severity of disease in K14CreRb\textsuperscript{fl}p130\textsuperscript{−/−} mice was not significantly different compared to that in either Rb\textsuperscript{fl}p130\textsuperscript{−/−} or K14CreRb\textsuperscript{fl} mice. In contrast, we found that half of K14CreERtmRb\textsuperscript{fl}p107\textsuperscript{−/−} mice had invasive carcinoma. Likewise, the severity of disease was significantly worse than that scored in Rb\textsuperscript{fl}p107\textsuperscript{−/−} mice as well as nontransgenic mice, but was not significantly different compared with that scored in K14E7 mice. These observations indicate that inactivation of p107 contributes more to carcinogenesis in head and neck tissues in the absence of pRb than does inactivation of p130.

Expression of biomarkers for HPV-positive HNSCC in both epithelium and tumors from mice deficient for both pRb and p107, or pRb and p130. p16 is highly expressed in HPV-positive human HNSCC (33-34). Moreover, both MCM7 and p16 are induced in head and neck cancers arising in the K14E7 mice (13). MCM7 is an E2F-responsive gene and therefore MCM7 up-regulation in HPV-positive HNSCC is
likely due to the inactivation of pocket proteins by E7 (19). It is also likely that p16 is highly expressed because of the dysregulation of p16-pRb pathway in E7-expressing cells (5-6). To determine if combined loss of pRb and p107, or pRb and p130, lead to dysregulation of these two biomarkers of human HNSCC, we performed MCM7 and p16 immunohistochemical staining on lingual epithelium as well as tumors arising in these mice (Fig. 4). Similar results were obtained data on esophageal epithelium and tumors (data not shown).

As expected (13), the expression of MCM7 was highly up-regulated in both lingual epithelium and tumors arising in K14E7 mice (Fig. 4, left panel). In nontransgenic mice, MCM7 was only expressed in the basal cell layer head and neck epithelia and poorly expressed in the tumors. MCM7 staining in head and neck epithelium of both Rb\(^{\text{fl/fl}}\)p107\(^{\text{-/-}}\) and Rb\(^{\text{fl/fl}}\)p130\(^{\text{-/-}}\) mice was robust in basal cells with some additional staining in parabasal cells, particularly in the Rb\(^{\text{fl/fl}}\)p107\(^{\text{-/-}}\) mice. MCM7 expression was clearly evident in the tumors from these mice as well. As seen previously (13), MCM7 staining was strong in tumors as well as lingual epithelium of K14CreRb\(^{\text{fl/fl}}\) mice, but not as strong and even throughout the full thickness of the epithelium compared to that seen in K14E7 mice. MCM7 staining in the K14CreER\(\text{tm}\)Rb\(^{\text{fl/fl}}\)p107\(^{\text{-/-}}\) and K14CreRb\(^{\text{fl/fl}}\)p130\(^{\text{-/-}}\) mice was most comparable to that in the E7 transgenic mice in terms of the extensiveness of the strong nuclear signal throughout the basal and spinous layers of the epithelium, as well as positive nuclear staining in the tumors.

When we performed p16-specific immunohistochemistry of head and neck tissues (Fig. 4, right panels), we observed low levels of cytoplasmic staining in the basal and parabasal layers of the epithelium as well as in the benign tumors arising in nontransgenic
mice. In E7 transgenic and to varying degrees in the mice inactivated for pocket proteins, we detected a higher intensity of staining for p16, often with increased nuclear signal. This was most pronounced with the mice inactivated for both p107 and pRb. These findings (see Fig. S1 for quantification of p16 positivity for each genotype) indicate that each pocket protein can modulate the expression of p16, but loss of Rb and p107 has the most dramatic effect on inducing expression of this Cdk inhibitor.

Together with the MCM7 staining results, we can conclude that the inactivation of pocket proteins is largely responsible for the unique pattern of staining for MCM7 and p16 in HPV-associated human cancers. To look further at the dysregulation of the p16-RB pathway in mice disrupted in expression of the pocket proteins, we monitored levels of expression of E2F1, itself an E2F responsive gene (35-36) (Supplementary Fig. S2). In nontransgenic mice E2F1 was detected at low levels in the basal compartment of head and neck epithelia whereas it was more strongly detected throughout the full thickness of the epithelium in K14E7 mice. Again, the inactivation of both p107 and pRb most closely reproduced the strong E2F1 staining pattern throughout the epithelium that was observed in E7 transgenic mice. Of the mice singly inactivated for a pocket protein, only the Rb deficient epithelium showed greatly extended E2F1 staining into the suprabasal compartment. Not surprisingly, these results parallel that observed for MCM7 (Fig 4).

EZH2 (enhancer of zeste homologue 2) has been recently identified as a novel target gene activated by HPV-16 E7. EZH2 (i.e., encoded by EZH2) is a component of the PRC2 histone methyltransferase complex, and is also a potential E2F-regulated gene (37). As for MCM7 and E2F1, staining patterns for EZH2 (Supplementary Fig. S2) showed that the mice inactivated for both p107 and pRb showed an EZH2 staining
pattern most similar to that seen in the E7 transgenic mice. These observations are consistent with previous finding that EZH2 induction by HPV-16 E7 is regulated through the inactivation of pocket proteins (38).
Discussion

Our observations demonstrate that the dual inactivation of pRb and p107 nearly fully recapitulates the highly potent oncogenic phenotypes of the HPV16 E7 oncoprotein in head and neck cancer. Indeed, the high incidence as well as high grade of neoplastic disease in the mice deficient for Rb and p107, argues that loss of function of these two genes accounts for E7’s ability not only to cause tumors to arise, but also to promote their progression to a fully malignant state. This is a highly significant given that E7 is the driver of HPV-associated head and neck carcinogenesis (13). It is also remarkable considering that HPV16 E7 has been identified to associate with over 100 other cellular factors, many of which have also been implicated in carcinogenesis.

Our findings do not necessarily discount a role of other associated cellular factors in HPV-associated carcinogenesis. Indeed, many of these interactions between E7 and cellular factors may contribute significantly to E7’s strong oncogenic potency even in ways related to the pocket proteins. This is because E7’s destabilization of the pocket proteins is not absolute. While E7 induces the destabilization of pocket proteins by stimulating their ubiquitination and consequent proteasome-mediated degradation, there are still residual levels of pocket proteins in HPV-positive cancers. But E7 has other means of inactivating pocket protein function. E7 associates with components of the cellular machinery that normally regulate pocket protein activity including Cyclin/Cdk complexes and Cdk inhibitors such as p21. E7 inhibits p21 thereby stimulating phosphorylation of pocket proteins and consequently their functional inactivation. Thus E7 dysregulates pocket protein function by multiple means. That E7 has evolved these multiple means likely reflects the importance of HPV being able to effectively
dysregulate pocket protein function in the context of the viral life cycle, wherein E7 drives the production of progeny virions in the differentiating epithelia (26). But it also may explain E7 strong potency in cancer, in that our study demonstrates that the complete inactivation of p107 and pRb can drive head and neck carcinogenesis.

Our study adds to a growing body of research indicating that p107 as well as p130 function as tumor suppressors in mice. \( Rb^{+/+}p107^{-/-} \) chimeric mice develop various types of tumors at a higher frequency than \( Rb^{+/+} \) mice, indicating that p107 act as a tumor suppressor in the context of \( Rb \)-heterozygosity (39). In context of pRb-deficient mice, p107 is able to act as a tumor suppressor in the context of both retinoblastoma and non–small cell lung cancer (40-41). In addition, p130 acts as a tumor suppressor in pRb/p53-deficient mice in the context of small-cell lung carcinoma and in non-small cell lung carcinomas in the context of the activation of oncogenic K-Ras in pRb-deficient mice (42-43).

Both distinct and redundant functions have been ascribed to the pocket proteins (44). In our studies, compensatory increases in p130 or p107 were observed in the mice doubly deficient in the other two pocket proteins (Fig 1). This raised the possibility that increased levels of one pocket protein could suppress tumorigenicity in the head and neck region. For this reason we generated mice deficient for all three pocket proteins by creating the following genotype: \( K14CreERtmRb^{f/f}p130^{f/f}p107^{-/-} \) mice. However, following treatment of young adult mice with tamoxifen to induce Cre activity, the mice showed severe morbidity including wrinkled skin, loss of coat hair and body weight, and they died within 4 weeks. Topical delivery of 4-OH tamoxifen to the oral cavity failed to reduce the mortality issues. These issues precluded us from carrying out head and neck
cancer studies on triple-null mice.

In human cancers, mutations in \(RB\) are frequently observed, but the same is not true for \(p107\) and \(p130\). This has raised the question whether \(p107\) and \(p130\) are tumor suppressors in the context of human cancers. For most human cancers the answer appears to be no. Yet inactivation of both pRb and the other pocket proteins is a hallmark of HPV-associated cancers, and based upon our findings inactivation of \(p107\) and pRb together both accounts for the potency HPV E7 in causing human cancer, and accounts for the unique biomarker patterns of HPV-associated human cancers. One might ask then, why is it that HPVs cause human cancer in an apparently unique manner. The answer may lie in the recent reports on the mutational profiles of human head and neck cancers. Two groups, using exome analysis, discovered that HPV+ HNSCC have much lower frequencies of mutations in cellular genes compared to HPV- HNSCC, even regardless of the smoking status of the patients with HPV+ HNSCC (45-46). This raises the interesting paradox that HR HPVs create a neoplastic environment that is more resistant to the accumulation of mutations than in other neoplasias. For cancers caused primarily by carcinogenic agents this would clearly be a disadvantageous environment. Another interesting observation made in the above exome analyses was that the Notch pathway is frequently mutated in HNSCC including HPV+ cancers (45-46). A recent study has demonstrated that inactivation of pocket proteins lends hepatocellular carcinoma cells sensitive to Notch-mediated tumor suppression (47). This may provide one explanation for why Notch mutations arise in HPV+ HNSCC.
Acknowledgements

We thank Dr. Julien Sage from Stanford University for his kindly sharing with us mice carrying the p107-null and p130-floxed alleles, and for critically reading the manuscript. This work was supported by grants from the NIH DE017315 and CA022443.
References


### Table 1.

Histopathology summary for the both pRb and p107 conditionally deficient mice treated with Carcinogen for 16 weeks.

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<thead>
<tr>
<th>Genotype</th>
<th>Grade of Tumor (# of mice)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No Tumor</td>
</tr>
<tr>
<td>NTG (n=10)</td>
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</tr>
<tr>
<td>K14E7 (n=10)</td>
<td>5</td>
</tr>
<tr>
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<tr>
<td>K14CreERtmRb&lt;sup&gt;E7&lt;/sup&gt;p107&lt;sup&gt;+/−&lt;/sup&gt; (n=10)&lt;sup&gt;1,3&lt;/sup&gt;</td>
<td>1</td>
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</tbody>
</table>

1. All mice were on the same CD1/129/C57 mixed genetic background (see supplemental information section for details on breeding scheme)
2. P=0.94 comparing the severity of head & neck disease in K14CreERtmRb<sup>E7</sup>p107<sup>+/−</sup> vs K14E7
3. P= 0.0005, 0.0051 comparing the severity of head & neck disease in K14CreERtmRb<sup>E7</sup>p107<sup>+/−</sup> vs NTG, Rb<sup>E7</sup>p107<sup>+/−</sup>

### Table 2.

Histopathology summary for the both pRb and p130 conditionally deficient mice treated with Carcinogen for 16 weeks.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Grade of Tumor (# of mice)</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>9</td>
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<tr>
<td>K14CreRb&lt;sup&gt;E7&lt;/sup&gt;p130&lt;sup&gt;−/−&lt;/sup&gt; (n=15)&lt;sup&gt;1,4&lt;/sup&gt;</td>
<td>5</td>
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</tbody>
</table>

1. All mice were on the same FVB/129/C57 mixed genetic background (see supplemental information section for details on breeding scheme)
2. P=0.0062 comparing the severity of head & neck disease in K14CreRb<sup>E7</sup>p130<sup>−/−</sup> vs K14E7
3. P= 0.0026 comparing the severity of head & neck disease in K14CreRb<sup>E7</sup>p130<sup>−/−</sup> vs NTG
4. P = 0.022, 0.11 comparing the severity of head & neck disease in K14CreRb<sup>E7</sup>p130<sup>−/−</sup> vs K14CreRb<sup>E7</sup>, Rb<sup>E7</sup>p130<sup>−/−</sup>.
Figure Legends

Figure 1. Evaluation of pocket protein expression patterns in head and neck epithelium. Representative sections of lingual epithelium from indicated mouse genotypes stained with anti-pRb antibody (left panel), anti-p107 antibody (middle panel) and anti-p130 antibody (right panel). Examples of strongly positive-stained cells are indicated by black arrows. Brown, positive staining; blue, hematoxylin counterstain. Magnification, x40; scale bar, 200 µm.

Figure 2. Acute phenotypes in head and neck epithelium conferred by deficiency in pocket proteins. In panel A and B are shown quantification of percentage of suprabasal cells supporting DNA synthesis in lingual and esophageal epithelium of 12 week old mice of each genotype (n=3 or 4) that were injected BrdU one hour prior to sacrifice, and paraffin-embedded sections from these mice were stained with anti-BrdU. Panel A: Analysis of mice deficient for p107 alone or both p107 and pRb in comparison to nontransgenic and E7 transgenic mice, all on a CD1/129/C57 mixed genetic background. The asterisk indicate that DNA synthesis level in suprabasal compartment is significantly different compared with either in K14E7 mice or nontransgenic (NTG) mice (P=0.05, two-sided Wilcoxon rank sum test). Panel B: Analysis of mice deficient for p130 alone, pRb alone or both p130 and pRb in comparison to nontransgenic and E7 transgenic mice, all on a FVB/129/C57 mixed genetic background. The double asterisk indicates that DNA synthesis level in suprabasal compartment is significantly different compared with either in K14E7 mice or nontransgenic (NTG) mice (P=0.0495, two-sided Wilcoxon rank sum test).
sum test). In panel C and D are shown the frequency of epithelial cells supporting DNA synthesis in lingual and esophageal epithelium of 12 weeks old mice of each genotype (n=3–4 for each genotype) that were exposed to 0 (white bars) or 12 (black bars) Gy ionizing radiation 24 hours before BrdUrd administration. BrdUrd staining frequency in basal cells is graphed. Stars indicate that basal DNA synthesis in 12 Gy is significantly lower than that counted in 0 Gy. **Panel C:** Analysis of mice deficient for p107 alone or both p107 and pRb in comparison to nontransgenic and E7 transgenic mice, all on a CD1/129/C57 mixed genetic background. **Panel D:** Analysis of mice deficient for p130 alone, pRb alone or both p130 and pRb in comparison to nontransgenic and E7 transgenic mice, all on a FVB/129/C57 mixed genetic background.

**Figure 3. Tumor incidence in carcinogen-treated mice.** Following treatment with 4-NQO for 16 wks and a hold period for 8 wks, animals of the indicated genotypes were sacrificed and overt tumors in their tongue and esophagus were scored. **Panel A:** Incidence of overt tumor in mice deficient for p107 alone or for both p107 and pRb, compared to E7 transgenic mice. Single asterisk indicates that incidence of overt tumors in $K14\text{Cre}E_{R\text{m}}Rb^{f/f}p107^{-/-}$ mice is significantly higher than either nontransgenic ($P = 0.41 \times 10^{-9}$, two-sided Fisher's exact test) or $Rb^{f/f}p107^{-/-}$ mice ($P = 0.57 \times 10^{-5}$), but is not significantly different compared with its seen in $K14E7$ mice ($P = 0.38$). All mice in panel A were on the same CD1/129/C57 mixed genetic background. **Panel B:** Incidence of overt tumor in mice deficient for p130 alone, pRb alone or for both p130 and pRb, compared to E7 transgenic mice. Double asterisks indicate that incidence of overt tumors in $K14\text{Cre}Rb^{f/f}p130^{-/-}$ mice is significantly higher than it observed in nontransgenic ($P$
=0.35x10^{-8}), Rb^{ff}p^{130^-}K14CreRb^{ff} (P =0.0013) mice, but is significantly lower than that seen in K14E7 (P =0.014). All mice in panel B were on the same FVB/129/C57 mixed genetic background.

Figure 4. Evaluation of MCM7 and p16 expression in both epithelium and tumors from head and neck of carcinogen-treated mice. Representative images stained with anti-MCM7 antibody (left) and anti-p16 antibody (right). Brown, positive staining; blue, hematoxylin counterstain. Magnification, x40; scale bar, 200 µm.
Figure 1

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Figure 2
Figure 3

(A) Tumor incidence in different genotypes:
- NTG
- Rb\(^{-/-}\)/p107\(^{+/+}\)
- K14creER/Rb\(^{-/-}\)/p107\(^{+/+}\)
- K1AE\(^{WR}\)

(B) Tumor incidence in different genotypes:
- NTG
- Rb\(^{-/-}\)/p130\(^{+/+}\)
- K14cre/Rb\(^{-/-}\)/p130\(^{+/+}\)
- K1AE\(^{WR}\)

* indicates a significant difference compared to NTG.
** indicates a highly significant difference compared to NTG.
Figure 4

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Pocket Proteins Suppress Head and Neck Cancer

Myeong-Kyun Shin, Henry C. Pitot and Paul F. Lambert

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