E7 PROTEINS OF HIGH-RISK (TYPE 16) AND LOW-RISK (TYPE 6) HUMAN PAPILLOMAVIRUSES REGULATE p130 DIFFERENTLY

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DEDICATION

This work is dedicated to my late grandmother Audrey Small, my parents Lorna Skeete and Peter Barrow, my husband Brian Laing and my two precious, beloved daughters Brianna and Lydia. Thank you for being what matters most in my life!

ABSTRACT

Lisa C. Barrow

<u>E7 Proteins of High-Risk (Type 16) and Low-Risk (Type 6) Human</u>

<u>Papillomaviruses Regulate p130 Differently</u>

Human papillomaviruses (HPVs) are one of the most common causes of
sexually transmitted disease in the world. HPVs are divided into high-risk (HR) or
low-risk (LR) types based on their oncogenic potential. HPVs 16 and 18 are
considered HR types and can cause cervical cancer. HPVs 6 and 11 are
classified as LR and are associated with condyloma acuminata (genital warts).
Viral proteins of both HR and LR HPVs must be able to facilitate a replication
competent environment. The E7 proteins of LR and HR HPVs are responsible for
maintenance of S-phase activity in infected cells. HR E7 proteins target all pRb
family members (pRb, p107 and p130) for degradation. LR E7 does not target
pRb or p107 for degradation, but does target p130 for degradation.
Immunohistochemistry experiments on HPV 6 infected patient biopsies of
condyloma acuminata showed that detection of p130 was decreased in the
presence of the whole HPV 6 genome. Further, the effect of HR HPV 16 E7 and
LR HPV 6 E7 on p130 intracellular localization and half-life was examined.
Experiments were performed using human foreskin keratinocytes transduced
with HPV 6 E7, HPV 16 E7 or parental vector. Nuclear/cytoplasmic fractionation
and immunofluorescence showed that, in contrast to control and HPV 6 E7-
expressing cells, a greater amount of p130 was present in the cytoplasm in the

presence of HPV 16 E7. The half-life of p130, relative to control cells, was decreased in the cytoplasm in the presence of HPV 6 E7 or HPV 16 E7, but only decreased by HPV 6 E7 in the nucleus. Inhibition of proteasomal degradation extended the half-life of p130, regardless of intracellular localization. Experiments were also conducted to detect E7-binding partners. Cyclin C and cullin 5 were identified as proteins capable of binding to both HPV 6 E7 and HPV 16 E7. Preliminary experiments showed that decreasing protein levels of p600, a binding partner of both HPV 6 E7 and HPV 16 E7, by RNA interference might affect p130 stability. Elucidating the mechanisms of p130 degradation may identify potential targets for preventing degradation of p130 and allowing restoration of cell cycle control.

Ann Roman, Ph.D., Chair

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LIST OF ABBREVIATIONS

°C	degrees Celsius
3-ATA	3-amino thioacridone
ATCC	American Type Culture Collection
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CCE	cornified cell envelope
Cdk	cyclin dependent kinase
CDP	CCAAT-displacement protein
CF	cytoplasmic fraction
CHX	cycloheximide
CIN	cervical intraepithelial neoplasia
CK	casein kinase
C-K-SFM	complete keratinocyte serum free medium
CMV	cytomegalovirus
CR	conserved region
CTD	carboxy terminal domain
C-terminus	carboxyl-terminus
DAPI	4′-6-Diamidino-2-phenylindole
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic aicd

dNTP	deoxnucleotide triphosphate
ds	double stranded
DTT	dithiothreitol
E6-AP	E6-associated protein
EBC	extraction buffer C
ECL	enhanced chemiluminescent
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G418	geneticin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GSK3	glycogen synthase kinase 3
GST	glutathione-sepharose transferase
h	hours
H & E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HA	hemagglutinin
HCl	hydrochloric acid
HDAC	histone deacetylase

HFK	human foreskin keratinocytes
HPV	human papillomavirus
HR	high-risk
HRP	horse radish peroxidase
HSIL	high-grade squamous intraepithelial lesion
IP	immunoprecipitation
KCl	potassium chloride
LAC	Latin American and the Caribbean
LCR	long control region
LMB	leptomycin B
LR	low-risk
LSIL	low-grade squamous intraepithelial lesion
MCM7	minichromosome maintenance 7
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimolar
Na_3VO_4	sodium vanadate
NaCl	sodium chloride
NaF	sodium fluoride
NES	nuclear export signal
NF	nuclear fraction

ng	nanogram
NLS	nuclear localization signal
NP-40	Nonidet P-40
N-terminus	amino terminus
O/N	overnight
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PP2A	protein phosphatase 2A
PV	papillomavirus
rpm	revolutions per minute
RT	room temperature
RT-PCR	real-time polymerase chain reaction
s	seconds
SCF	Skp-Cullin-F-box
SDS	sodium dodecyl sulfate
SFM	serum free medium
SRC1	steroid receptor coactivator 1
TBP	TATA box-binding protein
TBST	Tris-buffered saline Tween 20

TERT	telomerase reverse transcriptase
U	units
VLP	virus like particle
WCE	whole cell extract
WT	wild type
µg	microgram
µl	microliter
µM	micromolar

INTRODUCTION

Human papillomaviruses (HPV) have been established as the causative agent in over 99% of cervical cancers (Walboomers et al., 1999). High-risk (HR) types of HPV, such as HPV 16, 18, 31 or 33 are responsible for cervical cancer development. Annually, 470,000 new cervical cancer cases are reported worldwide. Low-risk (LR) types of HPV, such as HPV 6 and 11, are not capable of transforming cells but can cause condyloma acuminata (genital warts) (Longworth and Laimins, 2004; zur Hausen, 1996).

HPV E6 and E7 are known oncoproteins. HR HPV E6 targets p53 for degradation whereas HR HPV E7 targets the pRb family members, pRb, p107 and p130 for degradation (Wise-Draper and Wells, 2008). Although the LR type of HPV E7 does not degrade pRb, LR HPV 6 E7 has been reported to target p130 for degradation (Zhang et al., 2006). Degradation of p130 is found to correlate with delayed differentiation. HPV E7 increases the number of cells in S-phase, therefore the ability of both HR and LR HPV to target p130 may be important for the HPV life cycle (Zhang et al., 2006).

The focus of this thesis is to understand HPV E7 regulation of p130 and to identify potential binding partners of HPV E7 that may be involved in E7-mediated degradation. This chapter addresses general HPV biology, including the viral proteins with emphasis on HPV E7. Background information pertinent to the rationale of this study is addressed, including the information related to p130 intracellular localization, E7 localization and p130 degradation.

HPV and cervical cancer

In women, cervical cancer is the second most frequent cancer worldwide and the leading cause of cancer-related deaths in women in developing countries (Drain et al., 2002; Snijders et al., 2006). In fact, Latin American and the Caribbean (LAC) are reported to have the greatest incidence of HPV infection in the world (Almonte et al., 2008). There is a correlation between cervical cancer incidence and socio-economic sub-standards. Haiti has a cervical cancer incidence of 87.3 per 100,000 women, the highest of the LAC region. The incidence of HPV infection in Barbados is 26 per 100,000 women and in the US is significantly lower at 8.2 per 100,000 women (Almonte et al., 2008; Watson et al., 2008). There are also variations of cervical cancer incidence amongst ethnic groups with African American and Hispanic women being diagnosed at twice the rate of Caucasian women (Watson et al., 2008).

The incidence of cervical cancer has significantly decreased over the last 15 years in developed countries due to utilization of the Pap smear test, which allows for early detection (Singer, 1995). Nonetheless, cervical cancer is responsible for claiming 4,000 lives per year in the US and there are over 11,000 new cases yearly; therefore, it is not a trivial disease (Horner et al., 2009). Further, in developed countries HPV infection occurs at alarming rates: HPV causing the most common viral sexually transmitted disease in the US, with 1 to 5 million new infections yearly (Burd, 2003). It is estimated that over 75% of sexually-active individuals have been infected with HPV (Cates, 1999).

In the 1930s papillomaviruses (PVs) were identified as etiologic agents that could cause warts and mammalian cancer (Rous and Beard, 1934; Shope and Hurst, 1933). Richard Shope was the first investigator to determine that infection with cottontail rabbit papillomaviruses resulted in the development of cutaneous papillomas and to correlate infection of a virus to development of a mammalian cancer (Shope and Hurst, 1933; zur Hausen, 1996). In 1976 Miesels and Fortin observed koilocytes in human biopsies and determined them to be a characteristic of PV infection. These koilocytes were described as PV producing cells that acquired an owl-eye shape and had a halo around abnormally small nuclei. Miesels and Fortin also postulated that differentiation between benign lesions that do not progress to cancer and those that do was possible (Meisels and Fortin, 1976; Meisels et al., 1981; zur Hausen, 2002). HPV were initially cloned and identified in the laboratory of Harold Zur Hausen. HPV 6 was isolated by centrifugation of a genital wart extract (Gissmann and zur Hausen, 1980) and HPV 16 DNA was detected by low-stringency hybridization (Durst et al., 1983; Gissmann and zur Hausen, 1980).

Cervical neoplasia is classified based on the morphology of HPV infected cells. Cells can be obtained by a pap smear in order to perform cytology, whereas histology is performed on a biopsy of a patient's tissue. Mild dysplasia is defined as CIN1 by histology and low-grade squamous intraepithelial lesion (LSIL) by cytology and generally represents the morphologic abnormality associated with transient HPV infections. In CIN1, only a few cells are considered to be abnormal. Malignant precursors of squamous cervical carcinoma include

moderate and severe dysplasia, that are traditionally defined, respectively, as cervical intraepithelial neoplasia grade 2 (CIN2) and grade 3 (CIN3) by histology and as high-grade squamous intraepithelial lesions (HSIL) by cytology. In CIN2 approximate half of the cells in the tissue are considered as abnormal. In CIN3 virtually all of the cells are abnormal, and if left untreated will invade the basement membrane, resulting in invasive cervical cancer (Crum and McLachlin, 1995; Kurman et al., 1994; Solomon et al., 2002).

HPV classification and genome organization

HPV are non-enveloped, double-stranded DNA viruses with a genome of approximated 7.9 kb (zur Hausen, 1996). The genome organization of HPV is illustrated in Figure 1. Transcription occurs from one of the two strands of the genome. The genome is divided into three segments; the early region (encoding proteins necessary for replication and transcription), the late region (encoding the L1 and L2 capsid proteins) and the long control region (containing *cis* regulatory elements necessary for replication and gene expression) (Longworth and Laimins, 2004; zur Hausen, 1996).

Sequence analysis has allowed the identification of more than 200 different types of HPV with differentiation of HPV types based on less than a 90% corresponding sequence of the L1 ORF with any previously identified HPV types (Burd, 2003; Delius and Hofmann, 1994). Two to ten percent similarity is recognized as a sub-type and <2% a variant (Munger and Howley, 2002). Ninety
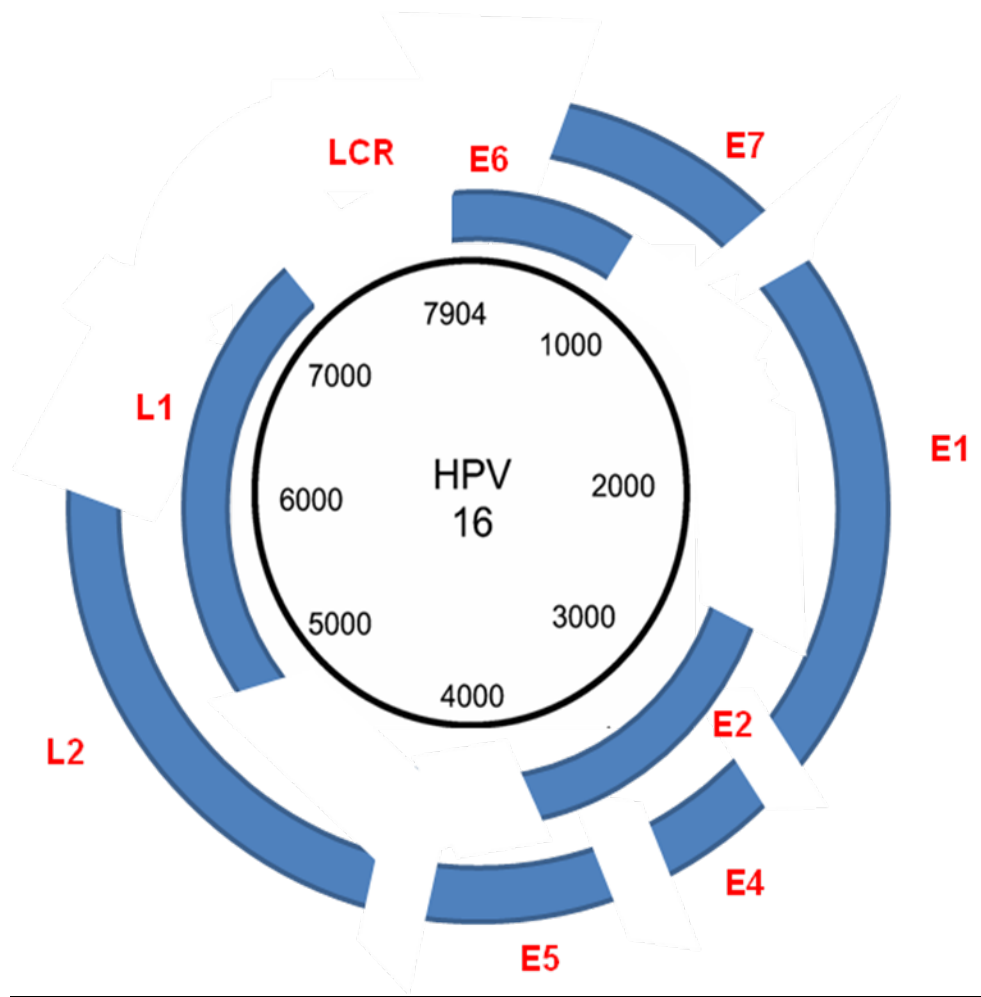
Figure 1. **The HPV genome**. The genome consists of an early region, late region and a long control region (LCR). The early region contains the E1, E2, E4, E5, E6 and E7 genes which regulate transcription, replication and cell cycle control. The late region encodes L1, and L2 capsid proteins. The LCR contains the origin of replication and *cis*-regulatory elements necessary for HPV transcription and replication. Figure adapted from Doorbar, 2006.

percent of HPV are classified into two major genera, Alpha and Beta. Alpha papillomaviruses include those HPV which cause genital/mucosal infections: HPV that infect the lining of the mouth, throat, respiratory tract, the anogenital or cervical epithelium (Burd, 2003; Doorbar, 2006). HPV 16 is the HR type of HPV responsible for more than 50% of cervical cancers (Hebner and Laimins, 2006). Beta papillomaviruses are associated with cutaneous infections, infecting the hands and feet. The remainder of the HPV are classified in Gamma, Mu and Nu genera and typically infect cutaneous sites (Doorbar, 2006).

HPV are associated with over 99% of all cervical cancer, 40-50% of penile and vulvar cancers and greater than 20% of head and neck cancers (D'Souza et al., 2007; Dillner et al., 2000; Hebner and Laimins, 2006; zur Hausen, 2002). HPV are classified as high-risk (HR) or low-risk (LR) depending on their ability to cause cancer. HPV 16, 18, 31, 33, and 45 and 59 are commonly associated with these malignancies (Burd, 2003; zur Hausen, 2000; zur Hausen, 2002). HPV 6, 11, 42, 43 and 44 are classified as LR, and can cause condyloma acuminata (genital warts) (Burd, 2003; Longworth and Laimins, 2004).

HPV life cycle

Completion of the HPV life cycle is dependent upon epithelial differentiation. In normal epithelium, cell proliferation occurs in the basal cell layer; as cells migrate upwards they exit the cell cycle and differentiate as shown in Figure 2 (zur Hausen, 1996). HPV gains entry into cells as a result of microabrasions in the epithelium (Longworth and Laimins, 2004). The receptor needed for HPV

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infection is not certain, but studies suggest that this receptor may be integrin α4ß6 (Munger et al., 2004). Heparin sulfate has also been reported to orchestrate attachment of the virions to cells (Giroglou et al., 2001; Joyce et al., 1999). However, the role of heparin sulfate has been contradicted by a report from the Ozbun's laboratory that heparin sulfate only played a role in COS-7 cells, a monkey kidney cell line (Smith et al., 2007). Further, they have reported that viral HPV 31 entry was caveolae-dependent, whereas HPV 16 entry was through clathrin-dependent endocytosis (Smith et al., 2007). An additional caveat to these studies is that, *in vivo*, HPV capsids have been shown to bind to the basement membrane therefore the *in vitro* studies discussed above do not truly recapitulate what occurs in a natural infection, since binding in these experiments is to the cell surface receptors (Horvath et al., 2010).

After virus infection, viral DNA is maintained extra-chromosomally at 50-100 copies in the basal cell layer (Hebner and Laimins, 2006; Longworth and Laimins, 2004). The infected cells then move to the differentiated suprabasal layer, where viral DNA is amplified and late genes are expressed resulting in mature virions (zur Hausen, 2002). Several studies indicate that both HR and LR HPV require the host cell to initiate cellular DNA replication for viral DNA amplification to occur (Hebner and Laimins, 2006; Longworth and Laimins, 2004; Snijders et al., 2006; zur Hausen, 2002).
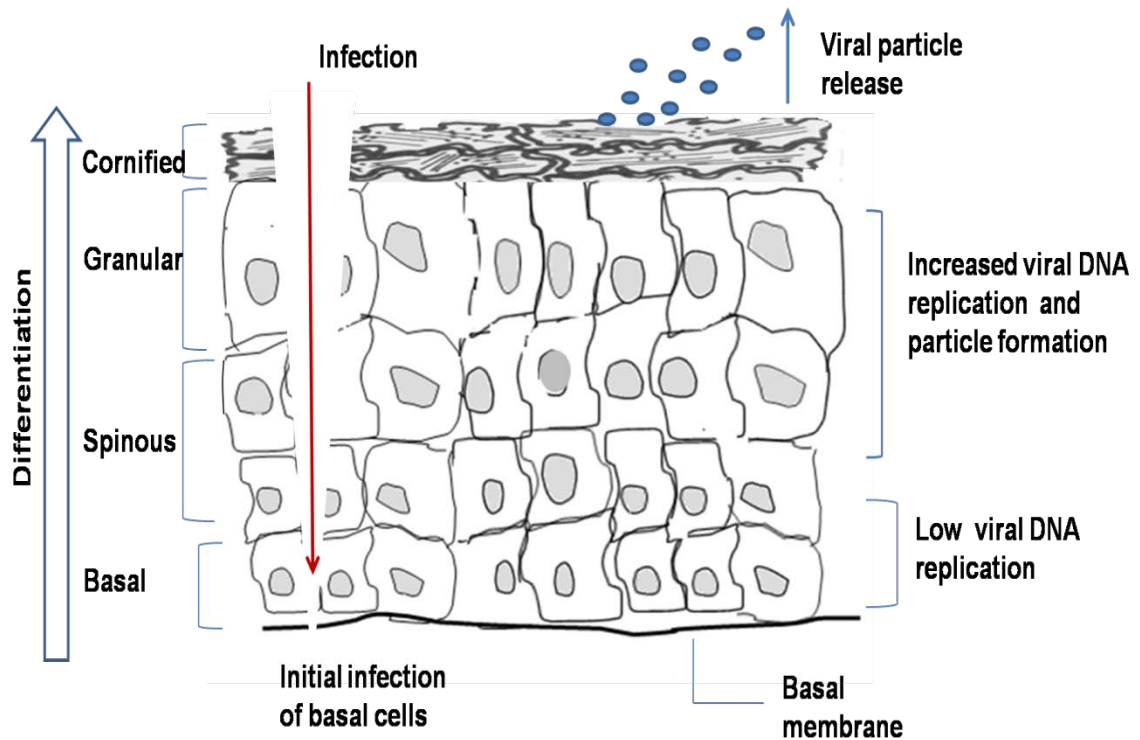
Figure 2. The HPV life cycle. HPV amplifies its genome and synthesizes

structural proteins within differentiated keratinocytes. Both HR and LR HPV must

either delay differentiation or induce differentiated keratinocyes to enter S phase.

Figure adapted from zur Hausen, 2002.

Viral proteins of both HR and LR HPV must be able to facilitate a replication competent environment within the differentiated compartment of the stratified squamous epithelium (Hebner and Laimins, 2006; Longworth and Laimins, 2004; Snijders et al., 2006). CCAAT displacement protein (CDP) is a protein that is highly expressed in undifferentiated cells of the basal cell layer, but as cells differentiate the expression of CDP significantly decreases. CDP negatively regulates the promoters of HPV E6, E7 and E1 and participates in the inhibition of HPV replication in undifferentiated cells (Ai et al., 1999). Therefore, HPV replication occurs in the stratified squamous epithelium where no CDP is present, but HPV still has the task of creating a replication competent environment in these cells that may be exiting the cell cycle. HPV E7 creates a replication competent environment by driving suprabasal cells into S-phase and causes unscheduled DNA synthesis (Collins et al., 2005; Munger et al., 2001). However, a recent report suggests that HPV may initiate viral DNA replication when the cells are in the G_2 phase (Wang et al., 2009). The E7 proteins of both HR and LR HPV are required for viral DNA maintenance and/or amplification (Flores et al., 2000; McLaughlin-Drubin et al., 2005; Oh et al., 2004b; Zhang and Roman, unpublished data). In the case of low-grade infections, after infection the viral genome is maintained as an extrachromosomal genome. In contrast, HPV DNA is integrated with host DNA in most cancerous lesions (Doorbar, 2006).

In HR HPV, transcripts are initiated at 2 major promoters, an early and late promoter. The early promoter is located upstream of the E6 open reading frame (ORF) whereas the late promoter initiates in the E7 ORF. In HPV 16 and 31 the

early promoter is p97, whereas in HPV 18 it is p105 (Hebner and Laimins, 2006). Initially after infection, E6, E7, E1 and E2 genes are transcribed from the early promoter. Activation of the late promoter and productive replication of HPV occurs simultaneously and E1, E2, E4, E5, as well as the capsid proteins L1and L2 are transcribed (zur Hausen, 1996).

HPV gene products and their activities

HPV E1 and E2

Replication factors E1 and E2 are initially expressed after infection and are required for maintenance of the extrachromosomal genome. HPV E1 is approximately 68 kDa in size (Longworth and Laimins, 2004). E1 has a lower affinity than E2 for binding to the origin; this ability is enhanced by E2. After initial binding of E1, additional E1 molecules bind in an ATP-dependent manner. E1 also possess 3'-5' helicase activity. E1 molecules form hexameric rings that, with chaperones, allow unwinding of supercoiled HPV DNA (Hughes and Romanos, 1993; Longworth and Laimins, 2004; Yang et al., 1993). DNA polymerase also binds to E1 (Hebner and Laimins, 2006).

HPV E2 is a 50 kDa protein. The N-terminus of HPV E2 plays a role in transactivation and the C-terminus is important for DNA binding and dimerization. These sequences are greater than 90% conserved in all HPV types (Blakaj et al., 2009; Longworth and Laimins, 2004; McBride et al., 1989; McBride et al., 1988). E2 binds to a consensus palindromic sequence (ACCGNNNCGGT) or the E2

binding site, at the origin of replication and as stated, recruits the E1 helicase via its C-terminus.

E2 also initiates transcription from the early promoter (Hegde, 2002; Longworth and Laimins, 2004). E2 represses binding of transcription factors such as TFIID and Sp1 when it is highly expressed, but at low expression activates the early promoter. The ability of E2 to act as an activator is due to binding to high-affinity sites, whereas E2 acts in a repressive manner when binding to low-affinity sites. In HPV E2 is mainly repressive. HPV-infected basal cells express low-levels of E2. As these cells differentiate the levels of E2 increase and so does viral copy number (Longworth and Laimins, 2004; Penrose and McBride, 2000). In cervical cancer, integration of HPV DNA occurs, interrupting the E2 ORF. This prevents E2-mediated transcription repression from occurring and allows E6 and E7 to be highly expressed (Doorbar, 2006; Longworth and Laimins, 2004; Shirasawa et al., 1987).

Reintroduction of bovine PV E2 or HPV E2 into HeLa carcinoma cell line represses E6 and E7 expression, resulting in restoration of p53 and pRb functions and causing cellular senescence (Dowhanick et al., 1995; Goodwin and DiMaio, 2000; Goodwin et al., 2000; Hwang et al., 1993). Recent studies also show that HPV 16 E2 interacts with p53 and CBP, a coactivator protein that plays a role in p53-dependent apoptosis, promoting apoptosis (Lee et al., 2000a; Massimi et al., 1999). However, it has been confirmed that LR HPV 11 E2 has no interaction with p53 (Parish et al., 2006).

HPV E1^E4

RNA splicing is responsible for the generation of E1^E4, a protein consisting of the first five E1 codons fused to the open reading frame of E4. Full - length E1^E4 is predominantly localized to the cytoplasm in the differentiated compartment of the HPV infected epithelium and is a 17 kDa protein (Doorbar et al., 1986; Jareborg and Burnett, 1991; zur Hausen, 1996). There are also 16 kDa, 11kDa and 10 kDa cleaved forms of E1^E4 (Doorbar et al., 1988).

The function of E1^E4 has not been clearly defined, but its expression had been shown to correlate with viral DNA amplification (Doorbar et al., 1997). However, a recent publication from Fang et al., showed that HPV 11 E1^E4 was not required for DNA amplification (Fang et al., 2006). E1^E4 is thought to supplement HPV E7's role in DNA synthesis activation. E1^E4 has been reported to induce the break-down of epithelial cytokeratins, permitting the escape of mature virions from the cornified envelope (Doorbar et al., 1991). Additionally, E1^E4 is associated with the cornified cell enveloped (CCE) (Bryan and Brown, 2000). This association disrupts the expression of loricrin (present in approximately 70% of healthy epithelium) and cytokeratin 10. This may result in the CCE becoming fragile and vulnerable to the escape of HPV, therefore allowing HPV to more readily infect new hosts (Bryan and Brown, 2000; Bryan and Brown, 2001). Both LR 11 HPV E1^E4 and HR 16 HPV E1^E4 are capable of altering the normal expression of cytokeratins (Bryan and Brown, 2000; Doorbar et al., 1997). Bryan et al. specifically showed that upon expression of

HPV 11 E1^E4, expression of loricrin and cytokeratin 10 was no longer

detectable (Bryan and Brown, 2000).

HPV E5

HPV E5 is a hydrophobic protein consisting of 85 amino acids. It is located

in the various membranes such as the Golgi, ER and plasma membranes

(Conrad et al., 1993). Bovine papillomavirus E5 is an oncogene and activates the

platelet derived growth factor receptor; however, both LR and HR HPV E5 have

meager transforming abilities but can co-operate with E7 to enhance its

transformation potential (Valle and Banks, 1995). HPV E5 associates with the

epidermal growth factor receptor (EGFR) (Hwang et al., 1995). Overexpression

of HR HPV 16 E5 results in an activating phosphorylation of EGFR, which

enhances its biological functions and prevents the receptor from being degraded

(Crusius et al., 1997; Pim et al., 1992; Zhang et al., 2002).

Detection of HPV E5 is very low in undifferentiated cells. It is thought that

HPV E5 may play a role in the later stages of the HPV life-cycle since it is only

detected in differentiated cells and disruption of HPV E5 expression interferes

with the life cycle of HR HPV (Fehrmann et al., 2003; Genther et al., 2003). It has

also been reported that expression of E5 correlates with a reduction in the

amount of HLA-A and HLA-B molecules (Ashrafi et al., 2005) and a decrease in

antigen presentation of major histocompatibility complex (MHC) class II

molecules (Zhang et al., 2003).

HPV L1 and L2

L1 and L2 are the two structural proteins of HPV. The L1 protein is the
major capsid protein and has a molecular weight of 55 kDa. The L2 protein is not
as abundant as the L1 protein and migrates between 72 and 78 kDa (Conway
and Meyers, 2009). 80% of the capsid consists of L1 pentamers. L1 alone or with
L2 can assemble into virus like particles (VLP) (Finnen et al., 2003; Rose et al.,
1993; Yuan et al., 2001; Zhou et al., 1991). VLP are particles that mimic the
external protein structure of a virus but do not contain genetic material that is
required for viral replication and are therefore not infectious (Campo and Roden,
2010; Finnen et al., 2003). HPV prophylactic vaccines based on VLP containing
only L1 are FDA-approved and are currently being marketed. These vaccines
have 100% efficacy at preventing HPV infections caused by the HPV types in the
vaccine (Campo and Roden, 2010). The efficiency of the HPV prophylactic
vaccines is due to generation of high titer type-specific neutralizing antibodies by
the immune system in response to exposure to VLP (Lowy and Schiller, 1998).
These vaccines are thought to provide long-term protection from HPV infection
(Conway and Meyers, 2009).

The HPV vaccine produced by Merck Sharp and Dohme offers specific
protection from the four mucosal types of HPV, HR HPV 16 and 18 and LR HPV
6 and 11, whereas the vaccine marketed by Glaxo Smith Kline protects against
HPV 16 and 18 only (Campo and Roden, 2010). The L1 capsid protein used to
make up the vaccine from Merck was produced using transgenic yeast and the
L1 capsid protein used in Glaxo's vaccine produced using a recombinant

baculovirus propagated in insect cells (Campo and Roden, 2010). Although the types of HPV included in these vaccines are the most prevalent types, there are obvious drawbacks. The vaccine does not protect against all HPV types. Another concern about the vaccine is that its production is expensive and therefore vaccines are not readily available in developing countries. Hopefully, there will be a broad spectrum vaccine manufactured in the near future that will also be relatively inexpensive to produce.

HPV E6

There are approximately 150 amino acids residues in the HPV E6 protein and it its approximate size is 18 kDa. The E6 protein consists of two zinc-binding domains, each containing a C-X-X-C-X_{29}-C-X-X-C sequence (Barbosa et al., 1989; Grossman and Laimins, 1989; Huibregtse et al., 1993). HR HPV E6 binds to E6-associated protein (E6-AP), a cellular ubiquitin-ligase, and targets the tumor suppressor p53 for degradation (Huibregtse et al., 1993; Scheffner et al., 1993). p53 plays many roles in cell-cycle regulation. It activates repair proteins in response to DNA damage, and if this damage is irreparable can induce cell arrest by activating p21, a cyclin kinase inhibitor (Hebner and Laimins, 2006; Levine, 1997).

The E6:E6-AP complex also targets NFX1-91 for degradation, enhances telomerase activity and increases cellular life-span (Gewin et al., 2004). Telomerase is an enzyme that is responsible for extending telomeric ends by the addition of hexamer repeats. NFX1-91 transcriptionally represses telomerase reverse transcriptase (TERT) expression (Gewin et al., 2004). E6:E6-AP complex

has also been reported to target E6TP1, MCM7 (minichromosome maintenance

7) and Bak for degradation (Munger et al., 2004; Wise-Draper and Wells, 2008).

Bak is a pro-apoptotic protein that is proteasomally degraded by HPV E6

(Thomas and Banks, 1999). MCM7 plays a role in guaranteeing that DNA

replication occurs only once per cycle. Therefore, interaction of HPV E6 with

MCM7 may result in over-duplication of chromosomes, contributing to genomic

instability (Kukimoto et al., 1998).

HR HPV E6 also binds directly to PDZ [PSD-95 (a 95 kDa protein involved

in signaling), Dlg (the *Drosophila* discs large protein), and ZO1 (the zonula

occludens 1 protein] domain-containing proteins via its C-terminal domain which

is highly conserved amongst HR HPV types (Glaunsinger et al., 2000; Lee et al.,

2000b; Nakagawa and Huibregtse, 2000; Thomas et al., 2001). LR HPV does not

possess the PDZ binding motif (Pim et al., 2000). PDZ proteins are necessary for

cell-cell adhesion and are implicated in cell signaling (van Ham and Hendriks,

2003). Binding of PDZ by HPV E6 is an important feature in progression to

carcinogenesis. HPV E6 mutants that can no longer bind PDZ are deficient for

E6-induced transformation in rodent cells and reduction of tumor development in

transgenic mouse models (James et al., 2006; Kiyono et al., 1997).

There are other proteins that have been discovered to interact with HPV

E6. Paxillin has been reported to bind to HR HPV E6, while both LR and HR HPV

E6 bind to p300, MCM7 and Bak. HR HPV E6 has a higher affinity for p300,

MCM7 and Bak (Kukimoto et al., 1998; Patel et al., 1999; Thomas and Banks,

1999; Thomas and Chiang, 2005; Tong and Howley, 1997; Zimmermann et al.,

2000). p300 functions as a transcriptional coactivator and a histone acetyltransferase (Iyer et al., 2004). Binding of p300 by HPV E6 prevents acetylation of p53 at p300-dependent sites, down-modulating p53-mediated expression (Zimmermann et al., 2000).

HPV E7

E7 proteins consist of approximately 100 amino acid residues and can be divided into three regions: conserved region 1 (CR1, amino acids 2-15), CR2 (amino acids 16-38), and the C-terminal zinc-binding region (amino acids 39-98) containing two Cys-X-X-Cys motifs. CR1 and CR2 are conserved with adenovirus E1A and SV 40 large T antigen. The zinc-binding C-terminal domain of E7 oncoprotein is proposed to be involved in homodimerization (Gage et al., 1990; Jewers et al., 1992; Munger et al., 2004; Munger et al., 2001). E7 proteins of HR HPV 16 and LR HPV 6 share 50% amino acid sequence identity and 15% conservative changes (Armstrong and Roman, 1992; Gage et al., 1990).

Both HR and LR HPV E7 proteins bind pRb family members through their LXCXE binding motif (Dyson et al., 1989). Furthermore, several *in vitro* studies have revealed that HPV 16 E7, as compared to HPV 6 E7, has a greater affinity for pRb, p107, and p130 (Ciccolini et al., 1994; Gage et al., 1990). HR HPV destabilize all pRb family members and this is a critical event that drives cellular transformation (Berezutskaya et al., 1997; Boyer et al., 1996; Davies et al., 1993; Gonzalez et al., 2001; Halbert et al., 1991; Helt and Galloway, 2001). The main contributing factor that results in enhanced binding of HR HPV E7 to pRb and its ability to target pRb for degradation is an aspartic acid versus glycine residue in

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HR vs. LR E7 proteins at the position immediately before the LXCXE binding motif. Although HPV 6 E7 has a lower affinity for binding p130 than HPV 16 E7, it is as efficient in targeting p130 for degradation (Zhang et al., 2006).

Significant differences between the HPV 16 E7 and HPV 6 E7 proteins have been reported such as HPV 16 E7, but not HPV 6 E7, can cooperate with ras to transform primary rodent cells, immortalize primary keratinocytes, and abrogate growth arrest mediated by DNA damage (Demers et al., 1996; Halbert et al., 1991; Jewers et al., 1992; Matlashewski et al., 1987; Storey et al., 1988; Watanabe et al., 1992). The molecular basis for the transformation ability of HR HPV E7 has been mapped to the amino-terminal half of the E7 protein (Heck et al., 1992; Phelps et al., 1992). The amino-terminal halves of HR and LR E7 proteins contain consensus recognition sequences for casein kinase II (CK II) (Barbosa et al., 1990; Firzlaff et al., 1989; Massimi and Banks, 2000). There are 2 serines in E7 that are specifically phosphorylated by CKII. When these sites are mutated the transforming ability of HPV E7 decreases (Barbosa et al., 1990). The E7 proteins of the HR HPV are phosphorylated *in vitro* at a higher rate than the LR HPV-encoded E7 proteins (Storey et al., 1988).

HR HPV E7 proteins have a number of cellular binding partners other than the pocket proteins. HR HPV E7 but not LR HPV E7 has been reported to interact with cyclin A/cyclin dependent kinase (Cdk) 2, cyclin E/Cdk2, PCAF, TATA box-binding protein (TBP), histone deacetylases (HDAC), E2F1, p21[CIP1] and p27[KIP1] via its C-terminus (Dell and Gaston, 2001; Munger et al., 2004; Wise-Draper and Wells, 2008). pRb is a substrate of both the cyclin A/Cdk2 and cyclin

E/Cdk2 complexes. HPV E7 interaction with these complexes results in reduction of Rb-associated transcriptional repression (McIntyre et al., 1996; Tommasino et al., 1993). Binding of HPV E7 to HDAC is indirect and is mediated by Mi2β (Brehm et al., 1999). p21^{CIP1} and p27^{KIP1} are cyclin kinase inhibitors and binding by E7 perturbs cell cycle inhibition (Funk et al., 1997; Zerfass-Thome et al., 1996). In differentiating cells, E7 binding to HDAC contributes to enhanced E2F-mediated transcription and increases proliferation (Hebner and Laimins, 2006).

pRb family members

The pRb family of proteins (pRb, p107 and p130) plays important roles in regulating cell cycle control and differentiation (Gonzalez et al., 2001; Munger et al., 2001). pRb family members are homologous in the "pocket" region, composed of subdomains A and B and separated by a spacer region that is highly conserved among each of the proteins. p130 and p107 share more homology than pRb. p130 and p107 contain a region between the A and B sub-domains that is responsible for inhibition of cyclin A/Cdk2 and cyclin E/CKD2 (Classon and Dyson, 2001; Claudio et al., 2002).

pRb family members each bind to specific members of the E2F family of transcription factors, which are responsible for the transcription of E2F-responsive genes, and hence S-phase entry (Cam and Dynlacht, 2003; Dimova and Dyson, 2005). The E2F family members, E2Fs 1-8 are transcription factors that are divided into two groups, activators and repressors. E2F1-E2F3 are activators and E2F4-E2F8 are repressors (Chen et al., 2009). E2F1-6, and form

heterodimeric complexes with the DP family of proteins, DP-1 and DP-2 whereas

E2F7 and E2F8 do not (Chen et al., 2009). E2F1, E2F2, and E2F3 are almost

exclusively regulated by pRb. p130 and p107 normally associate with E2F4 and

E2F5, and p130 associates with E2F4/5 at the G_0/G_1 stage of the cell cycle

(Dimova and Dyson, 2005; Helin et al., 1993). p130 and p107 may be able to

compensate for each other under certain conditions, but they both also have

independent roles. Of the pRb family members, p130 is highly expressed in

quiescent and differentiated cells, whereas p107 is primarily expressed during S

phase (Smith et al., 1996). It is important to note that p130-E2F complexes

appear to be fundamental in the permanent cell cycle withdrawal characteristic of

terminal differentiation. E2F4 and E2F5 lack nuclear localization signals (NLS).

These repressor E2Fs can be translocated to the nucleus by co-expression of

p130 or p107 (Lindeman et al., 1997).

   p130 levels, like the levels of other pRb family members, are regulated in

response to the proliferative state of cells and are controlled by Skp-Cullin-F-box

(SCF) complexes which mediate proteolysis in a phosphorylation-dependent

manner (Classon and Dyson, 2001); (DeCaprio et al., 1992; Tedesco et al.,

2002); (Shirodkar et al., 1992). p130 has been shown to be phosphorylated in

cycling cells by cyclin D/Cdk4 or Cdk6, cyclin A/Cdk2 and cyclin E/Cdk2 (Classon

and Dyson, 2001; Cobrinik, 2005). Cdk4/Cdk6, not Cdk2, is responsible for

targeting p130 for degradation in fibroblasts (Tedesco et al., 2002). In cycling

cells Cdk4/ Cdk6 phosphorylates p130 on Ser 672, resulting in a

hyperphosphorylated form of p130 that is targeted for degradation by an SCF

complex (Tedesco et al., 2002). In growth-arrested and terminally differentiated cells, p130 is phosphorylated by glycogen synthase kinase 3 (GSK3) in the loop region in the B subdomain and thus stabilized (Litovchick et al., 2004).

Proteasomal degradation

The majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway (Glickman and Ciechanover, 2002). The proteasome is a large 26S multisubunit complex that degrades polyubiquitylated proteins to small peptides. Proteasomes act on proteins marked specifically for degradation by a small protein called ubiquitin (Ciechanover et al., 2000). Ubiquitin is activated for transfer to substrate through the ATP-dependent formation of a thioester bond with the ubiquitin-activating (E1) enzyme and is subsequently transferred to a ubiquitin-conjugating (E2) enzyme. Finally, thioesterified ubiquitin is transferred to the target protein with the assistance of a ubiquitin ligase (E3). E3s bind directly to substrate, suggesting that they provide specificity in ubiquitylation reactions. It is likely that protein degradation *in vivo* is controlled primarily by regulating E3 activity or E3-substrate interaction (Ciechanover et al., 2000; Glickman and Ciechanover, 2002).

SCF complexes (E3 ubiquitin ligases) recognize and polyubiquitylate substrates in a phosphorylation-dependent manner, targeting them for degradation by the 26S proteasome (Deshaies, 1999). HPV 16 E7 and p130 both interact with and are ubiquitylated by SCF^{Skp2} complex (Oh et al., 2004a; Tedesco et al., 2002).

Nucleocytoplasmic shuttling

Nuclear localization signals (NLS) (K-K/R-X-K/R) are generally contained in proteins that are to be imported in the nucleus, whereas proteins to be exported from the nucleus contain nuclear export signals (NES) (L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI]) (Neufeld, 2009). Proteins are transported through the nuclear pore complex, and this import/export is mediated by karyopherins (Neufeld, 2009; Weis, 2003). Karyopherins that function in nuclear import are called importins (e.g. NTF2), whereas those that play a role in nuclear export are exportins (e.g. CAS). The karyopherins bind to their cargo proteins via the NLS or NES recognition sequence (Weis, 2003). Proteins that are imported in the nucleus are associated with the karyopherins until this karyopherin:cargo protein complex is bound to RanGTP which results in dissociation of the protein from karyopherin, as illustrated in Figure 3. Karyopherin is then recycled to the cytoplasm. Translocation of proteins that contain an NES from the nucleus to the cytoplasm occurs by these cargo proteins forming a complex with Crm1/RanGTP. In the cytoplasm this Crm1/RanGTP: cargo protein complex is disassociated by GTP hydrolysis facilitated by RanGAP (Pemberton and Paschal, 2005).

p130 localization

p130 contains three nuclear localization signals (NLS), two in the C-terminus and one in the loop region (Chestukhin et al., 2002). In undifferentiated cells, hypophosphorylated p130 is predominantly in the nucleus in the G_0/G_1
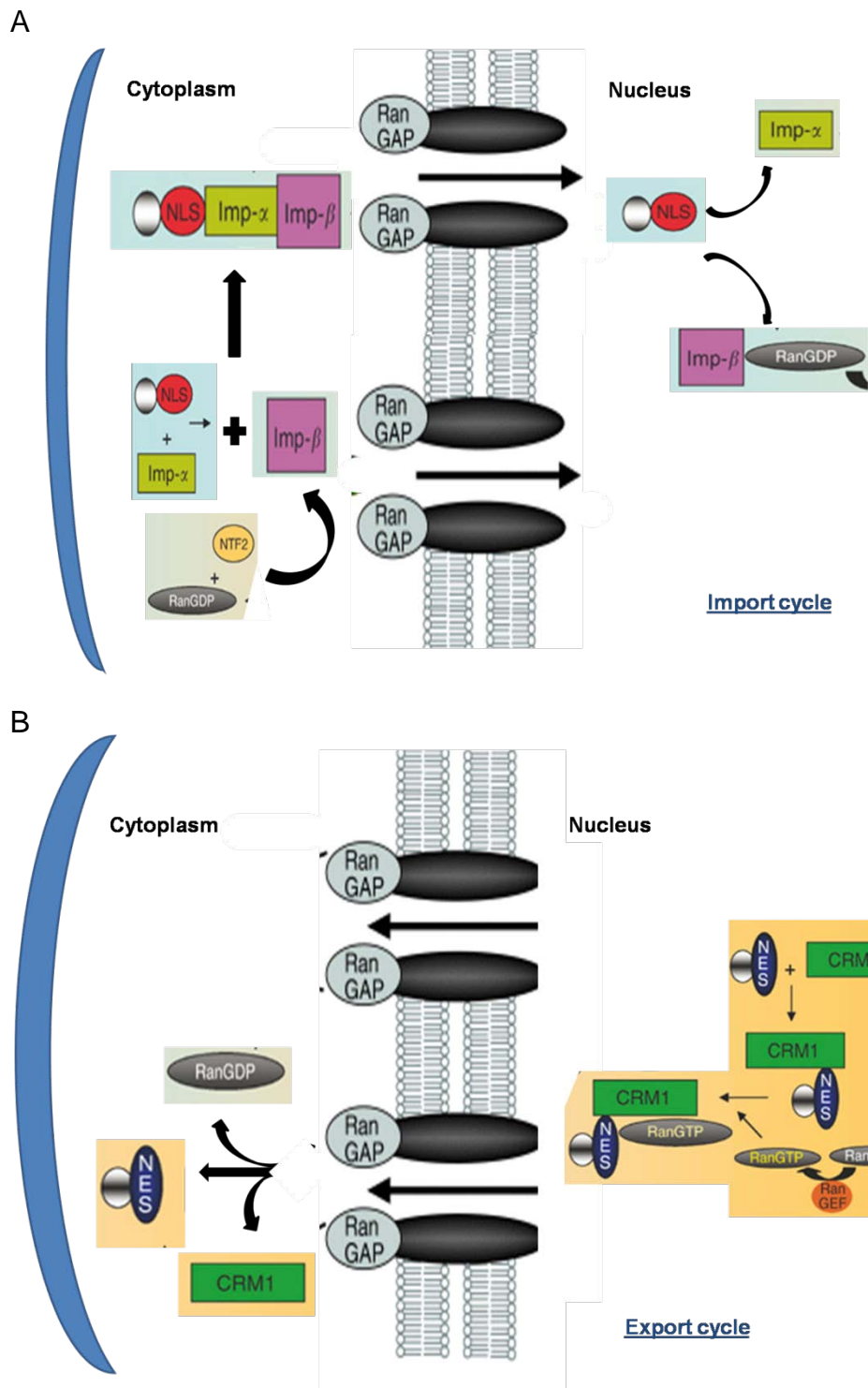
Figure 3. Nuclear import and export pathways. *(A)* Nuclear import of proteins

containing an NLS, mediated by the karyopherin-α: importin-β1 heterodimer

(Imp-α and Imp-β) and NTF2, which serves as a nuclear import receptor. Imp-α:

Figure 3 (con't). imp-β, NTF2 and the protein containing the NLS bind to Ran-guanosine diphosphate (Ran-GDP) and then bind to the nuclear pore. Entry into the nucleus occurs when Ran-GDP is exchanged for Ran-guanosine triphosphate (GTP) by Ran-guanine exchange factor (GEF). *(B)* Nuclear export of proteins containing an NES as mediated by Crm1, a nuclear export receptor. Crm1 recognizes the protein that contains the NES and binds to Ran-GTP. This complex is then shuttled to the cytoplasm. Figure adapted from Pemberton and Paschal, 2005 and Faustino et al., 2007.

phase of the cell cycle. In S-phase, p130 is typically phosphorylated and transported to the cytoplasm where it is targeted for degradation. Shuttling of p130 between the nucleus and the cytoplasm therefore provides a means of regulation (Chestukhin et al., 2002; Tedesco et al., 2002).

HPV E7 localization

Guccione et al. reported that HR and LR E7 proteins are detected predominantly in the nucleus by immunofluorescence (Guccione et al., 2002; Smith-McCune et al., 1999). Other studies, using subcellular fractionation, detected HPV 16 E7 in the cytoplasm and the nucleus (Nguyen et al., 2007; Smotkin and Wettstein, 1987). E7 is known to have both cytoplasmic and nuclear targets (Wise-Draper and Wells, 2008). In support of such observations, it has been reported that HPV 16 E7 has two NLS and one nuclear export signal (NES) (Knapp et al., 2009). HPV E7 has been shown to alter the localization of various proteins. Both HPV 6 E7 and 16 E7 relocalize steroid receptor coactivator 1 (SRC1) to the cytoplasm (Baldwin et al., 2006). HPV 16 E7 reduces the nuclear localization of p21^{Cip1} by a mechanism mediated by AKT (Westbrook et al., 2002).

Rationale for present studies

Data generated in the Roman lab have established that LR HPV 6 E7 is capable of targeting p130 for degradation through the proteasome pathway (Zhang et al., 2006). Zhang et al. 2006, have also published that there is a direct

correlation between a decrease in p130 expression and a delay in differentiation. The data suggest that p130 degradation is important for the HPV life cycle since both LR and HR HPV E7 have this ability and HPV 6 E7 mutants that could not target p130 for degradation also are not able to delay differentiation as efficiently as wild type (WT) HPV 6 E7 (Zhang et al., 2006).

In this thesis, to validate the loss of p130, immunohistochemical assays were performed on HPV 6 infected patient biopsies to determine the level of p130 expression *in vivo*. It was determined that there was a decrease of p130 levels in the presence of HPV 6, supporting the published retroviral transduction data.

Experiments were then conducted to determine the effect of LR and HR HPV E7 on p130 localization. Immunofluorescence and sub-cellular fractionation techniques were used. Both techniques established that there was an increase of cytoplasmic p130 in the presence of HR HPV 16 E7, but p130 localization of LR HPV 6 E7 transduced human foreskin keratinocytes was similar to the control cells. Treatment with leptomycin B (LMB), an inhibitor of Crm1/exportin 1, (a nuclear export protein) did not affect the HPV 16 E7-mediated cytoplasmic p130 localization. This suggests that HPV-E7 may retain p130 in the cytoplasm or else localization to the cytoplasm from the nucleus is via a Crm1/exportin 1 independent pathway.

Half-life studies were also conducted to address whether there was preferential degradation of p130 in either the cytoplasm or the nucleus in the presence of HPV 6 or 16 E7. The half-life of cytoplasmic p130 in the presence of

HPV 6 and 16 E7 was similar and shorter than the control. Interestingly, the half-life of nuclear p130 in HPV 6 E7 transduced cells was half as long as in HPV 16 E7 expressing cells and the control cells.

The mechanism of HPV E7-mediated p130 degradation was also investigated. p600 was previously identified as a cellular protein that binds to both HPV 6 E7 and HPV 16 E7 (Huh et al., 2005). p600 has been speculated to be an E3 ubiquitin ligase (Huh et al., 2005). Knock-down experiments using shRNA to p600 were performed. The results suggested that there was an increase in p130 stability. Earlier tandem-affinity purification experiments and mass spectrometry, from the Roman lab, showed binding of cyclin C to HPV 6 E7. Cyclin C/CDK3 is known to phosphorylate pRb resulting in G_0 exit (Ren and Rollins, 2004). HPV 6 E7 and HPV 16 E7 were confirmed to bind to cyclin C by glutathione-sepharose transferase (GST) pull-down experiments. Cullin 5 is a component of an E3 ubiquitin ligase. Adenovirus protein E4orf6 forms a complex with the Cullin5-ElonginB-ElonginC E3 ubiquitin ligase and targets p53 for polyubiquitination and proteasomal degradation (Luo et al., 2007). GST pull-down experiments showed that both HPV 6 and 16 E7 bound to cullin 5.

Thus, in this thesis, data show that LR HPV 6 E7 and HR HPV 16 E7 regulate p130 differently. HPV 6 E7 seems to have evolved to be able to target p130 in the nucleus. In contrast, HPV 16 E7 seems to sequester p130 in the cytoplasm, targeting it for degradation there, therefore providing HR HPV 16 E7 two ways of removing p130 from its nuclear targets. E7 binds to p600, cyclin C

and cullin 5; one or more of the latter proteins may participate in E7-mediated degradation.

MATERIALS AND METHODS

Preparation of human foreskin keratinocytes from neonatal foreskins

Human foreskin keratinocytes (HFKs) were isolated from neonatal foreskins obtained from routine circumcisions at Wishard Hospital (Rheinwald, 1980). Foreskins were washed with 1X PBS and fat and dermis removed using a sterile scalpel and scissors. Foreskins were washed again, minced and added to 5 ml of 37°C 1X trypsin-EDTA (Invitrogen, Carlsbad, CA) in a 25 ml flask containing a small stir-bar. HFKs were released from the minced tissue by incubation in trypsin/EDTA with gentle agitation with the stir bar, for 15 min in a 37°C, 5% CO_2 incubator. Cells were collected and transferred to a 15 ml conical tube containing 1 ml fetal bovine serum (FBS) (Invitrogen). Trypsin-EDTA was added to the foreskin tissue in the 25 ml flask and incubated in 37°C incubator twice more, stirring as before. The three tubes were spun for 2 min at 2000 rpm at room temperature (RT). Supernatant was aspirated and pelleted cells resuspended in E medium [(3 parts Dulbecco's Modified Eagles Medium (DMEM, Invitrogen), 1 part HAMS F12 (Invitrogen), 10% fetal calf serum (FCS) (Hyclone, Logan, UT), 0.4 µg/ml hydrocortisone, 0.1 nM cholera toxin, 5 µg/ml transferrin and 1X antibiotic-antimycotic solution containing 100 U penicillin/ml, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin B). HFKs were then plated in E medium on three 10 cm tissue culture dishes in the presence of 3 X 10^5 mitomycin C-treated J2 fibroblasts per plate. Media was changed after 3 days and EGF added to the E media. At 80% confluence, media was aspirated and the plate rinsed twice with 1X PBS. Five ml of 0.02% EDTA/PBS was added to

each plate and incubated for 2 min at RT. The J2 fibroblasts were removed by forcefully pipeting the 0.02% EDTA/PBS solution over the surface of the plate. The plate was then rinsed several times with 1X PBS. HFKs were trypsinized and plated 1:10 on 3 X 10^5 mitomycin C-treated J2 feeder cells in E medium. When HFKs became 80% confluent they were frozen in 1 ml DMEM containing 20% FBS and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) at one plate/vial, stored temporarily as stocks at -80°C and then transferred to liquid nitrogen tanks.

Production of retrovirus stock with stably transduced PA317 cells

PA317 LXSN retrovirus packaging cells and PA317 L(16E7)SN retrovirus packaging cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA). A vial of cells was thawed and plated on one 6 cm tissue culture dish in DMEM plus 10% FBS and 1 mg/ml geneticin (G418, Invitrogen; selection media). The next day the cells were transferred to a 10 cm tissue culture dish. Media was changed every three days until the plate was 90% confluent. The cells were then split 1:10 to 10 cm dishes and maintained in selection media until 80% confluent. The plates were then rinsed three times and the media changed to 5 ml of DMEM plus 10% FBS, in the absence of G418. Twenty-four hours later, virus was harvested and filtered through a 0.2 micron filter (Millipore, Billerica, MA) and 5 ml aliquots were frozen at -80°C. Fresh media were added to each plate and this process repeated once more to obtain two sets of virus.

Production of virus in transiently transfected SD3443 (Phoenix) cells

Viruses encoding HPV 6 E7, C-terminally tagged HPV 6 E7, C-terminally tagged 16 E7 and parental vector C-tap (Roman laboratory) and pCMV-HA-p130 (obtained from Dr. DeCaprio, Harvard University) were generated by transfection of the respective DNAs into Phoenix-ampho cells (ATCC). One vial of Phoenix-ampho cells (ATCC) was thawed to one 10 cm tissue culture dish, in DMEM plus 10% FBS. When cells were 90% confluent they were split to ten 10 cm dishes. Cells were transfected when they were 70% confluent. Media was removed from cells and 5 ml DMEM (serum and antibiotic free) containing 25 µM of chloroquine was added. Cells were then incubated at 37°C for approximately 15 min. For each plate to be transfected the following procedure was performed: 4 µg of DNA, 20 µl of plus reagent (Invitrogen) and 730 µl of DMEM were added to a 15 ml polystyrene Falcon conical tube. 30 µl lipofectamine (Invitrogen) and 720 µl of DMEM were added to another tube. The two solutions were mixed with a pipet and allowed to stand for 15 min (transfection solution). One and a half ml of transfection solution was then added to each plate containing DMEM plus chloroquine and the plate incubated for 3.5 h in the 37°C, 5% CO_2 incubator. After this period, 6.5 ml of DMEM plus 20% FBS was added. The following day, media were removed and 5 ml of DMEM plus 10% FBS added. Virus was harvested, filtered and stored on the third and fourth days following transfection as described above for the PA317 packaging cell line.

Titering retrovirus

NIH 3T3s were plated at 5 x 10^5 cells/6 cm tissue culture plate in 4 ml of DMEM plus 10% FBS. The next day, NIH 3T3s were infected with varying dilutions of virus (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) with 8 µg/ml polybrene, made up in 2 ml DMEM plus 10% FBS. The next day the cells were split 1:1 onto 10 cm dishes with 1 mg/ml G418 in DMEM containing 10% FBS. Three days later, the cells were fluid changed and this was repeated until colonies formed. Upon formation of colonies, the media was aspirated from the plates and the plates fixed and stained with 1% methylene blue/10% ethanol solution in water to visualize individual colonies. The plates were then rinsed with distilled water and allowed to air-dry overnight (O/N). The colonies were counted and viral titer calculated using the formula shown below.

$$\text{Infectious virus/ml} = \frac{\text{Number of colonies}}{\text{Virus volume (ml) x dilution factor of cells plated}}$$

Transfection of HFKs using FuGENE 6

At 50% confluence HFKs were transfected using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). HFKs were growing in 4 ml of C-K-SFM: serum free media (SFM) supplemented with bovine pituitary extract and human recombinant epidermal growth factor (EGF) (both purchased from Invitrogen) and 1:1000 dilution of 50 mg/ml gentamicin (Sigma-Aldrich). A 3:1 ratio of FuGENE 6 tranfection reagent: DNA complex (18 ul of transfection reagent: 6 µg of DNA) made up to 600 µl in C-K-SFM was incubated for 30 min and then added to the

HFKs. Forty-eight hours later the transfected cells were harvested. Rev-green fluorescent protein (GFP) expression plasmid (Ludwig et al., 1999) was obtained from Dr. Johnny He's laboratory; HA-p130 and HA-p130-Δ21-5A-m-NLS-1&2 DNAs were obtained from Dr. Jim DeCaprio, Harvard University (Chestukhin et al., 2002).

Retroviral infection of HFKs

Third-passage HFKs were grown to about 40% confluence in a 10 cm tissue culture dish in C-K-SFM. The media was aspirated and HFKs were infected with 5 ml of the recombinant retrovirus or parental virus, in the presence of 8 µg/ml of polybrene. Depending on the titer of the virus, cells were either infected once or twice, with the amount of virus needed to obtain approximately 5 X 10^6 viruses per 1.5 X 10^6 cells. After 6 h the cells were fed with C-K-SFM and kept at 37°C in 5% CO_2 for 48 h. The cells were then transferred to four 10 cm diameter dishes and selected with 200 µg/ml of G418 for 3 days. Selected cells were expanded and generally harvested at 80% confluence. For differentiation experiments, transduced HFKs were grown to 100% confluence and induced to differentiate in 2 mM $CaCl_2$ for 48 h.

Ten ng/ml leptomycin B (LMB) (Sigma-Aldrich; stock solution: 5.5 µg/ml), an inhibitor of CRM-1 mediated nuclear export, was used to address the effects of HPV E7 on p130 localization. Transduced HFKs were treated for 4 h with 10 ng/ml of LMB. For studies to determine the half-life of p130 in the cytoplasm and nucleus of HPV E7 transduced cells, the transduced HFKs were treated with 0.25

mM cycloheximide [(CHX, Sigma-Aldrich); stock solution 0.355 M, dissolved in DMSO] for 2h, 4h and 6h to inhibit protein synthesis. HPV E7 transduced cells were also treated with CHX plus 50 µM MG132 (Sigma-Aldrich; stock solution 50 mM, dissolved in DMSO) an inhibitor of proteasomal degradation for 2 h, 4h and 6h. For experiments to determine the effect of cyclin-Cdk activity on E7-mediated degradation, cells were treated with 12.5 µM roscovitine (Sigma-Aldrich), 50 µM flavopiridol (Sigma-Aldrich) or 12.5 µM 3-amino thioacridone (3-ATA) (Alexis Biochemicals) for 4 h. All Cdk inhibitors were dissolved in DMSO at a concentration of 25 mM for roscovitine and 3-ATA and 50 mM for flavopiridol.

Nuclear/cytoplasmic fractionation

For fractionation experiments, HFKs transduced with LXSN, L(6 E7)SN or L(16 E7)SN were washed twice with 1X PBS, trypsinized and pelleted. Cells were washed again with 1X PBS and 300 µl ice cold cell fractionation buffer (PARIS kit, AppliedBiosystems/Ambion, Austin, TX) containing 100 X protease inhibitor cocktail (Sigma-Aldrich) was added to the cell pellet. The mixture of fractionation buffer and cells were incubated on ice for 10 min; the suspension became clear rapidly, indicating lysis of the plasma membrane. Lysates were spun at 2000 rpm for 3 min in a microcentrifuge and the supernatant saved (cytoplasmic fraction). The pellet was washed in 300 µl ice-cold cell fractionation buffer and spun at the speed indicated above. Three hundred microliters of 2× Laemmli buffer [4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.125 M Tris–HCl) was then added to the pellet, and the pellet vortexed and vigorously pipeted

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to obtain the nuclear fraction. The nuclear fraction was incubated on ice for 10 min to ensure complete cell disruption. Cytoplasmic and nuclear fractions were clarified by centrifugation at 4°C for 1 min at top speed in a microcentrifuge.

Western blotting

For whole cell extracts (WCEs), cells were lysed with extraction buffer C (EBC: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40) or 2X SDS lysis buffer [(20% glycerol, 4% SDS, 120 mM Tris-HCl pH 6.8)]. Cells harvested with EBC buffer were scraped with a rubber policeman while the plate sat on ice. Cells harvested with 2X SDS lysis buffer were harvested at RT. Protease and phosphatase inhibitors were added before use: protease inhibitor cocktail,125 µM Na$_3$VO$_4$ (Sigma-Aldrich; stock solution 200 mM made up in ddH$_2$O) and 50 mM NaF (Sigma-Aldrich; stock solution 100 mM made up in ddH$_2$O). Protein concentrations were determined using the Bio-Rad DC protein assay kit. Proteins were separated on 6% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) at 130 V, using a mini-gel apparatus. The proteins were then transferred to nitrocellulose at 100 V for 1h with an ice pack. Following transfer, the nitrocellulose membranes were stained for 7 min at RT with ponceau red (Sigma-Aldrich) as a preliminary indicator of equal loading. Excess ponceau red was removed from the membrane with brief rinsing with 5% acetic acid (diluted in ddH$_2$O) followed by a ddH$_2$O rinse. The stained membrane was then scanned. Western analysis was performed using the established standard protocol. Membranes were blocked for at least 1 h at RT or O/N at 4°C in

blocking solution [TBS-T (20mM Tris-HCl ph 7.5, 500mM NaCl and 0.05% v/v Tween 20) that contained 5% wt/vol non-fat dry milk (Kroger brand)].

Monoclonal antibodies were generally incubated for 1h at the designated dilution in the above blocking solution. However, monoclonal p130 and pRb were incubated O/N at 4°C. Rabbit polyclonal antibodies were always incubated with the membrane O/N in 5% bovine serum albumin (BSA) (Sigma-Aldrich) in TBST. After addition of primary antibody, the blot was washed three times with 1X TBST, 10 min each wash. Goat anti-mouse-horse radish peroxidase (HRP) conjugated secondary antibody (Bio-rad, Hercules, CA) was used at a dilution of 1:8000 in 1X TBST and goat anti-rabbit secondary antibody (Bio-rad) was used at a dilution of 1:3000 in 1X TBST. Blots were incubated for 1h with secondary antibodies at RT and washed three times with 1X TBST. Blots were then developed using the enhanced chemiluminescent (ECL) Western blotting substrate kit (Thermo Scietific, Rockford, IL).

The following antibodies were used: monoclonal p130, 1:1000 dilution (cat # 610261) and pRb, 1:1000 dilution (cat # 554136) BD Biosciences, San Jose, CA. Polyclonal p130, 1:100 dilution (cat # sc-317), E2F5, 1:1000 dilution (cat # sc-599), GFP, 1:2000 dilution (cat # sc-9996), cyclin C, 1:500 dilution (cat # sc-5610), nucleolin, dilution 1:1000 (cat # sc-8031), Cdk8, 1:200 dilution (cat # sc-1521) and p600, 1:1000 dilution (cat # sc-100615), Santa Cruz. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), 1:30,000 dilution (cat # MAB374) and cullin 1, 1:1000 dilution (cat # MAB3783), Millipore. Tubulin, 1:3000 dilution (cat # T6074), Sigma-Aldrich. Lamin B, 1:2000 dilution (cat # AB16048), Abcam,

San Francisco, CA. HA, 1:1000 dilution (cat # 11583816001), Roche. Cdk3,

1:200 dilution (cat # AHZ0172), Invitrogen. Cullin 5, 1:700 dilution (cat # A302-

173A), Bethyl Laboratories, Montgomery, TX. For fractionation experiments used

to determine localization of proteins, equal volume fractions of cytoplasmic to

nuclear protein were used. For all other experiments equal quantity of protein

was analyzed and samples normalized to tubulin, for cytoplasmic fractions, lamin

B or nucleolin for nuclear fractions, or nucleolin or GAPDH for WCEs.

For experiments to determine the phosphorylation status of p130, lambda

protein phosphatase was used (New England Biolabs, Ipswich, MA). One

hundred units of λ-phosphatase were incubated in 1x λ-phosphatase reaction

buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM ethylene glycol tetraacetic acid, 2

mM dithiothreitol (DTT), 0.01% Brij 35) with WCEs (harvested in EBC buffer) for

30 min at 30°C. Fifty mM EDTA was then added to samples and incubated at

65°C for 1 h to heat inactivate phosphatase activity.

Immunofluorescence

Infected HFKs were grown on glass coverslips in C-K-SFM and

transduced with LXSN, L(6E7)SN or L(16E7)SN. Cells were fixed in 4%

paraformaldehyde for 10 min, rinsed and permeabilized with 0.1% Triton X-100 in

PBS for 10 min. The cells were immersed in 0.1% BSA/PBS for 30 min to block

nonspecific binding. Cells were incubated with polyclonal antibody to p130

(Santa Cruz) diluted in 0.1% BSA/PBS for 1.5 h at RT. Various concentrations of

p130 antibody were tested (1:50, 1:100 and 1:200); the 1:100 dilution was

determined to be the optimal concentration. Cells were then washed with 0.1%

BSA/PBS at RT and incubated with fluorescein isothiocyanate (FITC)-conjugated

secondary antibody (Santa Cruz) at a 1:200 dilution. Cells were counterstained

with 5 µg/ml of 4′-6-Diamidino-2-phenylindole (DAPI) for 7 min at RT, washed

again, mounted and stored in the dark for at least 2 h. Fluorescent images were

then taken with a Nikon Eclipse microscope (Dr. Johnny He's laboratory). For

experiments with the Rev-GFP plasmid (Ludwig et al., 1999), HFKs were

transfected with FuGENE 6 transfection reagent as described earlier.

Generation of HA-p130 mutants

Three putative nuclear export sequences were identified using the NES

predictor (www.cbs.dtu.dk/). Deletion mutants were generated in the individual

three NES (p130 Δ1, deleted 228-238; p130 Δ2, deleted 510-520; p130 Δ3,

deleted 850-860) and all combinations thereof. Two complimentary

oligonucleotides with the desired NES deleted were synthesized using the

QuikChange II XL site-directed mutagenesis kits (Agilent Technologies, Inc.,

Santa Clara, CA). The parental DNA used was pHA-p130, a 4.3 kb plasmid

obtained from Dr. Jim DeCaprio, Harvard University (Chestukhin et al., 2002).

The polymerase chain reaction (PCR) conditions were as follows. In a 1.5

ml eppendorf tube a 50 µl reaction was set up containing 10 ng of HA-p130

dsDNA template in 10x reaction buffer. To the template solution was added 1 µl

dNTP mix, 125 ng of oligonucleotide primers 1 and 2 and 3 µl of QuikSolution.

Two and a half units of PfuUltra HF DNA polymerase were added. The DNA

thermocycler was programmed for 1 min at 95°C, followed by 18 cycles of 50 s at 95°C (denaturation), 1 min at 55°C (annealing) and 5 min at 68°C (extension), followed by an additional 7 min extension reaction at 68°C. Listed below are the sequences of single stranded oligonucleotides used as primers for generation of HA-p130 deletion NES mutants.

p130 Δ1 sense, 5'-ATTTGGTCAATTCTTAATTCTTATCACCTGAATG CACTTCAGTGTTCTAATCG-3'

p130 Δ1 antisense, 5'-CGATTAGAACACACTGAAGTGCATTCAGGTGATAAGA ATTGACCAAAT-3'

p130 Δ2 sense, 5'-TTAGAATCTGTTATTGAGCAGGAAGGTATTCTGGAACAAG ATGCGTTC-3'

p130 Δ2 antisense, 5' GAACGCATCTTGTTCCAGAATACCTTCCTGCTCAATAACAG ATTCTAA-3'

p130 Δ3 sense, 5'-GTATACCATTTAGCAGCTGTCCGCTCAGATGAATTGAGG AAAAAAATC -3'

p130 Δ3 antisense, 5'- GATTTTTTTGGTCAATTCATCTGAGCGGACAGCTGC TAAATGGTATAC

Dpn I Digestion of the Amplification Products

Ten units of the Dpn I restriction enzyme was then added directly to each amplification reaction. The reaction was mixed by pipeting up and down several times. The reaction mixtures were then spun at RT at full speed for 10 s in a

microcentrifuge and the reaction incubated at 37°C for 1 h to digest the parental

supercoiled dsDNA.

Transformation of XL10-Gold Ultracompetent Cells

XL10-Gold ultracompetent cells were gently thawed on ice. Forty-five μl of

ultracompetent cells per reaction were aliquoted to a prechilled 15 ml BD Falcon

polypropylene tube. 2 μl of β-mercaptoethanol (Sigma-Aldrich), were added the

cells, contents were swirled gently and incubated on ice for 10 mins, swirling

every 2 mins. Two μl of the Dpn I-treated DNA was then transferred to the aliquot

of ultracompetent cells. The transformation reaction was mixed gently and

incubated on ice for 30 mins. The tubes were heat-pulsed in a 42°C water-bath

for 30 s and incubated on ice for 2 min. 500 μl of NZY$^+$ broth (NZB plus

supplements:10 g NZ amine, 5 g yeast extract, 5 g NaCl, 12.5 mM MgCl$_2$, 12.5

mM MgSO$_4$, 20 mM glucose) was added to each tube and incubated at 37°C for

1 h with shaking at 225-250 rpm. Two hundred and fifty μl of transformation

reaction was plated on an agar plate containing 100 μg/ml ampicillin per ml of NZ

agar. The transformation plates were incubated at 37°C for approximately 16 h.

Plasmid purification

Colonies were picked, grown up in 500 ml cultures and purified using

QIAGEN-tip 500^{TM} columns according to the manufacturer's recommendations

(QIAGEN, Inc., Valencia, CA). Ultraviolet absorbance at 260 nm was used to

determine DNA concentration using a NanoDrop 2000 spectrophotometer

(Thermo Scientific). Purity and quantity were verified by the OD_{260}/OD_{280} ratio and by examination of the electrophoretic pattern of the DNA in agarose mini-gels.

<u>DNA sequence analysis</u>

The DNA sequences of the mutants (p130 Δ1 NES, p130 Δ2 NES, p130 Δ3 NES, p130Δ1Δ2 NES, p130 Δ1Δ3 NES, p130 Δ2Δ3 NES, p130 Δ1Δ2Δ3) were provided by the DNA Core Sequencing Facility (IUSM Department of Biochemistry and Molecular Biology) using the following primers:

p130 Δ1 sense, 5' CAGCCCTGTACTGTGCTG 3'

p130 Δ1 antisense, 5'GCCATCATGTAAGGAACACAG 3'

p130 Δ2 sense, 5'CAGCATTTCCAGCCAGACGAG 3'

p130 Δ2 antisense, 5' GACGACCTCAAGGCAGCAGGC 3'

p130 Δ3 sense, 5' CAAGGCCAGTCTGTAACCAGC 3'

p130 Δ3 antisense, 5'GGTCCAGATGTCTGTCCATC 3'

All of the p130 mutants were confirmed to have been successfully generated.

<u>DNA *in Situ* hybridization</u>

Sections from biopsies of HPV 6 positive condyloma acuminata were used that have been typed by polymerase chain reaction in Dr. Darron Brown's laboratory. To identify individual cells that contain HPV DNA, DNA *in situ* hybridization was performed using an *in situ* typing assay (Enzo Diagnostics, Farmingdale, NY). Sections were deparaffinized in xylene followed by ethanol,

and treated with 25 µg/ml proteinase K for 5 min at 37° C. Sections were then treated with 3% H_2O_2 in methanol for 30 min to reduce endogenous peroxidase activity. Sections were washed in 1x PBS, dehydrated by incubation in ethanol, and dried. HPV 6 type specific probe was added at 5 ng/µl to DNA *in situ* hybridization buffer (Dako, Carpinteria, CA). Sections were then covered with a HybriStrip (Research Products International, Mt. Prospect, IL) and heated at 95°C for 10 min. Probe and target DNA were hybridized O/N at 37°C. Sections were washed two times in 2X SSC (diluted in ddH$_2$O from 20x SSC: 175.3 g NaCl, 88.2 g Na Citrate) plus 0.05% Tween-20 for 10 min at 37°C. Sections were blocked and probes detected using detection reagent, as instructed by the manufacturer. Sections were dehydrated in ethanol and mounted using Vectashield (Vector Laboratories, Burlingame, CA); slides were viewed by light microscopy.

Immunohistochemistry

Sections from biopsies were deparaffinized and treated with 3% H_2O_2 in methanol as described above. Sections were blocked with non-specific rabbit serum (Santa Cruz) and incubated with antibody against p130 at a 1:100 dilution in phosphate buffered saline (PBS) (Santa Cruz). HPV negative foreskin tissue was used as control. Antibodies to keratin 14 (a marker for stratified squamous epithelium) at a 1:100 dilution in PBS and proliferating cell nuclear antigen (PCNA; expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle) at a 1:100 dilution in PBS (both antibodies from Santa Cruz) were

used as positive controls to validate the assay. As a negative control primary antibodies were omitted. Antibody binding was detected using the Vectastain ABC detection system (Vector Laboratories) to yield a purple precipitate. Slides were then examined by light microscopy using a Nikon microscope (Dr. Johnny He's laboratory) and images captured.

Generation of glutathione-sepharose transferase (GST) fusion proteins

Five μl of *E.coli* DH5α bacterial cells containing plasmid that expressed GST or GST fusion proteins were grown O/N in 5 ml of NZB in the presence of 0.1 mg/ml of ampicillin. The next day, the 5 ml culture was added to 250 ml NZB containing 0.1 g/L of ampicillin. The bacteria were grown for 1 h at 37°C and then at RT for 2 h with continuous shaking. After 2 h, 1 mM isopropyl β-D-1-thiogalactopyranoside, dissolved in NZB, was added to the flask. Sixteen to eighteen hours later the bacteria were pelleted by centrifuging at 6000 rpm for 15 min. The cell pellet was resuspended in a volume of the detergent lysis buffer [200 mM NaCl, 50mM Tris-HCl pH 8.0, 1mM DTT and protease inhibitor tablet (Roche)] equal to 3X the size of the pellet. The bacteria were disrupted by processing 3 times in a French-press. Bacterial debris was pelleted at 15,000 rpm for 30 mins. Glutathione sepharose beads (GE Healthcare, Piscataway, NJ) were pre-equilibrated in binding buffer by washing the desired quantity of beads for 30 min with 1 ml of detergent lysis buffer, spinning down and 3000 rpm for 3 mins and aspirating detergent lysis buffer. This was done three times and an equal volume of detergent lysis buffer added to the beads (50% slurry). Five

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hundred μl of the 50% slurry was then added to the supernatant (containing unpurified GST or GST fusion proteins) and rocked O/N at 4°C. Beads were pelleted by centrifugation at 3,000 rpm for 5 min. The sepharose beads were washed three times with 1 ml of detergent lysis buffer, with rocking for 30 min at 4°C and aspiration of the supernatant between each wash. Two hundred and fifty μl of 20 mM glutathione (Sigma-Aldrich) in detergent lysis buffer was added to the beads with rocking for 30 min at 4°C, to elute the bound GST protein. The elution step was performed three times. The elutions were combined and concentrated using 10,000 molecular weight cut off ultra-centrifugal filtration devices (Millipore). GST fusion proteins were placed in dialysis cassettes (Thermo Scientific) and dialyzed O/N at 4°C in 1 L of detergent lysis buffer. Proteins were quantified, quick frozen using a dry/ice ethanol mix and stored at -80°C.

Glutathione-s-transferase (GST) pull-down assays

HeLa nuclear extract (Accurate, Westbury, NY) was pre-cleared O/N by rocking with 10 μg of GST protein and 25 μl of pre-equilibrated glutathione-sepharose beads 50% slurry. This solution was spun down and the supernatant used for the GST pull down assay. Ten μg of GST or GST fusion protein in binding buffer [20 mM Hepes, 150 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 0.02% Nonidet P-40 (NP-40), 10% glycerol, 0.035% 2-mercaptoethanol, mini protease inhibitor cocktail tablet (Roche: 1 tablet in 10 ml of solution)] was rocked with glutathione-sepharose beads for 1 h at 4°C. Precleared HeLa nuclear extract

(1000 µg) was added to each reaction and rocked for another hour. The beads

were then washed with 1 ml of washing buffer (20 mM Hepes, 150 mM KCl, 4

mM MgCl$_2$, and 1 mM EDTA, 0.1% NP-40, 10% glycerol, 0.035% 2-

mercaptoethanol, protease inhibitor cocktail tablet) three times, resuspended in

30 µl of 2x SDS sample buffer and boiled. Beads were spun down and

supernatant used to perform Western analysis. The proteins were separated by

SDS/PAGE. Proteins were transferred to the nitrocellulose membrane, stained

with Ponceau red, and probed with antibodies to cyclin C (Santa Cruz), Cdk3

(Invitrogen), Cdk8 (Santa Cruz), cullin 1 (Chemicon) and cullin 5 (Bethyl

Laboratories) as described under *Western Blot Analysis*.

<u>Immunoprecipitations</u>

For immunoprecipitations (IPs), HFKs were transduced with C-Tap 6 E7

(HA-myc-6 E7), C-Tap 16 E7 (HA-myc-16 E7), or control vector C-Tap (HA-myc-

LXSN) and harvested in IP lysis buffer (0.1% NP-40, 0.25 M NaCl, 50 mM

HEPES, 5 mM EDTA, 0.2 mM Na$_3$VO$_4$, 8 mM NaF, protease inhibitor cocktail).

Cell lysate was clarified by spinning for 10 s at full speed in a micro-centrifuge at

4°C. For IPs using rabbit polyclonal antibodies (cyclin C and cullin 5 antibodies),

lysates (1000 µg for each IP) were pre-cleared with 20 µl of protein-A beads

(protein-A beads used in this protocol were equilibrated in IP lysis buffer) and

1:100 dilution of rabbit IgG for 1h at 4°C. This mixture was then spun at 3000 rpm

for 1 min and supernatant transferred to a new eppendorf tube. Antibody was

added at a 1:100 dilution and rocked for 2 h at 4°C. Next, 50 µl of protein-A

agarose beads (Millipore) were added and rocked O/N. Lysate containing

antibody bound to agarose beads was spun at 3000 rpm for 1 min and the

supernatant transferred to a new eppendorf tube. This supernatant was saved as

input to insure that equal quantity of protein was used in each IP. Beads were

then washed three times, for 1 min, with cold IP lysis buffer. Thirty µl of 2x SDS

sample buffer was used to resuspend the beads, the beads boiled for 5 mins,

spun down for 10 s at 12,000 rpm and the supernatant ran on a 10% gel. For IPs

using mouse monoclonal antibody (HA antibody), no pre-clear step was

performed. Lysates were incubated with 50 µl of protein-G agarose beads

(Roche) instead of protein-A agarose beads.

RESULTS

Chapter 1

The Effect of HPV 6 on Expression of p130 (a pRb family member) *In Vivo*

The Roman laboratory has published data showing that HPV 6 E7 is capable of destabilizing p130 (Zhang et al., 2006). To further validate these results, the expression of p130 in HPV 6 infected condyloma acuminata was investigated. HPV 6 positive biopsies and control foreskin tissues were obtained from Dr. Darron Brown's laboratory. These biopsies were typed by polymerase chain reaction in the Brown laboratory and determined to contain only HPV type 6. Control foreskin tissue was not infected with HPV.

Seven samples were processed, 3 of the biopsies of condyloma acuminata were obtained from male patients and 4 from female patients. Tissues had to have their epithelium intact in order to be processed. *In situ* hybridization was performed as additional verification of the presence of HPV 6 DNA in the nuclei of the cells of the patient biopsies as well as to differentiate between HPV positive and HPV negative cells, as it was a possibility that the biopsies contained normal tissue as well (Figure 4). A dense nuclear signal was detected in the PCR typed HPV 6 positive condyloma acuminata after hybridization with HPV 6 probe. Cells were HPV 6 positive. No staining was detected in the control foreskin tissue.

Immunohistochemical analysis was performed to detect expression of p130 in the uninfected foreskin (Figure 5) and HPV 6 infected condyloma

100X   **400X**

HPV 6 probe — A, B

No probe — C, D

HPV 6 probe — E, F

No probe — G, H

Foreskin tissue

Biopsy #1505
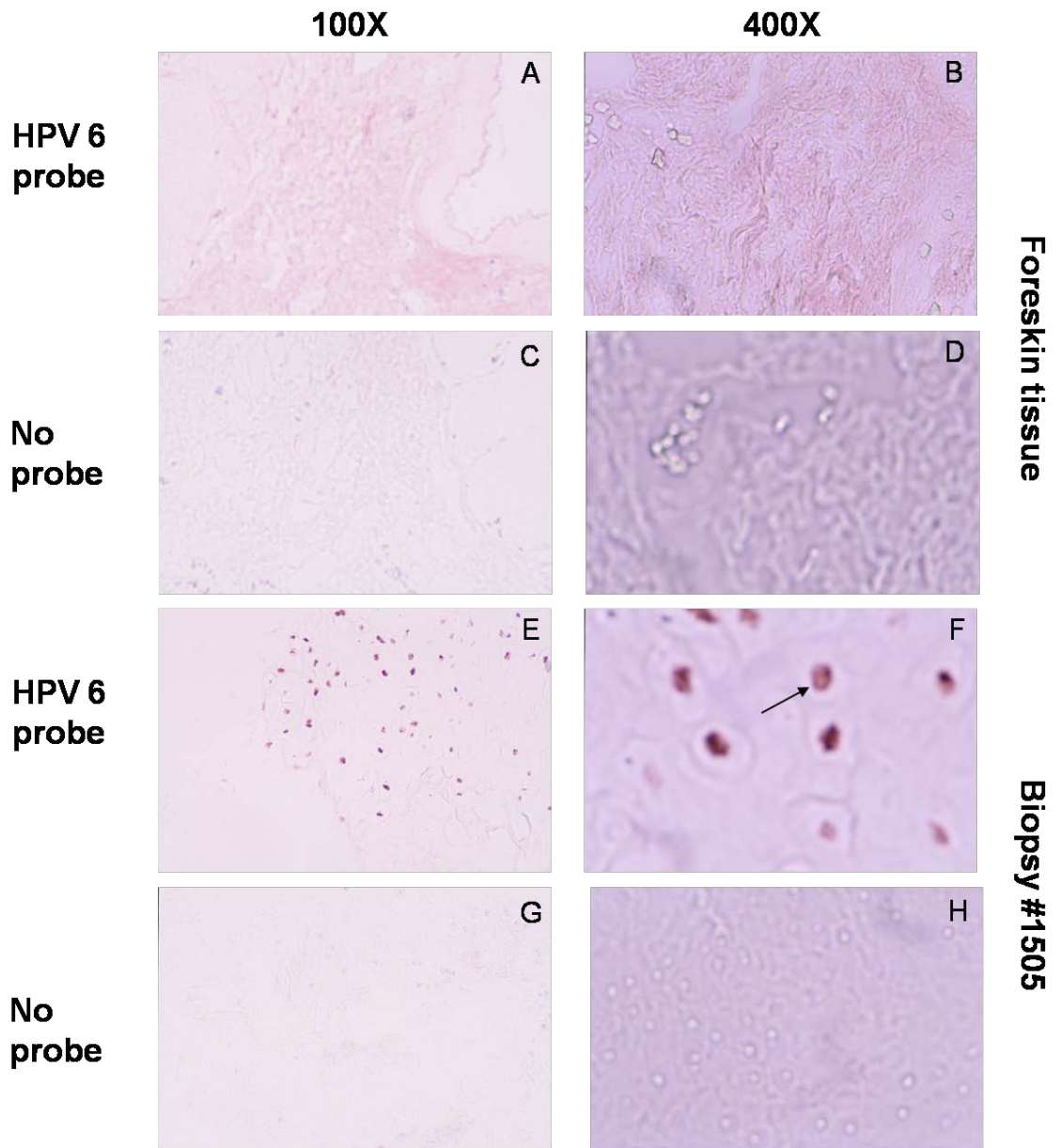
Figure 4. DNA *in situ* analysis of foreskin tissue and HPV 6 positive biopsy.

DNA in situ analysis was performed on uninfected foreskin tissue, using biotin-

labeled HPV 6 DNA probe *(A and B)*. As a negative control no probe was used

(C and D). DNA *in situ* analysis was then conducted on a biopsy of a condyloma

acuminatum from a female patient *(E and F)*, and without probe as a negative

control probe *(G and H)*. Arrow in panel F indicates an HPV 6 positive cell.

Original magnifications: 100x and 400x.

Foreskin tissue

100x   400x

H&E

Anti-p130

Anti-PCNA

Anti-k14

No 1°
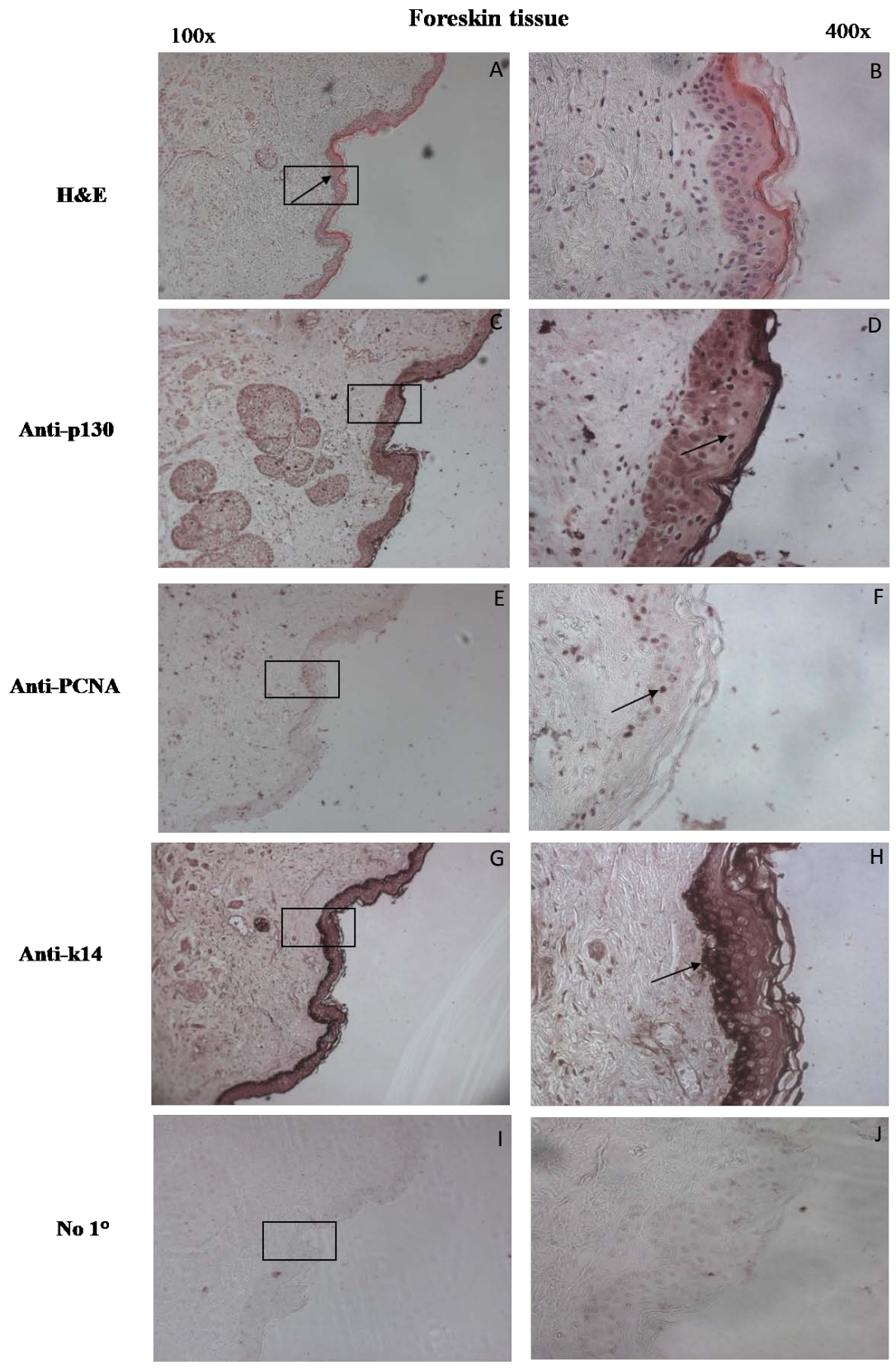
Figure 5. Immunohistochemical analysis of control foreskin tissue to determine p130 expression. Histology of the control foreskin tissue that is not infected with HPV is indicated by hematoxylin and eosin-stained sections *(A and B)*. The 100x magnification is shown in the left panel; the boxed region shows what is magnified at 400x and shown in the right panels. Immunohistochemical analysis was performed to detect p130, a pRb family member *(C and D)*; the arrow in panel D indicates the nucleus of a cell that stained positive for p130. PCNA staining was used as a positive control *(E and F)* and detected in the nuclei, indicated by the arrow in panel F. Keratin 14, a marker for stratified squamous epithelium was also used as a positive control *(G and H)*. The arrow in panel H points to the basement membrane. The bottom panels *(I and J)* are without primary antibody as a negative control.

acuminata (Figures 6, 7 and 8). Hematoxylin and eosin staining allowed for determination of the tissue's architecture. Antibodies to keratin 14 and proliferating cell nuclear antigen (PCNA) were used as positive controls for cytoplasmic staining and nuclear staining, respectively. Keratin 14 is specifically expressed in keratinocytes and forms keratin intermediate filaments. PCNA is a protein that is expressed in the nuclei of cells undergoing DNA synthesis. Keratin staining was detected throughout the stratified squamous epithelium in foreskin tissue and patient biopsies. PCNA was detected mainly in the nuclei of the basal cells and a few suprabasal cells in foreskin tissue. In infected tissue there was positive staining in the nuclei of cells in the basal layer and more staining in the suprabasal layer than foreskin tissue. This was expected since more cells are in S-phase. As a negative control, primary antibodies were omitted and no staining was detected in either patient biopsies or foreskin tissue. p130 was detected in the nuclei of basal and suprabasal cells in foreskin tissue. In contrast to foreskin tissue, there was complete absence of p130 staining in the patient biopsies assayed. This supports the retroviral data published by Zhang et al., that HPV 6 E7 destabilizes p130 (Zhang et al., 2006).
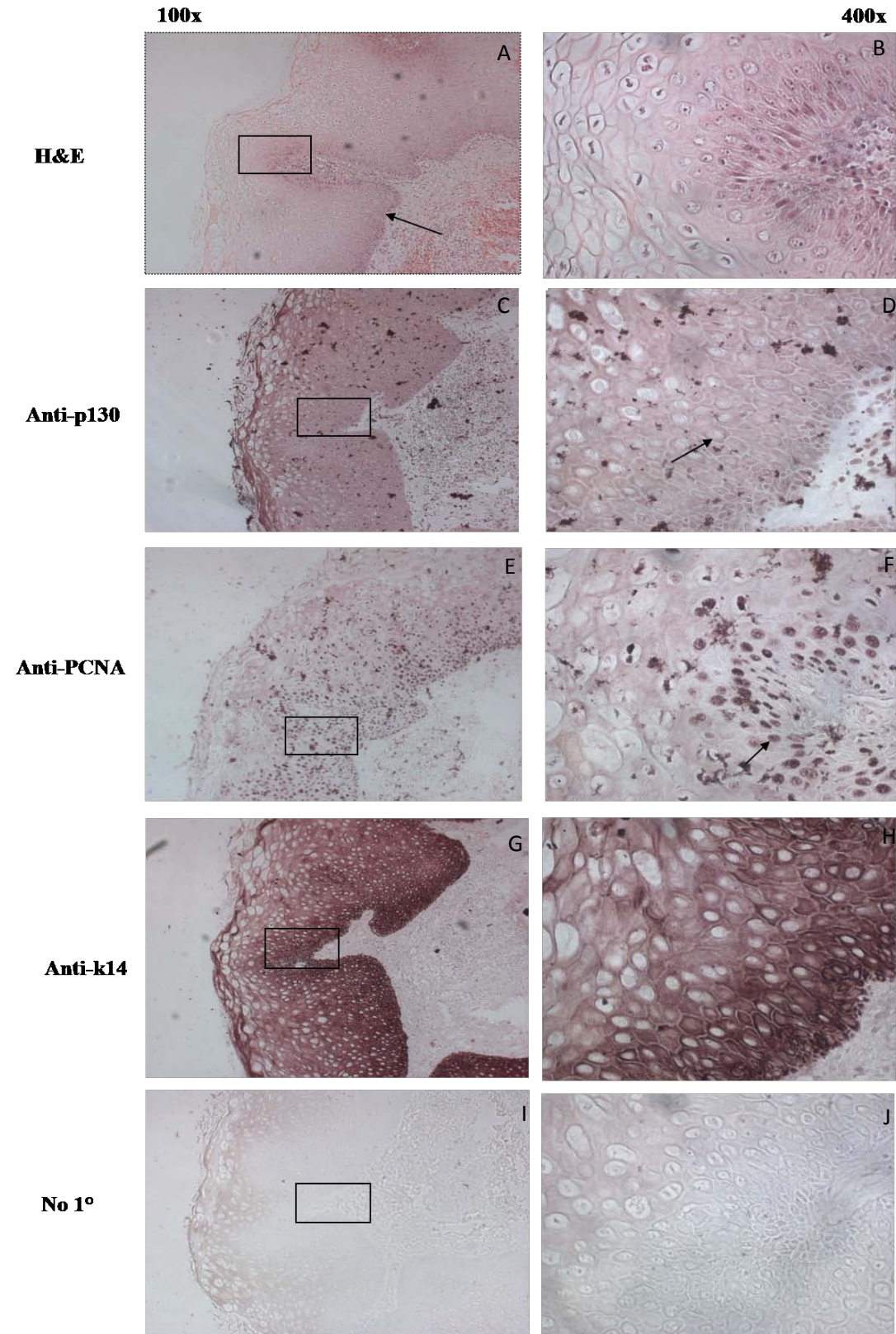
Figure 6. Immunohistochemistry to detect p130 expression levels in HPV 6 positive biopsy. Immunohistochemistry was performed on patient biopsy #1505 (a condyloma acuminatum from a female patient). Histology is indicated by hematoxylin and eosin-stained sections *(A and B)*. The 100x magnification is shown in the left panels and the 400x magnification in the right panels. Immunohistochemical analysis was performed to detect p130, a pRb family member *(C and D)*. The arrow in panel D points to a nucleus that is negative for p130 staining. PCNA and keratin 14 were used as positive controls as in Figure 5 *(E, F, G and H)*. The bottom panels *(I and J)* are without primary antibody as a negative control.

Biopsy #1184
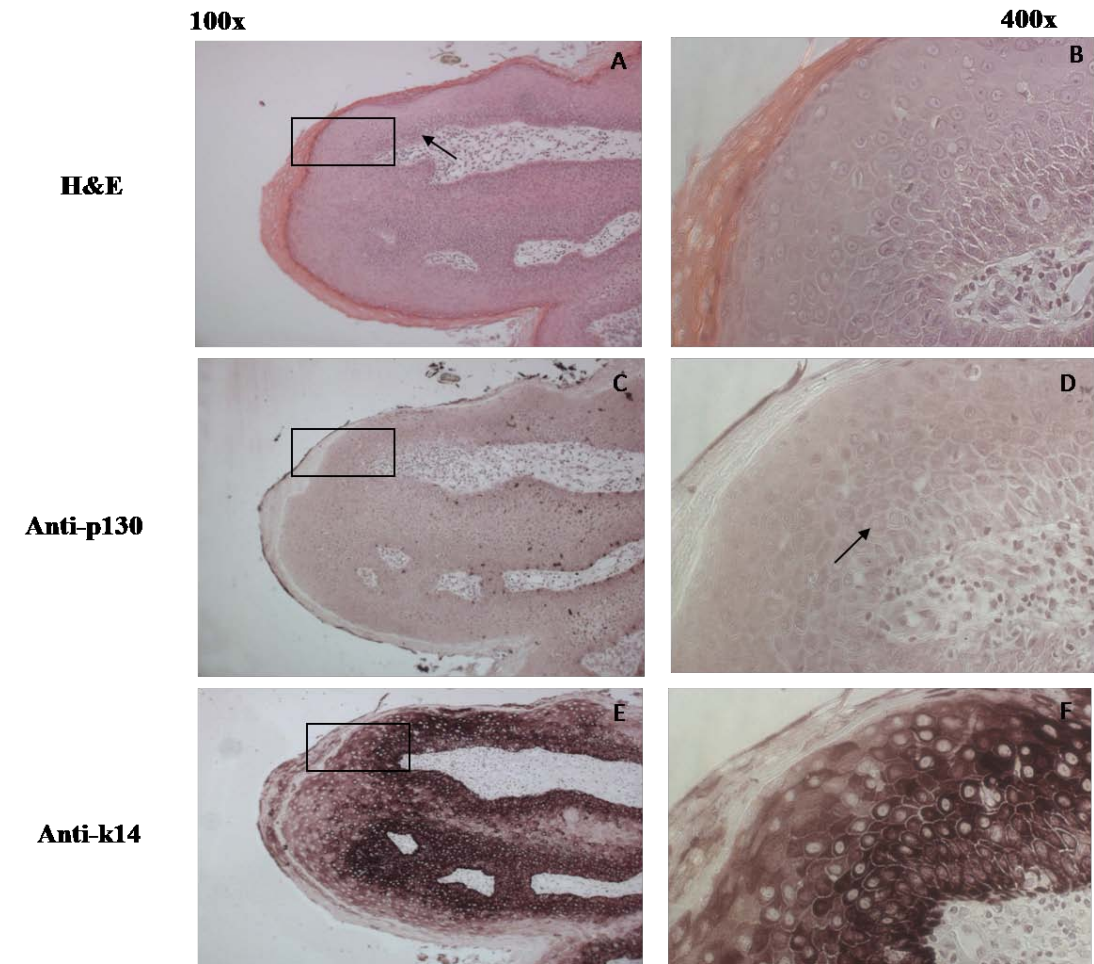
Figure 7. Immunohistochemical analysis of HPV 6 positive biopsy # 1184 to detect p130 expression. Immunohistochemistry was performed on patient biopsy #1184 (a condyloma acuminatum from a male patient). Histology is indicated by hematoxylin and eosin-stained sections *(A and B)*. The 100x magnification is shown in the left panels and the 400x magnification in the right panels. No staining of p130 was detected *(C and D)*. The arrow in panel D points to a nucleus with an absence of p130 staining. Keratin 14 was used as positive control as in Figure 6 *(E and F)*.

Biopsy #1390

100x   400x

H&E

Anti-p130

Anti-PCNA
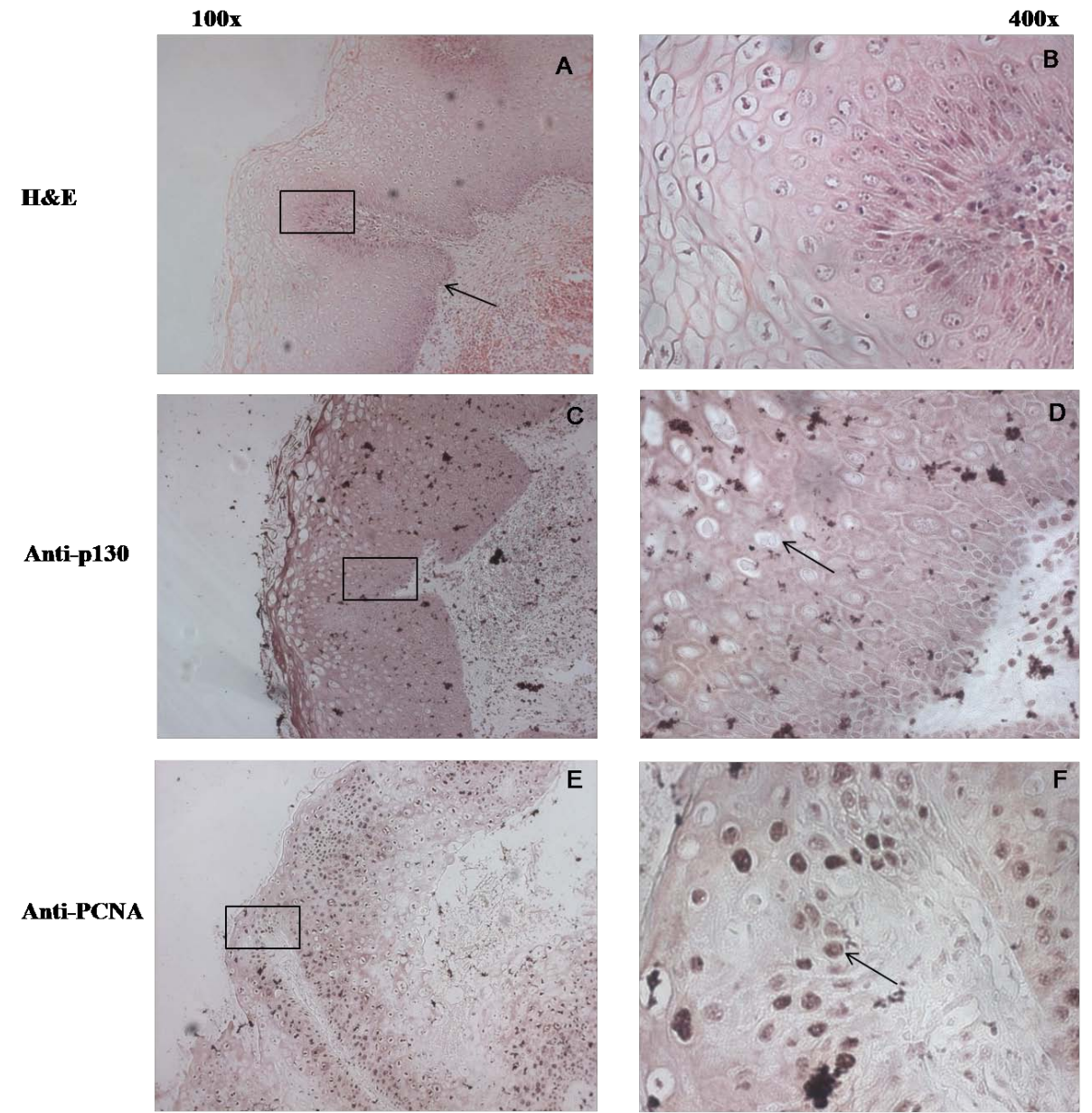
Figure 8. Immunohistochemical analysis of HPV 6 positive biopsy #

1390. Immunohistochemistry was performed on patient biopsy #1390 (a

condyloma acuminatum from a female patient).Hematoxylin and eosin-

staining is shown in the top panels sections *(A and B)*, the arrow points to the

basement membrane. This HPV 6 positive biopsy was also negative for p130 staining *(C and D)*. The arrow in panel *D* points to a nucleus with an absence of p130 staining. PCNA was used as a positive control *(E and F)*, the arrow points to a nucleus that stained positive for PCNA.

Chapter 2

p130 phosphorylated forms 1 and 2 are present in human foreskin keratinocytes (HFKs). It has been reported that p130 has over 22 serine/threonine phosphorylation sites, 3 of which are present in pRb and 10 in p107 (Hansen et al., 2001). p130 is phosphorylated by Cdk 4/6 at serine 672 and phosphorylation at this site targets p130 for degradation (Figure 9). The migration of p130 is affected by its phosphorylation: there are 3 migratory forms of p130, form 1, 2 and 3, with the assumption that form 3 is present when p130 is fully phosphorylated (Tedesco et al., 2002). Therefore, we were interested in investigating the phosphorylation status of p130 in HFKs retrovirally transduced with LXSN, or retrovirus encoding HPV 6E7 [L(6E7)SN] or HPV 16 E7 [L(16E7)SN]. HFKs were grown in C-K-SFM in undifferentiated conditions or induced to differentiated with 2 mM $CaCl_2$ for 48 h. Proteins were harvested with EBC buffer and 100 µg of cell lysate treated with 100 U of λ-phosphatase (a serine/threonine phosphatase). Treatment with λ-phosphatase decreased p130 phosphorylation, resulting in unphosphorylated p130 and p130 form 1 that migrated more quickly than cell lysates that were left untreated. The results of cells grown under undifferentiated (Figure 10) and differentiated conditions (data not shown) were similar. A sample prepared from cycling T98G human glioblastoma cells (obtained from Dr. Jim DeCaprio, Harvard University) was used as a control lysate. T98G cells are known to express p130 form
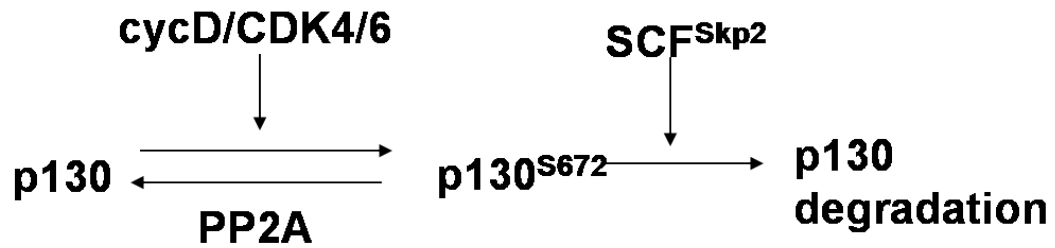
Figure 9. **Schematic figure of p130 regulation in normal cycling cells.** p130

is phosphorylated by cycD/Cdk 4/6 complex at serine 672 and targeted for

degradation by an SCF-Skp2 complex. Protein phosphatase 2A (PP2A) has

been reported to dephosphorylate p130 at the serine 672 resulting in its

stabilization (Garriga et al., 2004; Tedesco et al., 2002).
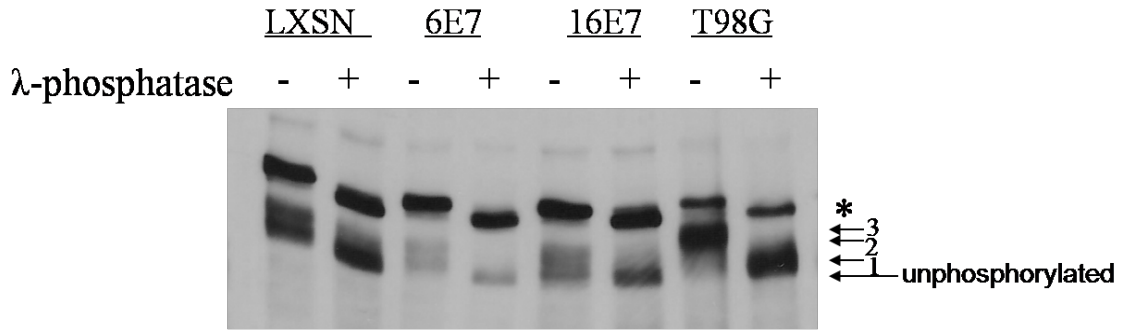
Figure 10. **Phosphorylated forms of p130 in HFKs**. HFKs were grown in C-K-SFM and infected with LXSN, L(6E7)SN or L(16E7)SN and selected for 3 days with G418. HFKs were harvested at 80% confluence in EBC buffer. One hundred µg of cell lysate (HFKs and T98G glioblastoma cells) were left untreated or treated with 100 U of λ-phosphatase for 30 min. Antibody to p130 was used and Western analysis performed to detect phosphorylated and unphosphorylated form of p130. ∗, unknown protein cross-reacting with anti-p130.

3 (Hansen et al., 2001). Only p130 phosphorylated forms 1 and 2 were detected in untreated retrovirally transduced HFKs (Figure 10).

Expression of p130 in HPV E7 expressing cells treated with cylin/Cdk inhibitors. p130 is unique in that it is the only pRb family member that is targeted for degradation by both HPV 6 E7 and HPV 16 E7 (Zhang et al., 2006). In uninfected cells, p130 phosphorylation by Cdk4/6 results in p130 being targeted for degradation by an SCF^{Skp2} complex in a cell-cycle dependant manner (Tedesco et al., 2002). To ascertain whether E7-mediated degradation of p130 is dependent on cyclin/Cdk activity, HFKs transduced with LXSN, L(6E7)SN or L(16E7)SN were treated with 12.5 µM 3-ATA (an inhibitor of Cdk4/6) (Kubo et al., 1999), 12.5 µM roscovitine (an inhibitor of Cdk1, 2 and 5) (Meijer et al., 1997) (Figure 11A) or 50 µM flavopiridol (an inhibitor of Cdk 1, 2, 4, 6, 7 and 9) (Chao et al., 2000; Dai and Grant, 2003; Sedlacek, 2001) (Figure 11B). Whole cell extracts (WCEs) were harvested using EBC and Western analysis performed with 40 µg of protein. Analysis of the phosphorylation status of pRb validated that the inhibitors were functional. Hypophosphorylated and hyperphosphorylated forms of pRb were present in cells expressing LXSN and treated with vehicle only. However, in the presence of each of the Cdk inhibitors, only the hypophosphorylated form of pRb was detected (Figure 11). This result was expected as pRb is known to be phosphorylated by cyclin D2-Cdk4, cyclin E-Cdk2, and cyclin A-Cdk2 (Cobrinik, 2005). The hyperphosphorylated band of was lost only upon treatment with flavopiridol (Figure 11B), but not 3-ATA or roscovitine (Figure 11A). The steady-state level of p130 in HPV expressing cells

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in the presence of each Cdk inhibitor was compared to LXSN expressing cells. In the presence of Cdk inhibitors, both HPV 6 E7 and HPV 16 E7 retained the ability to decrease the steady-state level of p130 (Figure 11).

p130 is located mainly in the cytoplasm in HFK transduced with HPV 16 E7. p130 is known to shuttle between the nucleus and cytoplasm (Chestukhin et al., 2002). HPV E7 has been shown to alter the localization of various proteins. Both HPV 6 and 16 E7 relocalize steroid receptor coactivator 1 (SRC1) to the cytoplasm (Baldwin et al., 2006), and HPV 16 E7 reduces the nuclear localization of p21^{Cip1} (Westbrook et al., 2002). Therefore, the effect of LR and HR HPV E7 on the intracellular localization of p130 was investigated to test the hypothesis that E7 could alter p130 localization (Figure 12). Subcellular fractionation was performed on HFKs transduced with LXSN (control), HPV 6 E7 or HPV 16 E7. Equal-volume fractions of cytoplasmic and nuclear extracts were obtained and Western analysis performed using monoclonal antibodies to p130, lamin B (marker for nuclear fraction) and tubulin (marker for cytoplasmic fraction). Endogenous p130 was detected at similar levels in the cytoplasmic and nuclear fraction in cells expressing LXSN and HPV 6 E7. In contrast, HFKs expressing HPV 16 E7 had significantly more p130 (three fold) in the cytoplasm (Figure 13).
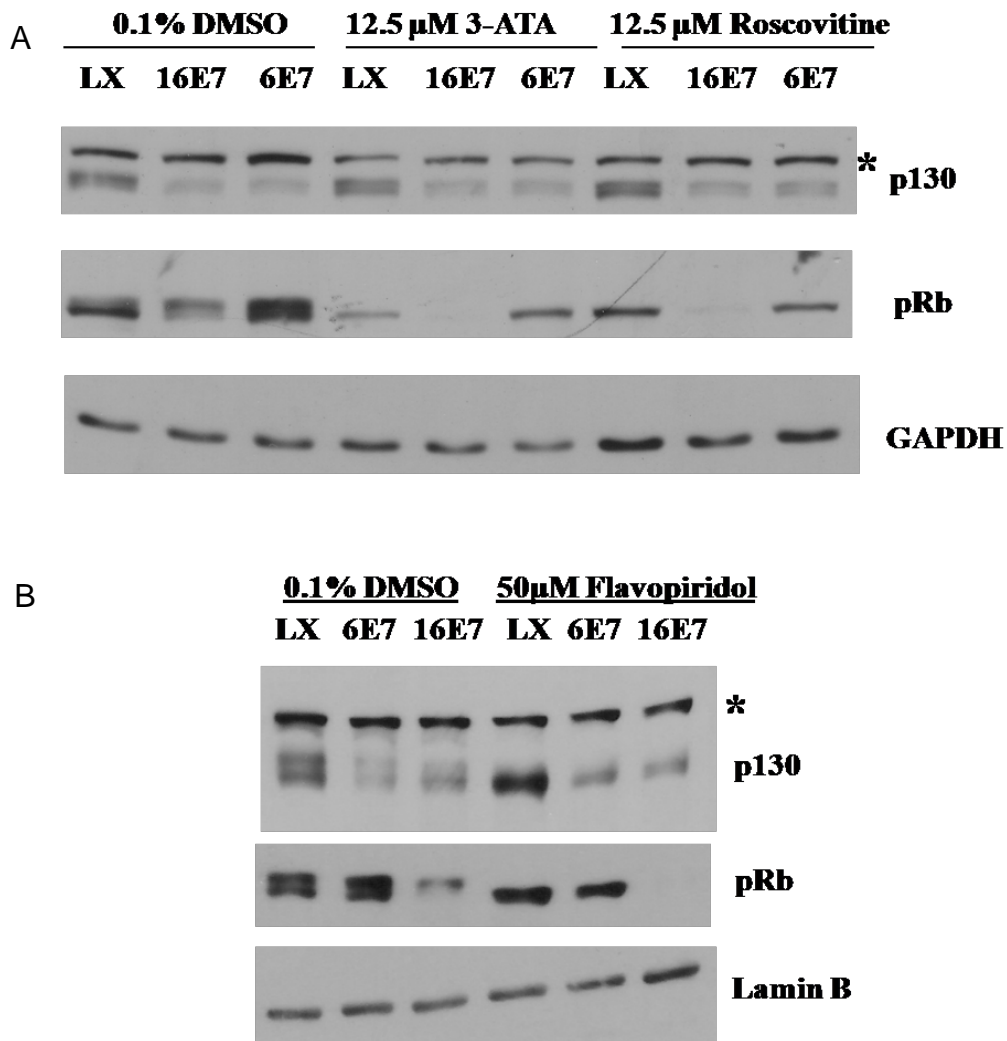
Figure 11. Effect of Cdk inhibitors on p130 expression. *(A)* HFKs were

infected as in Figure 10 and grown in C-K-SFM. Cells were then treated with 12.5

µM 3-ATA, 12.5 µM roscovitine or DMSO (vehicle). Experiment conducted by

Wei Chen. *(B)* Retrovirally transduced HFKs were treated with 50 µM flavopiridol

or DMSO (vehicle) as a negative control. Whole cell extracts (WCEs) were

harvested using EBC buffer and Western analysis performed using mouse

Figure 11 (con't). monoclonal antibodies to p130, pRb, GAPDH and lamin B. ∗,

unknown protein cross-reacting with anti-p130 (*A* and *B*).
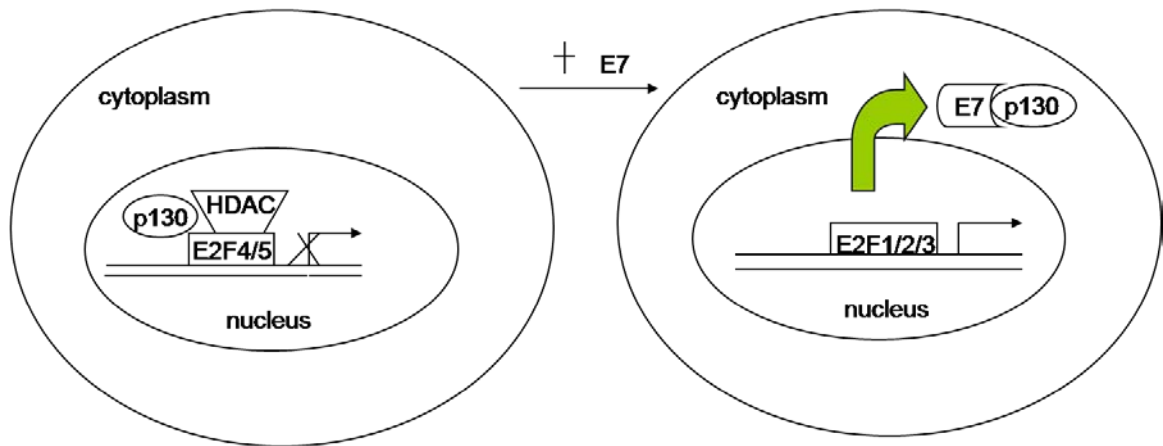
Figure 12. **Schematic of the possible effect of E7 on p130 intracellular localization**. HPV E7 may either be able to retain p130 in the cytoplasm or localize p130 from the cytoplasmic to the nucleus and thereby, allow for expression of E2F1/2/3-responsive genes which would drive the cell into S-phase.

Figure 13. Determination of p130 localization in nuclear and cytoplasmic fractions in undifferentiated cells. *(A)* HFKs were infected as in Figure 10 and grown in C-K-SFM. Cells were harvested using EBC buffer for whole cell

Figure 13 (con't). extracts [WCEs (left three lanes)], or cytoplasmic and nuclear buffers to harvest cytoplasmic fractions (CF) and nuclear fractions (NF). Equal-volume fractions of nuclear and cytoplasmic protein were analyzed using monoclonal antibodies to p130. Antibodies to E2F5 were used to determine where this protein localized in the presence of control (LXSN), 6E7 or 16E7 expressing cells. Antibodies to lamin B (nuclear marker) and GAPDH (cytoplasmic marker) were used to establish that the fractions were enriched. A representative experiment is shown. ∗, unknown protein cross-reacting with anti-p130. *(B)* Quantity one software was used for densitometric analysis. The nuclear fractions were then set to 1 and the ratio of cytoplasmic to nuclear p130 localization is shown. The average ± standard deviation of 4 independent experiments is shown, (++), $p < 0.01$. A student two-tailed T-test was performed to acquire statistical significance.

E2F5 was localized to the nuclear fraction in all cases. In differentiated cells there was more p130 (nearly 5 fold) in the cytoplasm in HPV 16 E7 expressing HFKs, whereas in LXSN and HPV 6 E7 expressing cells the ratio of cytoplasmic to nuclear p130 was similar (Figure 14). This result is similar to the results in undifferentiated cells.

The distribution of p130 in HFKs transduced with LXSN, L(6 E7)SN or L(16 E7)SN was also determined by immunofluorescence. Localization of p130 was mostly nuclear in HFKs transduced with LXSN and HPV 6 E7. p130 staining was mostly cytoplasmic in HFKs transduced with HPV 16 E7 (Figure 15). This difference in the intracellular localization of p130 in the presence of HPV 16 E7 compared to that seen in LXSN or HPV 6 E7 expressing cells is consistent with subcellular fractionation results.

To test whether HPV 16 E7 relocalizes p130 to the cytoplasm via Crm1-dependent nuclear export we took advantage of a specific inhibitor of Crm1, leptomycin B (LMB). LMB inhibits nuclear export of the human immunodeficiency virus Rev protein (Wolff et al., 1997). We verified that the concentration of LMB (10 ng/ml) used in our experiments was sufficient to inhibit Crm1-mediated nuclear export in HFKs by transfecting the cells with a Rev-GFP plasmid using FuGENE 6 transfection reagent. We also transfected Rev-GFP into COS-7 cells, since the concentration of LMB used was shown to be adequate at inhibiting
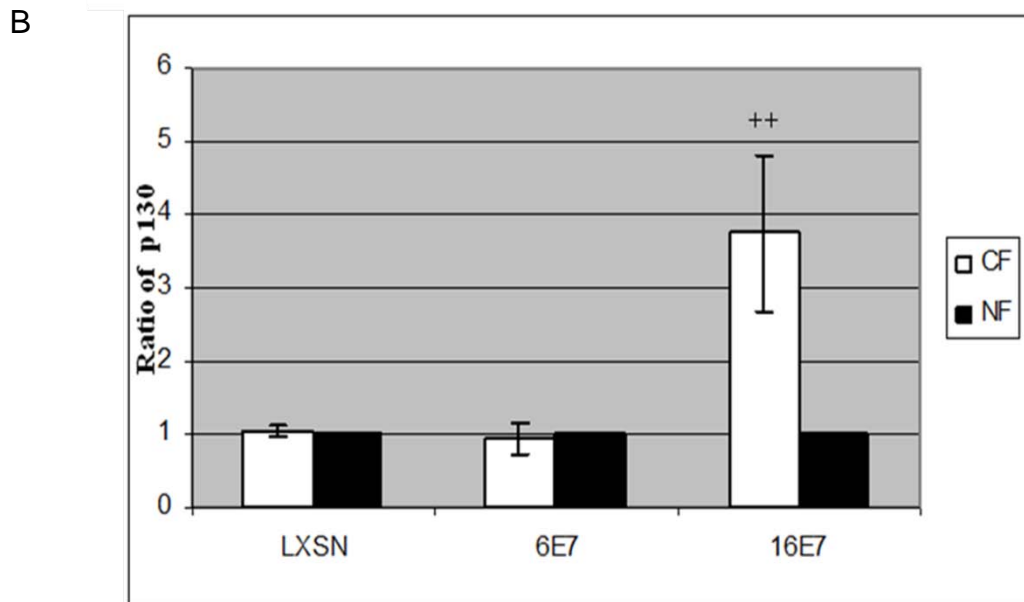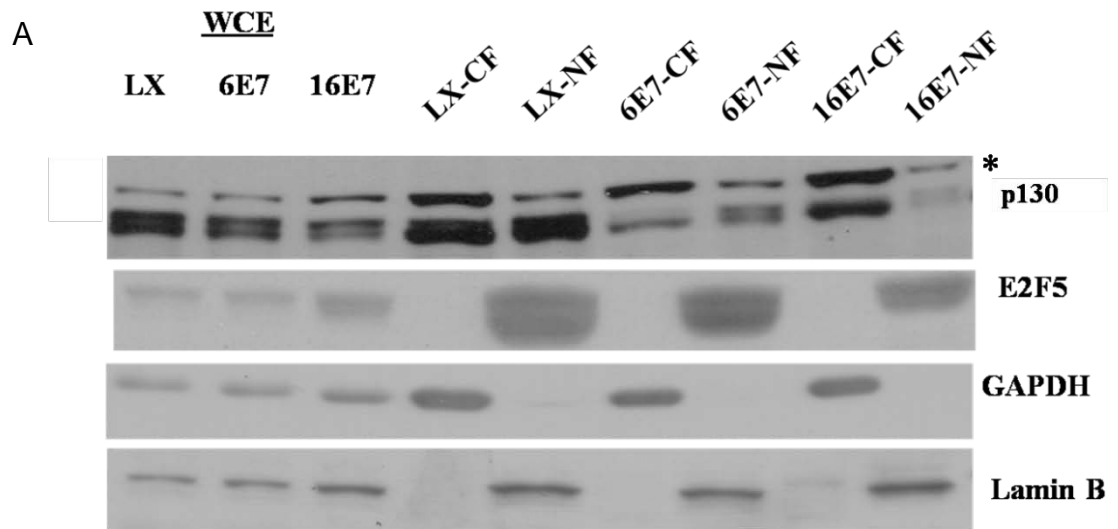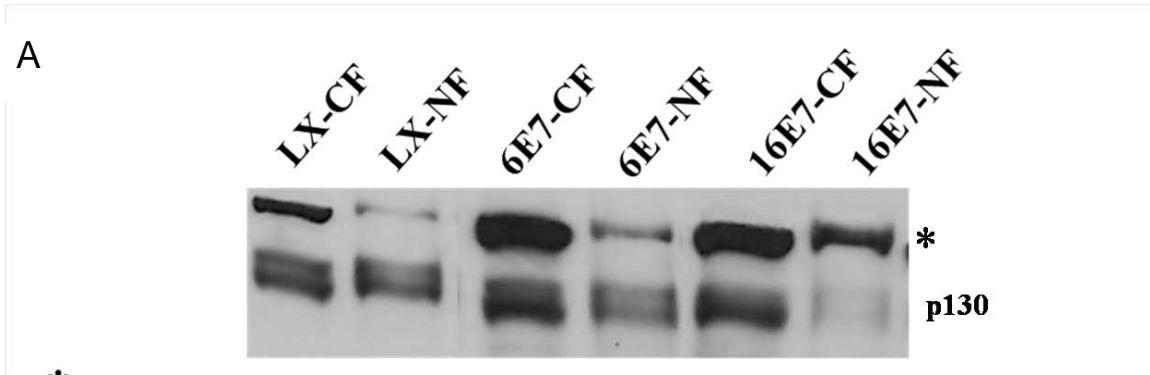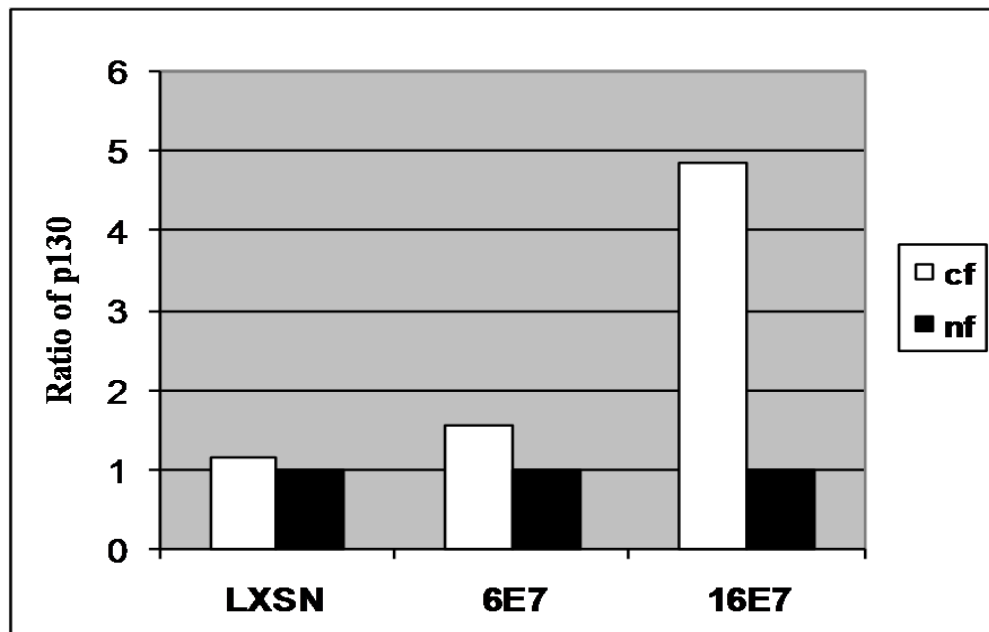
Figure 14. Determination of p130 localization in nuclear and cytoplasmic fractions in differentiated cells. *(A)* HFKs were infected as in Figure 10, differentiation was induced by the addition of 2 mM CaCl$_2$ for 48 h. Cytoplasmic fractions (CF) and nuclear fractions (NF) were obtained as in Figure 13. Equal-volume fractions of nuclear and cytoplasmic protein were analyzed using monoclonal antibodies to p130. ∗, unknown protein cross

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Figure 14 (con't). reacting with anti-p130. Antibodies to lamin B (nuclear

marker) and GAPDH (cytoplasmic marker) were used to establish that the

fractions were enriched (data not shown). A representative experiment of 2

performed is shown. *(B)* Quantity one software was used for densitometric

analysis. The nuclear fractions were then set to 1 and the ratio of cytoplasmic

to nuclear p130 localization is shown.
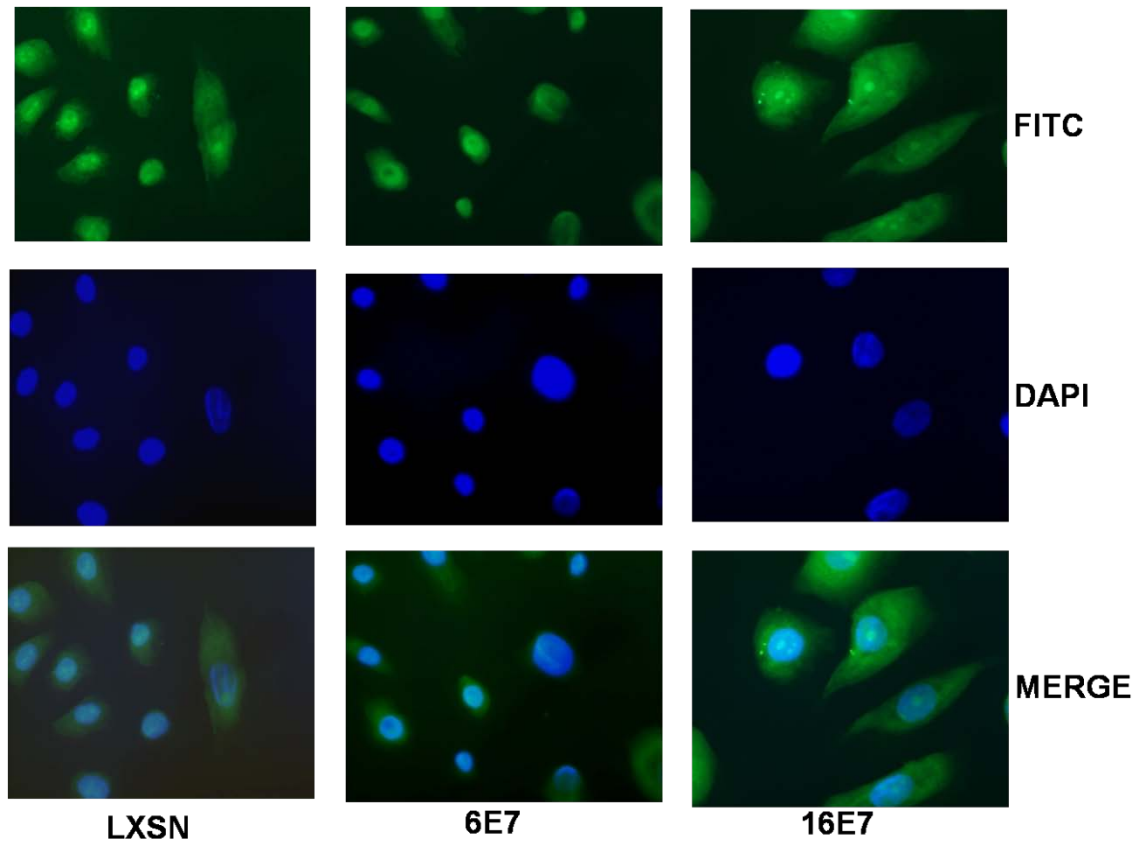
Figure 15. Determination of p130 localization by immunofluorescence.

Immunofluorescence studies were performed on HFKs infected as in Figure 10

and grown on coverslips in C-K-SFM. Cells were incubated with rabbit polyclonal

antibody to p130 and visualized by immunofluorescence using FITC-conjugated

secondary antibody. A representative experiment is shown. Magnification: 100x.

nuclear export in this cell type. Treatment of COS-7 cells (Figure 16) and HFKs

(Figure 17) with LMB resulted in the accumulation of Rev-GFP fusion protein in

the nucleoli of cells, as detected by immunofluorescence. Western analysis of

equal quantity of protein from cytoplasmic and nuclear subcellular fractions

obtained from COS-7 cells transfected with Rev-GFP, also confirmed that the

concentration of LMB was sufficient (Figure 18). However, LMB treatment did not

affect cytoplasmic localization of p130 in HPV 16 E7 expressing cells as shown

by subcellular fractionation (Figure 19). There was a 3 fold increase of p130 in

the cytoplasm compared to nuclear p130 in HFKs expressing 16 E7 whether

treated with LMB or left untreated. In contrast, the ratio of p130 in the cytoplasm

and nucleus of p130 in the LXSN control was similar.

To further address the effect of E7 on p130 localization, three putative

nuclear export sequences were identified in p130 using an NES predictor

(NESpredictor www.cbs.dtu.dk/) and deletion mutants of HA-p130 wild type (WT)

HA-p130 plasmid obtained from Dr. Jim DeCaprio) were generated using a

Quikchange mutagenesis kit (Figure 20). Individual deletion mutants and all

combinations of these deletions were successfully generated. HA-p130 NES Δ1,

HA-p130 NES Δ2 and HA-p130 NES Δ3 were characterized by

immunofluorescence. WT HA-tagged p130 and HA-p130-Δ21-5A-m-NLS-1&2

were used as controls. WT HA-tagged p130 was shown to be capable to shuttle

between the cytoplasm and nucleus (Chestukhin et al., 2002) and HA-p130-Δ21-

GFP

DAPI

MERGE

control   + LMB
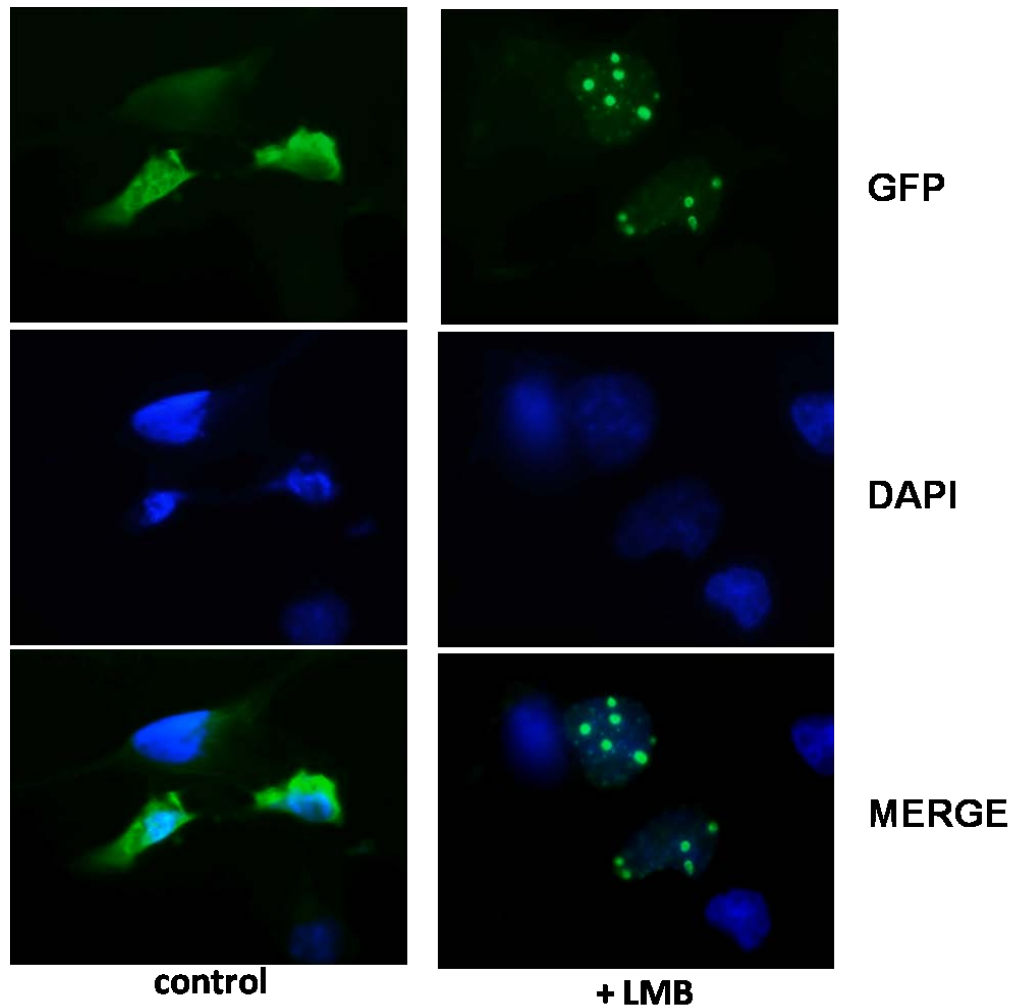
Figure 16. **Effect of LMB on COS-7 cells transfected with a plasmid**

encoding Rev-GFP. Transfected cells were grown on coverslips and treated or

not with LMB (10 ng/ml) for 4 h. Cells were fixed with 4% formaldehyde,

counterstained with 5 µg/ml of 4'-6-Diamidino-2-phenylindole (DAPI) for 7 min at

RT to label the nuclei, washed and mounted. Autofluorescence of Rev-GFP was

detected by fluorescence microscopy (above). Magnification: 100x.
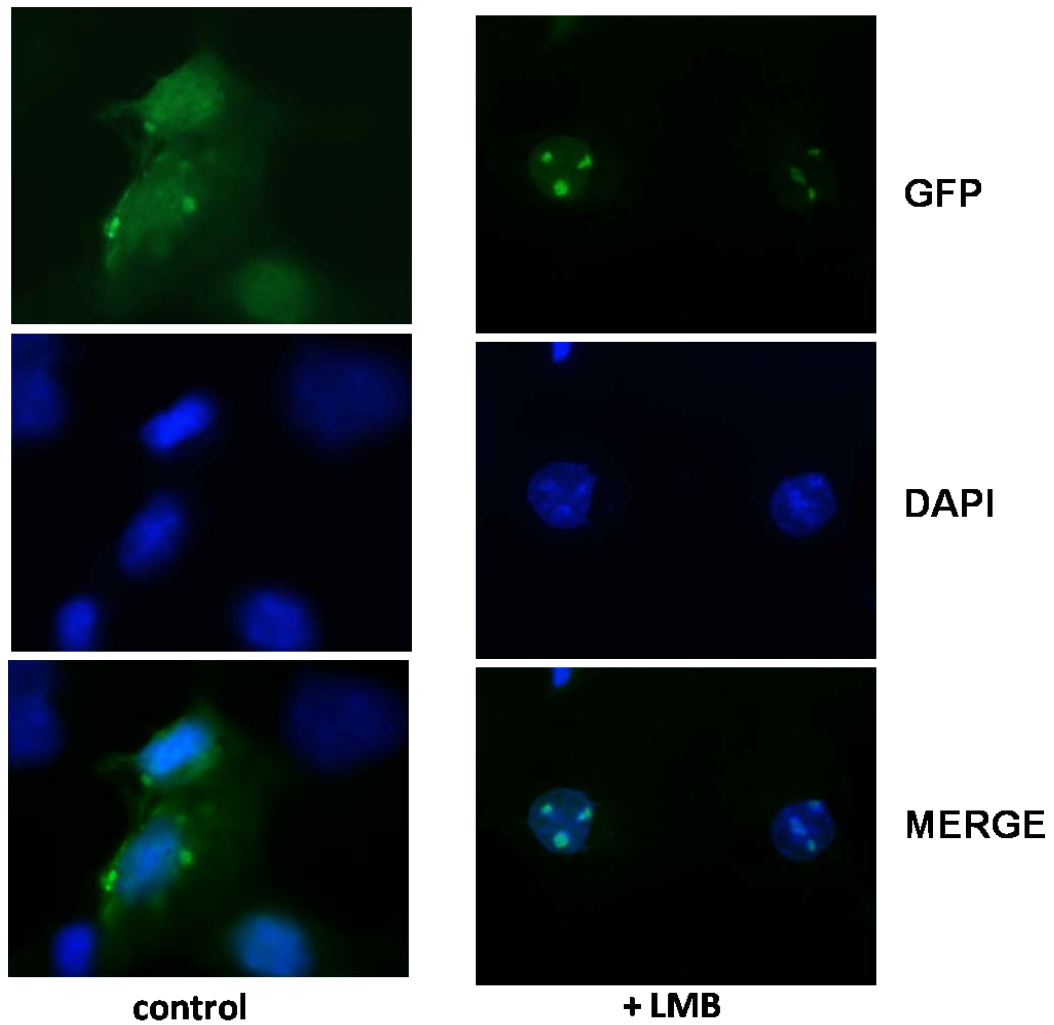
Figure 17. **Effect of LMB on the cytoplasmic localization of HFKs transfected with a plasmid encoding Rev-GFP.** Cells were treated and imaged as in Figure 16. Magnification: 100x.
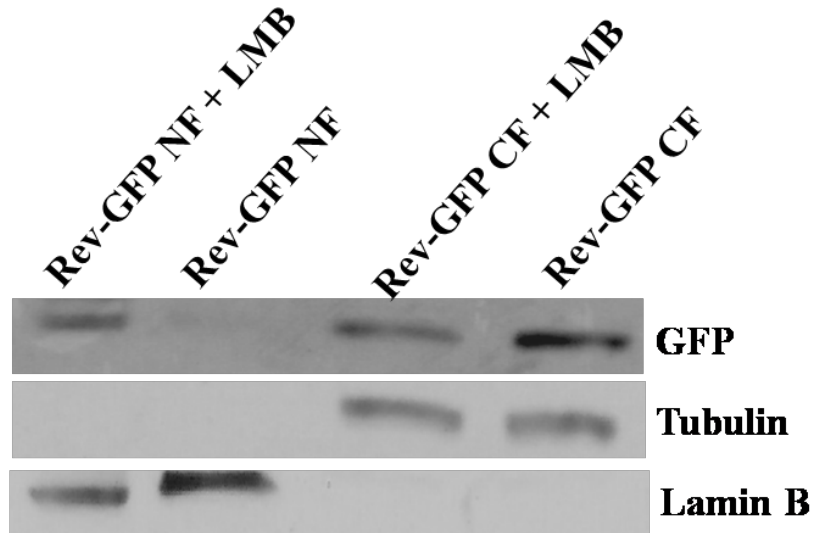
Figure 18. **Western Blot Analysis showing effect of LMB on Rev-GFP.** WCEs

were harvested with EBC buffer from COS-7 cells transfected with Rev-GFP.

Equal quantity of cell lysate was used and Western analysis performed using

antibodies to GFP, tubulin and lamin B.
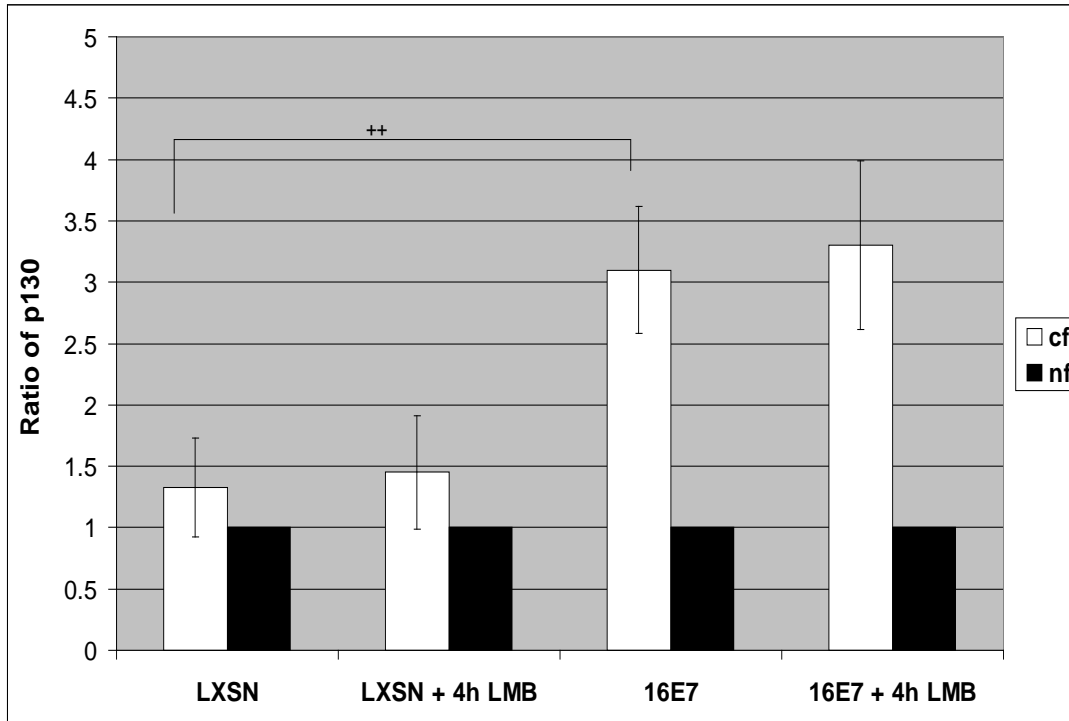
Figure 19. Effect of LMB on the cytoplasmic localization of p130 in HPV 16 E7 expressing cells. HFKs were infected as in Figure 10 and grown in C-K-SFM to 80% confluence. Cells were then treated with LMB (10 ng/ml) for 4 h and harvested for cytoplasmic and nuclear extracts. Equal-volume fractions of nuclear and cytoplasmic extracts were analyzed using antibodies to p130. Antibodies against lamin B (nuclear marker) and tubulin (cytoplasmic marker) were used as in Figure 13. Densitometric analysis was performed using Quantity one software and the result normalized as in Figure 13. The averages and standard deviation from 4 experiments are shown, (++), $p < 0.01$. Student's two-tailed T-test was performed to acquire statistical significance.
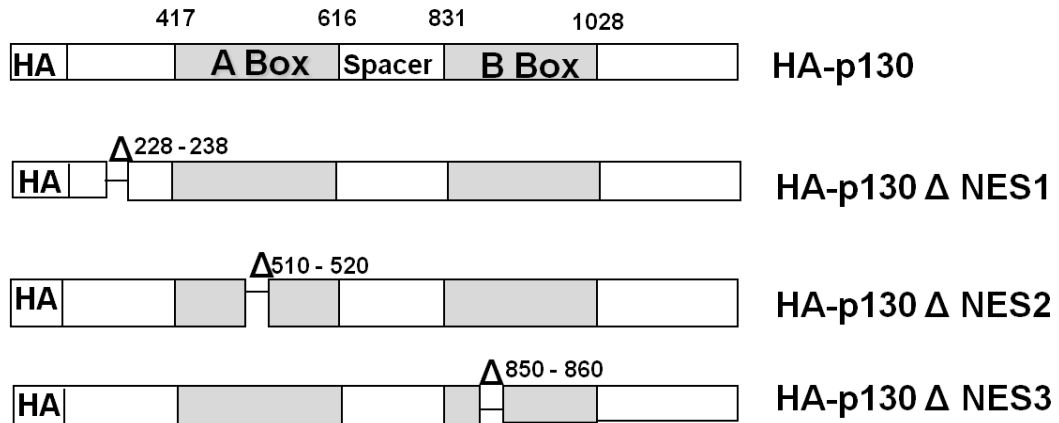
Figure 20. Schematic figure of HA tagged-p130 ΔNES 1, 2 and 3 constructs.

Deletion mutations were made in putative nuclear export sequences. Deletions
are denoted by Δ and the positions of the amino acids deleted are indicated.
Figure adapted from Canhoto et al., 2000.

5A-m-NLS-1&2, generated by DeCaprio's laboratory, was shown to an exclusively cytoplasmic mutant (Chestukhin et al., 2002). Fluorescence microscopy was performed using these two plasmids; the cytoplasmic mutant was specifically localized to the cytoplasm as previously reported. It was expected that WT HA-p130 would localize to the cytoplasm and nucleus, as did endogenous p130 (Figure 15, as in LXSN expressing cells). Surprisingly, HA-p130 was detected only in the nuclei of transfected HFKs (Figure 21). Following transfection, the new HA-p130 NES mutants 1, 2 and 3 were also found to be exclusively nuclear but it could not be verified whether these mutations were successful at preventing cytoplasmic localization since the WT plasmid did not behave as expected (Figure 22). We also obtained CMV HA-p130 from DeCaprio's laboratory and retrovirally transduced HFKs with this construct and selected with puromycin. Again, the tagged protein was only detected in the nucleus by flurorescence microscopy (data not shown).

The half-life of p130 in the nucleus is shorter in HFKs transduced with HPV 6 E7. In WCEs, the half-life of p130 in the presence of HPV 6 E7 and HPV 16 E7 is decreased relative to control cells (Zhang et al., 2006). The reduced half-life of p130 in the presence of HPV E7 is restored following treatment with proteasomal inhibitors, implying that E7 targets p130 for degradation through the proteasome pathway (Zhang et al., 2006). To determine the half-life of p130 in the cytoplasmic and nuclear fractions, HPV E7 expressing cells were treated with 0.25 mM cycloheximide (CHX), a protein synthesis
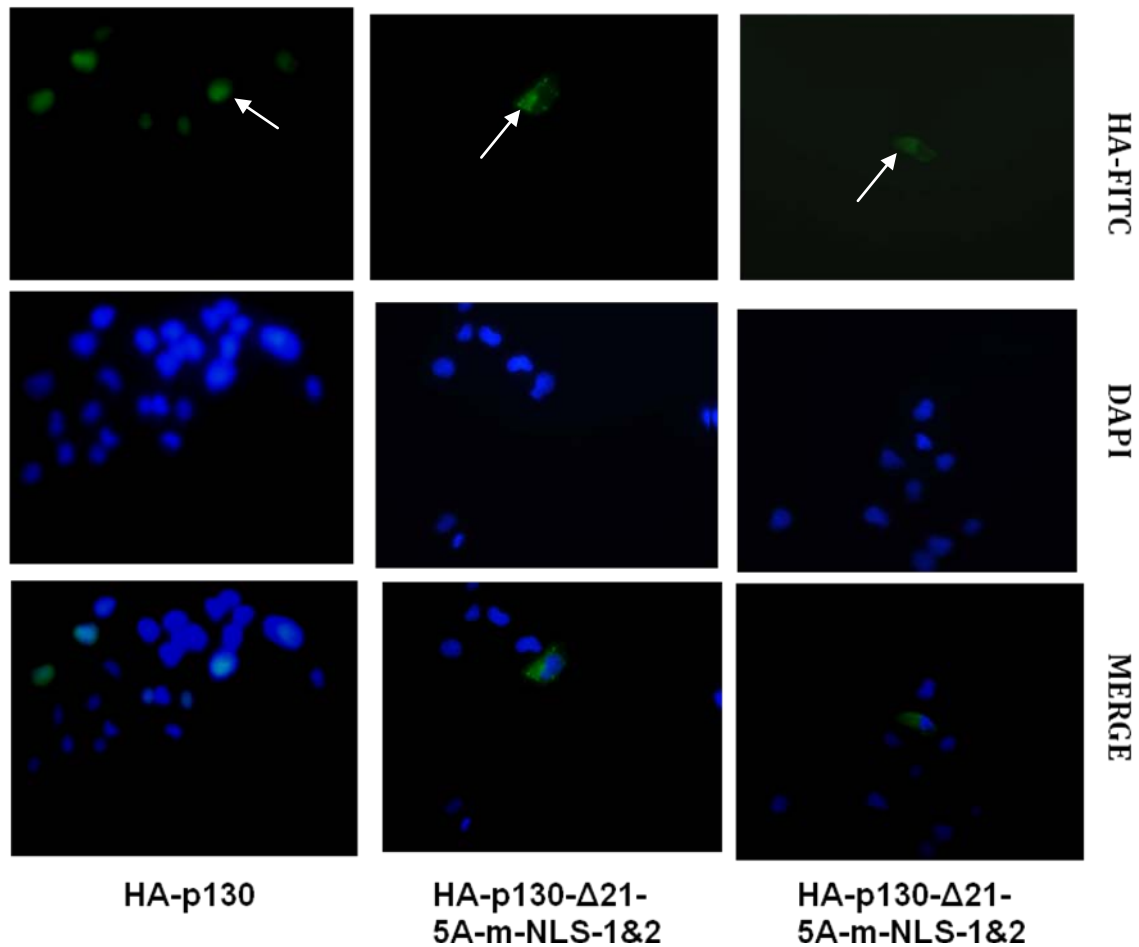
Figure 21. Localization of HA-p130 and HA-p130-Δ21-5A-m-NLS-1&2. HFKs

were transfected with HA-p130 and HA-p130 mNLS 1&2 Δ21 cytoplasmic mutant

(obtained from Dr. Jim DeCaprio). Cells were fixed with 4% formaldehyde and

HA-FITC antibody was used to detect the localization of the plasmids. Cells were

counterstained with 5 µg/ml of DAPI for 7 min at RT to label the nuclei, washed

and mounted. Fluorescence microscopy was used to image cells. Arrows

indicate cells that are expressing the plasmids. Magnification: 40x.
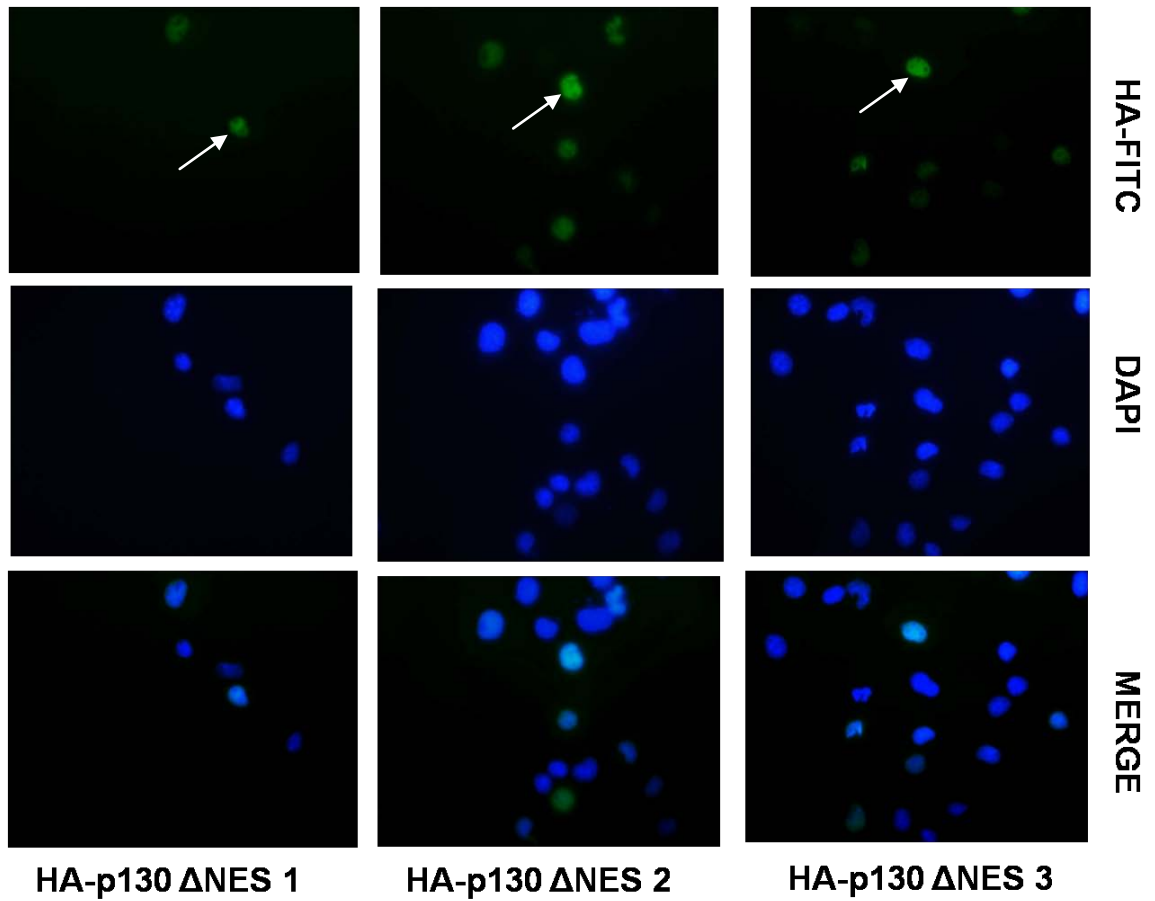
Figure 22. Detection of localization of HA-p130 ΔNES 1, 2 and 3 mutants.
HFKs were transfected with the HA-p130 NES mutants using FuGENE 6 and
cells were processed and visualized as in Figure 21. Arrows show cells that have
been successfully transfected and are expressing HA-p130 ΔNES 1, 2 or 3.
Magnification: 40x.

inhibitor. At 0, 2, 4, and 6 h post-treatment, p130 levels in cytoplasmic and nuclear fractions were determined by Western blot analysis. The half-life of p130 in the cytoplasmic fraction of HPV 6 E7 and 16 E7 expressing cells was ~2 h, significantly shorter than the > 6h half life in control cells (Figure 23). In a minority of cases, the half-life of p130 in the cytoplasm was shorter in HPV 16 E7 cells than HPV 6 E7 cells. The half-life of nuclear p130 was between 3 and 4 h in HPV 6 E7 expressing cell, less than half as long as in control and HPV 16 E7 expressing cells (Figure 24). To establish whether the decrease in p130 half-life by HPV E7 in the nuclear and cytoplasmic fractions was due to degradation through the proteasome pathway, HFKs were infected as above and treated with 0.25 mM CHX plus 50 µM MG132, a proteasome pathway inhibitor. After treatment with MG132 the ability of either E7 to shorten the half-life of p130 in the cytoplasmic and nuclear fraction was decreased (Figures 25 and 26). This suggests that although both HPV 6 E7 and HPV 16 E7 efficiently target p130 for proteasomal degradation in the cytoplasm, HPV 6 E7 is also very efficient at targeting p130 for proteasomal degradation in the nucleus.
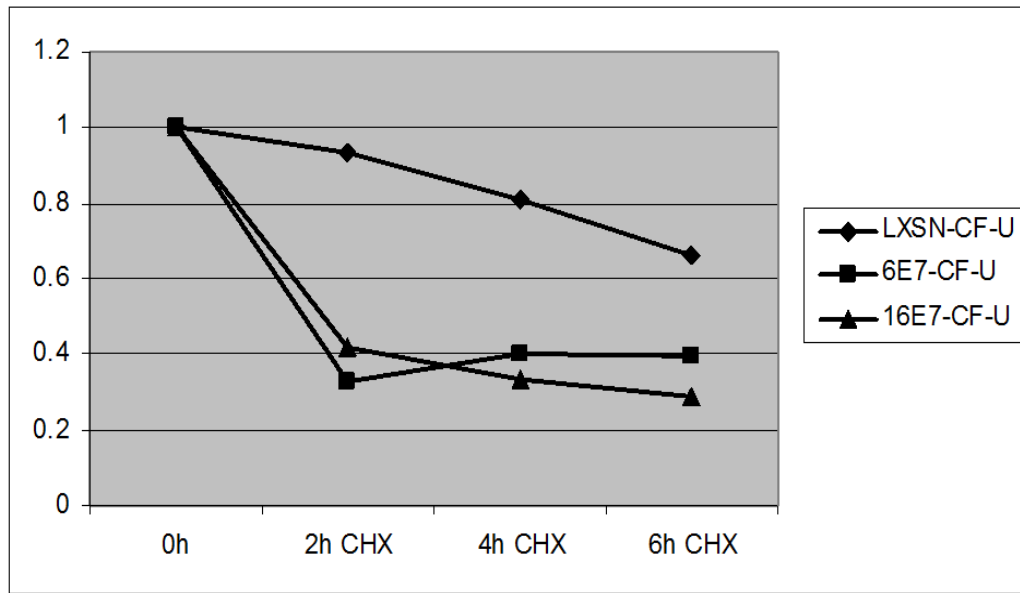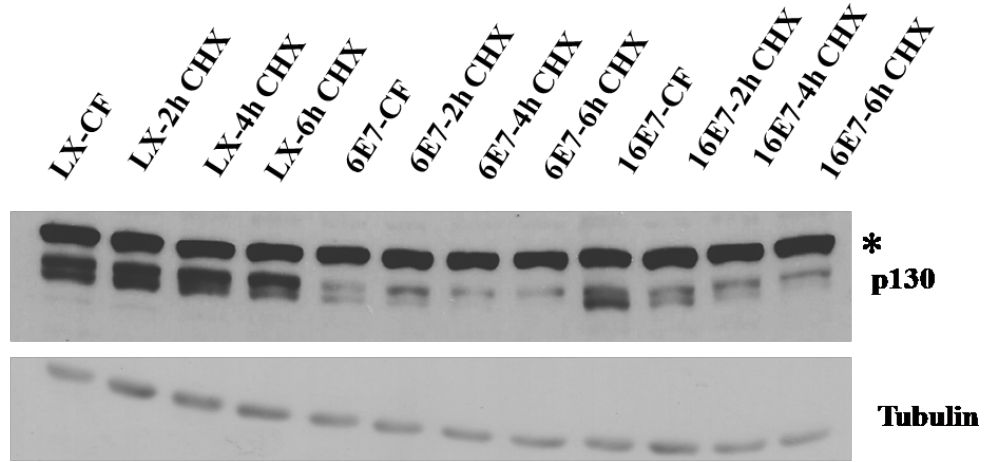
Figure 23. **Half-life of p130 in the cytoplasm in the presence of HPV 6 E7 and HPV 16 E7.** HFKs were infected as in Figure 10 and treated with 0.25 mM cycloheximide (CHX) for the indicated times. Immunoblots of cytoplasmic fractions (CF) (top) and densitometry (bottom) from a representative experiment of seven experiments are shown. Cytoplasmic fractions (CF) were corrected for

Figure 23 (con't). tubulin and are relative to untreated LXSN. ∗, unknown protein

cross-reacting with anti-p130.
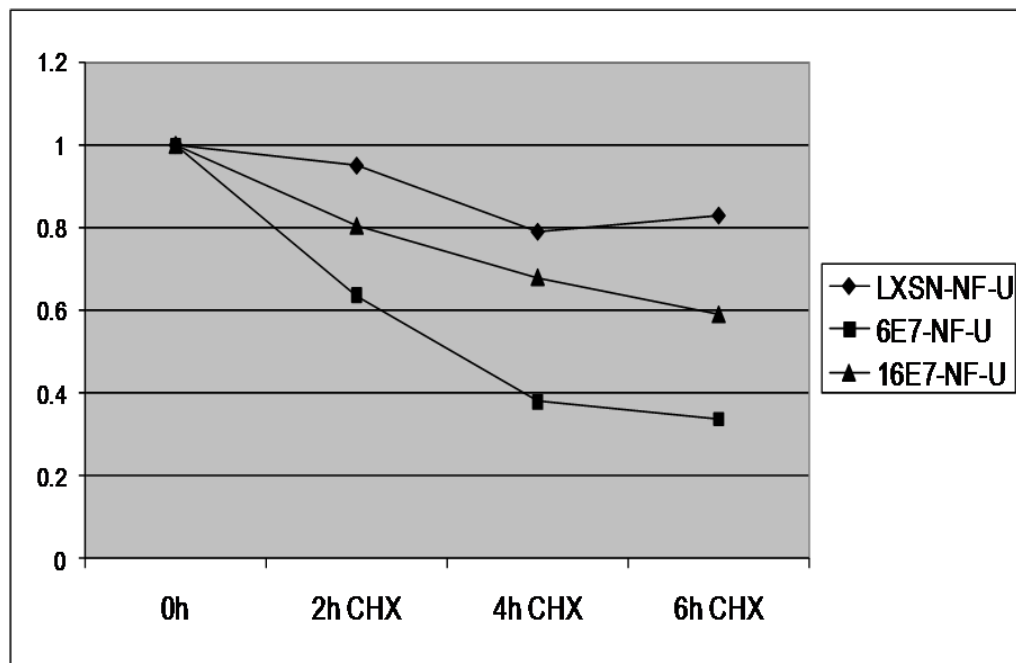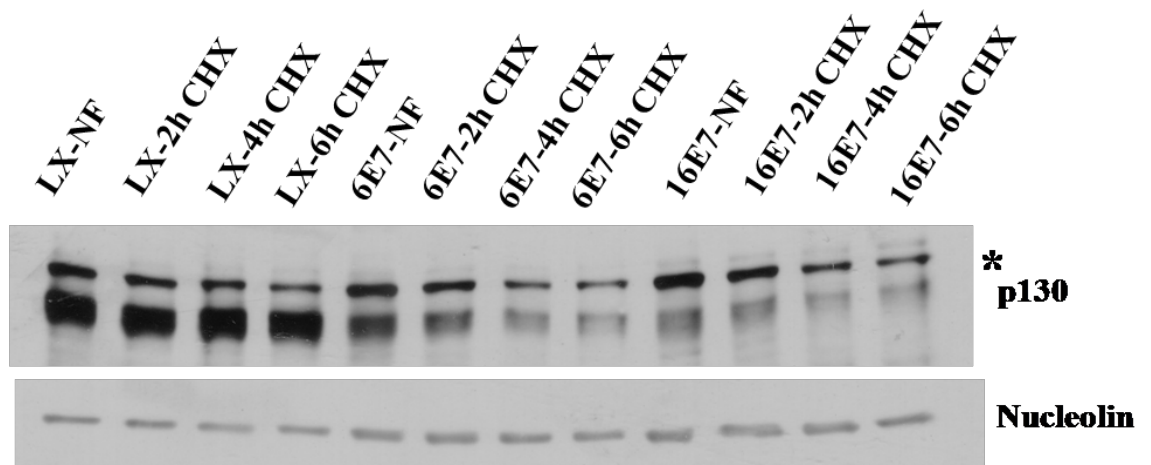
Figure 24. **Half-life of p130 in the nucleus in the presence of HPV 6 E7.**

HFKs were infected as in Figure 10 and treated with CHX for 2h, 4h or 6h or left

untreated. Immunoblots of nuclear fractions (CF) (top) and densitometry (bottom)

from a representative experiment are shown. Seven experiments were

performed. Nuclear fractions were corrected for nucleolin and are relative to

Figure 24 (con't). untreated LXSN. ∗, unknown protein cross-reacting with anti-

p130.
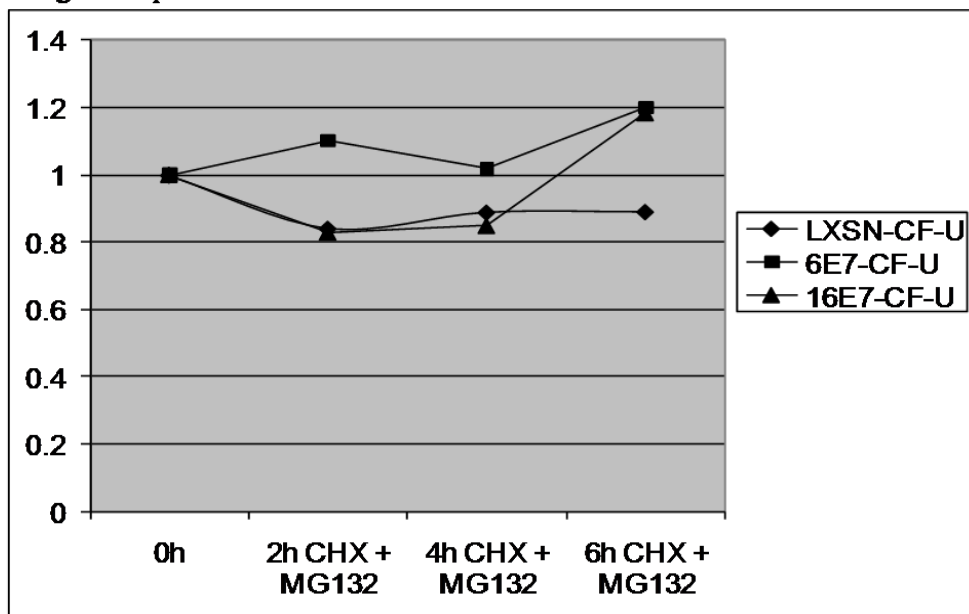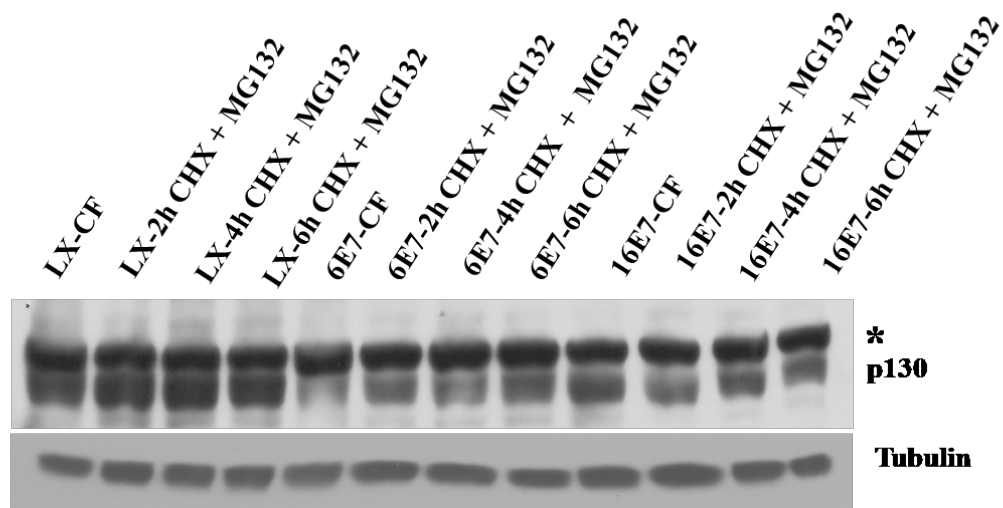
Figure 25. **Effect of MG132 on cytoplasmic p130.** HFKs were infected as in Figure 10 and treated with 0.25 mM CHX plus 50 µM MG132 for the indicated times. Immunoblots of cytoplasmic fractions (CF) (top) and densitometry (bottom) from a representative experiment (of 7) are shown.

Figure 25 (con't).∗, unknown protein cross-reacting with anti-p130. Cytoplasmic

fractions were corrected for tubulin and are relative to untreated LXSN.
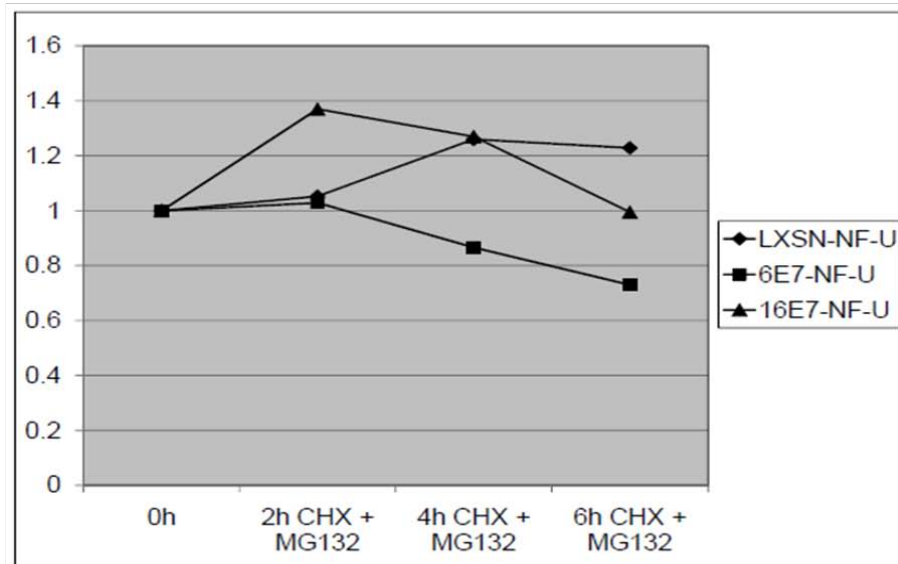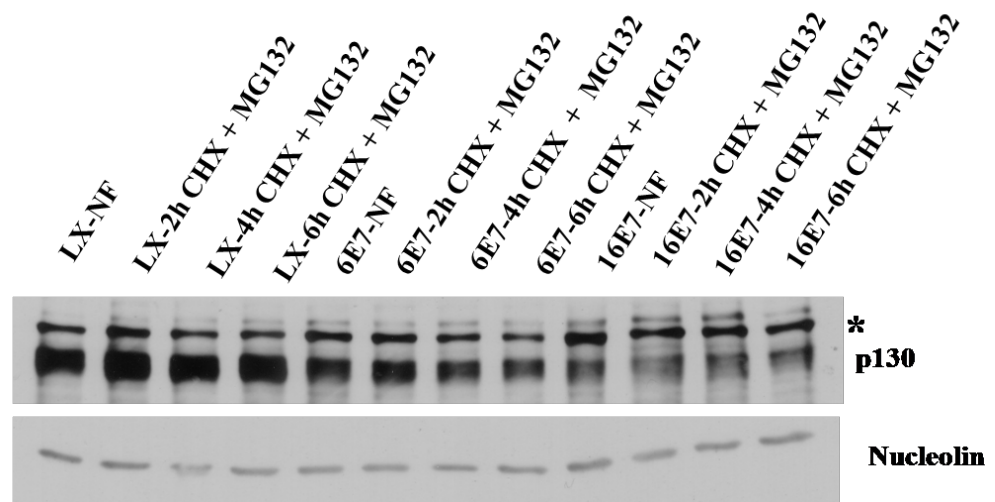
Figure 26. Effect of MG132 on nuclear p130. Experiments were conducted as in Figure 25. Immunoblots of nuclear fractions (NF) (top) and densitometry (bottom) from a representative experiment of 7 are shown. ∗, unknown protein cross-reacting with anti-p130.

Chapter 3

HPV E7 and cullin 5

Adenovirus E1A E4orf6 and E1B55K have been reported to reduce the half-life of p53, a tumor suppressor protein, and target it for degradation via a proteasomal pathway. E4orf6 and E1B55K bind to cullin 5, elongin B, elongin C and Rbx1, a complex with E3 ubiquitin ligase activity (Querido et al., 2001). The ability of E4orf6 and E1B55K to recruit this complex is also responsible for degradation of DNA ligase IV and the Mre11/Rad50/Nbs1 DNA repair complex (Blanchette and Branton, 2009). To determine whether E7 binds to cullin 5, glutathione sepharose transferase (GST) pull-down assays were performed. GST 16 E7 migrated more slowly than GST 6 E7 based on a negative charge due to aspartic acid at residue 4 (Armstrong and Roman, 1993). Binding of GST 6 E7 and 16 E7 to cullin 5 was detected by GST pull-down experiments; GST alone did not bind to cullin 5 (Figure 27).

HPV 6 E7 mutants HPV 6 E7 H2AR4AH5A (mutant in conserved region 1) and HPV 6 E7 L67R (mutant in C-terminal half) are loss of function mutants for p130 degradation although they still bind to p130. HPV 6 E7 C25A (mutant in conserved region 2) neither binds nor degrades p130 (Zhang et al., 2006) (Figure 28). Therefore, to determine whether binding of E7 to cullin 5 correlates with the ability of E7 to target p130 for degradation, E7 mutants were used. Using antibody to cullin 5 from Santa Cruz, both HPV 6 E7 H2AR4AH5A and C25A bound cullin 5 less well than wild type (WT) 6 E7 (Figure 29). Consistent with these findings, antibody to cullin 5 from Bethyl also showed that HPV
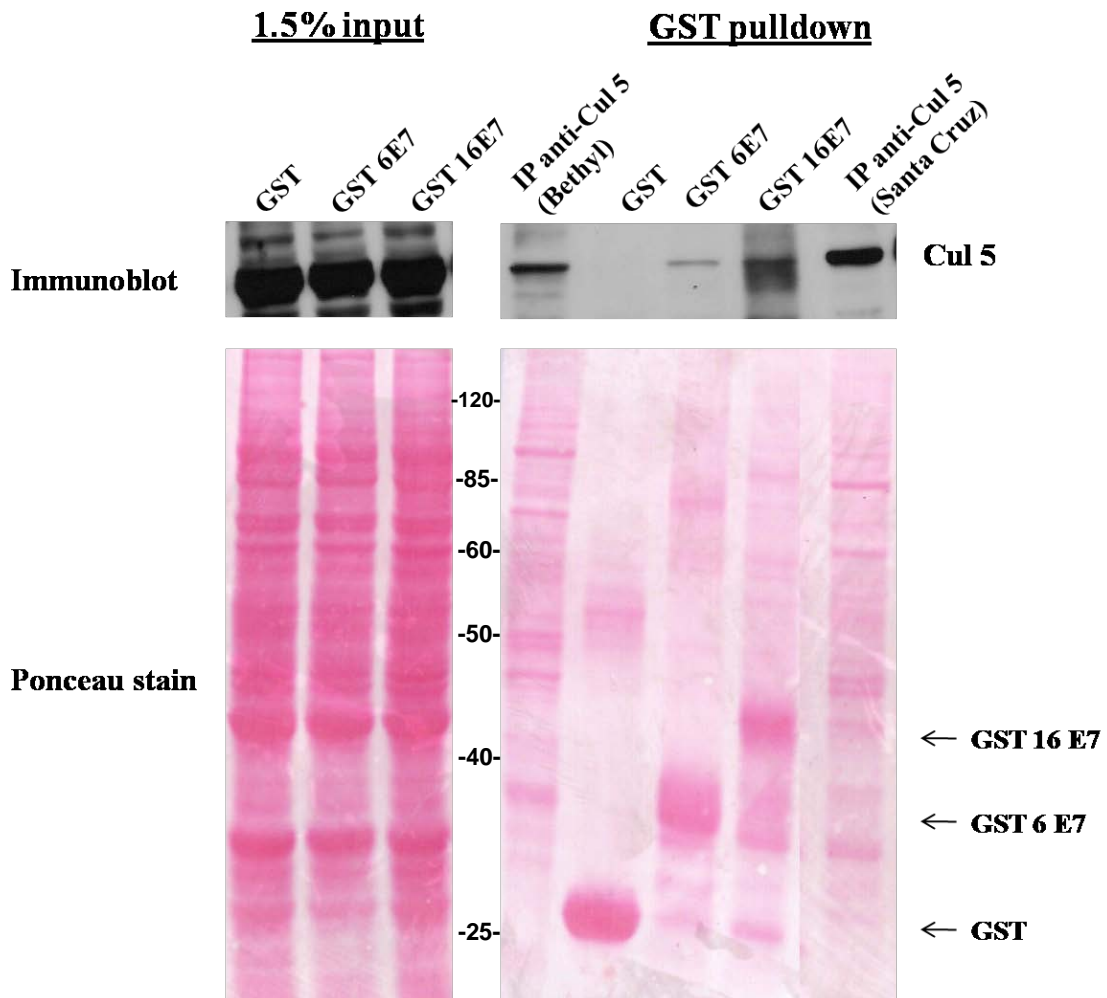
Figure 27. Characterization of HPV 6 and 16 E7 with respect to cullin 5 binding. GST, GST 6 E7, GST 16 E7 were incubated with 1 mg each of pre-cleared HeLa nuclear extract. Bound proteins were detected, after separation by SDS/PAGE and transfer to nitrocellulose membrane, by immunoblotting with antibodies to cullin 5 (Bethyl Laboratories). Ponceau red staining of the membrane before probing indicated that similar levels of GST or GST fusion proteins were present. The 1.5% input showed that the amounts of lysate used to

Figure 27 (con't). conduct each pull down were equal. Immunoprecipitations of cullin 5 were performed using 1 mg of pre-cleared HeLa nuclear extract per reaction and 1:100 dilution of antibody to cullin 5 from Bethyl Laboratories or Santa Cruz, first and last lanes respectively.

H2AR4AH5A   C25A
  A A A   A
  ↑ ↑ ↑   ↑
HPV6 E7 **MHGRH**VTLKD IVLDLQPPDP VGLHCYEQLV DSSEDEVDE. VDGQDSQPLK

                    L67R
                     R
                     ↑
HPV6 E7 QHFQIVTCCC GCDSNVRLVV QCTETDIREV QQLLLGTLNI VCPICAPKT

HPV 6 E7 Mutant	p130 binding	p130 degradation
H2AR4AH5A	Y	N
C25A	N	N
L67R	Y	N

Figure 28. HPV 6 E7 mutants. HPV 6 E7 mutants H2AR4AH5A, C25A and

L67R were generated in the Roman laboratory by site directed mutagenesis and

characterized with respect to p130 binding and degradation (Zhang et al., 2006).

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Figure 29. Characterization of HPV E7 with respect to cullin 5 binding using Santa Cruz antibody. GST, GST 6 E7, GST 16 E7, GST 6 E7 H2AR4AH5A and GST 6 E7 C25A were incubated with 1 mg each of pre-cleared HeLa nuclear extract. Bound proteins were detected after separation by SDS/PAGE and transfer to nitrocellulose membrane. The membrane was probed with antibodies to cullin 5 (Santa Cruz). Ponceau red staining of the membrane before probing indicated that similar levels of GST or GST fusion proteins were present in the precipitate. The input shows that the amounts of lysate used to conduct each pull down were relatively the same.

6 E7 C25A bound less efficiently than HPV 6 E7. However, the result with HPV 6 E7 H2AR4AH5A was inconsistent with the results obtained with the Santa Cruz antibody. In this GST pulldown assay the quantity of GST 6 E7 H2AR4AH5A was less than GST 6 E7, therefore GST 6 E7 H2AR4AH5A may bind to cullin 5 as well as GST 6 E7. Additional experiments will be needed to determine the efficiency of binding of GST 6E7 H2AR4AH5A to cullin 5.

Next, antibody to cullin 5 from Bethyl Laboratories was used to further confirm our results and to take advantage of a cullin 5 blocking peptide (Bethyl Laboratories). To confirm that the band identified as cullin 5 was indeed cullin 5 the blot was stripped and five times the amount of cullin 5 blocking peptide incubated with antibody to cullin 5 for 1 h and then incubated O/N with the blot. This confirmed that the band that was detected with antibody to cullin 5 was truly cullin 5 since no band is seen in the presence of blocking peptide (Figure 30). The implication of these results will be further addressed in the Discussion.

HPV E7 and cyclin C

Cyclin C was identified by the Roman laboratory as an HPV E7-associated cellular protein, by mass spectrometric analysis of proteins isolated by tandem-affinity purification, using epitope tagged HPV E7. Cyclin C/Cdk3 phosphorylates pRb at S807/811 during the G_0/G_1 transition; this is required for cells to effectively exit G_0 (Ren and Rollins, 2004). Interested in cyclin C as a candidate for p130 degradation, the hypothesis was formed that cyclin C/Cdk3 may be capable of phosphorylating p130 and contributing to its destabilization.
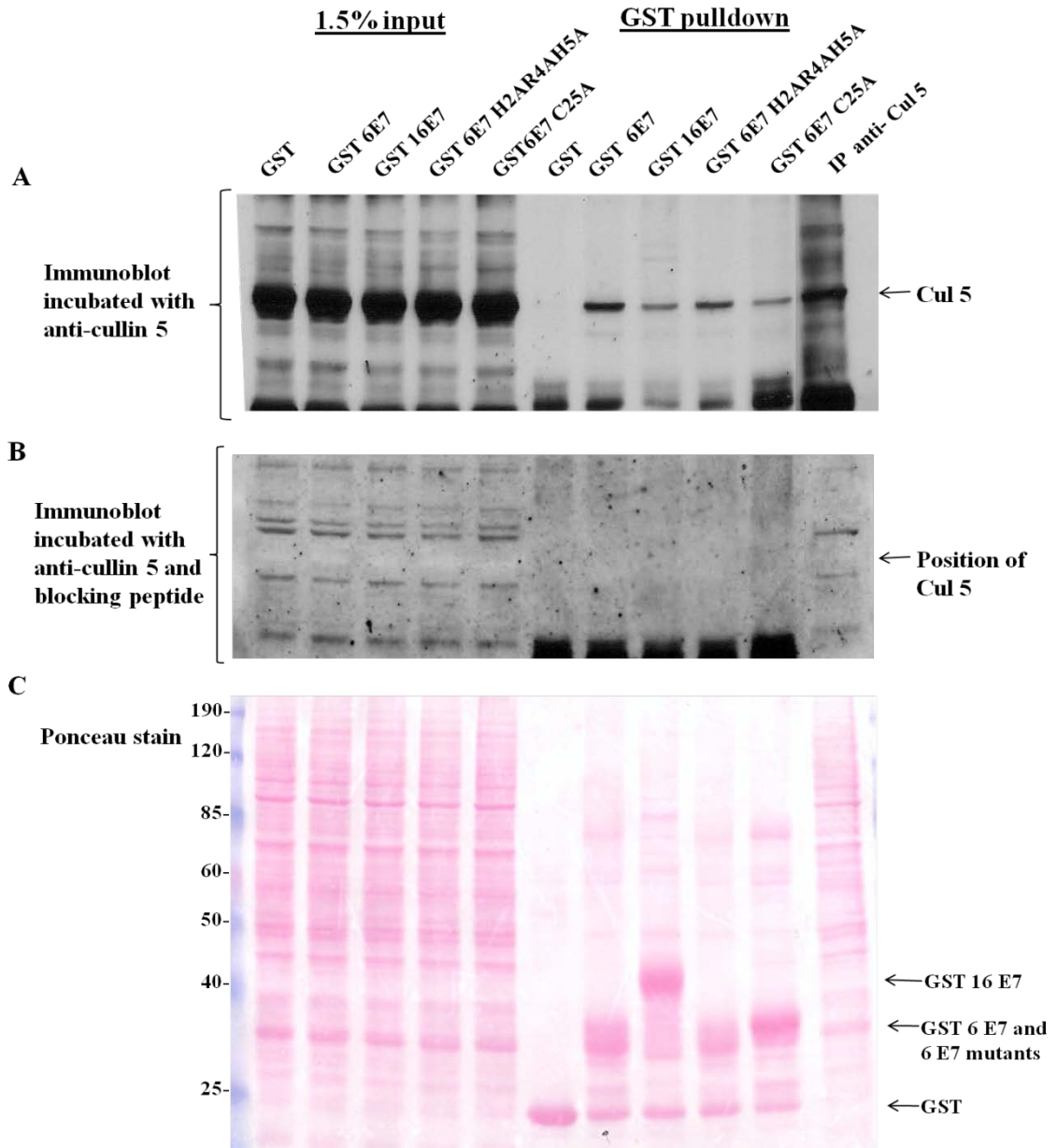
Figure 30. Characterization of HPV 6 E7 mutants with respect to cullin 5 binding using Bethly Laboratories antibody. *(A)* The ability of HPV E7 to bind cullin 5 using antibody to cullin 5 (Bethyl Laboratories) was investigated. GST, GST 6 E7, GST 16 E7, GST 6 E7 H2AR4AH5A and GST 6 E7 C25A were incubated with 1 mg each of pre-cleared HeLa nuclear extract as in Figure 25.

Figure 30 (con't). Immunprecipitation of cullin 5 was performed using 1 mg of pre-cleared HeLa nuclear extract and 1:100 dilution of antibody to cullin 5 (Bethyl Laboratories). *(B)* Blot was stripped and re-probed with antibody to cullin 5 incubated with cullin 5 blocking peptide (Bethyl Laboratories). The band that was detected as cullin 5 was no longer detected after incubation with blocking peptide. *(C)* Ponceau red staining of the membrane was performed as in Figure 27.

GST pull down assays were conducted to confirm the interaction detected by tandem affinity purification. GST 6 E7 and GST 16 E7 were shown to bind to cyclin C; there was no binding to the GST negative control (Figure 31). HPV 6 E7 mutants, H2AR4AH5A, C25A and L67R, were also analyzed with respect to cyclin C binding. GST 6 E7 H2AR4AH5A did not bind cyclin C as efficiently as GST 6 E7, whereas no decrease in binding of cyclin C to mutants GST 6 E7 C25A and 6 E7 L67R was observed (Figure 32). Immunoprecipitation experiments using antibody to HA to immunoprecipitate HA-tagged E7 from HFKs did not show binding to cyclin C. This may be due to the low expression of cyclin C in this cell type. We did not detect any binding of GST E7 to Cdk3 (Figure 33) or Cdk8 (data not shown) by GST pull down assays.

Effect of p600 knockdown on p130 stability

p600 was identified as a cellular protein that binds to both HPV 6 E7 and HPV 16 E7 (Huh et al., 2005). p600 has been speculated to be an E3 ubiquitin ligase (Huh et al., 2005), and has been reported to bind the N-terminus of HPV 16 E7. Therefore, p600 is a candidate regulatory protein needed for efficient p130 degradation by both HPV 16 E7 and HPV 6 E7. p600i (p600 knockdown) shRNA and p600r (reverse) shRNA retrovirus was obtained from Dr. Karl Munger's laboratory. HFKs were transduced with LXSN, L(6E7)SN or L(16E7)SN and selected with G418. They were then transduced with p600i or p600r (as a control) and selected with puromycin. At 80% confluence HFKs were harvested
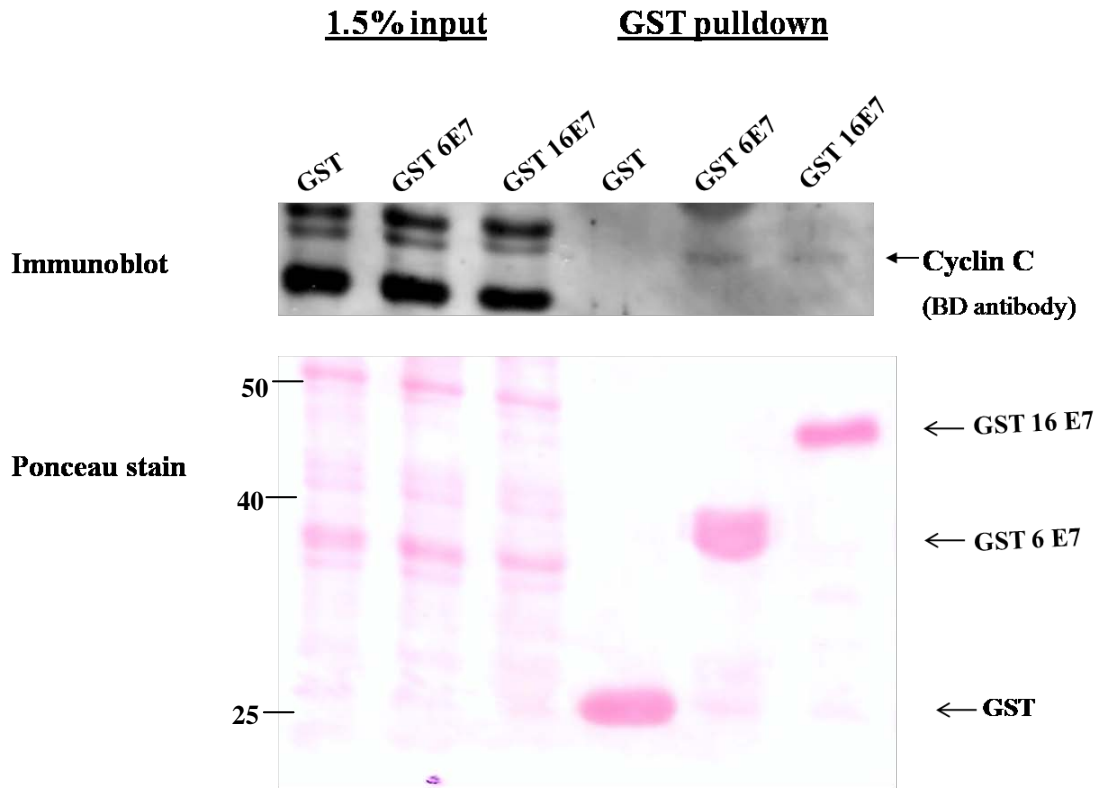
Figure 31. Validation of HPV E7 binding to cyclin C. GST pulldown

experiments were performed as in Figure 25 using GST, GST 6 E7 and GST 16

E7. Cyclin C was detected using antibody from BD Biosciences.
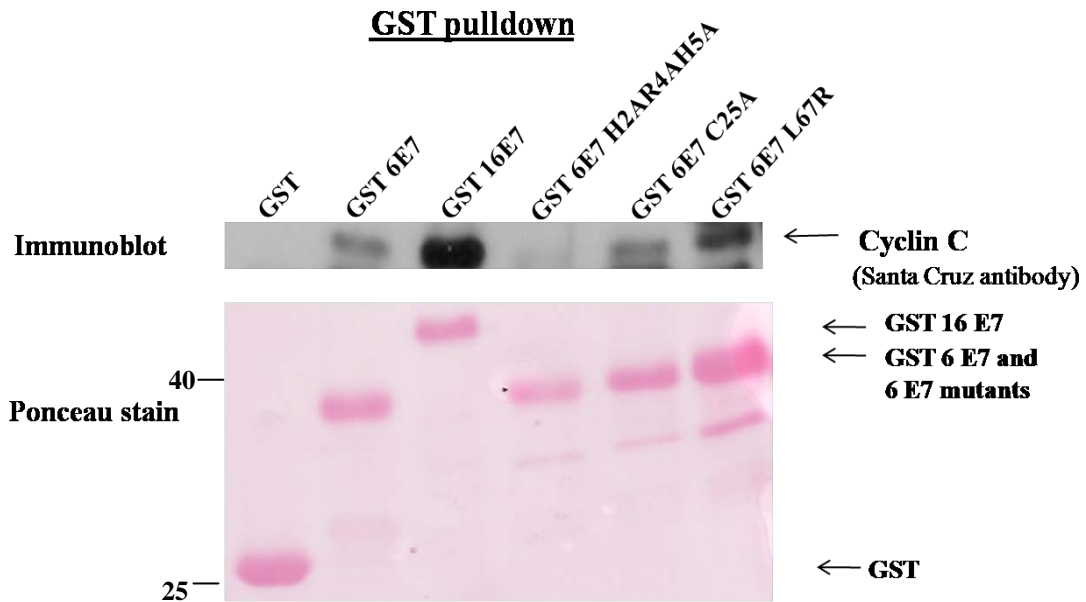
Figure 32. Characterization of the ability HPV 6 E7 mutants to bind to cyclin C. GST pulldown experiments were performed as in Figure 25 using GST, GST 6 E7 and GST 16 E7, GST 6 E7 H2AR4AH5A, C25A and L67R. Binding to cyclin C was detected using antibody from Santa Cruz.
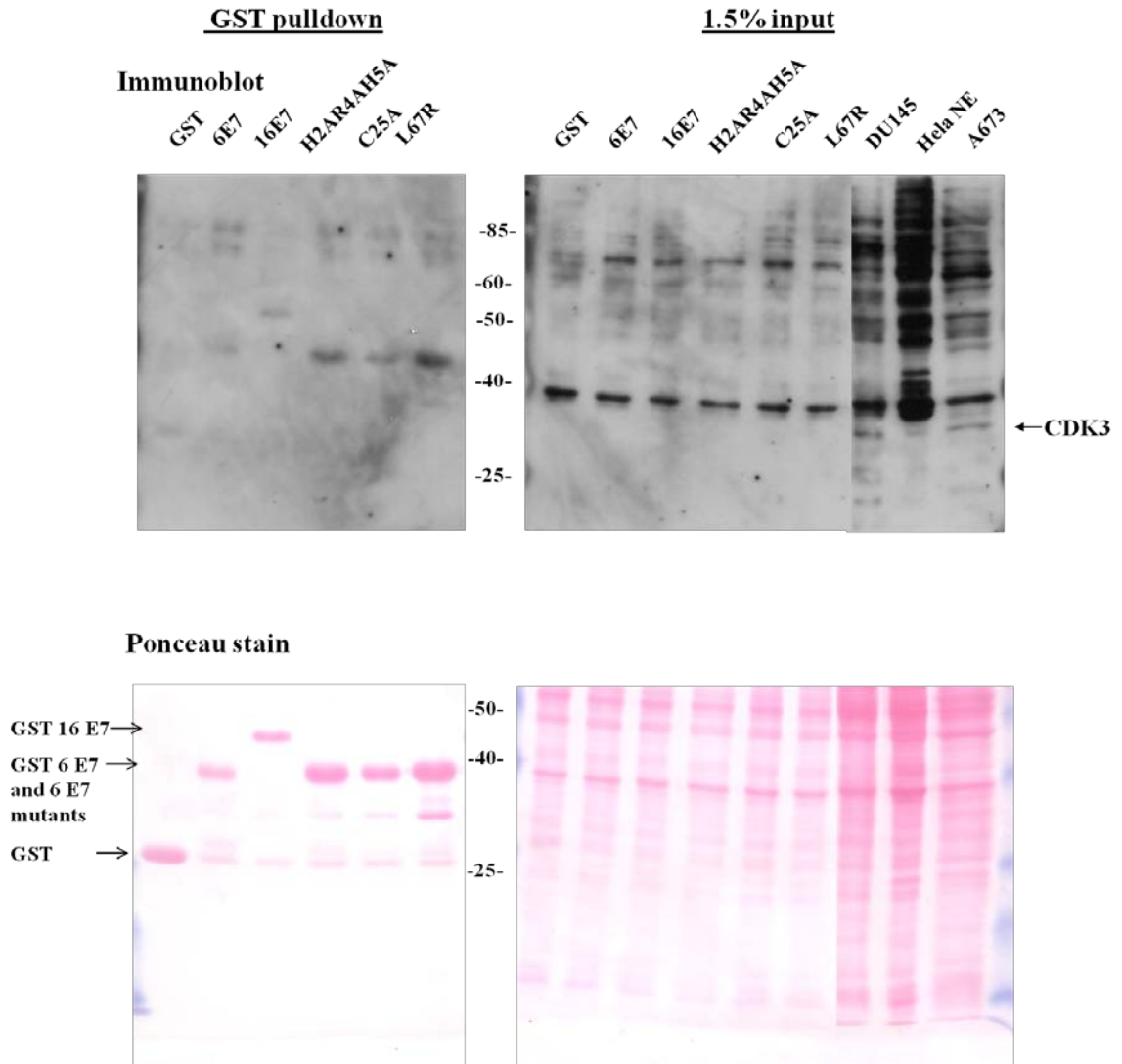
Figure 33. HPV E7 and Cdk3. GST pull-down assays were performed as in Figure 27.

and Western analysis performed, antibodies to p600, p130 and nucleolin were used.

Expression of p600 was decreased from 40-70% in cells expressing p600i when compared to HFKs expressing p600r (Figure 34). Decreased expression of p600 in HFKs transduced with HPV E7 resulted in some stabilization of p130 (Figure 35).

A

p600 i   p600 r

LX   6E7   16E7   LX   6E7   16E7

p600

Nucleolin

B
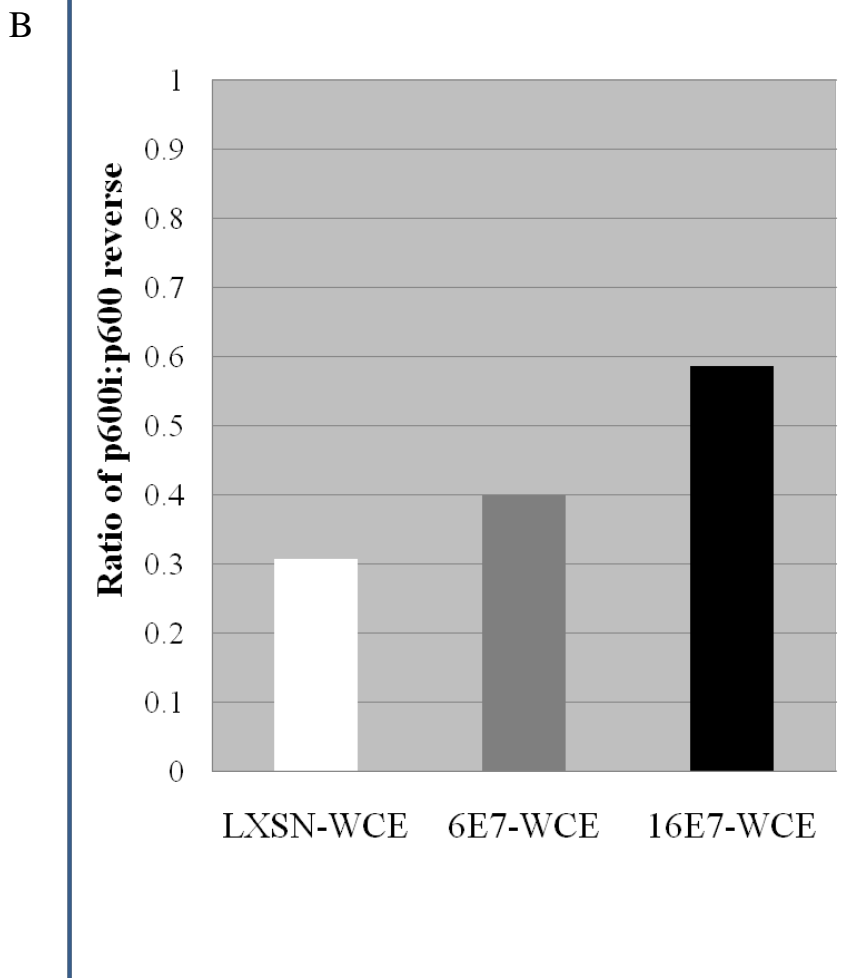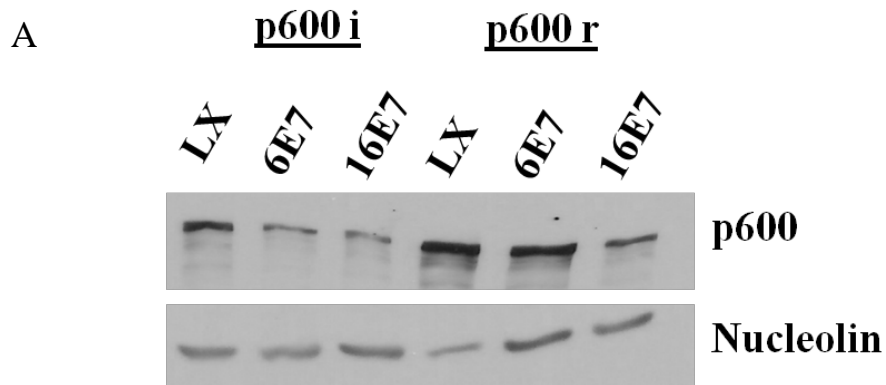
Figure 34. **Knock-down of p600 using p600 shRNA retrovirus**. *(A)* HFKs were

transduced with LXSN, L(6E7)SN or (L16E7)SN), selected with G418 and

transduced with p600i (p600 knock-down) or p600r (p600-shRNA retrovirus and

selected with puromycin. Cells were harvested and western analysis performed.

Antibodies to p600 and nucleolin (as a loading control) were used. *(B)* Ratio of

p600i:p600 reverse after densitometry and normalization to nucleolin. ∗,

unknown protein cross-reacting with anti-p130.
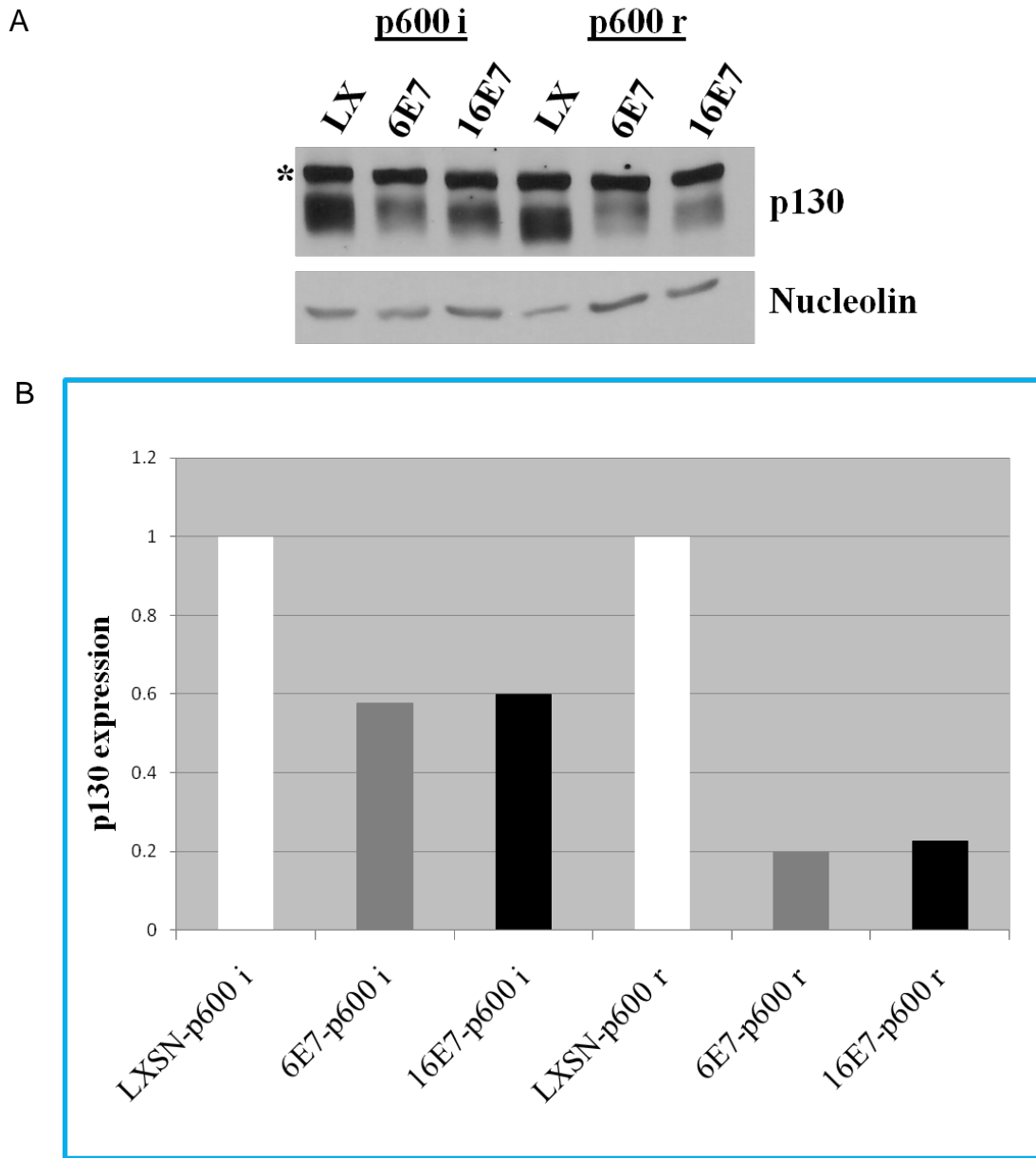
Figure 35. Effect of p600 knock-down on p130 stability. *(A)* HFKS were

transduced as in Figure 33. Western analysis was performed using antibodies to

p130 and nucleolin. *(B)* Quantity one software was used for densitometric

analysis. Whole cell extracts (WCEs) were normalized to nucleolin and LXSN-

600r and LXSN-p600i set to 1.

DISCUSSION

<u>Overview</u>

HPV 16 E7 targets pRb family members for degradation. The ability of HPV 16 E7 to target pRb for degradation in particular, is necessary for malignant transformation (zur Hausen, 2000). In contrast to HPV 16 E7, HPV 6 E7 is not transforming and does not affect the stability of pRb or p107 (Demers et al., 1994; Zhang et al., 2006); however it does target p130 for degradation (Zhang et al., 2006). pRb family members play a key role in regulating progression through the cell cycle. p130 is specifically up-regulated in G_0 and is responsible for keeping cells in a differentiated state (Cobrinik, 2005). The fact that both HR and LR E7 target p130 for degradation may indicate that p130 is important for the HPV life cycle. Targeting p130 for degradation may be conducive to creating an "S-phase-like" environment (Banerjee et al., 2006; Collins et al., 2005; Munger et al., 2001). Zhang et al. 2006, proposed that the mechanism of degradation of p130 by HPV 16 E7 and HPV 6 E7 might be similar based on mutational analysis (Zhang et al., 2006). However, the results presented in this thesis suggest that HR and LR E7 may achieve this by different mechanisms.

This thesis focuses mainly on understanding how p130 is regulated by HPV E7. Initial studies were conducted to investigate the expression of p130 in patient biopsies. Experiments were also conducted to determine the effect of HPV E7 on p130 localization and whether there was preferential degradation of p130 in the nucleus or cytoplasm in the presence of HPV 6 E7 or HPV 16 E7.

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Binding partners of HPV E7 were also identified and the possible effect of p600 (an established binding partner of HPV E7) on p130 stability was investigated. These findings will be discussed in detail in this chapter.

p130 is not detected by immunohistochemistry in HPV 6 positive biopsies

Patient biopsies infected with HPV 6 were analyzed for p130 expression to determine whether there would be a decrease in p130 expression, in the presence of HPV 6 genome. The results showed an absence of staining of p130. However, Doorbar reported that the expression of E7 in CIN1/CIN2 does not completely extend to the most differentiated epithelial layer (Doorbar, 2006). This may imply that p130 expression should be detected in the upper suprabasal layers. Nonetheless, in our experiments no p130 expression was detected in any of the cells in HPV 6 infected patient biopsies. The integrity of the nuclei of the cells was determined by using antibodies to proliferating cell nuclear antigen (PCNA). In agreement with the studies in this thesis, Wang et al. 2009, reported that organotypic raft cultures of HFKs infected with HPV 18 lacked p130 expression in basal and suprabasal cells (Wang et al., 2009).

Preliminary data shows that HPV E7 upregulates the p130-specific target, phospholipid scramblase 1

In an effort to further understand the implications of HPV E7-mediated degradation of p130, the effect of HPV E7 on p130 specific targets was investigated. Chip-on-chip experiments have shown phospholipids scramblase 1

(PLSCR1) and Treacher Collin syndrome factor 1 (TCOF1) to be bound exclusively by p130 (Balciunaite et al., 2005). PLSCR1 is involved in the processes of cell differentiation, apoptosis and proliferation. Of interest, cells that have a decreased expression of PLSCR1 are proliferate at a slower rate than wild type cells. TCOF1 is involved in ribosomal RNA gene transcription by interacting with an upstream binding factor. These genes are negatively regulated by p130 (Balciunaite et al., 2005). Loss of p130 should result in increased expression of such genes. RT-PCR using primers designed to amplify PLSCR1 performed during this thesis work showed that, in the presence of HPV 6 or 16 E7, there was an increase in the RNA expression of PLSCR1. This result was consistent with degradation of p130. One possibility is that the increasing PLSCR expression may result in more cells undergoing proliferation, allowing for increased replication of HPV. Results were inconclusive for TCOF1.

The subcellular location of E2F4 and E2F5 in the presence of HPV E7 was investigated by fractionation experiments. E2F4 and E2F5 associate with pRb, p130 and p107 and play a role in negatively regulating S-phase responsive genes (Deschenes et al., 2004). We hypothesized that in the presence of 16 E7, E2F4/5 may have an altered localization. However, E2F4/5 appeared to be predominantly nuclear in 16 E7 expressing cells, 6 E7 expressing cells and control cells. This may be due to the fact that E2F4/5 can enter the nucleus with pRb or p107 and, therefore, although p130 localization is altered, E2F4/5 localization is not.

HPV E7-mediated degradation of p130 is independent of cyclin/Cdk activity

Experiments were designed to test whether p130 degradation by HPV 6 E7 and HPV 16 E7 was dependent on cyclin/Cdk activity, since HPV E7 enhances entry of cells into the cell-cycle. An increase in the quantity of hyperphosphorylated p130 due to cell cycle entry might result in enhanced degradation of p130 (Tedesco et al., 2002). Treatment with three Cdk inhibitors, flavopiridol, 3-ATA and roscovitine, showed that both HR and LR HPV E7 retained their ability to target p130 for degradation (Figure 9). Therefore, destabilization of p130 by either HPV E7 may not simply be a result of entry into the cell cycle induced by E7. Rather, our results demonstrate that degradation of p130 by HPV E7 is independent of cyclin/Cdk activity. Previous studies have shown that, in human fibroblasts, inhibition of Cdk4/6 results in the loss of the hyperphosphorylated form of p130 (Tedesco et al., 2002). However, in E7 expressing HFKs treated with 3-ATA, a Cdk4/6 inhibitor, there was no difference in p130 phosphorylation status as compared to E7 expressing cells treated with DMSO control. Of the Cdks known to responsible for phosphorylating p130, flavopiridol and 3-ATA are both active against Cdk4/6, and flavopiridol and roscovitine are active against Cdk2. Treatment with flavopiridol resulted in the abolishment of the hyperphosphorylated form of p130. This suggests that in HFKs, hyperphosphorylated p130 may be derived from phosphorylation by both Cdk2 and Cdk4/6.

Detection of HPV E7 localization

HPV 16 E7 has been detected in the cytoplasm and in the nucleus. In most experiments where E7 has been reported to be in the nucleus, E7 was visualized by immunofluorescence (Smith-McCune et al.,1999, Guccione et al., 2002). Most times when E7 has been found in the cytoplasm, E7 has been detected by western analysis of subcellular fractions (Nguyen et al., 2007; Smotkin and Wettstein, 1987). The ability of HPV E7 to localize to both the cytoplasm and nucleus is supported by the fact that HPV E7 has both cytoplasmic and nuclear targets.

It is possible that HPV E7 appears mainly nuclear by immunofluorescence because the cytoplasmic signal is dispersed and therefore harder to detect, whereas the nuclear signal might be so dense and prominent that is obscures the cytoplasmic signal. Additionally, it has been proposed that cell fractionation disrupts the association of E7 with the nucleus if the association is weak and that is why HPV E7 appears to be more cytoplasmic by fractionation experiments (Smith-McCune et al., 1999). A recent study identified two nuclear localization signals and one nuclear export signal in HPV 16 E7 (Knapp et al., 2009), providing further evidence that HPV 16 E7 shuttles between the cytoplasm and nucleus. Data generated from this thesis support that HPV 16 E7 localizes to both the nucleus and cytoplasm, but HPV 16 E7 may preferentially localize to the cytoplasm, since p130 is mainly cytoplasmic in the presence of HPV 16 E7.

p130 is retained/relocalized to the cytoplasm in the presence of HPV 16 E7

p130 localized to the cytoplasm in the presence of HPV 16 E7. In contrast, the localization of p130 in the presence of HPV 6 E7 was similar to control cells. Identical results were also obtained in experiments where cells were grown under differentiated conditions. Analysis of putative NES (NES predictor www.cbs.dtu.dk/) suggests that HPV 6 E7 may have an NES in the N-terminus, in contrast to the identified NES in HPV 16 E7 which is in the C-terminus (Knapp et al., 2009). It is possible that upon binding to p130, a confirmation change occurs in HPV 6 E7 resulting in the NES being concealed. Alternatively, binding of HPV 16 E7 to p130 may result in a conformational change that causes the NLS to be inaccessible. Slight variations in the p130 localization results obtained by subcellular fractionation versus immunofluorescence (Figures 13 and 15) may be due to differences in the p130 epitopes recognized by the monoclonal and polyclonal antisera, p130 epitopes being masked in the cytoplasm and/or sensitivity issues with the immunofluorescence assay due to p130 being diffuse in the cytoplasm.

Treatment with LMB does not affect 16 E7-mediated p130 cytoplasmic localization

In HPV 16 E7 expressing cells, p130 localized mainly to the cytoplasm with or without LMB treatment (Figure 19). This may be because HPV 16 E7-mediated p130 nuclear export is Crm1-independent; alternatively, HPV 16 E7 may cause p130 to be retained in the cytoplasm. In published reports, p130

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cytoplasmic localization has been shown to be insensitive to treatment with LMB (Chestukhin et al., 2002). Additionally, although the C-terminus of HPV 16 E7 fused to GFP was shown to accumulate in the nucleus with LMB treatment (Knapp et al., 2009), no studies have shown that the subcellular localization of full length HPV 16 E7 protein is LMB sensitive.

HPV 6 E7 can target p130 for degradation in the nucleus

It has long been established that proteins can be targeted for degradation in the cytoplasm. There is robust evidence to support that proteins can also be targeted for degradation in the nucleus (von Mikecz, 2006). This is the first study, to our knowledge, to address the capacity of nuclear proteasomes in p130 degradation. The data presented here suggest that in the presence of HPV 16 E7, p130 degradation is mainly mediated by cytoplasmic proteasomes (Figures 23 and 25). However, for HPV 6 E7, nuclear proteasomes may also play a significant role (Figures 24 and 26).

HPV 16 E7 may preferentially target p130 for degradation in the cytoplasm after sequestering it there, whereas HPV 6 E7 may have evolved a mechanism for degrading p130 more efficiently in the nucleus. However, p130 degradation does not occur exclusively in either the nucleus or cytoplasm for HPV 6 E7 or HPV 16 E7. There may be more than one mechanism by which p130 is targeted for degradation by HPV 6 E7 and HPV 16 E7. Huh et al. reported that HPV 16 E7 recruits a cullin 2 E3 ubiquitin ligase complex that contributes to degradation of pRb. The authors suggest that this E3 ubiquitin ligase also contributes to the

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degradation of p130. HPV 6 E7 was not found to bind this cullin 2 complex (Huh et al., 2007). It is possible that HPV 6 E7 facilitates the interaction of another E3 ubiquitin ligase with p130 (discussed below). Additionally, HPV 6 E7, similar to human T-cell leukemia virus type 1 tax, may function as a molecular bridge (Kehn et al., 2005), or as a chaperone, like simian virus large T antigen (Srinivasan et al., 1997; Stubdal et al., 1997), to facilitate interaction of p130 directly with the proteasome.

Pitfalls of HA-p130 mutants

HA-p130 NES mutants were constructed to determine whether HPV 16 E7 could alter the localization of a p130 mutant that was located exclusively in the nucleus. Another purpose of generating HA-p130 NES mutants was to determine the ability of HPV 6 E7 to target them for degradation in the nucleus. These experiments would be important to perform because in the half-life experiments conducted in this thesis work, nucleocytoplasmic shuttling was a confounder to the interpretation of results. Using HA-p130 mutants that were exclusively nuclear would potentially avoid the caveat of p130 moving from the nucleus to the cytoplasm and therefore change in p130's location contributing to the decrease in p130 expression. These mutants were generated using a WT HA-p130 plasmid. It proved difficult to transfect HFKs. Approximately 30% transfection efficiency was obtained using FuGENE 6 transfection reagent. Immunofluorescence showed HA-p130 and HA-p130 mutants to be localized in the nuclei of cells, although p130 has been reported to be capable of shuttling

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between the cytoplasm and nucleus. It is possible that the tagged protein no longer functions like WT p130.

HPV 6 E7 and HPV 16 E7 bind to cullin 5

Human adenovirus E1B55K and E4orf6 target p53 for degradation by recruiting an E3 ubiquitin ligase containing cullin 5, elongin B/C, and the RING finger protein Rbx1 (Querido et al., 2001). Other proteins have also been described that are targeted by this cullin 5 complex as mentioned in Chapter 3. Cullin 2 and 5 both use elongin B/C adaptor proteins. Although HPV 16 E7 hijacks and directs the cullin 2 E3 ubiquitin ligase complex to target pRb family members for degradation, other HR (high-risk) and LR (low-risk) HPV E7 proteins do not, indicating that binding of cullin 2 is not an universal requirement for p130 degradation (Huh et al., 2007). Therefore, it is possible that the cullin 5 E3 ubiquitin ligase complex may be involved in targeting of p130 for degradation by HPV E7. To investigate whether p130 might be a substrate for cullin 5, binding of cullin 5 to HPV E7 was determined. Data generated from this thesis show that HPV 6 and HPV 16 E7 both are capable of binding to cullin 5 (Figures 27, 29 and 30). To determine whether binding of HPV E7 to cullin 5 correlated with the ability of HPV E7 to target p130 for degradation, HPV 6 E7 mutants were analyzed. HPV 6 E7 mutant H2AR4AH5A binds to p130 but does not degrade p130, whereas HPV 6 E7 mutant C25A does not bind or degrade p130 (Zhang et al., 2006). Preliminary results obtained by GST pull-down assays indicated GST 6 E7 C25A bound cullin 5 less efficiently than GST 6 E7. Results with GST 6 E7

H2AR4AH5A, however, were inconclusive. Further experiments to determine

whether cullin 5 is important for HPV-E7 p130 degradation need to be conducted

(Future Directions section). It is still an interesting finding that HPV E7 can bind

to cullin 5, as cullin 5 has been reported to regulate Hsp 90, Hif1 alpha (Ehrlich et

al., 2009) and Src tyrosine kinase (Laszlo and Cooper, 2009).

HPV 6 E7 and HPV 16 E7 bind to cyclin C

Cyclin C was found to bind to HPV 6 and HPV 16 E7 and bound HPV 16 E7

more efficiently than HPV 6 E7 (Figures 31 and 32). HPV 6 E7 mutant

H2AR4AH5A bound less efficiently than HPV 6 E7 (Figure 32). Mutant HPV 6 E7

H2AR4AH5A is a loss of function mutant for p130 degradation, although it binds

to p130 as efficiently as HPV 6 E7. This may indicate that the ability of HPV E7 to

bind to cyclin C is important for p130 degradation. Binding to cyclin C may allow

p130 to be phosphorylated by its binding partner CDK3, at serine 672 or another

phosphorylation site, that then results in p130 becoming unstable by being

targeted for degradation.

Cycin C also associates with Cdk8. The cyclin C/Cdk8 complex regulates

transcription. Reports indicate that the cyclin C/Cdk8 complex phosphorylates the

carboxy-terminal domain (CTD) of RNA pol II, resulting in premature

phosphorylation of CTD and inhibition of transcription initiation. In contrast, cyclin

C/Cdk8 can enhance transcription by phosphorylation of transcription factor Sip4

and by phosphorylation of TFIID (Hoeppner et al., 2005). HPV E7 may bind to

cyclin C, preventing it from interacting with CDK8 and thereby enhancing

transcription initiation. Conversely, HPV E7 may bind to the cyclin C/CDK8 complex and increase transcription of genes that are necessary for its life cycle.

Knockdown of p600 may stabilize p130

p600 was identified as a cellular protein that binds to both HPV 6 E7 and HPV 16 E7 (Huh et al., 2005). p600 is necessary for cellular transformation and anchorage-independent growth and has also been speculated as being an E3 ubiquitin ligase (Huh et al., 2005) because it contains a RING-finger domain. p600 was identified as a protein that associates with pRb although it binds E7 independently of pRb. Knockdown of p600 did not affect the stability of pRb (Huh et al., 2005). Here, experiments were conducted to determine the possibility that knockdown of p600 would destabilize p130. Preliminary data showed that there was an increase in p130 expression in HFKs transduced with HPV 6 E7 or HPV 16 E7 that were then transduced with p600 shRNA. This experiment, however, was only conducted once and needs to be repeated.

Ubiquitinated p130 has not been detected

Experiments to identify ubiquitinated p130 have been unsuccessful. In these experiments HFKs were transduced with LXSN, L(6E7)SN or L(16E7)SN selected and then transduced with a retrovirus encoding histidine-tagged ubiquitin (His-Ub: in order to express tagged ubiquitin). Cells were treated for 4 h with MG132 to inhibit proteasomal degradation and harvested in the presence of N-ethylmalamide to cross-link cysteine residues and inactivate deubiquitinases.

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p130 was immunoprecipitated with polyclonal antibody to p130 and immunoblotted with monoclonal antibodies to p130 and ubiquitin but no ubiquitinated p130 was identified, although p130 was detected. This inability to detect ubiquitinated p130 may signify that degradation of p130 is ubiquitin independent or that p130 is monoubiquitinated. The cytomegalovirus protein pp71 has been reported to target the pRb family members for degradation, in a ubiquitin-independent manner (Kalejta and Shenk, 2003). A monoubiquitinated form of p130 would be difficult to observe by Western analysis. It is also possible that the conditions of the assay are not optimized for finding ubiquitinated species.

Conclusions

In conclusion, the data presented in this thesis argue that although both LR and HR HPV target p130 for degradation, the mechanism by which the E7 proteins destabilize p130 may be different as presented by localization and half-life studies in Chapter 2. However, it is possible that there are some shared aspects of the mechanism used by HPV 6 E7 and HPV 16 E7 to target p130, since both of the E7 proteins bind cullin 5, cyclin C and p600. Additional experiments will be proposed in the Future Directions section to delineate the activities that are shared by the E7 proteins in regards to the identified binding partners mentioned above and p130 degradation. HPV 6 E7 seems to have evolved to co-exist better with its host cell, causing a productive infection (a benign condyloma) but not a lesion which is non-productive for the virus (a

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carcinoma). As such, HPV 6 E7 causes less perturbation of the Rb family circuitry than HPV 16 E7, consequently raising fewer distress signals for the cell to counter. Since HPV 6 E7 distinguishes between the Rb family members but HPV 16 E7 does not, the two E7 proteins may have evolved different mechanisms to deal with p130. By altering the intracellular localization of p130 so that more is found in the cytoplasm, 16 E7 has not only targeted p130 for degradation but also effectively removed p130 from its nuclear targets. Even though preliminary studies show that both HPV 6 E7 and HPV 16 E7 bind to cullin 5 and cyclin C remains to be determined what role, if any, binding to these proteins plays in the degradation of p130. Additional experiments to knockdown p600 need to be performed to confirm whether decreasing p600 expression truly plays a role in stabilizing p130 in the presence of HPV E7.

FUTURE DIRECTIONS

The major focus of this thesis work has been to compare the ability of LR HPV 6 E7 and HR HPV 16 E7 to regulate p130. In the presence of HPV 16 E7, more p130 was localized to the cytoplasm, whereas in HPV 6 E7 and LXSN transduced cells, p130 was mainly localized to the nucleus. HPV 16 E7 may have evolved to regulate p130 by not only targeting it for degradation, but by essentially removing it from its nuclear targets. In contrast, although HPV 6 E7 did not alter the nuclear localization of p130, HPV 6 E7 was found to preferentially target p130 for degradation in the nucleus. This is an interesting finding, since nuclear degradation of proteins has been relatively recently described. This data is novel as no other study has described p130 as being capable of being targeted for degradation in the nucleus.

. A caveat of the experiments described above is the fact that both localization and degradation events occur synchronously Therefore, it would be worthwhile to further delineate the role of these two processes in the regulation of p130 by HPV To atempt to separate these events HPV E7 transduced HFKs could be treated with MG132 (proteasomal inhibitor) and then performing immunofluorescence and subcellular fraction performed using antibodies to p130, p107 and pRb.

HA-tagged NES mutants were generated in an effort to more closely examine the ability of HPV 16 E7 to alter the intracellular localization of p130. However, the localization of these mutants could not be validated since the WT

HA-p130 appeared to be only nuclear by immunofluorescence, although it was expected to be expressed in both the cytoplasm and nucleus. It is possible that the HA-tag on p130 affected its normal function; therefore, it may be necessary to generate untagged p130 NES mutants to clarify these studies.

Tandem mass spectrometry data generated by the laboratory suggest that HPV E7 binds to cyclin C. GST pulldown experiments also suggest that HPV 6 E7 and HPV 16 E7 bind to cyclin C. More HPV 6 E7 and 16 E7 mutants could be tested for their ability to bind to cyclin C. Immunoprecipitation experiments using HA-myc tagged HPV E7 also could be performed to demonstrate *in vivo* binding of HPV E7 to cyclin C. Knock-down experiments using shRNA to cyclin C would provide greater insight into its potential role in p130 degradation.

Binding of HPV 6 E7 and HPV 16 E7 to cullin 5 was detected by GST pulldown experiments. Additional experiments using HPV 6 E7 and HPV 16 E7 mutants also could be conducted to determine whether loss of function mutants for p130 degradation can bind to cullin 5. As with cyclin C, immunoprecipitations of tagged HPV E7 to look for cullin 5 need to be pursued and knock-down of cullin 5 using RNA interference in HPV E7 expressing cells performed.

Preliminary data using shRNA of p600 indicated that, in HPV E7 expressing cells, knock-down of p600 may stabilize p130. Experiments need to be repeated to confirm this data. Also, HFKs, when transduced and selected twice, appeared to grow somewhat slowly. Changing to near-immortalized keratinocytes (NIKS) should be considered since these cells may be better at undergoing two rounds of selection.

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Initial immunofluorescence studies detected cyclin C mainly in the nucleus. Immunofluoresence and fractionation experiments can be performed to verify the location of cullin 5 and p600 in HPV E7 expressing cells. These experiments would provide insight into whether cyclin C, cullin 5 or p600 co-localize with p130. Also, if knock-down experiments to decrease expression of cyclin C, cullin 5 proved to also be successful, HPV 6 E7 and HPV 16 E7 expressing cells, knocked-down for each of p600, cyclin C or cullin 5 could be treated with cycloheximide (CHX) and studies performed to determine the half-life of p130 in these whole cell extracts, cytoplasmic and nuclear fractions. If one of these proteins were critical to E7-mediated p130 degradation the half-life of p130 would be extended upon its knock-down. If p130 half-life is extended upon knocking-down either cullin 5, cyclin C or p600 in HPV E7 expressing cells the effect on differentiation could be Whole cell extracts could be harvested from these HPV E7 cells knocked-down for the E7 binding partners stated above and Western analysis performed using antibodies to involucrin or keratin 14 (indicators or differentiation) to detect whether there was an increase in differentiation compared to control cells that express normal levels of cyclin C, cullin 5 or p600.

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CURRICULUM VITAE

Lisa C. Barrow

EDUCATION

Ph.D. 2010 Indiana University, Indianapolis, IN. Microbiology and Immunology
B.S. 2004 Claflin University, Orangeburg, SC. Biology, *summa cum laude*

PROFESSIONAL RESEARCH AND WORK EXPERIENCE

2004 – Present
Graduate Student: <u>Department of Microbiology and Immunology, Indiana University. Mentor: Ann Roman, Ph.D</u>. Research focused on determining the effect of human papillomavirus E7 on p130, a pRb family member.

SUMMER 2003
Research Assistant: <u>Department of Pathology and Microbiology, University of South Carolina. Mentor: Kim Creek, Ph.D</u>. Research to analyze the effect of human papillomavirus E6 on mRNA levels associated with epidermal growth factor receptor and the effect of p53 siRNA on the epidermal growth factor receptor promoter.

SUMMER 2002
Research Assistant: <u>Department of Microbiology and Immunology, Pennsylvania State University, Life Science Consortium Scholar. Mentor: Craig Meyers, Ph.D</u>. Research to investigate the effect of glucocorticoids on the upstream regulatory region of human papillomavirus type 18.

SUMMER 2001
Research Assistant: <u>Department of Psychology, University of South Carolina, Mentor: Donald Powell, Ph.D.</u> Participated in research funded by the South Carolina Alliance for Minority Participation (SCAMP). Research on eye-blink Pavlovian conditioning in New Zealand white rabbits.

2002-2004
Lab Assistant: Claflin University. Assisted in lab setup for experiments and described protocol to be performed during class for genetics and molecular biology labs.

2001-2004
Tutor: SCAMP, Claflin University. Tutored students in general biology, molecular biology, genetics and biochemistry.

TEACHING EXPERIENCE

FALL 2005
Teaching Assistant: Indiana University. Taught laboratory sections and met with student during office hours.

PUBLISHED WORKS

Barrow-Laing, L., Chen, W., and Roman, A. Low- and High-risk Human Papillomavirus E7 Proteins Regulate p130 Differently. Virology. 2010 May 10; 400(2):233-9.

Laing, B.M, Barrow-Laing, L., Harrington, M., Long, E.C., and Bergstrom, D.E. Properties of Double-Stranded Oligonucleotides Modified with Lipophilic Substituents. Bioconjugate Chem. 2010. In Press

Barrow-Laing, L., and Roman, A. E7 Proteins of High-risk and Low-risk Human Papillomaviruses Regulate p130 Differently. DNA Tumor Virus Meeting. July 2008, Madison, WI.

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Barrow, L., Bromberg-White, J., and Meyers, C. Effect of Glucocorticoids on the Upstream Regulatory Region of Human Papillomavirus. Annual Biomedical Research Conference for Minority Students. November 2002, New Orleans, LA.

HONORS, AWARDS AND SCHOLARSHIPS

- Dana Farber/Harvard Cancer Center Award Poster Presentation (2010)
- Indiana University School of Medicine Travel Award (2008)
- Travel Grant Award, DNA Tumor Virus Meeting (2006 and 2008)
- 1st Place Award, Oral Presentation, Research in Progress, Indiana University (2006)
- 2nd Place Award, Oral Presentation, Sigma XI Competition, Indiana University (2006)
- Summa Cum Laude Graduate, Claflin University (2004)
- 4th Place Award Poster Presentation, Undergraduate Science and Engineering Conference, Tuskegee University (2002)
- 2nd Place Award Poster Presentation (SCAMP) (2001)
- National Dean's List (2001-2004)
- Division of Natural Sciences and Mathematics, Claflin University Dean's List Certificate Recipient (2000-2004)
- Alice Carson Tisdale Honors College Scholar, Claflin University (2000-2004)

PROFESSIONAL AFFILIATIONS

- American Association for the Advancement of Science
- American Society for Microbiology