# Mechanism of Action of Human Papillomavirus Type 16 E7 in Human Papillomavirus-associated Carcinogenesis

by

Myeong-Kyun Shin

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The dissertation is approved by the following members of Final Oral Committee:
Paul F Lambert, Professor of Oncology
Norman R Drinkwater, Professor of Oncology
William Sugden, Professor of Oncology
Robert F Kalejta, Associate Professor of Molecular Virology and Oncology
Linda A Schuler, Professor of Department of Comparative Biosciences

# Abstract

## Mechanism of Action of Human Papillomavirus Type 16 E7 in Human Papillomavirus-associated Carcinogenesis

Myeong-Kyun Shin

Under the supervision of Professor Paul F. Lambert

at the University of Wisconsin-Madison

Persistent infections by high-risk Human Papillomavirus (HPV) types are associated with nearly all cervical cancer, a large majority of other anogenital cancers and a growing subset of head and neck cancers. Of these high-risk HPVs, HPV type 16 (HPV-16) is the genotype most commonly associated with these cancers. HPV-16 encodes three oncogene, *E5*, *E6*, and *E7* that are largely responsible for HPV-associated malignancies. In HPV transgenic mice, HPV-16 *E7* functions as the predominant oncogene in the context of cervical as well as head/neck cancers. However, the molecular mechanisms by which E7 causes cancers in these organs has remained undefined. HPV-16 E7 protein can interact with over 100 cellular proteins. Among these cellular factors, it is best known for its ability to bind to the tumor suppressor pRb and its related pocket proteins p107 and p130. Prior studies using genetically engineered mice demonstrated that inactivation of pRb alone is not sufficient to recapitulate the oncogenic properties of E7 in both cervical and

head/neck carcinogenesis. Based on these findings, the focus of my thesis was to identify other cellular factors that are important targets of E7 in causing cancers of the cervix and head/neck region. To perform my studies, I made use of mouse models for HPV-associated cervical and head/neck cancers previously established in the Lambert laboratory. In the context of head/neck cancer studies, I learned that combinatorial loss of pRb and p107 led to high susceptibility to head and neck cancer, and are therefore highly relevant E7 targets. In cervical cancer studies, I made two important observations. First, $p21^{Cip1}$ functions as a tumor suppressor in cervical carcinogenesis and its inactivation by HPV-16 E7 partially contributes to E7's oncogenic properties in the cervix. Secondly, in contrast to what I observed in the head/neck region, combinatorial loss of two or even all three pocket proteins was not sufficient to induce cervical cancer, though it did lead to the development of high grade dysplasia in the female reproductive tract. These findings provide evidence for a role of targets other than the pocket proteins and the p21 in mediating E7's oncogenic properties in cervical cancer.

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This thesis is dedicated to

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## Poem by my Supervisor, Dr. Paul F. Lambert

### The impossible dream

To Myeong Kyun I proposed,
three genes you knock out
then watch  those mice closely
for tumors shall sprout

But of these genes I chose
One, Rb, had an essential role
How could the mice survive
with their cells out of control

Such was Myeong Kyun's plight
Achieving my dream
He pondered many a tact
Then conjured a scheme

In their mouths tumors grew
With just two genes lost
This was easy he thought
And little effort did it cost

Then to the cervix he looked
but few tumors were seen
What was he to do
There must be another gene!

Indeed he already knew
a role for another gene, p21
And so you will see
A complicated tail he has spun

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# List of abbreviations

Note: this list does not contain the names of oligonucleotide primers.

BrdU- 5-bromo-2-deoxyuridine

Cdk- cyclin dependent kinase

Cdki- cyclin dependent kinase inhibitor

CC- cervical cancer

CIN- cervical intraepithelial neoplasia

CIS- carcinoma *in situ*

DAB- 3,3'-diaminobenzidine

flox, floxed- flanked by loxP sites

H- normal hyperplasia

HCL- hydrochloric acid

HDAC- histone deacetylase

H&E- hematoxylin and eosin

HNSCC- head and neck sqaumous cell carcinoma

HPV- human papillomavirus

HPV-16- human papillomavirus type 16

IHC- immunohistochemistry

i.p.- intraperitoneal

K14- keratin 14

LIC- large invasive cancer.

*loxP*- locus of excision of phage P, recognition sites for the Cre recombinase

Mcm- minochromosome maintenance

MEF- mouse embryonic fibroblast

MIC- microinvasive cancer

ORF- open reading frame

PCR- polymerase chain reaction

PBS- phosphate-buffered saline

PVDF- polyvinylidene fluoride

*Rb*- the retinoblastoma tumor suppressor gene

pRb- the retinoblastoma tumor suppressor gene product

RIPA- radioimmunoprecipitation assay

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

VC- vaginal cancer

VIN- Vaginal intraepithelial neoplasia

WT- wild-type

$^{137}$Cs- cesium-137

4-NQO- 4-Nitroquinoline 1-oxide

# Chapter 1:

# Introduction

**Basic virology of Human Papillomaviruses**

Human papillomaviruses (HPVs) are small non-enveloped viruses harboring double-stranded circular DNA genomes. There are currently identified approximately 200 HPV genotypes (1) that are formally distinguished by differences in the nucleotide sequence within a portion of the L1 gene encoding the major capsid protein (2). HPVs are epitheliotropic viruses and cause a number of different types of epithelial lesions as a consequence of their infection (3, 4). According to their tissue tropism, HPVs are classified as ‘cutaneous’ if they primarily infect skin, or mucosal if they primarily infect mucosal epithelia lining the anogenital tract and the head/neck region (e.g. oral cavity, oropharynx, larynx). Mucosotropic HPV types can be further subdivided into "low-risk" and “high-risk” HPVs based upon their association with human cancers. Low-risk HPVs, such as HPV6 and 11, cause genital warts and condyloma, but are rarely associated with malignant lesions (3). In contrast, infection of high-risk HPV types often cause clinically inapparent infections yet are associated with the vast majority of cervical cancers, a large fraction of cancers at other anogenital sites, and a growing subset of head and neck cancers.

The viral life cycle of HPV is tightly linked to the cell differentiation process of host cells it infects, and, because HPVs genomes are too small to encode many genes, the viral life cycle including the replication and transcription of the HPV genome largely relies upon host machinery. The initial step in the viral life cycle is thought to involve infection of cells within the basal compartment of stratified epithelia, which are accessed through wounds or micro-abrasions. Basal cells are the normally proliferating component of stratified epithelia, and progression of

the cell through mitosis has been found to be critical for the establishment of the viral genome as a nuclear plasmid in the infected cell (5). Following virus entry, the HPV genomes become established as low-copy number, nuclear plasmids, and then are stably maintained within the basal cells during cell proliferation/division. Upon basal cell division, some daughter cells become detached from the basement membrane normally bound by basal cells. This detachment initiates the terminal differentiation of the host cell. Differentiation of HPV-positive epithelial cells in turn triggers the productive stage of the viral life cycle, in which the viral genome is amplified, late genes encoding structural and other proteins are expressed, and progeny virus is made. The amplification of the viral genome again requires cellular DNA synthesis machinery, but this machinery is normally shut off in differentiating epithelial cells. To re-activate the host cellular replication machinery, early gene products of HPVs disrupt the normal differentiation program of host cells and drive the cells back into the cell cycle. Coincident with this genome amplification, late gene products are expressed, newly synthesized viral genomes are encapsidated and progeny virus particles are then released from the uppermost layers of the epithelium into the environment where they can infect another host.

**Viral proteins of Human Papillomavirus**

HPVs genomes in general encode eight translation open reading frames (ORFs) that, depending upon splicing and sites of transcriptional initiation, encode one or more viral proteins. According to their pattern of expression in the viral life cycle, viral proteins are designated as E (for “early”) and L (for “late”) protein. Late proteins are ones normally only expressed in the

terminally differentiated epithelial cells in the upper layers of a stratified epithelium; whereas early proteins are expressed throughout the life cycle beginning early after the initial infection of basal cells. The viral late proteins include L1, the major capsid protein, which can self assemble into virus like particles which are used for the generation of the currently available VLP based HPV vaccines (6), and L2, the minor capsid protein that is thought to facilitate encapsidation of viral DNA and viral infectivity (7). Of the early viral proteins, E1 and E2 are required for viral genome replication and maintenance. E1 has both adenosine triphosphatase (ATPase) activity and associated DNA helicase activity both of which contribute to the replication of the viral DNA. E2 has high affinity for DNA sequence elements on the viral genome. In addition to acting as a transcriptional regulator, E2 helps recruit E1 to the viral genome in the initial steps leading to viral DNA replication. E2 also plays a critical role in facilitating the inheritance of the viral genomes to daughter cells during cell division. E4's function is poorly understood at present. While named an early protein its expression pattern is more consistent with that of late viral proteins. It is argued to play a role in facilitating amplification of the viral genome and egress of progeny virus from terminally differentiated cells.

The E5, E6, and E7 proteins of high-risk HPV types in addition to contributing to the viral life cycle confer transforming properties to cells in tissue culture and tumorigenic properties in vivo in the context of laboratory animal-based studies. The E5 gene of mucosotropic HPVs such as HPV-6, and HPV-16, cause morphological transformation in rodent and human keratinocytes arguably by increasing the activity of epidermal growth factor receptor (EGFR), thereby leading to stimulation of cellular DNA synthesis (8-11). HPV-16 E5 also has been shown to enhance the immortalization potential of HPV-16 E6 and E7 in primary human

keratinocytes (12), and to stimulate the proliferation of human and mouse primary cells in cooperation with E7 (13, 14). Interestingly, the E5 ORF is not present in all HPV genotypes. Nonetheless, HPV-16 *E5* clearly has been shown to act as an oncogene in the context of skin and cervical carcinogenesis studies in mice (15, 16), and to induce epithelial hyperplasia, which is diminished in the context of mice expressing a dominant negative form of EGFR (17). In the context of the HPV-16 life cycle, E5 plays a quantitative role in augmenting the level of viral genome amplification within the differentiating compartment of the stratified squamous epithelia (17).

The HPV-16 E6 and E7 genes encode proteins that have much stronger transforming properties in tissue culture than does HPV-16 E5. Their role in human cancers is supported by the observation that E6 and E7 are commonly found expressed in these cancers, in many cases to the exclusion of other viral genes. Indeed, in many HPV-associated cancers, integration of the viral genome into the host genome leads to the selective retention of intact E6 and E7 genes, which continue to be expressed. In the case of the cervix, HPV-16 integration events have been shown to provide a powerful growth advantage over cells retaining the HPV-16 genome as a nuclear plasmid (18), and this correlates with increased expression of E6 and E7 owing at least in part to increased mRNA stability (19) and likely also to the loss of expression of the E2 transcriptional regulator which is known to inhibit transcription of the E6 and E7 genes (20). Loss of expression of E6 and E7 in HPV-positive cervical cancer derived cell lines leads to their undergoing senescence or apoptosis (21, 22), supporting the premise that these cancer cell lines remain addicted to these viral oncogenes. HPV-16 E6 and E7 cooperate to induce immortalization of human oral, cervical and foreskin keratinocytes, mammary epithelial cells,

bladder epithelial cells and a number of other epithelial as well as non-epithelial cell types (23). E6 and E7 can also cooperate with other known oncogenes, e.g. myc and activated ras, to transform a variety of cell types (23). In mice, expression of high risk HPV E6 and E7 genes leads to tumors in many tissues in which they are expressed (24). Thus there is an abundance of data to support the role of E6 and E7 in oncogenesis.

HPV-16 E6 and the E6 of other high risk HPVs are best known for their ability to bind to the tumor suppressor p53 in a ternary complex with E6AP, an ubiquitin ligase, leading to proteasome-mediated degradation of p53. E6 thus is able to inhibit the functions of p53 in controlling cell growth and triggering apoptosis in response to cell stress including DNA damage, and this is argued to allow for the accumulation of cellular mutations. E6's ability to inactivate p53 clearly contributes to some of its transforming properties in tissue culture (25, 26) and its tumorigenic properties in mice (27, 28). E6's inactivation of p53 is also believed to play a critical role in the HPV-16 viral life cycle, as viral genomes carrying mutations that render E6 unable to bind to p53 fail to become established as nuclear plasmids (Lorenz et al., unpublished data). High-risk HPV E6 can bind other cellular proteins including PDZ proteins. These latter interactions contribute to E6's transforming activities in tissue culture and tumorigenic properties in vivo in the context of mice. (29-34).

High-risk HPV E7 plays important functions not only in the viral life cycle, but also in the malignancies arising from HPV-infected cells. In the viral life cycle, HPV E7 plays the critical role of creating a cellular environment permissive for amplification of the viral genome within the differentiating compartment of the stratified epithelia. Specifically, it was shown that an E7-

defiicent HPV-16 genome fails to induce DNA synthesis within the suprabasal compartment and fails to amplify its genome in the context of epithelial organotypic cultures of human foreskin keratinocyte cells (35). Moreover, a E7-deficient HPV-16 genome expressed reduced levels of the capsid protein L1 (35). These observations indicate that the expression of E7 is necessary for the productive stage of the viral life cycle. This critical role of E7 in the viral life cycle correlates with its ability to inactivate pRb and the other pocket proteins (36).

HPV-16 E7 is a potent transforming protein. It was shown first to cooperate with activated ras to transform rodent cells in tissue culture. It was subsequently found to synergize with E6 to immortalize a number of different epithelial cell types. In the context of oncogenesis, studies in mice have demonstrated E7 to be the dominant HPV-16 oncogene in the context of cervical (37), anal (38) and head/neck (39) carcinogenesis. However, it is still unknown which cellar targets of HPV E7 contribute to E7's role in HPV-associated carcinogenesis. The goals of this thesis are to define the mechanism of action of HPV-16 E7 in the context of HPV-associated cancers.

**HPV-associated cancers**

It has been estimated that HPV infection accounts for approximately 5 percent of all cancers worldwide (40). High-risk HPV types have been found to be associated with several types of cancer: cervical, vulvar, vaginal, penile, anal, and a subset of head and neck cancers (40-44). In the US alone, there are more than 20,000 HPV-associated cancers arising each year in women. Of these, cervical cancer is the most common, with more than 12,000 cancers arising each year.

More than 11,000 men in the United States are diagnosed with HPV-associated cancers cancer each year, with oropharyngeal cancers being the most common (45).

Cervical cancer is the second most common cancer among women worldwide, although both incidence and mortality rates have declined over the past several decades in advanced countries including the United States (40, 45, 46). The prevalence of Papanicolaou cytology (i.e. Pap smear), which is a screening method to detect cervical intraepithelial neoplasia (CIN) or invasive cervical cancer, led to a dramatic reduction in cervical cancer morbidity and mortality in developed countries over the last 50 years. However, the incidence of cervical cancer has remained high in the developing countries, which lack the resources for widespread screening programs, such as Pap smear and more recently HPV DNA tests.

Persistent infection with high-risk HPVs is thought to be necessary factor in the development of cervical cancer, although it is certainly not sufficient to cause cervical carcinogenesis. Of high-risk HPV types, HPV type 16 is responsible for approximately 50-60% of cervical cancers depending upon the geographical location. HPV-18 is the next most common genotype and is found in approximately 14% of cervical cancers, predominantly in adenocarcinomas (41). In addition to the infection with high-risk HPVs, several cofactors have been associated with the increased risk of persistent infection of high-risk HPVs and/or progression to high-grade CIN or invasive cancer, including smoking, long-term oral contraceptive pill use, high parity, multiple sexual partners, infection with human immunodeficiency virus (HIV) or chlamydia trachomatis(CT), immune suppression as well as several nutritional and dietary factors (47).

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer by incidence worldwide. It can arise in the oral cavity, oropharynx, larynx and hypopharynx (48). The most important risk factors of HNSCCs are tobacco use and alcohol consumption, which seem to have a synergistic effect. Beyond these risk factors, high-risk types of HPVs are etiologically linked to approximately 20% of head and neck squamous cell carcinomas (43, 44). Of these, HPV type 16, a high-risk human papillomavirus (HR-HPVs), is associated with the vast majority of HPV-positive HNSCC (43, 44). HPV-associated HNSCCs primarily arise in the oropharynx and the base of the tongue. In the oropharynx, HPV-associated HNSCC accounts for about 60% of the squamous cell carcinoma (43, 49). Recently, it has been reported that HPV-associated head and neck cancer is increasing in its incidence in U.S. and Europe, despite the fact that overall incidence of head and neck cancer has been slowly decreasing since 1980s (45).

HPV-associated HNSCCs are distinct from HPV-negative HNSCCs at multiple levels including the age of the patients, histopathology and gene expression profiles of the cancers and clinical prognosis. Incidence of HPV-associated head and neck cancer is relatively high in young individuals and less tightly linked with alcohol assumption and smoking (50, 51). In clinical outcome, HPV-associated HNSCCs have a more favorable prognosis. Patients with HPV-associated HNSCC have higher survival rates compared to those with HPV-negative cancers when treated with either radiotherapy or chemo/radiation combination therapy, the two standard-of-care therapeutic regimens. At a molecular level, mutations in the p53 tumor suppressor gene are infrequently observed in HPV-associated head and neck cancers, whereas these cancers are frequently observed to express high levels of p16. In comparison 60-80% of HPV-negative HNSCC have mutations in p53, and are mostly negative for p16 (51). HPV-associated HNSCC

have a gene expression profile distinct from HPV-negative HNSCC and more similar to that of HPV-associated cervical cancer (52). These molecular differences are likely to be largely attributed to the activities of the HPV-encoded oncogenes, *E6* and *E7*.

**Oncogenic properties of high-risk HPV E7**

HPV type 16 encodes three oncogenes; *E5*, *E6*, and *E7*. Of these viral oncogenes, E7 seems to be the most potent oncogene in HPV-associated carcinogenesis. Over the past two decades, a number of research studies have demonstrated the oncogenic potential of high-risk HPVs E7 in carcinogenesis. In tissue culture studies, high risk HPV E7 is able to induce immortalization in primary human keratinocytes, coexpressed with E6 (53). Furthermore, high risk HPV E7 can dysregulate a wide variety of cellular processes, such as gene transcription, aberrant DNA synthesis, protein degradation, epigenetic reprogramming, genomic integrity, and cellular metabolism (54). In the context of transgenic mouse models for HPV-associated human cancers, HPV-16 *E7* acts as the dominant viral oncogene in cervical, anal, and head/neck carcinogenesis (33, 37). However, it remains largely unclear by what mechanism(s) E7 induces cancers. HPV-16 E7 can associate with over 100 cellular proteins (55). Thus it is reasonable to predict that E7 causes cancer though multiple functions.

**Relevant targets of HPV 16-E7 in HPV-associated carcinogenesis**

Of the over 100 cellular proteins known to complex with HPV-16 E7, the retinoblastoma protein is the best characterized. The retinoblastoma tumor suppressor protein (pRb) is a member of a family of three closely related proteins that includes p107 and p130, which have a highly conserved sequence in their so-called pocket domain. pRb is a critical cell-cycle regulator, that regulates the transition of cells from $G_1$-to-S at least in part by binding to and modulating activity of members of the E2F family of transcription factors (56). Under normal conditions (i.e. when its activities and its regulation are not altered), pRb contributes to the suppression of cellular proliferation (56), stimulation of differentiation and senescence (57, 58), cell survival (59) and maintenance of stem cell quiescence (60). In addition, pRb functions as a tumor suppressor in various types of tissues(61). When bound by the high risk HPV E7 oncoproteins, pRb is dissociated from E2F transcription factors and degraded through a proteasome-dependant degradation pathway (62-64). E7's inactivation of pRb, has been argued to be needed for many of the phenotypes caused by E7 *in vivo* in the context of the mouse skin, including E7's dysregulation of the cell cycle, disruption of terminal differentiation, inhibition of DNA damage response, and induction of genomic instability (65, 66) . These conclusions came from the use of mice genetically modified to express a mutant form of pRb that E7 cannot bind. However, other studies that made use of conditional null pRb mice argued that inactivation of pRb is not sufficient to account for E7's oncogenic potential in the context of cervical as well as head/neck carcinogenesis (39, 67). These studies argued that other biochemical activities of E7 contribute to E7-mediated carcinogenesis and set the stage for my own thesis studies.

**Thesis studies**

The goals of my thesis studies have been to define what biochemical activities of E7, other than its inactivation of pRb, contribute to its oncogenic properties. I chose to focus my efforts on biochemical activities of E7 that relate to its dysregulation of the cell cycle. I made this choice for several reasons. Firstly, cell cycle dysregulation is frequently documented in cancer (68). Secondly a major cellular process dysregulated in HPV-associated cervical and head/neck cancers is the cell cycle (52). And thirdly many of the changes in the cell cycle regulation seen in these HPV-associated cancers are thought to be caused by E7. Thus I placed most of my effort at investigating known targets of E7 that are involved in the control of the cell cycle. These included the other pocket proteins, p107 and p130 and the cdk inhibitor, p21. The rationale for their choice is provided below.

One of my main directions of research in this thesis was to investigate the importance of pocket proteins other than pRb as relevant targets of E7 in carcinogenesis. E7 can bind to and degrade both p107 and p103, the two other members of a pocket proteins family. Both p107 and p130 originally were identified as targets of viral oncoproteins encoded by other small DNA tumor viruses with high transforming potential, specifically Adenovirus E1A, and SV40 large T antigen (69-72). All three pocket proteins share some important biochemical functions. p107 and p130 share a high degree of sequence homology with pRb in the large C-terminal domain. All three pocket proteins associate with members of the E2F family of transcription factors. And all three pocket proteins are similarly regulated in their biochemical activities through their phosphorylation by cyclin/cdk complexes. However, there are several distinct differences among

pocket proteins. First, their expression patterns in the context of the cell cycle and cell type are distinct (73). Additionally, whereas pRb primarily binds to transcription activator members of the E2F family, E2F1-3, p107 and 130 primarily bind to transcription repressor members of the E2F family, E2F4 &5. In terms of the role of pocket proteins in human cancer, there are also clear differences. Whereas genetic or epigenetic alterations of *RB* has been reported in various types of human cancers (61), such changes in *p107* and *p130* are rarely observed. That being said, there is clear experimental evidence in mice that p107 and/or p130 can function as tumor suppressors in the context of some tissues (74-78). That and the fact that HPV E7 oncoprotein can inactivate p107 and p130 made them a reasonable focus of my thesis studies.

Another potentially relevant target of HPV-16 E7 that plays an important role in regulating the cell cycle is the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1}$. HPV-16 E7 has been shown to bind to and inactivate $p21^{CIP1}$ (79, 80). The activities of pocket proteins are normally controlled through their phosphorylation by cyclin/cdk complexes, which are in turn are regulated by cdk inhibitors (81). $p21^{CIP1}$ specifically inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4/6 complexes which are involved in regulating entry into S in part through their phosphorylation of pRb. $p21^{CIP1}$ is also an important regulator of DNA stress responses; it is transcriptionally activated by p53, and mediates DNA damage-induced, p53-dependent arrest of cellular DNA synthesis. $p21^{Cip1}$ is also known as a tumor suppressor (82); loss of $p21^{Cip1}$expression is observed in breast cancer and oral/esophageal cancers (83-86). Mice deficient for p21 have increased incidence of spontaneous tumors compared with p21-sufficient mice, and are more susceptible to chemically induced skin cancers (87, 88). Given this

knowledge, we tested the hypothesis that E7's inactivation of p21 contributes to cervical carcinogenesis.

# Chapter 2:
# Materials and Methods

**Transgenic mice.** *K14E7* mice have been previously described (89, 90). *K14Cre* mice were obtained from Dr. Anton Berns at the Netherlands Cancer Institute (60). The *Rb* floxed mice were generated by Julien Sage and Tyler Jacks (91). Briefly, this mutant strain contains *lox P* sites flanking *Rb* exon 3, with no other additional sequences. Expression of Cre recombinase was shown to lead to the excision of the sequence flanked by *lox P* sites and lead to a subsequent frame-shift resulting in a null *Rb* allele.

$Rb^{f/f}p107^{-/-}$,$Rb^{f/f}p130^{-/-}$, $Rb^{f/f}p130^{f/f}p107^{-/-}$ mice were kindly provided by Dr. Julian Sage and previously described (91-93). *KRT14-cre/Esr1* (*K14CreERtm*) was obtained from Jackson Laboratory.

To generate mice inactivated for both pRb and p130, $K14CreRb^{f/f}$ (maintained on the inbred FVB/N genetic background), which have been described previously (39), were crossed to $Rb^{f/f}p130^{-/-}$ mice (on the inbred 129/C57 genetic background) and $K14Cre^{+/-}Rb^{f/+}p130^{+/-}$ offspring then crossed to $Rb^{f/+}p130^{+/-}$ mice to generate $Rb^{f/f}p130^{-/-}$, $K14CreRb^{f/f}$, and $K14CreRb^{f/f}p130^{-/-}$ 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.

To generate mice inactivated for both pRb and p107, *KRT14-cre/Esr1*(i.e., $K14CreERtm^{+/+}$ on inbred CD1 genetic background, The Jackson Laboratory) were crossed to $Rb^{f/f}p107^{-/-}$ mice (on a mixed 129/C57 genetic background) and $K14CreERtm^{+/-}Rb^{f/+}p107^{+/-}$ offspring then crossed to $Rb^{f/f}p107^{-/-}$ mice to generate $K14CreERtmRb^{f/f}p107^{-/-}$and $Rb^{f/f}p107^{-/-}$ 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 for pRb, p107 and p130, *K14CreERtm*$^{+/+}$ were crossed to *Rb*$^{f/f}$*p130*$^{f/f}$*p107*$^{-/-}$ mice (on a mixed 129/C57 genetic background) and *K14CreERtm*$^{+/-}$ *Rb*$^{f/+}$*p130*$^{f/+}$*p107*$^{+/-}$ offspring then crossed to *Rb*$^{f/f}$*p130*$^{f/f}$*p107*$^{-/-}$ mice to generate *K14CreERtm Rb*$^{f/f}$*p130*$^{f/f}$*p107*$^{-/-}$mice on a CD1/129/C57 mixed genetic background. *K14E7*$^{CVQ68\text{-}70AAA}$ (*K14E7*$^{CVQ}$) transgenic mice were generated by mutating amino acids 68-70 in the *K14E7*$^{WT}$ plasmid by PCR. The transgene cassette was excised, purified, and microinjected into FVB/N embryos, which were implanted into pseudopregnant female mice. Founder mice and their offspring were screened by PCR and Southern blot to identify lines with single, stable transgene integration sites. E7 western blots on vaginal tissue lysates identified one line, number 204, which expressed E7$^{CVQ}$ at levels similar to E7$^{WT}$ in *K14E7* line 2304.

All studies in Chapter 3, 4 were performed on a mixed FVB/129/C57 or CD1/129/C57 backgrounds, with all genotypes within each experiment bred to contain the same levels of genetic heterogeneity. All studies in Chapter 5 were performed on FVB backgrounds.

All mice were genotyped by PCR using the following primers: for E7 or E7$^{CVQ}$, oligos 709-1 (5'-GGCGGATCCTTTTATGCACCAAAAGAGAACTG-3') and 709-4 (5'-CCCGGATC CTACCTGCAGGATCAGCCATG-3'); for Cre, oligos Cre-5 (5'-GCACGTTCACCGGCATCA AC-3') and Cre-3 (5'-CGATGCAACGAGTGATGAGGTTC-3'); for Rb$^{flox}$, oligos 5'-lox (5'-CTCTAGATCCTCTCATTCTTC-3') and 3'-lox (5'-CCTTGACCATAGCCCAGCAC-3'); for p107, oligos p107-NEO (5'-ACGAGACTAGTGAGACGTGC-3'), p107-WT (5'-TGTCCTGA

GCATGAACAGAC-3', and p107-COM (5'-TCGCTGGCAGTCTGAGTCAG-3'); for p130, oligos RB-PGK3' (5'-GAAGAACGAGATCAGCAGC-3'), 122-WT (5'-TACATAGTTT CCTTCAGCGG-3'), and p130-C1 (5'-ACGGATGTCAGTGTCACG-3') ; and for $p130^{flox}$, oligos 5'-lox (5'-CTCTAGATCCTCTCATTCTTC-3') and p130-4768r (5'-GAC TGC TGG TAT TAG AAC CC-3')

One hour prior to sacrifice, all mice were intraperitoneally injected with bromodeoxyuridine (BrdU, 10 µl per g body weight of 12.5mg/ml solution). For the irradiation studies, mice were exposed to 0 or 12 Gy ionizing radiation from a $^{137}Cs$ source 24 hours before mice were injected IP with BrdU (10ion studies, mice were exposed to 0 or 12 Gy ionizing radiation from a GTTTriment bred to contain the same levels of genetic heterogeneity. Allned in the American Association for 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 cervical stratified epithelia of *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, 6 week oldmice were intraperitoneally injected with tamoxifen (4mg of tamoxifen dissolved in corn oil (Fisher Scientific Inc.)/day) for 5 consecutive days. To generate the Rb-nulligenic and p130-nulligenic states in the cervical stratified epithelia of *K14CreERtmRb$^{f/f}$p130$^{f/f}$p107$^{-/-}$* mice, 6 week old mice 4-hydroxytamoxifen (4-OHT) (0.05mg of 4-hydroxytamoxifen dissolved in corn oil (Fisher Scientific Inc.) /day) was applied topically to the vaginal cavity 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 (39). 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 (5ign-keys><key app="EN 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 me. Final diagnoses were made by H.C.P., a mouse pathologist, who was blinded to the mouse genotype.

**Immunohistochemical analysis.** Tissue samples were fixed in 4% paraformaldehyde/1X PBS, embedded in paraffin, and cut into 5μm sections. For cervical analyses, histological sections were selected which provide a clear view of the endo- and exocervical epithelium as well as the "transformation zone" where cervical stratified squamous epithelium meets uterine glandular epithelium. Sections were used for hematoxylin and eosin staining (H&E), immunohistochemical staining for pRb, p53, p21, Mcm7, cyclin E, and BrdU, and immunofluorescent staining for keratin 14 (K14).

For immunohistochemical stains, sections were deparaffinized in xylenes and rehydrated through a graded series of ethanol/water solutions. Endogenous peroxidase activity was quenched by treatment in 3% $H_2O_2$ in methanol for 10-20mins. Slides were washed in PBS and heated in boiling 10mM sodium citrate pH6.0 for 20 mins. And then, further unmasking was achieved with 20 minutes of immersion in 2N HCl. Samples were blocked for 30 min at ambient

temperature in 5% horse serum in PBS (for pRb) or 5% horse serum/5% milk/PBS (for p21, MCM7, p16, p107, p130). 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) for 12 hours at room temp. After washes in PBS, biotinylated secondary antibody and streptavidin-peroxidase conjugate were applied according to the Vectastain ABC kit instructions (Vector Labs cat#PK-6200). Staining was developed in 3,3f-diaminobenzidine (DAB) solution (Vector Labs cat#SK-4100) for 2-5 minutes, then quenched in $H_2O$. Slides were counterstained with hematoxylin, dehydrated through a series of ethanols and xylenes, and coverslipped.

**Quantitation of cell proliferation.** BrdU incorporation into newly synthesized DNA was used as a measure of keratinocyte proliferation by counting BrdU-stained tongue/esophagus and cervical sections. All keratinocyte nuclei in ten visual fields were scored as either positive (brown) or negative (blue) for BrdU incorporation in both basal and suprabasal layers in stratified epithelium. For counting purposes, even slightly brown cells were counted as positive. Three to four mice were counted per genotype, and results are presented as the mean +/- standard deviation.

**Statistical analysis.** A two-sided Fisher's exact test was used to determine the significance of differences in the incidence of cervical cancer 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 performed.

**Estrogen treatment and cervical carcinogenesis.** All cervical analyses were performed in estrogen-treated mice. For studies of acute cervical phenotypes, 6-7 week old mice were inserted with a 60 day time-release 0.05mg 17-β-estradiol pellet beneath the dorsal skin (Innovative Research of America, cat #SE-121). Six weeks later, mice were sacrificed and complete reproductive tracts were obtained, fixed 24-48 hours in 4% paraformaldehyde in PBS, and paraffin-embedded. One hundred 5μm sections spanning the thickness of the reproductive tract were made for each mouse. For cervical cancer studies, mice were inserted with one estradiol pellet at 5-7 weeks of age, followed by two more pellets at 60 day intervals. 60 days after administration of the third pellet, mice were sacrificed and as described above and cervical sections were made. Every tenth section was then stained with hematoxylin and eosin and analyzed for the presence of cervical dysplasia or neoplasia. Criteria for histopathological diagnoses have previously been described (37). Briefly, cervical intraepithelial neoplasia (CIN) lesions grade 1-3 were characterized by progressively worse abnormalities in epithelial differentiation, nuclear atypia, and epithelial architecture. Invasive squamous cell carcinomas were diagnosed when nests of squamous cells were seen to have invaded the dermis. Microinvasive cancers were smaller than 0.5 $mm^2$ at their largest cross-section; large invasive cancers were larger. Lesions were only diagnosed if present in 2 or more consecutive H&E stained sections. Uncertain or borderline diagnoses were clarified by consultation with a professional histopathologist. Each mouse was assigned a diagnosis according to the worst cervical lesion detected. Lower uterine cancers including adenocarcinomas and adenosquamous

carcinomas were also detected in several genotypes of estrogen-treated mice including nontransgenic controls. Uterine adenosquamous carcinomas were distinguished from cervical squamous cell carcinomas by the presence of acini of invasive glandular cells. Uterine cancers were excluded from our analysis; throughout Chapter 4 and 5, "cancer" refers to cervical squamous cell carcinoma. Cross-sectional areas of invasive cancers were determined using Zeiss Axiovision 3.1 software on a Zeiss Axioskop microscope.

**E7 western blot.** Mice were treated with estrogen for six weeks as described above. Mice were sacrificed, and the vaginal wall was isolated and homogenized in cold RIPA buffer with protease and phosphatase inhibitors, sonicated briefly, agitated 30 mins at 4C, and spun 20 mins at full speed in a microfuge. Supernatant was collected, and the protein concentration determined by Bradford assay. Samples were run on 15% SDS-PAGE gels, transferred to PVDF membrane, and probed for E7 with a mixture of antibodies (1:150 Zymed 8C9 and 1:200 Santa Cruz ED17). Band intensities were quantified using NIH ImageJ software.

# Chapter 3:

# Pocket Proteins Suppress Head and Neck Cancer

This chapter is accepted in ***Cancer Research*** and currently in press (Myeong-Kyun Shin, Henry C. Pitot, and Paul F. Lambert. **2012** Pocket Proteins Suppress Head and Neck Cancer. ***Cancer Research***). $Rb^{flox}$ $p130^{-/-}$, $Rb^{flox}$ $p107^{-/-}$ mice were kindly provided by Dr. Julien Sage. Various grades of carcinomas in tongue and esophagus were finally verified by Dr. Henry C Pitot. All other experiments were performed by me.

## 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 HPV-16 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 HPV-16 E7 in its contribution to HPV-positive HNSCC.

## Introduction

Head and neck squamous cell carcinomas (HNSCC) are 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 (43, 44). 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 (45). HPV type 16, a high-risk human papillomavirus (HR-HPVs), is associated with the vast majority of HPV+HNSCC (43, 49).

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 (50, 51). 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 (51). 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 (94). HPV+HNSCC also have gene expression profiles very distinct from HPV-HNSCC and more similar to that of HPV-positive cervical cancer (95). These molecular differences have been largely attributed to the activities of the HPV-encoded oncogenes.

HR-HPVs such as HPV-16 encode for three oncogenes; E5, E6, and E7. Of these, E6 and E7 are commonly found to be expressed in HPV+ HNSCC (96), much like in cervical cancer (97). 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 (3, 98). Previously, we reported that HPV-16 E7 is the dominant viral oncogene in HPV-associated HNSCC, based on studies in which HPV-16 transgenic mice were treated with the chemical carcinogen, 4-nitroquinoline 1-oxide (4-NQO) (39, 99). 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 (39). 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.

## 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 (39). HPV-16 E7 has been reported to bind and degrade other pocket protein family members, p107 and p130 (100). 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 $Rb^{f/f}p107^{-/-}$ mice, carrying a conditionally homozygous null alleles of *Rb* in which exon 3 is flanked with *loxP* sites (101). The use of an inducible Cre was necessitated by the fact that $K14CreRb^{f/f}p107^{-/-}$ 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 (60)). Young adult $K14CreERtmRb^{f/f}p107^{-/-}$ 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 $Rb^{f/f}p130^{-/-}$ mice to *K14Cre* mice. Mortality/high morbidity issues were not observed with the mice. To verify the expression level of each pocket protein in $K14CreERtmRb^{f/f}p107^{-/-}$, $K14CreRb^{f/f}p130^{-/-}$, we performed pRb, p107

and p130 immunohistochemical staining on the lingual and esophageal epithelium from these transgenic mice as well as nontransgenic, *Rb$^{f/f}$p107$^{-/-}$*, *Rb$^{f/f}$p130$^{-/-}$*, *K14CreRb$^{f/f}$*, *K14E7* transgenic mice (Figure 3.1, data not shown in esophageal epithelium). As expected, pRb staining was infrequently detected in the tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice as well as the *K14CreRb$^{f/f}$* and *K14CreRb$^{f/f}$p130$^{-/-}$* indicating that the floxed *Rb* allele had undergone recombination. In contrast, pRb staining was detected in Cre-negative *Rb$^{f/f}$* mice, such as *Rb$^{f/f}$p107$^{-/-}$, Rb$^{f/f}$p130$^{-/-}$* mice, as well as in nontransgenic mice (Figure 3.1, left panel). As expected, we could not detect p107 in lingual epithelium from *Rb$^{f/f}$p107$^{-/-}$* and *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, but it was detected in lingual epithelium from *Rb$^{f/f}$p130$^{-/-}$*, *K14CreRb$^{f/f}$, K14CreRb$^{f/f}$p130$^{-/-}$* and nontransgenic mice (Figure 3.1, middle panel). Interestingly, levels of p107 were elevated in *Rb*-deficient tissues from *K14CreRb$^{f/f}$*, and *K14CreRb$^{f/f}$p130$^{-/-}$* mice. Moreover, p107 staining was more strongly and evenly detected in *K14CreRb$^{f/f}$p130$^{-/-}$* mice compared to *K14CreRb$^{f/f}$* mice. p130 staining was not detectable in lingual epithelium nulligenic for p130, such as *Rb$^{f/f}$p130$^{-/-}$*, *K14CreRb$^{f/f}$p130$^{-/-}$* mice (Figure 3.1, right panel). We also observed an increased expression of p130 in *K14CreRb$^{f/f}$, K14CreERtmRb$^{f/f}$p107$^{-/-}$* 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 (35, 102, 103) including head and neck epithelia (39).

Loss of pRb in head and neck epithelia recapitulated some but not all E7-mediated effects on DNA synthesis (39). 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 *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, we observed a significant increase in suprabasal DNA synthesis compared to either nontransgenic or *Rb$^{f/f}$p107$^{-/-}$* mice but this induction of suprabasal DNA synthesis was significantly lower than that observed in *K14E7* mice (Figure 3.2A). In *K14CreRb$^{f/f}$* mice, pRb inactivation alone induced a significant increase in suprabasal DNA synthesis compared to either nontransgenic or *Rb$^{f/f}$p130$^{-/-}$* mice but this induction was less than that quantified in either *K14CreRb$^{f/f}$p130$^{-/-}$* mice or *K14E7* mice (Figure 3.2B), and is consistent with our prior study(39). Similarly, with tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, we observed an increase of suprabasal DNA synthesis in *K14CreRb$^{f/f}$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 (Figure 3.2B). 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 (104-106). 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 (65-67, 107). 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 (Figure 3.2C&D). In *K14CreRb*$^{f/f}$, no effect of pRb inactivation alone was seen in blocking DNA-damage induced arrest of DNA synthesis (Figure 3.2D). Similarly inactivation of either p107 (Figure 3.C) or p130 (Figure 3.2D) failed to influence the DNA damage response. However, this response was as completely inhibited in tamoxifen-treated *K14CreERtmRb*$^{f/f}$*p107*$^{-/-}$ mice (Figure 3.2C) and in *K14CreRb*$^{f/f}$*p130*$^{-/-}$ mice (Figure 3.2D) 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 (Figure 3.2C) or p130 (Figure 3.2D)

alone compared to nontransgenic (p107 and p130 sufficient) mice, but this reduction was not seen in tissues deficient for pRb alone (Figure 3.2D). 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 Figure 3.1).

**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 (107). This protocol was applied to groups of mice with the following genotypes: nontransgenic, $Rb^{f/f}p107^{-/-}$, $Rb^{f/f}p130^{-/-}$, *K14CreRb*$^{f/f}$, *K14CreERtmRb*$^{f/f}p107^{-/-}$, *K14CreRb*$^{f/f}p130^{-/-}$, and *K14E7*. Consistent with our prior studies (39), *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 (Figure 3.3A, Figure 3.3B). *K14CreRb*$^{f/f}$ mice had a significant increase in the incidence of overt tumors compared with that observed in the nontransgenic mice (Figure 3.3B; $p<0.05$), but significantly lower incidence than that developed observed in the *K14E7* mice (Figure 3.3B; $p<0.05$). $Rb^{f/f}p130^{-/-}$ mice had no significant increase in the incidence of overt tumors compared with that observed in the nontransgenic mice (Figure 3.3B; $p=0.26$). $Rb^{f/f}p107^{-/-}$ mice had a marginally significant increase in the incidence of overt tumors compared with that observed in the nontransgenic mice (Figure 3.3A; $p=0.064$). In contrast, *K14CreRb*$^{f/f}p130^{-/-}$ mice had a significant increase in the incidence of overt tumors compared to that observed in *K14CreRb*$^{f/f}$ mice (Figure 3.3B; $p<0.05$), but was

still significantly lower than the incidence observed in *K14E7* mice (Figure 3.3B, $p<0.05$). *K14CreERtmRb$^{f/f}$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 *K14E7* mice (Figure 3.3A; $p=0.69$). 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 (39), half or more of the *K14E7* mice developed invasive carcinomas (50% on CD1/129/C57 mixed background; Table 3.1, 66.7% on FVB/129/C57 mixed background; Table 3.2) and they also developed more severe disease overall compared to nontransgenic mice (Tables 3.1 and 3.2). Mice inactivated for either pRb or p107 did not develop any invasive cancers (Tables 3.1 and 3.2); 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$^{f/f}$p130$^{-/-}$* 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$^{f/f}$* mice (Table 3.2). In *K14CreRb$^{f/f}$p130$^{-/-}$* mice, 13 out of 15 mice had either no tumor or just a benign papilloma, and the other two mice had invasive carcinoma (Table 3.2). The severity of disease in *K14CreRb$^{f/f}$p130$^{-/-}$* mice was not significantly different compared to that in either *Rb$^{f/f}$p130$^{-/-}$* or *K14CreRb$^{f/f}$* mice. In contrast, we found that half of *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice had invasive carcinoma. Likewise, the severity of disease was significantly worse than that scored in *Rb$^{f/f}$p107$^{-/-}$* 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 (108, 109). Moreover, both MCM7 and p16 are induced in head and neck cancers arising in the K14E7 mice (39). 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 (67). It is also likely that p16 is highly expressed because of the dysregulation of p16-pRb pathway in E7-expressing cells (50, 51). 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 (Figure 3.4). , Similar results were obtained data on esophageal epithelium and tumors (data not shown).

As expected (39), the expression of MCM7 was highly up-regulated in both lingual

epithelium and tumors arising in *K14E7* mice (Figure 3.4, 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 $Rb^{f/f}p107^{-/-}$ and $Rb^{f/f}p130^{-/-}$ mice was robust in basal cells with some additional staining in parabasal cells, particularly in the $Rb^{f/f}p107^{-/-}$ mice. MCM7 expression was clearly evident in the tumors from these mice as well. As seen previously (39), MCM7 staining was strong in tumors as well as lingual epithelium of $K14CreRb^{f/f}$ 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 $K14CreERtmRb^{f/f}p107^{-/-}$ and $K14CreRb^{f/f}p130^{-/-}$ 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 (Figure 3.4, 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 (Figure 3.5 for quantification of p16 positivity for each genotype) indicate that each pocket protein can modulate the expression of p16, but loss of pRb 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 (110, 111)(Figure 3.6). 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 pRb deficient epithelium showed greatly extended E2F1 staining into the suprabasal compartment. Not surprisingly, these results parallel that observed for MCM7 (Figure 3.4).

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 (112). As for MCM7 and E2F1, staining patterns for EZH2 (Figure 3.6) 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 (113).

## Discussion

Our observations demonstrate that the dual inactivation of pRb and p107 nearly fully recapitulates the highly potent oncogenic phenotypes of the HPV-16 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 (39). It is also remarkable considering that HPV-16 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 (35). 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 (74). 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 (76, 78). 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 (75, 77).

Both distinct and redundant functions have been ascribed to the pocket proteins (73). In our studies, compensatory increases in p130 or p107 were observed in the mice doubly deficient in the other two pocket proteins (Figure 3.1). 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 (114, 115). 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 (114, 115). A recent study has demonstrated that inactivation of pocket proteins lends hepatocellular carcinoma cells sensitive to Notch-mediated tumor suppression (116). This may provide one explanation for why Notch mutations arise in HPV+ HNSCC.

**Table 3.1 Histopathology summary for the both pRb and p107 conditionally deficient mice treated with Carcinogen for 16 weeks.**

| Genotype[1] | Grade of Tumor (# of mice) | | | | |
|---|---|---|---|---|---|
| | No Tumor | Papilloma/ Polyps | Grade 1 | Grade 2 | Grade 3 |
| NTG (n=10) | 9 | 1 | | | |
| K14E7 (n=10) | | 5 | 3 | 2 | |
| $Rb^{f/f}p107^{-/-}$ (n=10) | 6 | 4 | | | |
| $K14CreERtmRb^{f/f}p107^{-/-}$ (n=10)[2,3] | 1 | 4 | 2 | 2 | 1 |

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$^{f/f}$p107$^{-/-}$* vs *K14E7*
3. P= 0.0005, 0.0051 comparing the severity of head & neck disease in *K14CreERtmRb$^{f/f}$p107$^{-/-}$* vs *NTG*, *Rb$^{f/f}$p107$^{-/-}$*

**Table 3.2 Histopathology summary for the both pRb and p130 conditionally deficient mice treated with Carcinogen for 16 weeks**.

| Genotype | Grade of Tumor (# of mice) | | | | |
|---|---|---|---|---|---|
| | **No Tumor** | **Papilloma/polyps** | **Grade 1** | **Grade 2** | **Grade 3** |
| NTG (n=12) | 11 | 1 | | | |
| K14E7 (n=12) | 1 | 3 | | 4 | 4 |
| K14CreRb$^{f/f}$ (n=15) | 11 | 4 | | | |
| Rb$^{f/f}$p130$^{-/-}$ (n=15) | 9 | 4 | 1 | 1 | |
| K14CreRb$^{f/f}$p130$^{-/-}$ (n=15)[2,3,4] | 5 | 8 | 1 | | 1 |

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$^{f/f}$p130$^{-/-}$* vs *K14E7*
3. *P* =0.0026 comparing the severity of head & neck disease in *K14CreRb$^{f/f}$p130$^{-/-}$* vs *NTG*
4. *P* = 0.022, 0.11 comparing the severity of head & neck disease in *K14CreRb$^{f/f}$p130$^{-/-}$* vs *K14CreRb$^{f/f}$*, *Rb$^{f/f}$p130$^{-/-}$*.

**Figure 3.1**

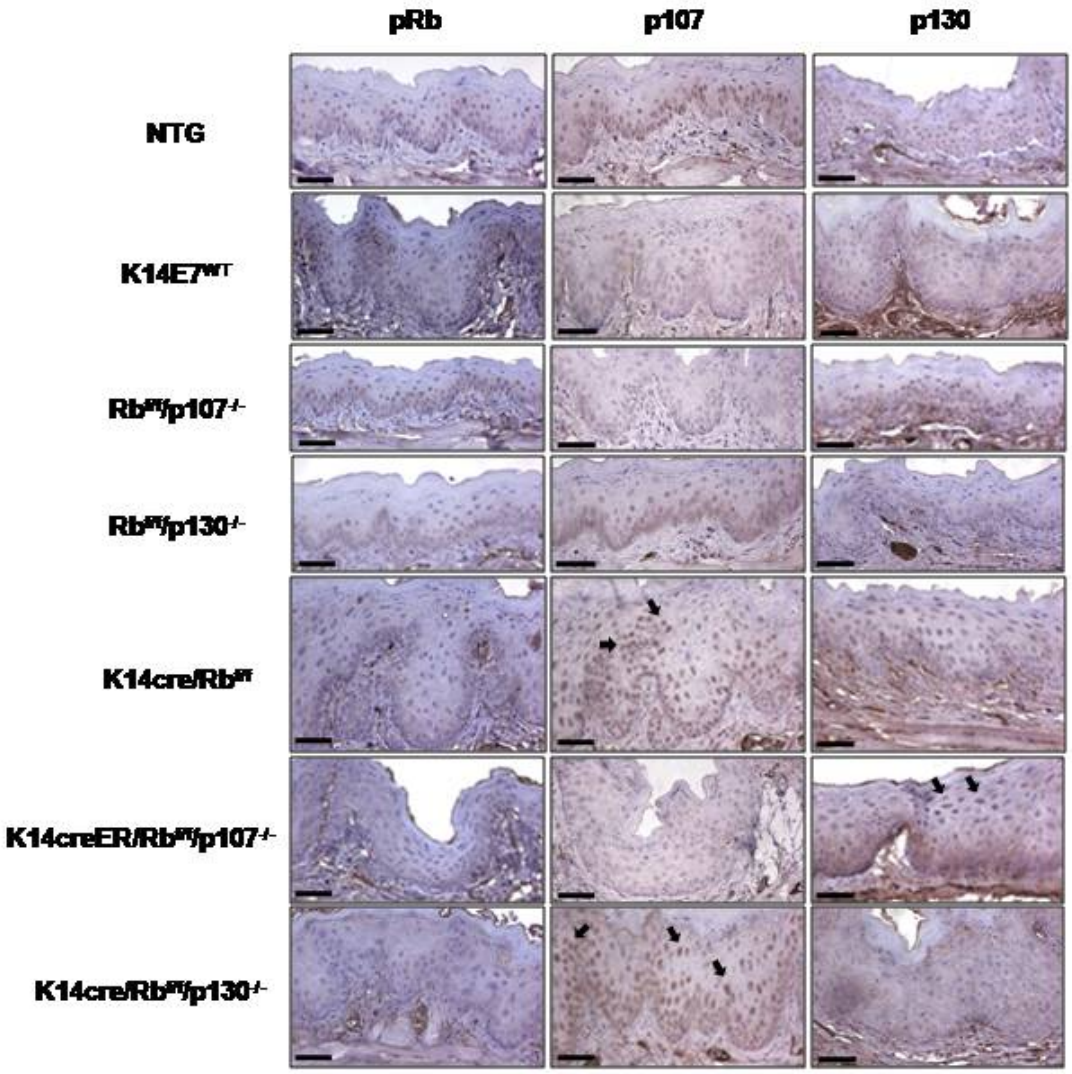

pRb
p107
p130
NTG
K14E7WT
Rbf/f/p107-/-
Rbf/f/p130-/-
K14cre/Rbf/f
K14creER/Rbf/f/p107-/-
K14cre/Rbf/f/p130-/-

**Figure 3.1 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 3. 2**

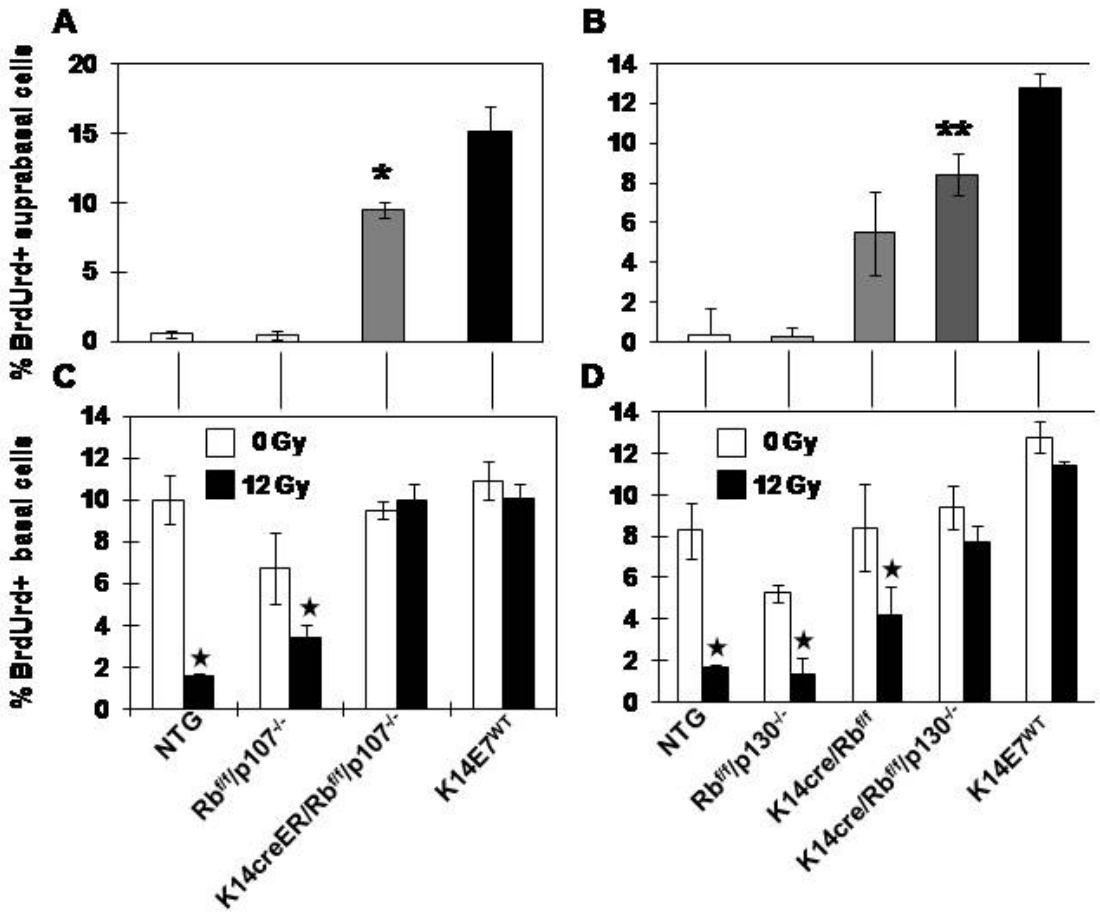

A
B
C
D
% BrdUrd+ suprabasal cells
% BrdUrd+ basal cells
0 Gy
12 Gy
NTG
Rb^f/f/p107^-/-
K14creER/Rb^f/f/p107^-/-
K14E7^WT
Rb^f/f/p130^-/-
K14cre/Rb^f/f
K14cre/Rb^f/f/p130^-/-

**Figure 3.2 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=3or 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). 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 3.3**

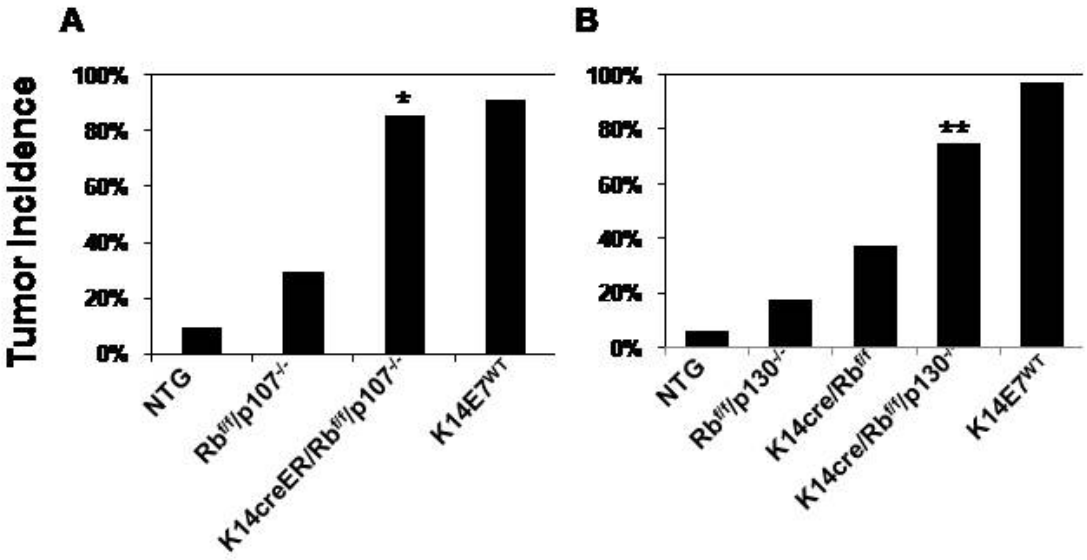

A
B
Tumor Incidence
100%
80%
60%
40%
20%
0%
±
±±
NTG
Rb^f/f/p107^-/-
K14creER/Rb^f/f/p107^-/-
K14E7^WT
Rb^f/f/p130^-/-
K14cre/Rb^f/f
K14cre/Rb^f/f/p130^-/-

**Figure 3.3 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 *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice is significantly higher than either nontransgenic ($P$ =0.41x10$^{-9}$, two-sided Fisher's exact test) or *Rb$^{f/f}$p107$^{-/-}$* mice ($P$ =0.57x10$^{-5}$), 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 *K14CreRb$^{f/f}$p130$^{-/-}$* mice is significantly higher than it observed in nontransgenic ($P$ =0.35x10$^{-8}$), *Rb$^{f/f}$p130$^{-/-}$*,( $P$ =0.1461x10$^{-5}$) *K14CreRb$^{f/f}$* ($P$ =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 3.4**

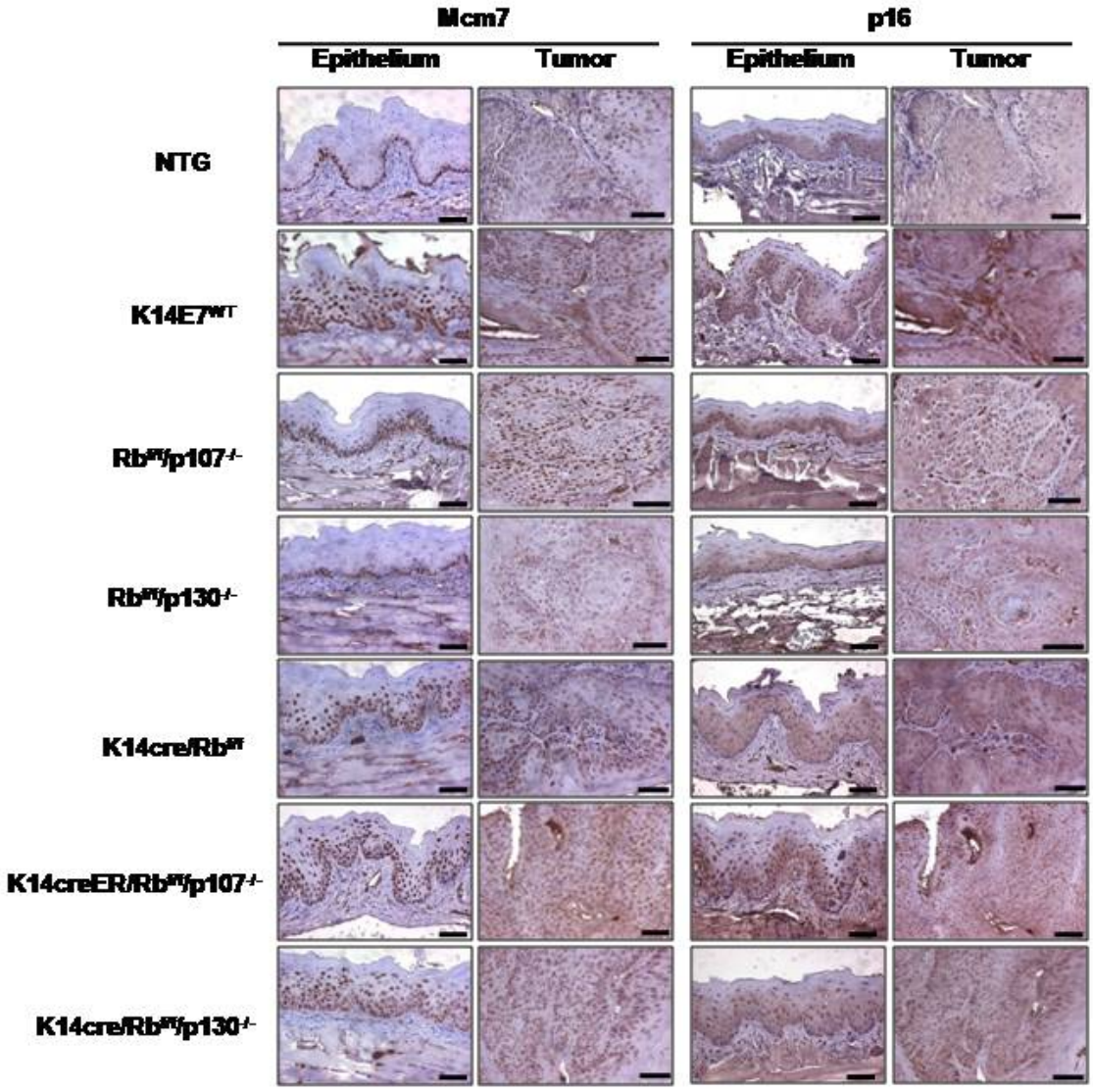

Mcm7
p16
Epithelium
Tumor
Epithelium
Tumor
NTG
K14E7WT
Rbfl/fl/p107-/-
Rbfl/fl/p130-/-
K14cre/Rbfl/fl
K14creER/Rbfl/fl/p107-/-
K14cre/Rbfl/fl/p130-/-

**Figure 3.4 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 3.5**

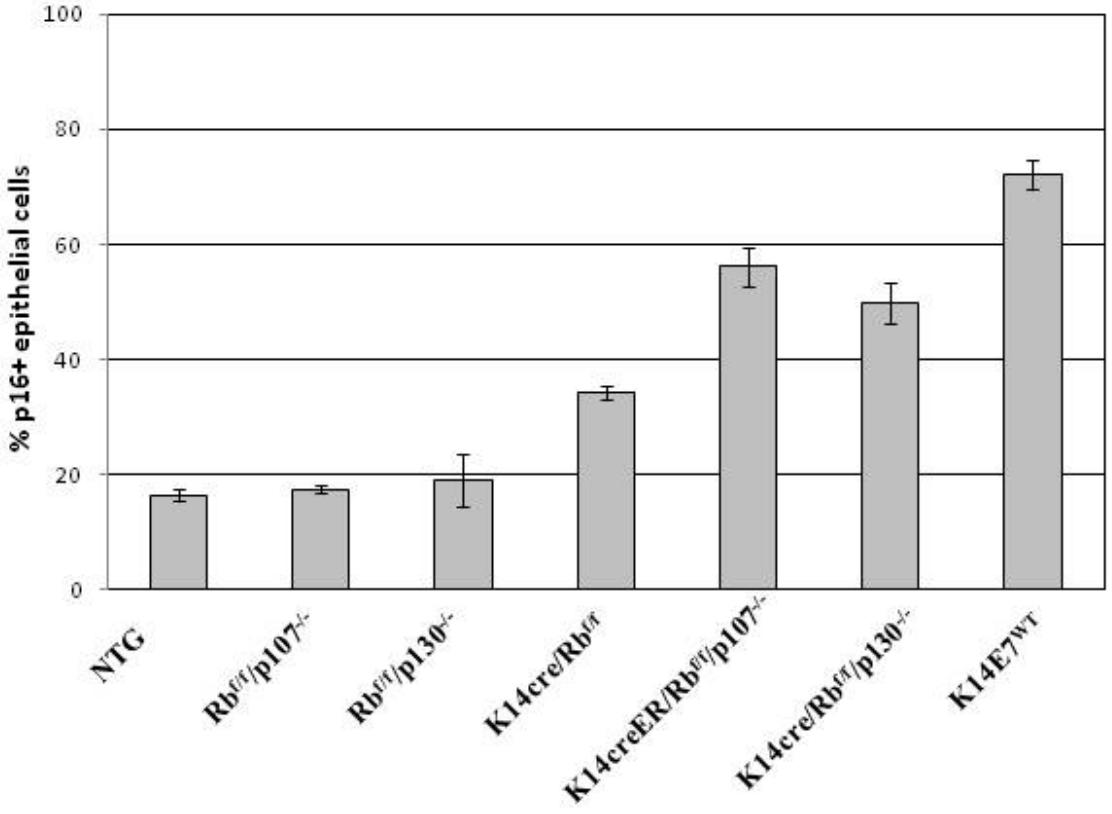

100
80
60
40
20
0
% p16+ epithelial cells
NTG
Rbf/f/p107-/-
Rbf/f/p130-/-
K14cre/Rbf/f
K14creER/Rbf/f/p107-/-
K14cre/Rbf/f/p130-/-
K14E7WT

**Figure 3.5 Evaluation of p16 expression in lingual epithelium.** Shown graph is the quantification of the percentage of p16-positive stained cells in lingual epithelium of 4-NQO treated mice of each genotype (n=3).

**Figure 3.6**

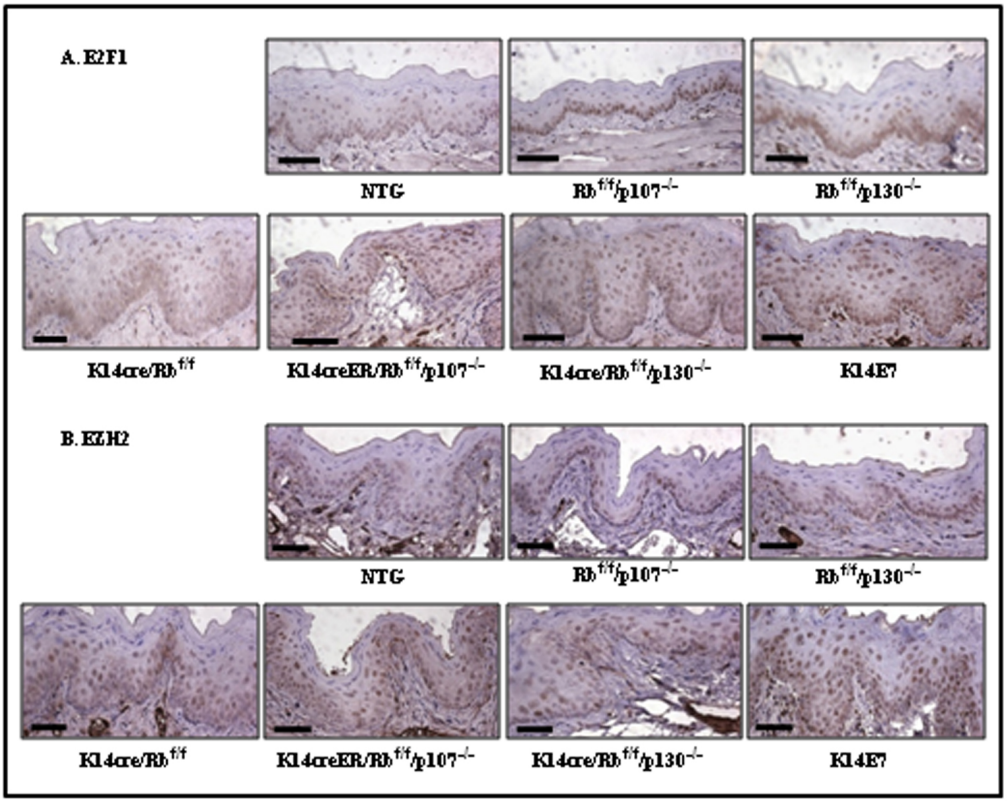

A. E2F1
NTG
Rb$^{f/f}$/p107$^{-/-}$
Rb$^{f/f}$/p130$^{-/-}$
K14cre/Rb$^{f/f}$
K14creER/Rb$^{f/f}$/p107$^{-/-}$
K14cre/Rb$^{f/f}$/p130$^{-/-}$
K14E7
B. EZH2
NTG
Rb$^{f/f}$/p107$^{-/-}$
Rb$^{f/f}$/p130$^{-/-}$
K14cre/Rb$^{f/f}$
K14creER/Rb$^{f/f}$/p107$^{-/-}$
K14cre/Rb$^{f/f}$/p130$^{-/-}$
K14E7

**Figure 3.6. Evaluation of E2F1 and Ezh2 expression in lingual epithelium of carcinogen-treated mice.** Representative sections stained with anti-E2F1 antibody (A) and anti-Ezh2 antibody (B). Brown, positive staining; blue, hematoxylin counterstain. Magnification, x40; scale bar, 200 μm.

# Chapter 4:

# Inactivation of Pocket Proteins Is Not Sufficient to Efficiently Induce Cervical Cancer in Mice

This chapter will be submitted for publication to ***Cancer Research*** (Myeong-Kyun Shin, Julian Sage and Paul F. Lambert). $Rb^{flox}$p130$^{-/-}$, $Rb^{flox}$p107$^{-/-}$, $Rb^{flox}p130^{flox}$ p107$^{-/-}$, mice were kindly provided by Dr. Julien Sage. All other analyses were performed by me.

## Abstract

HPV-16, which is associated etiologically with approximately 60% of cervical cancers, encodes three oncogenes: *E5*, *E6* and *E7*. Of these HPV-16 oncogenes, *E7* has been found to be the dominant viral oncogene to induce cervical cancer in mice. Over 100 cellular proteins can associate with HPV-16 E7. E7 is known to dysregulate the cell cycle at least in part through its capacity to bind and induce the degradation of pRb and its related pocket protein family members, p107 and p130. Importantly, combination of these activities has correlated with E7's ability to induce cancer in mouse head and neck region. We previously showed that the inactivation of pRb is itself not sufficient to recapitulate the oncogenic properties of E7 in cervical carcinogenesis. In this study, we examined the susceptibility of cervical cancer in mice deficient in the expression of pRb and either of two related "pocket" proteins, p107 and p130, or in mice deficient in the expression of all three pocket proteins. We report here that combinatorial loss of two or all pocket proteins is not sufficient to account for the oncogenic potential of E7 in cervical carcinogenesis. These data indicate the oncogenic properties of HPV-16 E7 in cervical carcinogenesis involve its ability to disrupt genes that are not limited to pRb and its related pocket proteins.

## Introduction

Cervical cancer remains a major woman's health problem worldwide, although both incidence and mortality rates have declined over the past several decades in developed countries including the United States owing to screening efforts (45, 46) . Infections by a subset of human papillomavirus (HPV) are the primary cause of cervical cancer and, among this 'high-risk' subset of human mucosal HPVs, HPV type 16(HPV-16) is responsible for approximately 60% of cervical cancers (117). HPV-16 encodes three oncogenes; *E5, E6,* and *E7* (8, 118). Of these, *E6* and *E7* are commonly found expressed in HPV-associated cervical cancers (119). *E7* was identified to be the predominant oncogene in terms of its ability to induce cervical cancer in transgenic mouse models (15, 37).

In tandem affinity purification/mass spec analyses, HPV-16 E7 protein has been found associated with over 100 different cellular proteins (55). Through these associations, E7 has been implicated in dysregulating a wide range of cellular processes, such as gene transcription, DNA synthesis, protein degradation, epigenetic reprogramming, genomic integrity, cellular metabolism, and so on (54). Of these cellular targets of E7, the best known are the tumor suppressor pRb and its related pocket protein family members, p107 and p130 (69, 120, 121). pRb is the best known member of pocket protein family. It is an important regulator of the cell cycle in the transition point from the G1 phase to the S phase at least in part because it can bind to an inactivate a family of transcription factors called E2Fs. In response to mitogenic stimuli, pRb is post-translationally modified via phosphorylation by cyclin/cdk complexes, resulting in its release from, and consequent activation of these E2F transcription factors that are key

regulator of expression of genes involved in cell cycle progression (56). Previously, it has been shown that E7's binding to pRb leads to pRb's inactivation and degradation through the proteasome-dependent degradation (62). As a result, the activation of theE2F transcription factors results in the transition of cell cycle from G1 to S. Inactivation of pRb by E7 also contributes to alterations in other cellular processes including differentiation, DNA damage responses, centrosome synthesis and tumorigenesis (66, 105, 122, 123). Importantly, pRb is defined as a tumor suppressor. In various types of human cancers, either genetic or epigenetic inactivation of *RB* has been reported (61). These findings support the hypothesis that pRb inactivation by E7 likely is an important contributor to the oncogenic potential of HPV-16 E7 in cervical carcinogenesis. However, in our prior studies, we discovered that inactivation of pRb is not sufficient to account for E7's ability to induce cervical cancer in mice (67). This observation led us to the present studies directed at asking what other cellular targets of E7 contribute to cervical carcinogenesis.

One key property of E7 is its ability to dysregulates the cell cycle. Global gene expression analysis in HPV-associated cancers indicated that many of the cell cycle regulatory genes induced in HPV-positive cancers are E2F-responsive genes (52), For this reason we focused our attention on other cellular targets of E7 that are involved in cell cycle regulation, specifically other members of the pocket protein family, p107 and p130 (100). p107 and p130 have similarities with pRb, both in their overall structure, their ability to bind and inactivate E2Fs, as well as sequence homology in a large C-terminal domain known to mediate their interaction with viral oncoproteins such as SV-40 LT, Adenovirus E1A, and HPV E7 (69-72). There is clearly functional overlap among the pocket proteins, as demonstrated by genetic compensation among

pocket protein family members: pRb, p107 and p130 (73). Despite these similarities to pRb, the tumor suppressive activity of either p107 or p130 remains largely questioned, because genetic alteration in either p107 or p130 is not commonly found in human cancers (61, 124). Nevertheless, the concept that p107 and/or p130 function as tumor suppressors in the context of cervical carcinogenesis has remained a popular hypothesis because HPV-16 E7 is able to inactivate both p107 and p130 as it can pRb. Furthermore, multiple studies in mice have shown that p107 as well as p130 can function as tumor suppressors in different tissue contexts (74-78), including our own studies in the context of head and neck carcinogenesis (Shin et al, in press).

In this study, we made use of genetically engineered mice to determine whether the combined loss of function of multiple pocket proteins is sufficient to account for the oncogenic properties of HPV-16 E7in cervical carcinogenesis. Our results demonstrate that the combinational inactivation of two or all three pocket proteins is not sufficient to induce cervical cancer. These findings indicate that the other cellular target(s) of E7 must contribute to cervical carcinogenesis. However, we did find that the combinational inactivation of pocket proteins does account for certain acute phenotypes conferred by HPV-16 E7 in the context of cervical epithelium and also we found evidence for genetic compensation among the pocket protein members in the murine cervix.

## Results

**Conditional inactivation of both *Rb* and *p107*, or *Rb* and *p130* in cervical epithelia.** HPV-16 E7 has been well characterized for its ability to bind and degrade pocket protein family members such as pRb, p107 and p130 (100). We have shown previously that pRb inactivation alone is not sufficient to reproduce the oncogenic phenotypes induced by E7 in context of cervical carcinogenesis (67). Thus, we asked if combinatorial loss of two pocket proteins is sufficient to reproduce the oncogenic effect by E7. To study combined loss of pRb and other pocket protein member, either p107 or p130, contributes to cervical carcinogenesis, we generated mice that were inactivated for both pRb and p130, or pRb and p107 in stratified cervical epithelia. This was achieve using a conditional knockout allele of *Rb*, together with a germline knockout allele for *p130*. Specifically, to knock out both *Rb* and *p130* in the cervical epithelium, we crossed $Rb^{f/f}p130^{-/-}$ mice to *K14Cre* mice, a transgene that directs expression of constitutively active Cre to all stratified squamous epithelia of the adult mouse. The same strategy could not be used to generate mice knocked out both *Rb* and *p107* because $K14CreRb^{f/f}p107^{-/-}$ mice display high rates of mortality at a very early age, which we ascribe to difficulty in the mice drinking water or eating food owing to hyperplasia leading to occlusion of the esophagus (Shin et al., in press, (60)). Therefore, to generate mice inactivated for both pRb and p107 in cervical epithelium, we crossed $Rb^{f/f}p107^{-/-}$ mice to *K14CreERtm*, a transgenic mouse strain that expresses a tamoxifen-inducible form of Cre in stratified squamous epithelia of the adult mouse. We then administered tamoxifen to adult $K14CreERtmRb^{f/f}p107^{-/-}$ mice, at 6 weeks of age, well after the

age when mortality was observed in the *K14CreRb$^{f/f}$p107$^{-/-}$* mice. This strategy eliminated mortality issues presumably because the adult esophagus is sufficiently large that the epithelial hyperplasia resulting from inactivation of both pRb and p107 does not occlude it. To verify the efficiency of Cre or CreERtm-induced disruption of pRb expression in cervical stratified epithelium of the *K14CreRb$^{f/f}$p130$^{-/-}$*and *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, we performed pRb, as well as p107 and p130 immunohistochemical staining on the female reproductive tracts of these mice**,** as well as that of nontransgenic, *Rb$^{f/f}$p130$^{-/-}$*, *Rb$^{f/f}$p107$^{-/-}$*, *K14CreRb$^{f/f}$*, and *K14E7* transgenic mice (Figure 4.1). As expected, pRb staining in cervical epithelium was sharply reduced in the *K14E7*, *K14CreRb$^{f/f}$*, *K14CreRb$^{f/f}$p130$^{-/-}$* and the tamoxifen-treated *K14CreERtm-Rb$^{f/f}$p107$^{-/-}$*mice, while still observed in Cre-negative *Rb$^{f/f}$* mice, such as *Rb$^{f/f}$p130$^{-/-}$*, *Rb$^{f/f}$p107$^{-/-}$* mice (Figure 4.1, left panel). These observations indicate that the floxed *Rb* allele in cervical stratified epithelium had undergone recombination by K14 promoter-driven Cre or CreER transgene. Likewise, we could not detect p107 in cervical stratified epithelium nulligenic for p107, such as *Rb$^{f/f}$p107$^{-/-}$* and tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, as well as *K14E7* mice, but it was detected in the *Rb$^{f/f}$p130$^{-/-}$*, *K14CreRb$^{f/f}$*, *K14CreRb$^{f/f}$p130$^{-/-}$*, and nontransgenic mice (Figure 4.1, middle panel). It is interesting to note that the expression of p107 was increased in both *K14CreRb$^{f/f}$*, *K14CreRb$^{f/f}$p130$^{-/-}$* mice, which are conditionally pRb-deficient tissues, over that observed in *Rb*-sufficient nontransgenic control mice. Moreover, p107 positive-stained cells were more uniformly observed in the stratified epithelium of the*K14CreRb$^{f/f}$p130$^{-/-}$* mice compared to that of the*K14CreRb$^{f/f}$* mice. These results indicate that compensatory increases in expression of p107 can arise in cervical epithelium when the tissue is deficient in the expression of pRb alone, and more so when it is deficient in expression of both pRb and p130. A

similar trend was observed for p130. It was not detectable in cervical epithelium from both *Rb$^{f/f}$p130$^{-/-}$* and *K14CreRb$^{f/f}$p130$^{-/-}$* mice, which are harboring the *p130* null allele (Figure 4.1, right panel), and was increased in pRb-deficient tissue, though not nearly as strongly as was seen for p107. Taken together, these data indicate that the K14driven Cre and CreERtm transgenes were effective at inducing recombination of the floxed *Rb* allele in the cervical epithelia within the female reproductive tract, and demonstrate that compensatory increases in expression of functional pocket proteins can arise in cervical stratified epithelium.

**Suprabasal DNA synthesis in cervical stratified squamous epithelium deficient in pocket proteins.** HPV-16 E7 confers acute changes to the cervical epithelium including the reprogramming suprabasal cells to support DNA synthesis, and disrupting DNA damage-induced cell cycle arrest. These effects of E7 may contribute to aberrant cell proliferation, the accumulation of mutations and carcinogenesis (35, 75, 103, 104, 106). As previously described (67), pRb inactivation alone was not sufficient to cause these same phenotypes in cervical epithelium. To determine if the combined loss of pRb and p130, or pRb and p107 in cervical stratified epithelia could confer one these phenotypes, the induction of DNA synthesis in suprabasal cells, we analyzed by immunohistochemistry (67, 125), 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. In *Rb$^{f/f}$p130$^{-/-}$*, tamoxifen-treated *Rb$^{f/f}$p107$^{-/-}$*, and *K14CreRb$^{f/f}$* mice, there was no significant induction of suprabasal DNA synthesis in cervical stratified epithelia compared to nontransgenic mice (Figure 4.2A, B), indicating that inactivation of any one pocket protein is not able to induce a suprabasal DNA synthesis in cervical stratified epithelia. The data obtained with the *K14CreRb$^{f/f}$* mice was consistent with our prior study (67).

In contrast, in *K14CreRb$^{f/f}$p130$^{-/-}$* mice, we observed a significant increase in suprabasal DNA synthesis compared to either *Rb$^{f/f}$p130$^{-/-}$* or *K14CreRb$^{f/f}$* mice though this induction of suprabasal DNA synthesis was significantly lower than that observed in *K14E7* mice (Figure 4.2A). Similarly, we observed an increase of suprabasal DNA synthesis in tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice compared to either nontransgenic or tamoxifen-treated *Rb$^{f/f}$p107$^{-/-}$* mice, but the frequency of BrdUrd-positive cells was less than that observed in *K14E7* mice (Figure 4.2B). These observations demonstrate that the combined loss of pRb and either p107 or p130 partially account for the DNA synthesis in suprabasal compartment induce by E7.

**Abrogation of DNA damage response in irradiated cervical epithelia deficient for pRb and p130, or pRb and p107.** To determine if the combined inactivation of pRb and either p107 or p130 is sufficient to account for HPV-16 E7's ability to inhibit DNA damage response, mice were exposed to ionizing radiation from a $^{137}$Cs source, injected with BrdUrd 24 hours later, and sacrificed 1 hour after injection. The prevailing effect of ionizing radiation on cervical epithelia is an arrest in DNA synthesis within the normally proliferating basal layer of the cervical epithelium, which is maximally observed at 24 hours post-irradiation. We therefore scored the frequency of BrdUrd-positive basal cells at this time point in the cervical epithelia of the different mouse strains. As expected, there was a significant radiation-induced arrest in DNA synthesis after irradiation in nontransgenic mice, but this effect of radiation was absent in the *K14E7* mice (Figure 4.2 C&D), consistent with prior studies demonstrating that E7 abrogates this DNA damage response (104-106). In mice singly deficient for pocket protein (*Rb$^{f/f}$p130$^{-/-}$*, tamoxifen-treated *Rb$^{f/f}$p107$^{-/-}$*, *K14CreRb$^{f/f}$* mice), we also observed a significant decrease in DNA synthesis after irradiation, indicating that single inactivation of pocket proteins is not able

to inhibit DNA damage responses in cervical epithelium. For the *K14CreRb$^{f/f}$* mice, this result was consistent with our prior study (67). However, in both *K14CreRb$^{f/f}$p130$^{-/-}$* and tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, we saw an abrogation of the DNA damage response much like that seen in *K14E7* mice. Thus combined loss of pRb and either p107 or p130 is sufficient to recapitulate E7's ability to inhibit DNA damage response in cervix. These result also support the premise that there is some degree of functional overlap among pocket proteins in the context of radiation-induced cessation in cellular DNA synthesis, as inactivation of individual pocket proteins failed to lead to abrogation of DNA damage responses.

**Contribution of combinatorial inactivation for either pRb and p107, or pRb and p130 on cervical carcinogenesis.** To learn if combined loss of pRb and either p107 or p130 is also able to recapitulate the oncogenic potential of E7 in cervical carcinogenesis, we exposed mice of the different genotypes described above to exogenous estrogen for 6 months. Exogenous estrogen sufficient to induce continuous estrus acts as a co-carcinogen in the mouse cervix, leading to high incidence of progressive neoplastic disease culminating in cervical cancer in HPV-16 transgenic mice. E7 transgenic mice are particularly sensitive to estrogen-induced cervical cancers (37). After treatment of mice with estrogen for 6 months, we evaluated the incidence of cervical cancer and precancerous cervical lesions in cohorts of mice of the following genotypes: nontransgenic, *Rb$^{f/f}$p130$^{-/-}$*, tamoxifen-treated *Rb$^{f/f}$p107$^{-/-}$*, *K14CreRb$^{f/f}$*, *K14CreRb$^{f/f}$p130$^{-/-}$*, tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$*, and *K14E7* mice. Note that because mice deficient for pRb and p130 were generated on one mixed genetic background, and mice deficient for pRb and p107 were generated on a slightly different genetic background (see Materials and Methods), separate sets of positive and negative control groups (e.g. nontransgenic,

E7 transgenic, and mice disrupted for individual pocket proteins) were generated on each of these two genetic backgrounds, and the histopathology results for each genetic background are reported in separate tables (Tables 4.1 and 4.2). The frequency of cervical cancer and overall severity of disease between the negative controls (i.e. comparing nontransgenics in Table 4.1 to nontransgenics in Table 4.2 p = 1, 1) and between the positive controls (i.e comparing *K14E7* in Table 4.1 to *K14E7* in Table 4.2, p = 0.7722, 0.9921) on the two genetic backgrounds was not significantly different (see Tables 4.1 and 4.2) indicating that these two genetic backgrounds had little difference in their susceptibility to cervical carcinogenesis. Consistent with our prior studies (37, 67), nearly all K14E7 mice developed severe cervical dysplasia and/or invasive cervical cancers, while most nontransgenic mice failed to develop high-grade cervical dysplasia or cancer (Tables 4.1 and 4.2). Neither high-grade dysplasia nor cervical cancer was observed in estrogen treated $Rb^{f/f}p130^{-/-}$, $K14CreRb^{f/f}$ (Table 4.1) and tamoxifen-treated $Rb^{f/f}p107^{-/-}$mice (Table 4.2), indicating that inactivation of single pocket proteins is not sufficient to induce either severe cervical intraepithelial neoplasia (CIN) or cancer. Interestingly, in the $K14CreRb^{f/f}p130^{-/-}$ and tamoxifen-treated $K14CreERtmRb^{f/f}p107^{-/-}$ mice, we failed to see a significant increase in frequencies of cancer compared to that observed in the single inactivation of pocket proteins (Tables 4.1 and 4.2) as further discussed below.

In $K14CreRb^{f/f}p130^{-/-}$ mice, only one mouse out of 29 mice develop invasive cervical cancer (Table 4.1); the incidence of cervical cancer in these $K14CreRb^{f/f}p130^{-/-}$ mice was not significantly different compared to that in either $Rb^{f/f}p130^{-/-}$ (p=0.4677), $K14CreRb^{f/f}$ (p= 0.4085), or the nontransgenic (p=1) mice; whereas it was significantly lower than that seen in the K14E7 mice (p=0.014). The severity of cervical disease, which takes into account the frequencies of not

only cancer but also precancerous lesions, in *K14CreRb$^{f/f}$p130$^{-/-}$* mice was significantly worse compared to that in *Rb$^{f/f}$p130$^{-/-}$* ($p=7.7 \times 10^{-7}$), *K14CreRb$^{f/f}$* ($p=1.3 \times 10^{-7}$), and nontransgenic ($p=8.2 \times 10^{-6}$) mice, but still not as severe as that observed in *K14E7* mice ($p=1.7 \times 10^{-10}$). These results indicate that the combined loss of pRb and p130 contributes to development of precancerous lesions in cervix, but is not sufficient to cause cancer to develop.

In tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice, 2 out of 23 mice developed cervical cancer (Table 4.2); this incidence of cervical cancer was not significantly different compared to that in tamoxifen-treated nontransgenic ($p=0.49$) or tamoxifen-treated *Rb$^{f/f}$p107$^{-/-}$* ($p=0.49$) mice. However, the severity of cervical disease in tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice was significantly worse than that observed in either tamoxifen-treated nontransgenic ($p=1.2 \times 10^{-9}$) or tamoxifen-treated *Rb$^{f/f}$p107$^{-/-}$* ($p=5.0 \times 10^{-9}$) mice. All of tamoxifen-treated *K14CreERtmRb$^{f/f}$ p107$^{-/-}$* mice developed moderate and/or severe dysplasia (Table 4.2), whereas most of *K14CreRb$^{f/f}$p130$^{-/-}$* mice developed only low to moderate grade dysplasia (Table 4.1). This difference was significant ($p=3.49 \times 10^{-7}$), suggesting that inactivation of pRb and p107 leads to a greater propensity for neoplastic progression than does inactivation of pRb and P130. Nevertheless, the severity of disease in tamoxifen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice was not nearly as severe as that in tamoxifen-treated *K14E7* mice ($p=5 \times 10^{-4}$). To verify that the short-term (consecutive 5 days) treatment with tamoxifen used to induce CreERtm activity prior to initiating treatment with estrogen does not, in of itself, have an effect on cervical carcinogenesis, we also examined both the incidence of cervical cancer and severity of cervical disease from each of non tamoxifen-treated mouse of following genotypes: nontransgenic, *Rb$^{f/f}$p107$^{-/-}$*, *K14CreERtmRb$^{f/f}$p107$^{-/-}$*, *K14E7* mice. No effect of short-term treatment with tamoxifen was

observed in terms of either severity of disease or frequency of cancers (Table 4.3). Together, these data demonstrate that combined loss of pRb and either p107 or p130 is not able to recapitulate the oncogenic potential of E7 in cervical carcinogenesis, but it partially account for the potential of HPV-16 E7 to develop moderate and/or severe grade of cervical intraepithelial neoplasia.

Treatment of K14E7 transgenic mice with estrogen induces not only cervical cancers but also vaginal cancers consistent with the role of high risk HPVs in vaginal cancer in women. We therefore evaluated the susceptibility of the above described cohorts of mice inactivated for one or two pocket proteins to vaginal neoplastic disease. A similar result was observed as seen for the cervix; inactivation of two pocket proteins was not sufficient to induce vaginal neoplasia efficiently as seen in K14E7 transgenic mice (Tables 4.4 and 4.5).

**Contribution of the inactivation for three pocket proteins, pRb, p107, and p130on cervical carcinogenesis.** Because combined loss of pRb and either p107 or p130 was not sufficient to recapitulate E7's efficiency in causing neoplastic disease in the cervix, we asked if the inactivation for all three pocket proteins was able to recapitulate the oncogenic properties of E7. To generate conditional nulligenic mice for three pocket proteins, we treated *K14CreERtmRb$^{f/f}$p130$^{f/f}$p107$^{-/-}$* mice topically with low doses of 4-OH tamoxifen (4-OHT) to recombine the floxed alleles of *pRb* and *p130* selectively in the lower female reproductive tract. This low dose topical treatment with the active metabolite of tamoxifen was necessitated because systemic delivery of even low doses of tamoxifen to these *K14CreERtmRb$^{f/f}$p130$^{f/f}$p107$^{-/-}$* mice led to high morbidity/mortality (data not shown). Topical treatment with 4-OHT did not lead to

morbidity/mortality issues. To determine the efficiency of CreERtm-induced recombination by 4-OHT in cervical stratified epithelium from these mutant mice, we performed pRb, p107, p130 and MCM7 immunohistochemical staining on the cervical epithelium from 4-OHT-treated *K14CreERtmRb$^{f/f}$p130$^{f/f}$p107$^{-/-}$* mice. From these immunohistochemical staining, we observed that with topically applied 4-OHT *K14CreERtm* mediated disruption of pRb and p130 expression was observed throughout the lower reproductive tract including the vagina and the majority of the cervix. In the cervical septum, which is higher up in the female reproductive tract than the rest of the cervix, the pattern of loss of pRb and p130 expression was mosaic (approximately 50% of the epithelium in this part of the cervix was disrupted for pRb and p130 as compared to 100% in other portions of the cervix and the vagina). That we saw this mosaic pattern was not surprising as the cervical septum likely was not exposed to the 4-OHT as effectively as to the lower portion of the cervix and the vagina despite efforts to deliver the drug to the cervico-vaginal canals surrounding the cervical septum.

To determine the influence of the combined loss of all three pocket proteins on cervical carcinogenesis, we evaluated the incidence of cervical cancer and severity of cervical disease in 4-OHT-treated *K14CreERtmRb$^{f/f}$p130$^{f/f}$p107$^{-/-}$* mice, after the treatment with the co-carcinogen estrogen for 6 months. Surprisingly, none of 4-OHT-treated *K14CreERtmRb$^{f/f}$p130$^{f/f}$p107$^{-/-}$* mice developed cervical cancer, though a high percentage of developed high grade dysplasia (CIN3) (Table 4.2). A similar result was observed in the vagina (Table 4.5). These carcinogenesis studies demonstrate that inactivation of pocket proteins is not sufficient to account for HPV-16 E7-16nogenesis studies demonstrate that inactivation of pocket proteins is not sufficient to account importance of other cellular targets of E7 in mediating oncogenic potential.

**Expression of MCM7in cervical stratified epithelium from the mice deficient for both pRb and p107, pRb and p130 and three pocket proteins.** Previously, it has been reported that MCM7 is a potent biomarker for the detection and diagnosis of the progressive cervical disease in HPV-16 transgenic mice and cervical cancer in women (126). MCM7 is a component of a cellular DNA helicase (127), and its expression is regulated by E2F-transcription factors (128, 129), which are negatively regulated by pocket proteins (130). MCM7 induction by E7 correlates at least in part with pRb inactivation (67). Here we performed MCM7 immunohistochemical staining on the cervical epithelium from mice deficient for multiple pocket proteins (Figure 4.3). Consistent with our previous observations, the expression of MCM7 was robustly up-regulated in the whole thickness of the cervical stratified epithelium in *K14E7* mice, whereas expression of MCM7 in nontransgenic mice was only detected in basal cells. MCM7 staining in cervical epithelium of both $Rb^{f/f}p107^{-/-}$ and $Rb^{f/f}p130^{-/-}$ mice also was mainly restricted to basal cells with some of positive staining in parabasal cells. MCM7 staining was more strongly up-regulated in cervical epithelium of $K14CreRb^{f/f}$ mice compared to that detected in either $Rb^{f/f}p107^{-/-}$ or $Rb^{f/f}p130^{-/-}$ mice, but not as strong and even throughout the whole epithelium compared to that seen in *K14E7* mice. MCM7 staining in the $K14CreERtmRb^{f/f}p107^{-/-}$ and $K14CreRb^{f/f}p130^{-/-}$ mice was more abundant than seen in $K14CreRb^{f/f}$ mice, particularly for the $K14CreERtmRb^{f/f}p107^{-/-}$ mice. This result indicates that combinatorial loss of both pRb and p107 has more potential to regulate the induction of MCM7 than the combined loss of both pRb and p130. MCM7 staining in the $K14CreERtmRb^{f/f}p130^{f/f}p107^{-/-}$ mice was most comparable to that seen in *K14E7* mice in terms of the extensiveness of the strong nuclear signal from the basal cells to uppermost cell layer in cervical stratified epithelium. These observations indicate that each pocket protein can

partially regulate the expression of MCM7, but inactivation of single or combinations of two pocket proteins is not sufficient to recapitulate fully the induction of MCM7 expression seen with HPV-16 E7.

**Correlation of combinational inactivation of pocket proteins, arising from leaky CreERtm activity, with cervical dysplasia; .**It has been reported previously that a low level of Cre activity can be detected in the absence of tamoxifen induction in the skin of *K14creERtm* mice (101). Therefore, we were interested in learning whether the same was true in the epithelial mining the lower female reproductive tract. We observed a mosaic pattern of the expression of pRb(data not shown) and MCM7 (Figure 4.4A) expression in cervical epithelium from 6 month estrogen-treated *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice that were not treated with tamoxifen, indicating that Cre recombinase activation indeed had occurred in absence of tamoxifen-treatment. More interestingly, we found that the areas with high MCM7-positive cells displayed moderate to high grade cervical dysplasia (Figure 4.4C and Figure 4.4F), while areas largely negative for MCM7 staining did not show evidence for moderate/high grade dysplasia. No cancers arose in these mice. The frequency of these moderate to high grade dysplasia was not as frequent in the *K14CreERtmRb$^{f/f}$p107$^{-/-}$*mice that were not treated with tamoxifen (Table 4.3) compared to the same mice treated with tamoxifen (Table 4.2). These findings provide further evidence for the leakiness of the *K14creERtm* allele. But more importantly to the goals of this study, these observations demonstrate further that inactivation of multiple pocket proteins is sufficient to drive efficient development of dysplasia, but not cancer.

## Discussion

**The oncogenic potential of combinational inactivation of pocket proteins in cervical carcinogenesis.** In this study, we observed that the combinational inactivation of two or even all three pocket proteins failed to develop an invasive cervical cancer in estrogen-treated transgenic mouse model. However, inactivation of pRb and p107, or all three pocket proteins was sufficient to drive development of high grade dysplasia (Tables 4.1 and 4.2). These findings also provide evidence that p107, and perhaps p130 to a lesser degree, acts as a tumor suppressor in the mouse cervix, at least in concert with pRb. Furthermore, the combined loss of two pocket proteins, either pRb/p107 or pRb/p130, recapitulated at least in part the acute phenotypes induced by E7 in cervical stratified epithelium (Figure 4.2). We conclude that these acute phenotypes of E7 are driven by inactivation of pRb and at least one other pocket protein, and correlate with the development of precancerous lesion, but not cancer itself. In combination, these observations support the hypothesis that inactivation of pocket proteins drive development of benign lesions, but that target(s) of E7 other than the pocket proteins are necessary to convert precancerous lesions into malignant cervical cancer. Consistent with this hypothesis, HPV 16 E7 was observed to cause cervical cancer in mice expressing a mutant form of *Rb* that E7 cannot bind (67). Cancers did not form in these same *Rb* mutant mice without the expression of E7, arguing that an pRb-independent activity(s) of E7 contributes to cervical carcinogenesis. Given this and our observations made in the present study that cervical cancers did not arise in the mice deficient for all three pocket proteins, we conclude that pocket proteins-independent activities of E7 contribute to cervical carcinogenesis.

**Redundancy of pocket protein family members in cervix.** Studies have reported functional overlap between pocket protein family members in the context of development and cell cycle regulation. This functional overlap may explain both genetic redundancy and functional compensation among pocket protein family members (73). The results obtained from our acute phenotype studies can likewise be explained were there functional overlap between pocket proteins. Mice deficient for both pRb and p107, or pRb and p130 in their cervical epithelia displayed increases in suprabasal DNA synthesis and abrogation of DNA damage responses compared to mice deficient for any one pocket protein (Figure 4.2). Moreover, we observed that the expression of p107 and to a lesser degree p130 are increased in cervical epithelium deficient for pRb (Figure 4.1). A compensatory induction of p107 in response to the inactivation of pRb has been previously reported in several other mouse tissues ((75, 131, 132), Shin et al. in press). However, it still remains unclear whether the compensatory induction in expression of pocket proteins in cervical epithelium necessarily informs us on functional compensation.

**Other cellular target(s) of E7, except for pocket proteins, in cervical carcinogenesis.** Using tandem affinity purification/mass spec analyses, over 100 different cellular factors have been found associated with E7 protein (55). Of these which contribute to E7 mediated cervical carcinogenesis remains largely unclear. We have previously demonstrated a role of E7‘s inactivation of p21, one such target of E7 and a known cellular tumor suppressor, in cervical carcinogenesis (125). Thus there is at least one non-pocket protein target of E7 that contributes to E7’s oncogenic potential in this tissue. But, there are likely more. An interesting set of targets of E7 that may have global effects on cells are cellular factors that regulate epigenetic

reprogramming. E7 can associate with Mi2β and histone deacetylase (133), and these interactions have led to the prediction that E7 can broadly dysregulate transcription of cellular genes through chromatin remodeling. More recently, HPV-16 E7 was discovered to associate with E2F6-containing polycomb transcriptional repressor complexes (134) that control the expression of a variety of genes mainly though histone modification (135), and to induce the expression of histone demethylase (136). Aberrant DNA methylation patterns are known to be frequent events in cancers. DNA methylation is thought to be important in many processes including DNA repair, genome stability, and chromatin structure (137, 138). Recently, it has been reported that E7 directly associates with and alters the activity of Dnmt1, a DNA methyltransferase known as, the main 'maintenance' methyltransferase (139). It also has been shown that Dnmt1is highly over-expressed in HPV-associated cancers compared to that normal tissue (52). It is intriguing to speculate that E7's modulation of the epigenome may contribute to its role in cervical cancer in a manner that synergizes with its other capabilities of dysregulating the cell cycle via its interactions with the pocket proteins and other cell cycle regulatory machinery (e.g. p21). Our current studies strongly suggest that inactivation of the pocket protein efficiently drives early stages of neoplasia in the cervix but is not sufficient to cause malignant progression. A compelling reason for considering the role of epigenetic reprogramming by E7 in malignant progression is that such reprogramming is thought to be a relatively late step in neoplastic progression (54, 140). Consistent with this concept, the number of genes dysregulated in the progressive disease leading to cervical cancer most dramatically rises in the transition between CIN3 and cervical cancer.

**Differences in the importance of pocket proteins in E7-driven carcinogenesis in different tissues.** What is striking about the findings of this study is that they differ so greatly from our prior results in the context of head and neck cancer, another cancer type caused by high risk HPVs, most commonly HPV-16. We previously determined that, of the HPV-16 oncogenes, E7 is the primary driver of head and neck carcinogenesis in mice (39). And as was observed in the cervix (67), inactivation of pRb was not sufficient to fully account for E7's potency in causing head and neck cancer. However, inactivation of pRb and p107 together were found to induce susceptibility to head and neck cancers as efficiently as did E7 expression (Shin et al., in press). A less dramatic synergy was seen with inactivation of pRb and p130. This is in striking contrast to what we report in the current study investigating cervical carcinogenesis. In the cervix inactivation of all three, let alone two pocket proteins was unable to efficiently induce cervical cancer. What can explain this difference? One possibility is the manner in which cancer re induced in the different tissues. For cervical cancer we use estrogen which likely acts primarily as a reversible promoter (141) through its activation of its receptor ERα (142). In contrast, in the head and neck mouse model we use a mutagen, 4-NQO that likely is driving the accumulation of genetic changes in cells. Another likely possibility is that this difference in susceptibility reflects differences in the tissue type. For instance, p107 is able to act as a tumor suppressor in the context of both retinoblastoma and non-small cell lung cancer in an *Rb*-deficient background (76, 78). Whereas, p130 can act as a tumor suppressor in the context of small-cell lung carcinoma as well as in non-small cell lung carcinomas in an *Rb/p53*-deficient background (75, 77). These findings support our hypothesis that tumor susceptibility may differ depending on the tissue context.

**Table 4.1 Histopathology summary in cervix for the both pRb and p130 conditionally deficient mice treated with estrogen for 6 months.**

| Genotype[1] | Grade of Cervical Disease (# of mice) | | | | | Cancer Incidence (%) |
|---|---|---|---|---|---|---|
| | H | CIN1 | CIN2 | CIN3 | CC | |
| NTG(n=22) | 19 | 2 | 1 | | | 0 |
| $K14E7^{WT}$ (n=32) | | | | 23 | 9 | 28.1 |
| $K14CreRb^{f/f}$ (n=42) | 33 | 9 | | | | 0 |
| $Rb^{f/f}p130^{-/-}$ (n=33) | 26 | 7 | | | | 0 |
| $K14CreRb^{f/f}p130^{-/-}$ (n=29)[2,3,4] | 6 | 11 | 10 | 1 | 1 | 3.4 |

1. All mice were on the same FVB/129/C57 mixed genetic background (see material and method section for details on breeding scheme)
2. P=0.014comparing the incidence of cervical cancer in *K14CreRb$^{f/f}$p130$^{-/-}$*vs*K14E7*
3. P=1.7 x $10^{-10}$comparing the severity of cervical disease in *K14CreRb$^{f/f}$p130$^{-/-}$*vs *K14E7*
4. P=8.2 x $10^{-6}$, 7.7 x $10^{-7}$, 1.3 x $10^{-7}$comparing the severity of cervical disease in *K14CreRb$^{f/f}$p130$^{-/-}$*vs *NTG*, *Rb$^{f/f}$p130$^{-/-}$*, *K14CreRb$^{f/f}$*, respectively.

**Table 4.2 Histopathology summary in cervix for the pRb/p107, pRb/p107/p130 conditionally deficient mice treated with estrogen for 6 months.**

| Genotype[1,2,3] | Grade of Cervical Disease (# of mice) | | | | | Cancer Incidence (%) |
|---|---|---|---|---|---|---|
| | H | CIN1 | CIN2 | CIN3 | CC | |
| NTG(n=22) | 19 | 3 | | | | 0 |
| $K14E7^{WT}$ (n=24) | | | 1 | 15 | 8 | 33.3 |
| $Rb^{f/f}p107^{-/-}$ (n=20) | 15 | 5 | | | | 0 |
| $K14CreERRb^{f/f}p107^{-/-}$ (n=23)[4,5,6] | | | 7 | 14 | 2 | 8.7 |
| $K14CreERRb^{f/f}p130^{f/f}p107^{-/-}$ (n=15)[7] | | | 1 | 14 | | 0 |

1. All mice were on the same CD1/129/C57 mixed genetic background (see material and method section for details on breeding scheme)
2. All of *NTG*, *K14E7*, $Rb^{f/f}p107^{-/-}$, $K14CreERtmRb^{f/f}p107^{-/-}$ mice were treated with TAM at starting point of this study
3. All of $K14CreERtmRb^{f/f}p130^{f/f}p107^{-/-}$ mice were treated with 4-hydrixy tamoxifen at starting point of this study
4. P=0.072comparing the incidence of cervical cancer in $K14CreERtmRb^{f/f}p107^{-/-}$ vs*K14E7*
5. P=5.3 x $10^{-3}$comparing the severity of cervical disease in $K14CreERtmRb^{f/f}p107^{-/-}$ vs*K14E7*
6. P=1.2 x $10^{-9}$, 5.0 x $10^{-9}$comparing the severity of cervical disease in $K14CreERtmRb^{f/f}p107^{-/-}$ vs*NTG*, $Rb^{f/f}p107^{-/-}$, respectively
7. P=0.022comparing the severity of cervical disease in $K14CreERtmRb^{f/f}p130^{f/f}p107^{-/-}$ vs*K14E7*

**Table 4.3. Histopathology summary in cervix for the both Rb and p107 conditionally deficient mice treated with estrogen for 6 months. (Without treatment of TAM)**

| Genotype[1,2] | Grade of Cervical Disease (# of mice) | | | | | Cancer Incidence(%) |
|---|---|---|---|---|---|---|
| | H | CIN1 | CIN2 | CIN3 | CC | |
| NTG(n=11) | 11 | | | | | 0 |
| $K14E7^{WT}$ (n=16) | | | 1 | 11 | 4 | 25 |
| $Rb^{f/f}p107^{-/-}$ (n=20) | 15 | 4 | 1 | | | 0 |
| $K14CreERRb^{f/f}p107^{-/-}$ (n=20) | 6 | 6 | 2 | 6 | | 0 |

1. All mice were on the same CD1/129/C57 mixed genetic background (see material and method section for details on breeding scheme).
2. All of *NTG*, *K14E7*, $Rb^{f/f}p107^{-/-}$, $K14CreERtmRb^{f/f}p107^{-/-}$ mice were not treated with TAM.

**Table 4.4 Histopathology summary in vagina for the both pRb and p130 conditionally deficient mice treated with estrogen for 6 months.**

| Genotype[1] | Grade of Vaginal Disease (# of mice) | | | | | Cancer Incidence(%) |
|---|---|---|---|---|---|---|
| | H | VIN1 | VIN2 | VIN3 | VC | |
| NTG(n=22) | 18 | 3 | | | 1 | 4.50 |
| $K14E7^{WT}$ (n=32) | | | 1 | 22 | 9 | 28.1 |
| $K14CreRb^{f/f}$ (n=42) | 33 | 9 | | | | 0 |
| $Rb^{f/f}p130^{-/-}$ (n=33) | 27 | 6 | | | | 0 |
| $K14CreRb^{f/f}p130^{-/-}$ (n=29) | 7 | 8 | 12 | 1 | 1 | 3.4 |

1. All mice were on the same FVB/129/C57 mixed genetic background (see material and method section for details on breeding scheme)

**Table 4.5 Histopathology summary in vagina for the pRb/p107, pRb/p107/p130conditionally deficient mice treated with estrogen for 6 months.**

| Genotype[1,2,3] | Grade of Vaginal Disease (# of mice) | | | | | Cancer Incidence(%) |
|---|---|---|---|---|---|---|
| | H | VIN1 | VIN2 | VIN3 | VC | |
| NTG(n=22) | 19 | 3 | | | | 0 |
| $K14E7^{WT}$ (n=24) | | | 2 | 14 | 8 | 33.3 |
| $Rb^{f/f}p107^{-/-}$ (n=20) | 17 | 3 | | | | 0 |
| $K14CreERRb^{f/f}p107^{-/-}$ (n=23) | | 2 | 5 | 15 | 1 | 4.35 |
| $K14CreERRb^{f/f}p130^{f/f}p107^{-/-}$ (n=15) | | | 2 | 12 | 1 | 6.67 |

1. All mice were on the same CD1/129/C57 mixed genetic background (see material and method section for details on breeding scheme)
2. All of *NTG*, *K14E7*, $Rb^{f/f}p107^{-/-}$, $K14CreERtmRb^{f/f}p107^{-/-}$mice were treated with TAM at starting point of this study
3. All of $K14CreERtmRb^{f/f}p130^{f/f}p107^{-/-}$mice were treated with 4-hydrixy tamoxifen at starting point of this study

**Figure 4.1.**

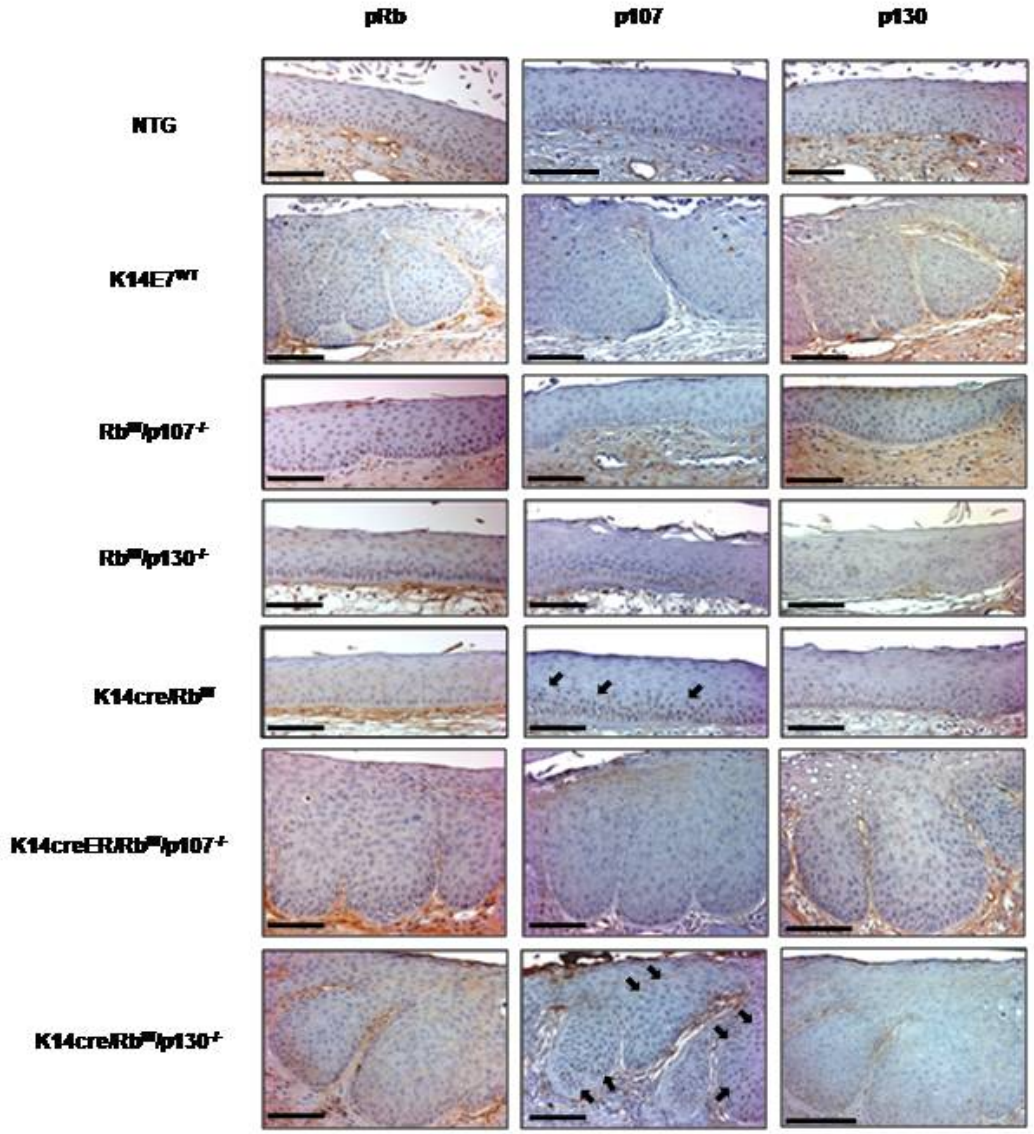

pRb
p107
p130
NTG
K14E7WT
Rb-/-/p107-/-
Rb-/-/p130-/-
K14creRb-/-
K14creERRb-/-/p107-/-
K14creRb-/-/p130-/-

**Figure 4.1. Evaluation of pocket protein expression patterns in cervical epithelium.** Representative sections of cervical 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 4.2**

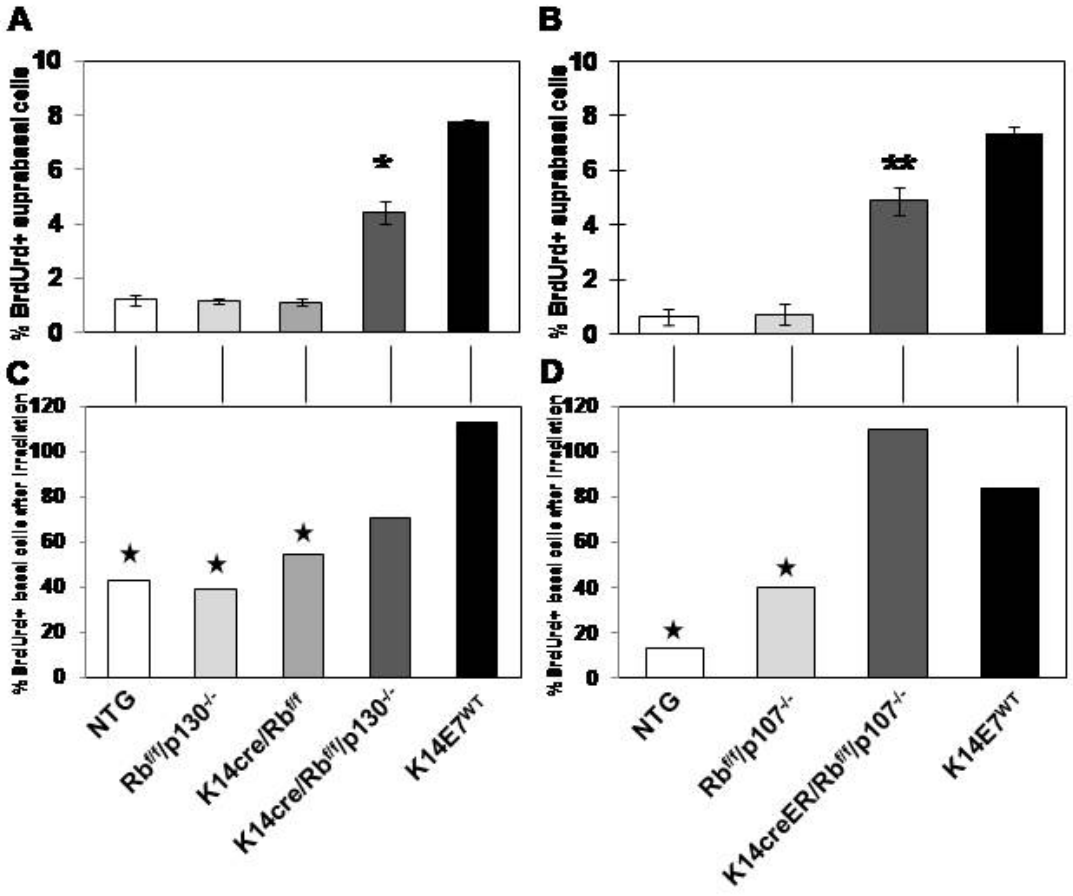

A
B
C
D
% BrdUrd+ suprabasal cells
% BrdUrd+ basal cells after irradiation
NTG
Rb^fl/fl/p130^-/-
K14cre/Rb^fl/fl
K14cre/Rb^fl/fl/p130^-/-
K14E7^WT
Rb^fl/fl/p107^-/-
K14creER/Rb^fl/fl/p107^-/-

**Figure 4.2. Acute phenotypes in cervical epithelium conferred by deficiency in pocket proteins.** In panel A and B are shown quantification of percentage of suprabasal cells supporting DNA synthesis in cervical epithelium of 12 week old mice of each genotype (n=3) 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 p130 alone, pRb alone or both p130 and pRb in comparison to nontransgenic and E7 transgenic mice, all on a FVB/129/C57mixed 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 =0.0495, two-sided Wilcoxon rank sum test). Panel B: 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 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). In panel C and D are shown the quantification of epithelial cells supporting DNA synthesis in cervical epithelium of 12 weeks old mice of each genotype (n=3 for each genotype) that were exposed to 0 Gy versus 12 Gy ionizing radiation 24 hours before BrdUrd administration. The ratio of BrdUrd-positive staining 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 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.Panel D: 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.

**Figure 4.3**

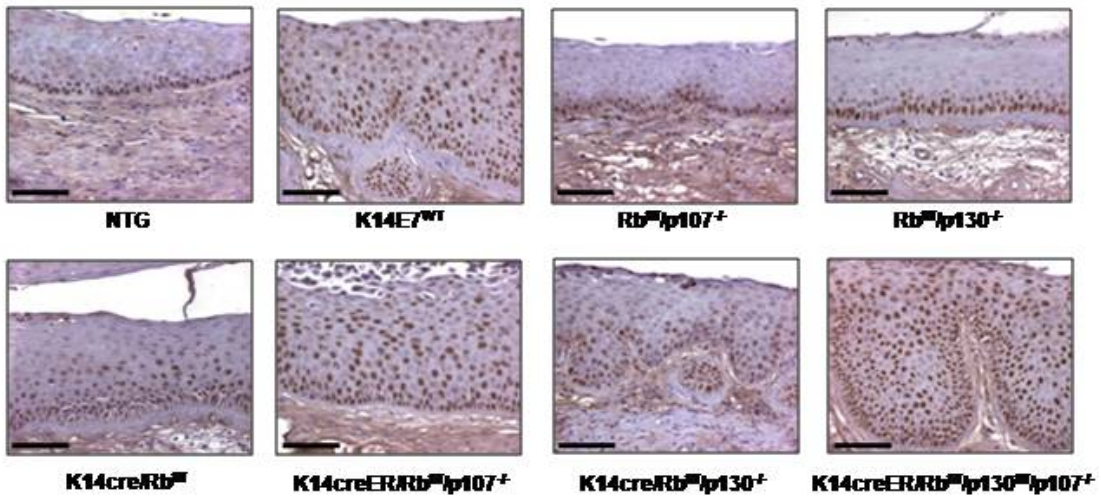

NTG
K14E7WT
Rb-/-/p107+/-
Rb-/-/p130+/-
K14creRb-/-
K14creER/Rb-/-/p107+/-
K14creRb-/-/p130+/-
K14creER/Rb-/-/p130-/-/p107+/-

**Figure 4.3. Evaluation of MCM7 expression in cervical epithelium from estrogen-treated mice.** Representative images stained with anti-MCM7 antibody. Brown, positive staining; blue, hematoxylin counterstain. Magnification, x40; scale bar, 200 μm.

**Figure 4.4.**

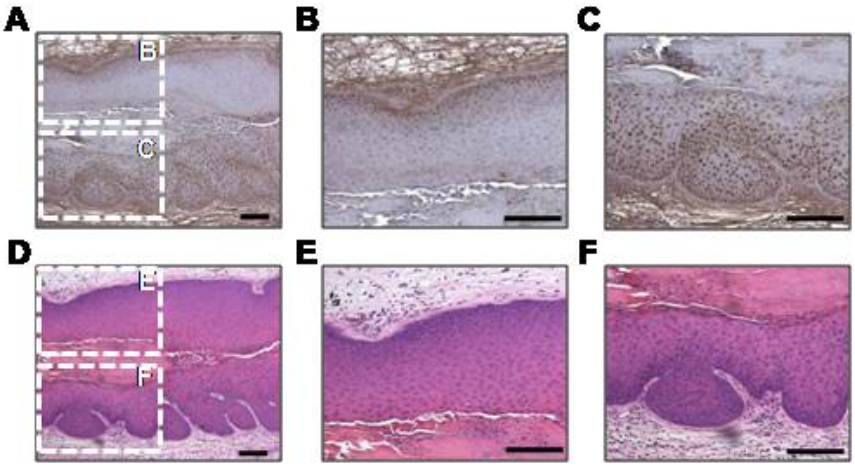


**Figure 4.4. Evaluation of MCM7 expression in the cervical epithelium from *K14CreERtmRb$^{f/f}$p107$^{-/-}$* mice in the absence of tamoxifen-treatment.** Representative histopathology and images stained with anti-MCM7 antibody of cervical stratified epithelium after 6 months of estrogen treatment in *K14CreERtmRb$^{f/f}$p107$^{-/-}$*mice in the absence of tamoxifen-treatment. A~C. Sections were stained with antibodies specific to MCM7. Magnification, x10; scale bar, 100 μm. Magnified images of areas highlighted in the white-dashed boxes are shown to the right, in B&C, Magnification, x20; scale bar, 100 μm. D~F. Sections were stained with H&E. Magnification, x10; scale bar, 100 μm. Magnified images of areas highlighted in the white-dashed boxes are shown to the right, in E&F, Magnification, x20; scale bar, 100 μm. Note that the area with abundant staining for MCM7 in cervical stratified epithelium(C) has shown more dysplastic lesion (F).

# Chapter 5:

# Human Papillomavirus E7 Oncoprotein Overrides $p21^{Cip1}$'s Tumor Suppressor Activity in Cervical Carcinogenesis

This chapter is published (Myeong-Kyun Shin, Scott Balsitis, Tiffany Brake and Paul F. Lambert. Human Papillomavirus E7 Oncoprotein Overrides $p21^{Cip1}$'s Tumor Suppressor Activity in Cervical Carcinogenesis ***Cancer Research***; **69**: (14). July 15, **2009**). Tiffany Brake generated the *K14E7p21*$^{-/-}$ mice and performed 6 months cancer studies. Scott Balsitis generated the *K14E7*$^{CVQ68\text{-}70AAA}$ mice and performed acute phenotypes studies. All other analyses were performed by me.

## Abstract

The E7 oncoprotein of the high risk human papillomaviruses (HPVs) is thought to contribute to cervical carcinogenesis at least in part by abrogating cell cycle regulation. E7 can dysregulate the cell cycle through its interaction with several cellular proteins including the retinoblastoma suppressor protein, pRb as well as the cdk inhibitor, $p21^{Cip1}$. Inactivation of pRb in cervical epithelia is not sufficient to explain E7's ability to cause cervical cancers in transgenic mice. In the current study, we focused on the role of $p21^{Cip1}$ in cervical cancer. Cervical disease was significantly increased in $p21^{-/-}$ mice compared to $p21^{+/+}$ mice, demonstrating that $p21^{Cip1}$ can function as a tumor suppressor in this tissue. Importantly, E7's ability to induce cervical cancers was not significantly enhanced on the p21-null background consistent with the hypothesis that E7's ability to inhibit $p21^{Cip1}$ contributes to its carcinogenic properties. Further supportive of this hypothesis, cervical carcinogenesis in mice expressing a mutant form of HPV-16 E7, $E7^{CVQ}$, that fails to inactivate $p21^{Cip1}$, was significantly reduced compared to that in $K14E7^{WT}$ mice expressing wild type HPV-16 E7. However, $K14E7^{CVQ}$ mice still displayed heightened levels of cervical carcinogenesis compared to that in nontransgenic mice indicating that activities of E7 besides its capacity to inactivate $p21^{Cip1}$ also contribute to cervical carcinogenesis. Taken together, we conclude that $p21^{Cip1}$functions as a tumor suppressor in cervical carcinogenesis and that $p21^{Cip1}$ inactivation by HPV-16 E7 partially account for to the contribution of HPV-16 E7 to cervical carcinogenesis.

## Introduction

Among the mucosotropic human papillomaviruses (HPVs), a subset, called the high-risk HPVs, are the major causative factor in cervical cancer, the second most common cancer among women worldwide (46). Of these high-risk HPVs, HPV type 16 (HPV-16) is the most common, being found in 60% for all cervical cancers (117). High-risk HPVs encode three oncogenes, *E5*, *E6* and *E7,* that independently and synergistically transform and/or immortalize murine fibroblasts and/or human keratinocytes in tissue culture (53, 98, 143) and induce skin cancers in mice (16, 89, 90, 144). Based on studies in mouse models, E7 appears to be the most potent of these three oncogenes in inducing cervical carcinogenesis (15, 37). This study focuses on the carcinogenic properties of E7 in the cervix.

HPV-16 E7 is a multifunctional protein with potent transforming and oncogenic properties that is capable of overriding the normal differentiation process and disrupting cell cycle regulation (100). HPV-16 E7 is able to dysregulate the cell cycle by binding to several cellular proteins, including the pocket protein family members, pRb, p107 and p130, and the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Of these, the best characterized cellular target is retinoblastoma tumor suppressor or pRb, (69). pRb, when bound by E7, is dissociated from E2Fs and degraded by the proteasome (62, 98). As a result, the functions of pRb in cell cycle regulation are disrupted by E7 (66, 100). While these data support the hypothesis that pRb inactivation could account for E7′s ability to transform/immortalize cells in tissue culture and cause cancer in vivo, we recently learned that pRb inactivation is not sufficient to explain E7′s abilities to cause cervical dysplasia or invasive cervical cancer, even though pRb inactivation by

E7 is necessary for the induction of cervical dysplasia (67). Thus, non-pRb target(s) of E7 are likely important for E7′s contribution to cervical carcinogenesis.

The cyclin-dependent kinase (cdk) inhibitor $p21^{Cip1}$ is another cellular target of HPV-16 E7 (79, 80). Induction/over-expression of $p21^{Cip1}$ results in the inhibition of cyclin E/cdk2 complexes that are involved in the phosphorylation of pocket family proteins (145). This growth-arresting activity of $p21^{Cip1}$ correlates with its induction during growth arrest by TGF-β, cell differentiation, and in senescent cells (146, 147). In humans, loss of $p21^{Cip1}$ expression correlates with breast cancer and oral/esophageal cancers (83, 84, 86). In mouse model studies, *p21*$^{-/-}$ mice have an increased incidence of spontaneous tumors compared with p21-sufficient mice (148) and an increased susceptibility to chemically induced skin carcinogenesis (87, 88). Thus, $p21^{Cip1}$ is considered to be a tumor suppressor. In addition, $p21^{Cip1}$ is inhibited by E7, despite the fact that the expression level of p21 is up-regulated in E7-expressing cells. HPV-16-E7 can bind to and inactivate p21′s function to inhibit cdk2 in human keratinocytes (80) and block PCNA-dependent DNA replication *in vitro* (79). These results implicate $p21^{Cip1}$ as a relevant target of E7 in its role in HPV-associated carcinogenesis.

In this study, we investigated the role of $p21^{Cip1}$ in cervical carcinogenesis and examined the importance of $p21^{Cip1}$ as a target of HPV-16 E7 in cervical cancer using mouse models. Our observations indicate that $p21^{Cip1}$ has a tumor suppressive activity in cervical carcinogenesis and that E7 overrides $p21^{Cip1}$′s tumor suppressive function; however, $p21^{Cip1}$′s inactivation alone is not sufficient to explain fully the oncogenic potential of HPV-16 E7. These data are consistent

with $p21^{Cip1}$ being an important target for E7 in carcinogenesis and for E7 being a multifunctional oncogene.

## Results

**P21 expression level is induced by HPV-16 E7 in murine cervix**. The levels of expression of p21 are known to be up-regulated in HPV-16 E7-expressing human keratinocytes (79, 80) as well as in high grade cervical intra-epithelial neoplastic squamous epithelium of human (149) , and this appears to be mediated by E7-epithelial neoplastic (65). To examine whether p21 expression is increased by HPV-16 E7 in the murine cervix, we performed p21 immunohistochemical staining on the cervical epithelium from nontransgenic and *K14E7* transgenic mice (Figure 5.1). The level of expression of p21 was significantly increased in the stratified epithelium of the *K14E7* mice compared to that of the nontransgenic mice. This induction was most evident in the suprabasal compartment of the stratified epithelium of the cervix, much like what was observed in the skin of these mice (65) and in the raft cultures of E7 positive human keratinocytes (79, 80). In addition, we confirmed (data not shown) that p21 was induced in HPV-positive human cervix tissues, both in cervical intra-epithelial neoplasia (CIN) lesions and in squamous cervical carcinoma (149, 150). These data demonstrate that HPV-16 E7 induces p21 expression in the cervix of transgenic mice as is apparent in the cervix of HPV-positive lesions in women.

**p21 functions as a tumor suppressor in the mouse cervix, but its tumor suppressive activity is suppressed by HPV-16 E7.** It previously has been shown that p21 functions as a tumor suppressor in skin carcinogenesis (87, 88) . We wanted to determine whether p21 has a tumor suppressive effect on cervical carcinogenesis, especially in light of the fact that E7 is known to inactivate p21 function. To address this question, we evaluated the incidence of

cervical cancer and precancerous cervical lesions in *p21*$^{-/-}$, *p21*$^{+/+}$, *K14E7p21*$^{-/-}$ and *K14E7p21*$^{+/+}$ mice treated with exogenous estrogen for six-months. Estrogen has been shown to be a critical cofactor in HPV-associated cervical carcinogenesis in mouse models (37, 141).

As predicted based upon prior studies (37), *p21*$^{+/+}$ mice did not develop high-grade cervical dysplasia or cervical cancer (Table 5.1). In contrast, a subset of *p21*$^{-/-}$ mice developed CIN3, CIS or cervical cancer (Table 5.1). This increase in cervical disease in the *p21*$^{-/-}$ mice compared to *p21*$^{+/+}$ mice was significant (p=0.05), consistent with p21 functioning as a tumor suppressor in this tissue. Given this finding, it was notable that the incidence of cervical disease in the *K14E7p21*$^{-/-}$ was not significantly higher than that observed in the *K14E7p21*$^{+/+}$ mice (p=0.3). This data is consistent with the hypothesis that E7 overcomes the tumor suppressive effect of p21. Also of note, the *p21*$^{-/-}$ mice had a reduced incidence of cervical disease compared to that observed in the *K14E7p21*$^{-/-}$ mice (p=0.06), consistent with the hypothesis that E7 possesses oncogenic activities in addition to its ability to suppress p21, thumor suppressive activities.

**Generation of a transgenic mouse expressing a form of HPV-16 E7 unable to inactivate p21.** The above-described data is consistent with HPV-16 E7 directly suppressing the tumor suppressive activity of p21; however, it is also possible that HPV-16 E7 is overriding p21's function via E7's ability to inactivate pRb and the other pocket proteins, the phosphorylation of which is normally regulated by p21. To distinguish between these two hypotheses, we generated transgenic mice expressing the HPV-16 *E7*$^{CVQ68\text{-}70AAA}$ mutant (referred to hereafter as *E7*$^{CVQ}$, consistent with prior literature) which in tissue culture is able to bind and destabilize pRb, p107, and p130 as efficiently as wild-type HPV-16 E7, but is deficient in its ability to inactivate p21

(151, 152). $E7^{CVQ}$ contains alanine substituted at amino acid positions 68-70 in HPV-16 E7, in place of cysteine, valine and glutamine residues, respectively. We identified a line of $K14E7^{CVQ}$ transgenic mice (line 204) expressing the HPV-16 $E7^{CVQ}$ mutant protein at levels similar to the levels of expression of wild-type HPV-16 E7 protein found in our reference $K14E7^{WT}$ mouse line (line 2304) used in our prior and current cervical carcinogenesis studies (Figure 5.2A-B). The $K14E7^{CVQ}$ mice displayed overt phenotypes including wrinkled skin, ruffled fur, and cataracts, similar to that observed in $K14E7^{WT}$ mice (data not shown).

**The ability of HPV-16 E7 to inactivate p21 partially contributes to hyperplasia in the cervical stratified epithelium.** A property of E7 is its ability to induce epidermal hyperplasia manifest in its induction of DNA synthesis within suprabasal compartment (35). E7 can induce suprabasal DNA synthesis, for which the inactivation of pRb is necessary but not sufficient(67). To determine if the inactivation of p21 by E7 affects suprabasal DNA synthesis, we examined the frequency of BrdU-positive cells in cervical stratified epithelium of six-week old (sexually mature) female mice that were treated with estrogen for either a short period (six weeks) that is sufficient to place the mice in estrus (data not shown) or for six months, the treatment period used in our cervical cancer studies. DNA synthesis within suprabasal layers in six week-estrogen treated $K14E7^{CVQ}$ mice was not significantly different compared to that in $K14E7^{WT}$ mice (Figure 5.2C) but was significantly lower amongst the $K14E7^{CVQ}$ mice compared to $K14E7^{WT}$ mice treated for six months with estrogen ($p=0.05$, Figure 5.2D). These data indicate that p21's inactivation by E7 contributes to suprabasal DNA synthesis in the presence of exogenous estrogen but this is only observed in older female mice that have undergone estrogen treatment for six months, the treatment period used in our cervical cancer studies. One possible explanation

is that there is selection for cells that spend more time in S phase or less time in the other parts of the cell cycle in these mice.

**HPV-16 E7$^{CVQ}$ is able to inactivate pRb, whereas it is unable to inactivate p21 function in the stratified epithelium in murine cervix.** To examine whether HPV-16 *E7$^{CVQ}$* mutant is able to bind and target pRb for degradation in our transgenic mice, we performed pRb immunohistochemistry. Both wt E7 and *E7$^{CVQ}$* mutant proteins were able to cause decreased steady state levels of pRb (Figure 5.3A) as well as the other two pocket proteins p107 (Figure 5.5A) and p130 (Figure 5.5B). Not surprisingly we also could not detect phospho-pRb(S807/S811) in the cervical epithelium of either the *K14E7$^{WT}$* and *K14E7$^{CVQ}$* mice (data not shown). These data are consistent with tissue culture based studies indicating that this mutant retains wild type E7 ( ability to bind and degrade the human pocket proteins (151, 152). E7's ability to induce the expression of E2F-responsive gene *Mcm7* is largely the consequence of its inactivation of pRb(67). Therefore, the induction of MCM7 can be used as a marker to assess E7's ability to inactivate pRb. Consistent with the ability of *E7$^{CVQ}$* mutant to cause a decreased steady state level of pRb (Figure 5.3A), we observed an induction in the levels of MCM7 in *K14E7$^{CVQ}$* mice similar to that in *K14E7$^{WT}$* mice (Figure 5.3B). The only difference in the MCM7 expression pattern between the *K14E7$^{WT}$* and *K14E7$^{CVQ}$* mice was the intensity of staining in the uppermost epithelial cell layers, wherein, the high induction of MCM7 was absent in the mutant E7 expressing tissue. Interestingly, we previously observed the similar pattern of MCM7 expression in cervix of *K14creRb$^{f/f}$* mice which is conditionally deleted *Rb* (67). This

could indicate some importance of p21's inactivation in permitting for the continued high level expression of MCM7 in terminally differentiated cells.

We next examined whether *K14E7$^{CVQ}$* mice have a deficiency in their ability to inactivate p21 in stratified epithelia of female reproductive tract. In previous studies, it has been demonstrated that p21 activity is associated with dephosphorylation of cyclin-dependent kinase 2 (81) and also reported that p21 blocks the phosphorylation of CDK-2 by CDK-activating kinase (81, 153). Thus, we looked at phospho-cdk2 expression in the epithelia of reproductive tract in *p21$^{-/-}$* mice and *p21$^{+/+}$* mice to confirm whether phospho-cdk2 expression is regulated by p21 function *in vivo*. As expected, phospho-cdk2 expression is up-regulated in *p21$^{-/-}$* mice compared to *21$^{+/+}$* mice (Figure 5.6). Next, we evaluated both total cdk2 and phospho-cdk2 expression in the epithelia of reproductive tract in non-transgenic mice, *K14E7$^{CVQ}$* mice and *K14E7$^{WT}$* mice. While total cdk2 was present in all three genotypes (Figure 5.3C), phospho-cdk2 was only evident in the cervical epithelium of *K14E7$^{WT}$* mice, not that of either nontransgenic or *K14E7$^{CVQ}$* mice (Figure 5.3D). This is consistent with the previous findings by Helt and Galloway that the HPV-16 *E7$^{CVQ}$* mutant is deficient in inactivating p21 (151, 152).

**The HPV-16 E7$^{CVQ}$mutant that is deficient in its ability to inactivate p21 has a lower incidence of cancer in murine cervix**. To determine if p21 inactivation by HPV-16 E7 contributes to cervical carcinogenesis, we evaluated the incidence of cervical cancer in *K14E7$^{CVQ}$* and *K14E7$^{WT}$* mice when treated with exogenous estrogen pellet for six-months. Consistent with our previous study (37), all *K14E7$^{WT}$* mice developed high-grade cervical dysplasia and/or invasive cervical cancer (Table 5.2). In contrast, *K14E7$^{CVQ}$* mice displayed a

significantly reduced incidence of frank cancer compared to *K14E7*$^{WT}$ mice (p=0.008) (Table 5.2). Tumor multiplicity in *K14E7*$^{CVQ}$ mice was also reduced (P=0.001) compared to that in *K14E7*$^{WT}$ mice (Table 2). The mean size of the cancers (i.e. mean cross-sectional area) was significantly smaller (p=0.0012) in the *K14E7*$^{CVQ}$ mice (0.39 $mm^2$) compared to the *K14E7*$^{WT}$ mice (2.55$mm^2$); and correspondingly, *K14E7*$^{CVQ}$ mice also had a significantly lower incidence of large invasive cancers when compared to *K14E7*$^{WT}$ mice (p=0.004) (Table 5.2). In addition, we observed less severe overall disease in *K14E7*$^{CVQ}$ mice compared to that in *K14E7*$^{WT}$ mice (p=0.001; here we compared the worst grade of disease amongst each cohort of mice) . All of these results together indicate that E7's ability to inactivate p21 correlates with its oncogenic properties.

Our data also support the conclusion that the inactivation of p21 alone is insufficient to fully account for E7's oncogenic potential as *K14E7*$^{CVQ}$ mice displayed a significant increase in overall cervical disease (p=0.000001) and a marginally significant increase in cancer incidence (p=0.07) compared to non-transgenic mice. We conclude that the inactivation of p21 by E7 contributes to, but alone is insufficient to account fully for, E7's role in cervical carcinogenesis.

**HPV-16 E7$^{CVQ}$ mutant induces expression level of the E2F-responsive gene, *Mcm7* and cyclin kinase inhibitor *p16* in cervical cancer similar to that observed in HPV-16 E7$^{WT}$ induced cervical cancers.** Both MCM7 and p16 have been used as robust biomarkers for HPV-associated cervical cancer in humans and mice (126, 154) , and their induction correlates strongly with the expression of E7 (126). We therefore compared the expression patterns of these same two biomarkers in the cancers arising in the *K14E7*$^{CVQ}$ and *K14E7*$^{WT}$ mice. Similar

to the prior analysis of cervical epithelia (Figure 5.3), MCM7 expression was induced in the tumors arising in *K14E7*$^{CVQ}$ mice much like that in tumors from *K14E7*$^{WT}$ mice (Figure 5.A). Likewise we saw heightened levels of p16 in tumors from mutant E7 transgenic mice (Figure 5.4B), though it was slightly reduced compared to tumors in wild type E7 transgenic mice.

## Discussion

**P21 is a tumor suppressor in cervical cancer.** In mice treated with estrogen for six months, we observed that some of *p21*$^{-/-}$ mice developed cervical cancer in contrast to none of *p21*$^{+/+}$ mice (Table 5.1). Furthermore, disease progression in the cervix was significantly increased in *p21*$^{-/-}$ mice (Table 5.1). These results are consistent with studies on chemically induced skin carcinogenesis in which p21 was found to function as a tumor suppressor in the epidermis (87, 88). However, we also found that the disruption of the tumor suppressive activity of p21 alone is not sufficient to account fully for cervical carcinogenesis induced by HPV-16 E7, because *K14E7p21*$^{-/-}$ mice had a significantly higher incidence of cervical disease compared to *p21*$^{-/-}$ mice (Table 5.1). That we observed that the tumor suppressive activity of p21 was not effective in the context of E7-expressing mice (*K14E7p21*$^{+/+}$ mice versus *K14E7p21*$^{-/-}$ mice, Table 1) is consistent with the hypothesis that E7 inactivates p21′s tumor suppressive activity (79, 80).

In a prior study, pRb inactivation together with exogenous estrogen treatment was found to be insufficient to cause the development of high-grade cervical dysplasia or cervical cancer (67). One possible explanation for this finding is that inactivation of multiple pocket proteins (e.g. pRb, p107 and p130), all of which are normally targeted by E7, are necessary for tumorigenesis in the cervix, and studies are underway to test this possibility. In contrast, p21 inactivation together with exogenous estrogen treatment was sufficient to cause the development of high-grade cervical dysplasia or cervical cancer, albeit at lower penetrance compared to that in E7

transgenic mice. This observation and the fact that E7 could override p21′s tumor suppressive activity indicate that p21 is a critical but certainly not the sole target for E7.

**The characterization of HPV-16 E7$^{CVQ}$ mutant′s phenotype in the cervical stratified epithelium of transgenic mice.** In previous studies in human foreskin keratinocytes, HPV-16 E7$^{CVQ}$ mutant was demonstrated to destabilize pRb and its related pocket proteins as efficiently as wild-type E7 while being defective for its ability to inactivate p21 (151, 152). A recent study has shown that the cullin-2 ubiquitin-ligase complex contributes to HPV-16 E7-mediated pRb destabilization, and that the E7$^{CVQ}$ mutant was reduced in its association with the cullin-2 ubiquitin-ligase complex (155). In our study, the E7$^{CVQ}$ mutant retained the ability to cause decreased steady state levels of pRb (Figure 5.3A). Furthermore, the heightened level of MCM7 expression, which is known to correlate with pRb inactivation, was not greatly different between wild-type E7 and E7$^{CVQ}$ mutant expressing mouse tissues (Figure 5.3B). These results are consistent with the hypothesis that E7$^{CVQ}$ mutant is able to efficiently degrade pRb and supports the hypothesis that an additional E7-associated ubiquitin ligase(s) contributes to HPV-16 E7-mediated pRb degradation or that the E7$^{CVQ}$ protein retains sufficient ability to bind cullin-2 to allow it to effectively degrade pRb.

The relationship between E7 and p21 is paradoxical. On one hand, E7 causes an increased level of p21 in human and mouse cells/tissues (65, 79, 80). On the other hand E7 can inactivate p21 (79, 80). In our study we observed that E7$^{CVQ}$′s ability to inactivate p21 was compromised based upon the fact that the activated form of cdk2, phospho-cdk2, was not induced in *K14E7$^{CVQ}$* mouse tissues, in contrast to what we observed in *K14E7$^{WT}$* tissues. This observation in mouse

tissues is consistent with the prior observation that $E7^{CVQ}$ is defective for its ability to inactivate p21 in human cells (151). However, we still noted an increase in the steady state levels of p21 in *K14E7*$^{CVQ}$ mouse tissues. This indicates that the induction of p21 by E7 is separable from E7′s ability to inactivate p21. This finding is consistent with the hypothesis (65) that p21′s induction by E7 is a consequence of E7′s ability to inactivate pRb, an activity that is retained by the $E7^{CVQ}$ mutant (Figure 5.1).

**The oncogenic potential of HPV-16 E7*$^{CVQ}$* mutant in cervical carcinogenesis.** By utilizing p21-null mice, we observed a tumor suppressive activity of p21 in cervical carcinogenesis. Thus, we investigated the importance of p21′s inactivation by E7 in cervical carcinogenesis by performing a phenotypic comparison between *K14E7*$^{WT}$ mice and *K14E7*$^{CVQ}$ mice. In *K14E7*$^{WT}$ mice treated with estrogen for six months, all of *K14E7*$^{WT}$ mice developed high grade squamous intraepithelial lesions, as described previously (37). *K14E7*$^{CVQ}$ mice showed a significant reduction in the incidence of both cervical cancer and disease progression compared to that in *K14E7*$^{WT}$ mice (Table 5.2). Furthermore, tumor multiplicity in *K14E7*$^{CVQ}$ mice was also reduced compared to that in *K14E7*$^{WT}$ mice (Table 5.2) and the mean size of the cancers was significantly smaller in the *K14E7*$^{CVQ}$ mice compared to the *K14E7*$^{WT}$ mice. These observations are consistent with the hypothesis that E7′s ability to inactivate p21 contributes to its oncogenic potential.

While our data with the *K14E7*$^{CVQ}$ mouse tissues are consistent with a role of E7′s inactivation of p21 contributing to cervical carcinogenesis, other interpretations cannot be discounted. For instance, wild type E7 is known also to inhibit another cdk-inhibitor that inhibits

cdk2 activity, p27 (156). It is not known whether the CVQ mutant retains the ability to inhibit p27 (Denise Galloway, personal communication). Also, E7 can directly interact with cdk2 and this could lead to E7′s modulation of this kinase′s activity (132). It is not known whether the CVQ mutation alters E7′s direct interaction with cdk2. Regardless, our results indicate that an alteration in the activity of cdk2 by E7, whether by its inhibition of p21, p27 and/or its direct modulation of cdk2, correlates with its role in cervical carcinogenesis.

Another interesting finding is that cervical disease and incidence of cancer in *K14E7*$^{CVQ}$ mice was significantly higher than in non-transgenic mice. This data supports the hypothesis that other activities of E7 also contribute to cervical carcinogenesis. Current studies are attempting to determine if these other activities include E7′s abilities to inactivate multiple pocket proteins.

**The molecular function of p21 on cervical carcinogenesis.** Previous studies have shown that p21 both inhibits cyclin-dependent kinases (145) and regulates PCNA (proliferating cell nuclear antigen)-dependent DNA replication (157, 158). The cdk2-inhibitory activity of p21 correlates with Rb-dependent E2F transcription regulation (159). In the current study, we observed that expression of *Mcm7*, an E2F responsive gene, was induced in cervical epithelium of *p21*$^{-/-}$ mice, consistent with the hypothesis that p21 indirectly regulates E2F activity, but this induction was less than that seen in *K14E7*$^{WT}$ mice. Given that E7 not only inactivates p21 but also the pocket proteins and thereby modulates E2F activity by multiple means, this result suggests that pRb remains partially active in p21 nulligenic cells. We also observed that MCM7, while induced in the *K14E7*$^{CVQ}$ mouse tissues, was not induced in the most terminally

differentiated cells (Figure 5.3B). This observation is consistent with the hypothesis that p21′s inactivation by E7 contributes partially to E7′s activation of E2Fs. Loss of pRb protein alone is not sufficient to completely account for E7′s ability to upregulate E2F-dependent transcription, particularly in the suprabasal compartment because MCM7 expression was less frequently observed in the suprabasal cells of the cervix of *K14creRb*$^{f/f}$ mice compared to that in *K14E7* mice (67). These data raise the possibility that E7′s inactivation of p21 may play a more important role in augmenting E7′s ability to suppress the E2F inhibitory function of p107 and/or p130. Alternatively the influence of E7′s inactivation of p21 may be independent of a disruption of pocket protein function. p21 can also inhibit PCNA-dependent DNA synthesis and E7 can override this inhibition (79). Levels of PCNA expression were higher in E7-expressing keratinocytes in tissue culture (80) and also in human patient samples (160). It is also possible that the inhibition of p21's cdk inhibitory activity alters the phosphorylation status on proteins in addition to the pocket proteins. Of potential relevance in this context is the observation that p16 induction is attenuated in tumors arising in *K14E7*$^{CVQ}$ mice. Recently, it has been described that E7 associates with polycomb group proteins including E2F6 and Bmi1 the latter a transcriptional repressor of p16; and E7's interaction with E2F6 was unaffected by the CVQ68-70AAA mutation (134). This raises the interesting possibility that p21 inactivation by E7 contributes to E7's modulation of epigenetic regulation of cellular genes. Therefore, it is likely that E7's inhibition of p21 has multiple effects on the cell cycle that are not restricted to the modulation of pocket protein function.

In summary, our data demonstrate that that p21 functions as a tumor suppressor in cervical carcinogenesis, that p21′s inactivation by E7 correlates with E7′s induction of carcinogenesis, but that p21′s inactivation cannot account fully for E7′s oncogenic potential.

**Table 5.1. Incidence of cervical disease in p21 deficient or sufficient mice treated 6 months with estrogen**

| Genotype | Grade of Cervical Disease (# of mice) | | | | | |
|---|---|---|---|---|---|---|
| | **H** | **CIN1** | **CIN2** | **CIN3/CIS** | **MIC** | **LIC** |
| $p21^{+/+}$ (n=8) | 7 | 1 | | | | |
| $p21^{-/-}$ (n=10)[1] | 5 | | | 2 | 3 | |
| K14E7$p21^{+/+}$ (n=9) | | | | 6 | 3 | |
| K14E7$p21^{-/-}$ (n=12)[2,3] | | | | 8 | 3 | 1 |

1. p=0.05 comparing incidence of cervical disease in *$p21^{-/-}$* vs *$p21^{+/+}$* mice.
2. p=0.06 comparing incidence of cervical disease in *K14E7$p21^{-/-}$* vs *$p21^{-/-}$* mice.
3. p=0.3 comparing incidence of cervical disease in *K14E7$p21^{-/-}$* vs *K14E7$p21^{+/+}$* mice

**Table 5.2. Incidence of cervical disease in *K14E7$^{WT}$* versus *K14E7$^{CVQ68\text{-}70AAA}$* mice treated 6 months with estrogen**

| Genotype | Grade of Cervical Disease (# of mice) | | | | | | Cancer | LIC | Tumor |
|---|---|---|---|---|---|---|---|---|---|
| | H | CIN1 | CIN2 | CIN3/ CIS | MIC | LIC | Incidence (%)[1] | Incidence (%)[2] | Multiplicity[3] |
| NTG (n=12) | 12[4] | | | | | | 0 | 0 | 0 |
| K14E7$^{WT}$ (n=8) | | | | 1 | 3 | 4 | 87.5 | 50 | 4.13 |
| K14E7$^{CVQ}$ (n=19) | | | 3 | 11 | 5 | | 26.3 | 0 | 0.84 |

1. p=0.008 comparing incidence of cervical cancer in *K14E7$^{CVQ}$* vs *K14E7$^{WT}$* mice
2. p=0.004 comparing incidence of large invasive cancers (LIC) in *K14E7$^{CVQ}$* vs *K14E7$^{WT}$* mice
3. p=0.001 comparing tumor multiplicity in *K14E7$^{CVQ}$* vs *K14E7$^{WT}$* mice
4. Data from prior study (141)

**Figure 5.1**

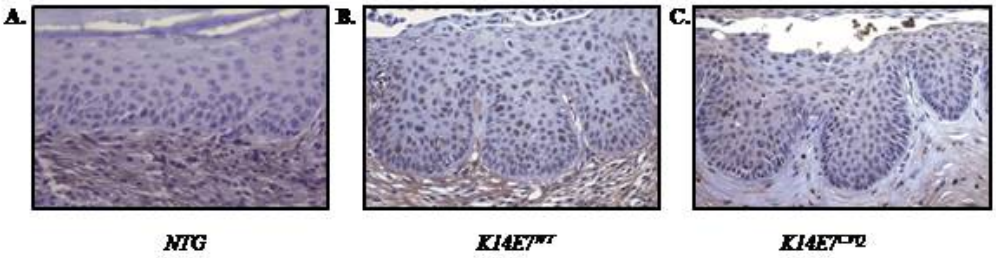


**Figure 5.1. p21 expression level in cervix of mice treated with estrogen for six months.** Images shown are from representative sections stained with anti-p21 antibody (brown) and counterstained with hematoxylin (blue) from (A) *non-transgenic (NTG)*, (B) $K14E7^{WT}$ and (C) $K14E7^{CVQ}$ mice.

**Figure 5.2**

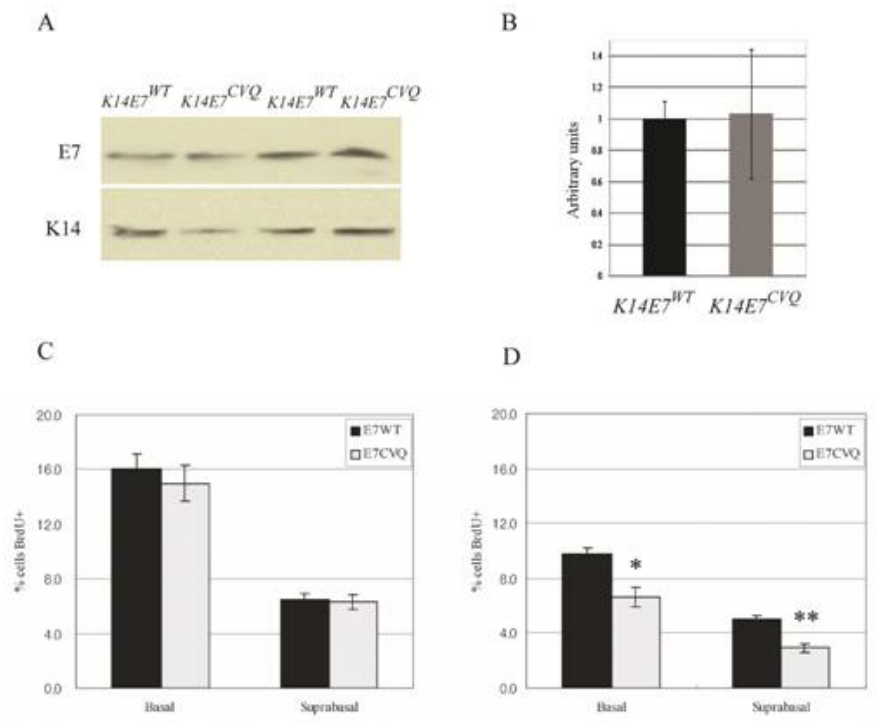

A
K14E7WT K14E7CVQ K14E7WT K14E7CVQ
E7
K14
B
Arbitrary units
K14E7WT K14E7CVQ
C
E7WT
E7CVQ
% cells BrdU+
Basal
Suprabasal
D
E7WT
E7CVQ
% cells BrdU+
*
**
Basal
Suprabasal

**Figure 5.2. E7 expression in *K14E7$^{WT}$* and *K14E7$^{CVQ68\text{-}70AAA}$* mice.** Mouse lower reproductive tract protein lysates were made as described in materials and methods and analyzed by E7-specific Western blot analyses. A representative experiment is shown in panel A. Top: E7 Western blot; bottom, K14 blot to verify equal loading. In panel B is shown the quantification of multiple E7 Westerns (n=3 for each genotype). Intensities of E7 specific bands were quantified and normalized to K14 expression. In panel C and D are shown quantification for DNA synthesis in stratified cervical epithelium of mice treated with estrogen for six weeks or six months. Estrogen-treated mice of each genotype (n=3 for each genotype) were injected BrdU one hour prior to sacrifice, and paraffin-embedded sections from these mice were stained with anti-BrdU. Shown in the graphs are the percentage BrdU incorporation in mice treated with estrogen for six weeks (C) and or six months (D). Asterisks indicate that DNA synthesis level is significantly reduced in suprabasal layer as well as basal layer in *K14E7$^{CVQ}$* mice compared to *K14E7$^{WT}$* mice ($P = 0.05$, two-sided Wilcoxon rank sum test).

**Figure 5.3**

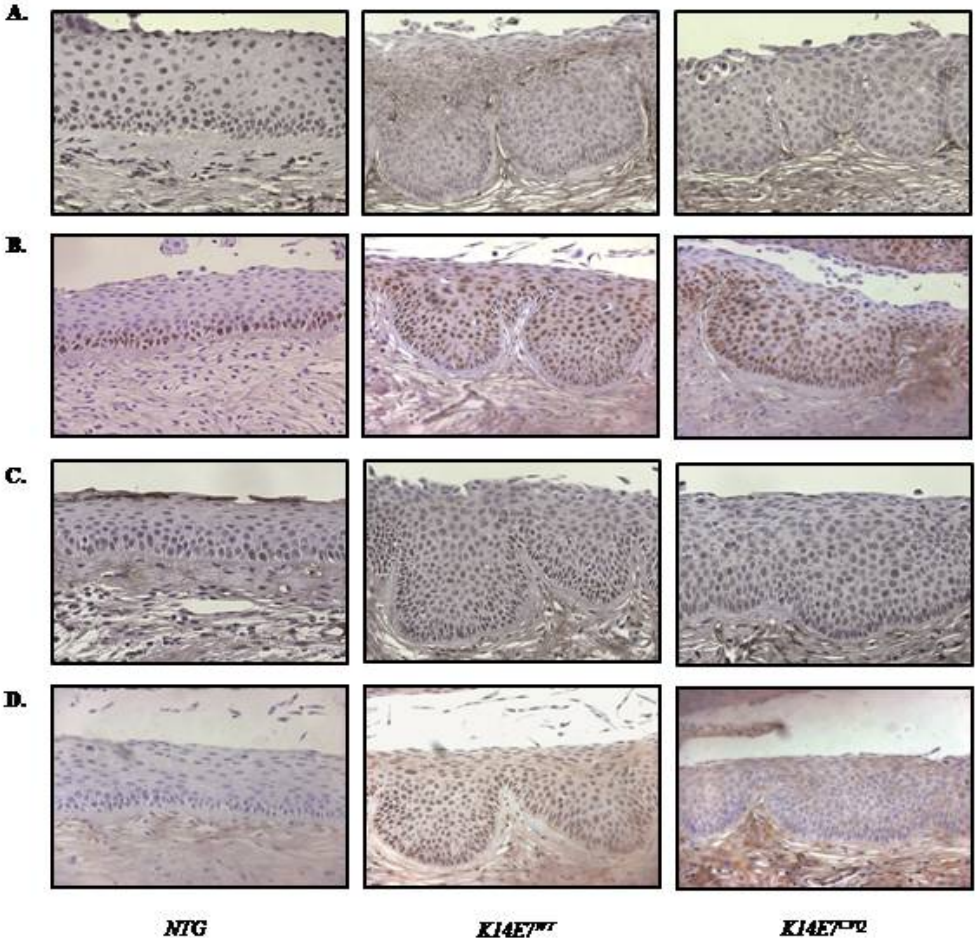


**Figure 5.3. Examination of pRb, MCM7, CDK2 and phospho-CDK2 expression in stratified cervical epithelium of mice treated with estrogen for six months.** Shown are representative images from sections stained with anti-pRb (A), anti-MCM7 (B), anti-CDK2 (C), and anti-phospho-CDK2 (D) antibody (black or brown signal) and counterstained with hematoxylin (blue signal).

**Figure 5.4**

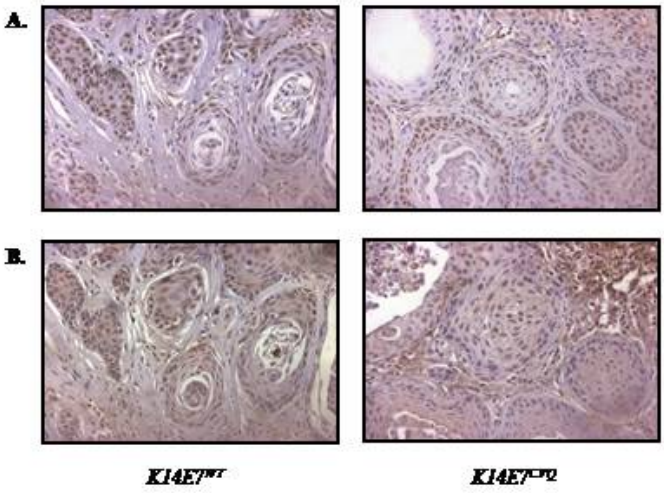


**Figure 5.4. Evaluation of MCM7 and p16 expression in tumors from the reproductive tract of female mice treated with estrogen for six months.** Shown are representative images from sections stained with anti-MCM7 (A) or anti-p16 (B) antibody (brown signal) and counterstained with hematoxylin (blue signal).

**Figure 5.5**

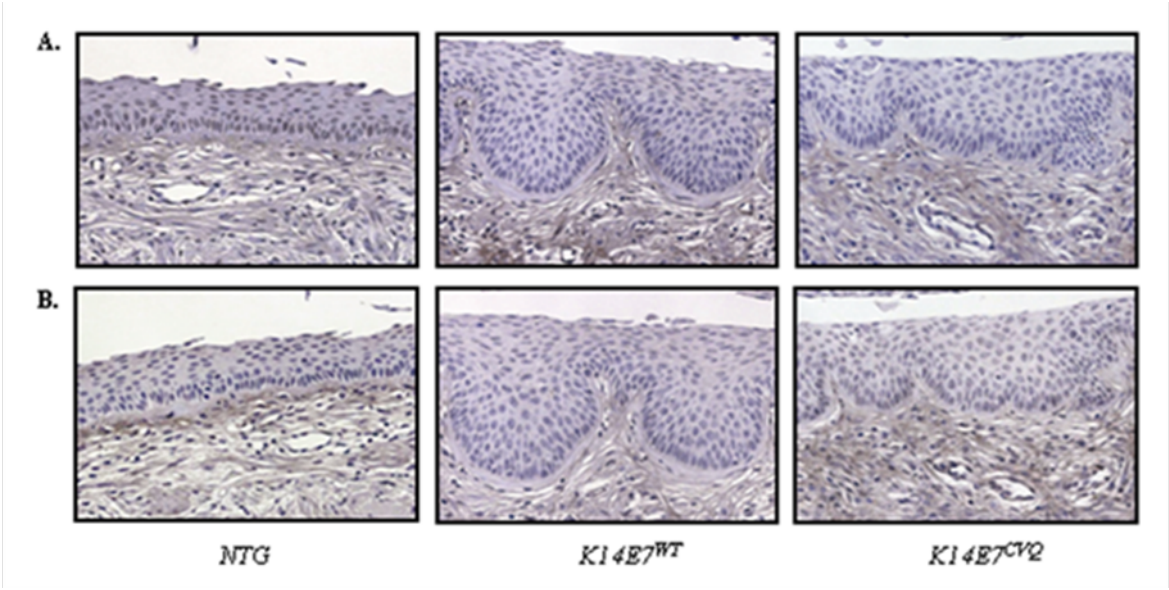
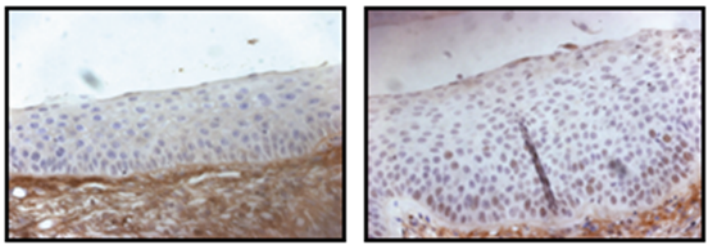


**Figure 5.5 Examination of p107 and p130 expression in stratified cervical epithelium of mice treated with estrogen for six months.** Shown are representative images of cervical epithelia stained with anti-p107 (A) or anti-p130 (B) antibodies (black to brown signal) and counterstained with hematoxylin (blue signal). Note the similar reduction in p107 (A) and p130 (B) staining in both *K14E7*$^{WT}$ and *K14E7*$^{CVQ}$ tissues compared to that in nontransgenic (NTG) tissue. These results are like that seen with antibodies to pRb (Fig 5.3A) and confirm that the CVQ mutant E7 protein retains the ability to bind and degrade all three mouse pocket proteins, as was previously demonstrated to be the case in human epithelial cells.

**Figure 5.6**



*p21*$^{+/+}$ *p21*$^{-/-}$

**Figure 5.6. Examination of phospho-CDK2 expression in stratified cervical epithelium of mice treated with estrogen for six months.** Shown are representative images of tissue sections from p21-sufficient ($p21^{+/+}$) or p21-nulligenic ($p21^{-/-}$) stained with anti-phospho-CDK2 antibody (brown signal) and counterstained with hematoxylin (blue signal).

# Chapter 6:

# Summary and Future Directions

**Summary and Discussion**

In recent years, the Lambert laboratory reported two important observations made through their characterization of mouse models for HPV-associated cancers in which the viral E7 oncoprotein is the dominant oncogene driving cancer. First, they reported that inactivation of the tumor suppressor pRb, a well known target of E7, was not sufficient to induce either severe cervical intraepithelial neoplasia (CIN) or invasive cancer, which was in stark contrast to the highly susceptible nature of HPV-16 E7 transgenic mice to cervical cancer (67). This was followed by a similar study in the context of head and neck cancer in which they reported that inactivation of pRb alone while inducing some tumors, did not lead to the high penetrance of tumors seen in HPV-16 E7 transgenic mice (39). Thus in two mouse tissues in which HPV-associated cancers arise in humans, the cervix and the head/neck region, inactivation of the tumor suppressor pRb was not sufficient to account for E7's potent oncogenic properties, Based on these findings, the goal of my thesis was to identify the non-pRb target(s) of HPV-16 E7 that are critical in E7 driving cervical and head/neck carcinogenesis.

**Identification of non-pRb targets of HPV-E7 in HPV-associated carcinogenesis.** I identified p21 as a tumor suppressor in cervical carcinogenesis and determined that E7's ability to inactivate p21 contributes partially to E7's oncogenic potential in this tissue (Chapter 5). These were the first studies to demonstrate that HPV-16 E7 can override the tumor suppressive activity of p21 *in vivo*. In contrast to the susceptibility of $p21^{-/-}$ mice to cervical cancer, we failed to see cervical cancer in pocket protein deficient mice, although combinatorial inactivation of pRb and p107 or all three pocket proteins was sufficient to induce high grade dysplasia (Chapter

4). One interpretation of the latter results is that inactivation of the pocket proteins by E7 drives early stages in neoplasia, but is in of themselves insufficient to cause malignant progression. If true then one would predict that other activities of E7 are critical for the latter steps in carcinogenic progression. One other observation consistent with this premise comes from earlier studies performed by Scott Balsitis in the Lambert lab. He monitored the carcinogenic properties of HPV-16 E7 on a genetic background in which the wild type *Rb* allele was replaced with a mutant allele encoding a form of the pRb protein that cannot be bound by E7 (the $Rb^{\Delta LXCXE}$ or $Rb^{\Delta}$ allele). There were two interesting phenotypes seen in his studies. First, *K14E7* mice, on this genetic background failed to develop cervical dysplasia. This is consistent with our conclusion that inactivation of pocket proteins, including pRb, drives formation of cervical dysplasia. Secondly, these same $K14E7Rb^{\Delta/\Delta}$ mice developed cervical cancer, albeit at a reduced frequency from that seen with K14E7 mice on a wild type *Rb* background. This argues that activities of E7 other than its ability to bind/inactivate pRb drive E7's induction of cervical malignancy, even in the absence of rampant cervical dysplasia. While the inactivation of p21 by E7 might represent one activity of E7 that drives conversion of precancerous lesions to malignancy, certainly other targets of E7 are of potential importance, as discussed below.

It is important to recognize that in HPV-induced cervical cancers E7 is always found to be co-expressed with a second viral oncogene, E6. E6 alters many cellular processes related to carcinogenesis including but not limited to DNA damage responses, apoptosis, epithelial to mesenchymal transition (EMT), angiogenesis and immortalization, only some of which relate to E6's ability to inactivate p53 (1). In estrogen-treated mice, E6 alone induces cervical cancers, albeit at a reduced efficiency compared to E7, and when co-expressed with E7 contributes to

increases in the size and multiplicity of cervical cancers (29). It is therefore reasonable to predict that the impact of E7's inactivation of pocket protein function on cervical carcinogenesis is influenced by the activities of E6.

In HPV-associated head/neck cancer, my studies demonstrated that both p107 and p130 have a tumor suppressive properties in concert with pRb. In particular, the combinatorial inactivation of pRb and p107 largely recapitulated the cancer susceptibility in this tissue seen with *K14E7* transgenic mice expressing HPV-16 E7. Furthermore, combined loss of pRb and p107 or pRb and p130 led to increased susceptibility to head and neck cancers compared to that seen with inactivation of any one pocket protein. These findings were in stark contrast with my observations in cervix (Chapter 3, 4).

**The different behaviors of pocket proteins depending on the different tissue context.** In tongue and esophagus, I observed a stronger oncogenic consequence of combinatorial inactivation of two pocket proteins than in cervix. As I discussed earlier in chapter 4, one of possibilities may be differences in the co-carcinogens used in the mouse model for cervical cancer versus head and neck cancer. Alternatively, the molecular function of pocket proteins may differ depending on the tissue context. For example, in the context of scoring suprabasal DNA synthesis, I observed different results with the inactivation of pRb alone in cervical epithelium versus lingual/esophageal epithelium. Consistent with the cancer phenotype studies, suprabasal DNA synthesis in lingual/esophageal epithelium from the mice deficient for pRb alone was significantly increased compared to that observed in nontransgenic mice (Chapter 3), but it was not significantly different in cervical epithelium (Chapter 4). Thus different actions of

the same molecule depending on the tissue may explain, at least in part, the differences in cancer susceptibility. It is interesting to consider that such differences could have influences on clinical response to therapies used in treating HPV-associated cancers. Clinical response to radiation therapy for HPV-associated head and neck cancer is more favorable compared to that for cervical cancer, although these cancers are both caused by the same HPV, primarily HPV-16.

**Functional compensation of pocket protein family members.** Two interesting issues in the context of studies on pocket proteins are genetic redundancy and functional compensation among pocket protein family members. Compensatory induction in expression of p107 and/or p130 in response to the inactivation of pRb have been previously observed in several other mouse tissues (75, 131, 132). Consistent with these observations, the results in this thesis also clearly showed that compensatory increases in expression of functional pocket proteins can arise in cervical stratified epithelium as well as lingual and esophageal epithelium, particularly when pRb is deficient (Chapter 3&4). In addition to the observed compensatory changes in levels of protein expression, the acute phenotype studies also support the possibility of functional compensation among pocket protein family members (Chapter 3&4). Further study is needed, however, to test the hypothesis that compensatory increases in expression of pocket proteins reflect actual functional compensation. To address this, it might be useful to examine the expression of several targets of E2F1-3 transcription factors, which are known to be negatively regulated by pRb. These results could tell us whether compensatory increases in expression of pocket proteins functionally rescue the inactivation of pRb. Many DNA viruses (papillomaviruses, polyoma virus, adenovirus, herpes viruses) target pocket proteins and do so in a similar manner, by expressing proteins that bind pocket proteins via a conserved LXCXE

amino acid motifs. A number of cellular proteins also bind to pocket proteins via the same LXCXE motif. Their binding to the pocket proteins is thought to be important in mediating the role of pocket proteins in multiple cellular processes including cellular responses to DNA damage (161, 162). Thus, by utilizing LXCXE motifs, many viruses have learned to exploit an important property of pocket proteins to themselves bind and inactivate these critical cellular factors.

**Other contributions of HPV-16 E7 in HPV-associated carcinogenesis besides its dysregulation of the cell cycle.** My cervical cancer studies strongly suggest that target(s) of HPV-16 E7 besides the pocket proteins contribute specifically to malignant conversion in cervical carcinogenesis. Of course one of these targets could be p21, which also is involved in cell cycle regulation and I have shown is a relevant target of E7 in the context of cervical cancer. But there are other reported cellular processes dysregulated by HPV-16 E7 and these consequences of E7 on the cell also might contribute to HPV-associated carcinogenesis. One intriguing possibility is epigenetic reprogramming by HPV-16 E7. This hypothesis was already discussed in chapter 4. Another effect of high-risk HPV E7 that may contribute to HPV-associated carcinogenesis is its induction of genome instability. Prior studies have documented that cervical cancer cells accumulate a wide range of chromosomal abnormalities and these genetic abnormalities may contribute to malignant progression (134, 163-166). HPV-16 E7 has been shown to induce a chromosomal instability, and this effect is at least in part independent of its ability to inactivate pRb family members (167, 168). Thus it is reasonable to hypothesize that genomic instability induced by HPV-16 E7 through mechanism independent of its inactivation of pocket proteins may contribute to malignant conversion in HPV-associated carcinogenesis.

**Challenges in working with pocket protein deficient mice.** In my effort to identify non-pRb target(s) of E7 in HPV-associated carcinogenesis, I focused on other pocket protein family members, p107 and p130. To generate mice deficient for deficient in expression of multiple pocket proteins, I depended upon the use of the Cre-lox based system to conditionally inactivate pRb in relevant tissues because of the embryonic lethal phenotype of the germline null allele of *pRb*. Specifically, I used the K14 promoter-driven Cre (K14cre) transgene to induce recombination of a floxed allele of *pRb* in relevant stratified squamous epithelia including the cervical stratified epithelium as well as lingual/esophageal epithelium. This permitted me to generate viable *K14creRb*$^{f/f}$ and *K14creRb*$^{f/f}$*p130*$^{-/-}$ mice to study. However, it had been already reported there is a mortality/morbidity issue in *K14Crep107*$^{-/-}$*Rb*$^{f/f}$ mice (60). To avoid this issue, I used K14 promoter-driven CreER*tm,* which encodes an inducible Cre-recombinase. CreER*tm* is a fusion protein, which consists of Cre fused to a mutated estrogen receptor ligand binding domain that is selectively bound by 4-hydroxytamoxifen (4-OHT), the active metabolite of tamoxifen. In the presence of 4-OHT, CreER*tm* translocates to the nucleus and catalyzes recombination (101). Using *K14CreERtmp107*$^{-/-}$*Rb*$^{f/f}$ mice, I was able to generate viable mice deficient in their expression of pRb and p107 in both the cervical and lingual/esophageal epithelium, when these mice were treated with either tamoxifen or 4-hydroxytamoxifen. Interestingly, I also observed a low level of Cre activity in the absence of tamoxifen induction (Chapter 4), which was predicted given that the K14creER*tm* transgene is known to be leaky (i.e. it shows some sporadic Cre in the absence of inducer) in other tissues. I was able to use this leaky phenotype to provide further evidence in support of our hypothesis that inactivation of multiple pocket proteins correlates with induction of preneoplastic disease in the cervix. In the

future, we may utilize this "leaky" Cre activity to provide further insight into the roles of genes in cancer because it allows one to create mosiacism for a gene targeted for recombination using Cre within an epithelial tissue.

The CreERtm transgene was also essential in my being able to generate mice inactivated for all three pocket proteins. Here I had to resort to topical applications of low doses of 4-OHT to the lower female reproductive tract of *K14CreERtmp107*$^{-/-}$*Rb*$^{f/f}$p130$^{f/f}$ mice that allowed for the efficient cre-mediated recombination of the Rb and p130 alleles within the vaginal and cervical epithelium while retaining viability. These mice were critical in allowing us to prove that inactivation of all three pocket proteins is not sufficient to induce cervical cancer formation, though it did efficiently induce cervical dysplasia. Systemic delivery of tamoxifen or 4-OHT or topical delivery of higher doses of 4-OHT led to the same morbidity/mortality issues observed in *K14Crep107*$^{-/-}$*Rb*$^{f/f}$ mice. I was unable to generate viable mice inactivated in all three pocket proteins in the head/neck epithelium owing to the same morbidity/mortality issues arising even in mice given the lowest doses of 4-OHT topically in the oral cavity. Fortunately, the strong cancer phenotypes of the double null reduced the importance of studying the phenotypes of the triple null mice in this tissue context.

**Future Directions**

The studies presented in this thesis identified at least in part non-pRb targets of E7 that contribute to HPV-associated carcinogenesis. Through the combinational inactivation either pRb and p107, or pRb and p130 in cervical and lingual/esophageal epithelium, I learned that the pocket protein family members, p107 and p130, have tumor suppressive activity in head and neck cancer and in cervical carcinogenesis, at least in terms of early steps in neoplastic development. (Chapter 3-4). Additionally, other studies demonstrated that p21 also acts as a tumor suppressor in cervical carcinogenesis, and that E7's inactivation of p21 partially accounts for E7's oncogenic potential in cervical carcinogenesis (Chapter 5). However, from these studies it is reasonable to conclude that other cellular target(s) of HPV E7 contribute to HPV-associated carcinogenesis. Additional studies are needed to determine the other relevant cellular target(s) of HPV E7.

**cyclin-dependent kinase (CDK) inhibitor $p27^{KIP}$, as a relevant target of E7 in HPV-associated carcinogenesis.** Previously, it has been shown *in vitro* assays that HPV-16 E7 is able to interact with cyclin-dependent kinase(CDK) inhibitor $p27^{KIP}$ and directly override its function, which is to block the kinase activity of cyclinE/cdk2 complex that is involved in cell cycle control (156). In order to determine if p27 functions as a tumor suppressor in HPV-associated cancers, I earlier had proposed monitoring the susceptibility of nontransgenic, *p27*$^{-/-}$, *K14E7*, and *K14E7*p27$^{-/-}$ mice to cervical cancer. Unfortunately, I learned that *p27*$^{-/-}$ and *K14E7p27*$^{-/-}$ mice display high degree of morbidity/mortality when placed on exogenous estrogen, such that by three months into the standard 6-month estrogen treatment regimen used for our cervical

carcinogenesis studies, all mice had to be sacrificed. At this time one could instead pursue studies in the context of head and neck cancer model but this has not been initiated.

Interestingly, p27 is now recognized to have a second activity relevant to cancer that is tumor promoting in nature, rather than tumor suppressive. Specifically, cytoplasmic localization of p27, which is triggered upon phosphorylation at T157 within its nuclear localization sequence by AKT, can promote cell migration and is associated with metastasis (169-171). In this context, two independent studies demonstrated that HPV-16 E7, through its ability to activate AKT, drives cytoplasmic localization of 27, and this correlates with increases in cell migration in human foreskin keratinocytes and mouse embryonic fibroblasts (172, 173). Given that cell migration is associated with malignant properties of cells, the ability of E7 to modulate p27 localization could play a role in late stages of malignant progression. To examine the molecular functions of p27 as a relevant target of E7 in HPV-associated carcinogenesis, I propose first to determine whether in vivo in the context of our mouse models for HPV-associated cancer, we observe the re-localization of p27 to the cytoplasm. For such studies p27-specific immunofluorescence studies would be carried out on cervical and the head/neck epithelium from nontransgenic and K14E7 transgenic mice (with tissues from p27-null mice used as a negative control to assess antibody specificity). I would also carry out parallel studies to confirm that AKT is increased in its activity and that p27 is specifically phosphorylated at the NLS site using abs specific to phosphorylated-AKT (S473) and phospho-p27 (T157). E7 is thought to activate AKT through its inactivation of pRb. Loss of pRb function activates the mTOR pathway which in turn leads to activation of AKT via phosphorylation of S473 (174). Thus it also would be interesting to learn if AKT activation and p27 cytoplasmic localization occur in both head/neck

epithelium and cervical epithelium of mice deficient for one or more of the pocket proteins, or if in fact there are tissue type differences that correlate with the differential susceptibility of these mice to head/neck cancers.

**Dissection of the functions of pocket proteins.** In this thesis, I mainly focused on determining if a non-pRb target acts as a tumor suppressor in HPV-associated carcinogenesis, but did not examine the detailed molecular function of combinatorial inactivation for pocket proteins. Therefore, in order to understand the molecular function of pocket proteins, I propose several biomarker studies on apoptosis, E2F-responsive genes, hypoxia-inducible factors and related proteins, the elements of DNA damage response pathway, and polycomb transcriptional repressor complexes. To address this, groups of mice with the following genotypes are needed: nontransgenic, $Rb^{f/f}p130^{-/-}$, tamoxifen-treated $Rb^{f/f}p107^{-/-}$, $K14CreRb^{f/f}$, tamoxifen-treated $K14CreERtmp130^{f/f}p107^{-/-}$, $K14CreRb^{f/f}p130^{-/-}$, tamoxifen-treated $K14CreERtmRb^{f/f}p107^{-/-}$, tamoxifen-treated $K14CreERtmRb^{f/f}p130^{f/f}p107^{-/-}$, and *K14E7* mice. Tissues from all of these mouse groups are currently available for biomarker studies. These suggested studies would extend our understanding of the consequences of inactivating pocket proteins on multiple processes related to carcinogenesis, lead to the identification of useful diagnostic markers for identifying cancers disrupted in pathways regulated by pocket proteins, and develop new approaches for treating HPV-associated disease.

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