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# Low- and high-risk human papillomavirus E7 proteins regulate p130 differently

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ABSTRACT

The E7 protein of high-risk human papillomaviruses (HR HPVs) targets pRb family members (pRb, p107 and p130) for degradation; low-risk (LR) HPV E7 only targets p130 for degradation. The effect of HR HPV 16 E7 and LR HPV 6 E7 on p130 intracellular localization and half-life was examined. 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. These results suggest that there may be divergent mechanisms by which LR and HR HPV E7 target p130 for degradation.



## Introduction

In women worldwide, cervical cancer ranks second in cancer incidence; in women in developing countries cervical cancer ranks first in cancer-related deaths (Drain et al., 2002; Snijders et al., 2006). Human papillomaviruses (HPVs) are associated with over 99% of all cervical cancer (Snijders et al., 2006). HPVs are classified as high-risk (HR) or low-risk (LR) depending on their pathogenicity. HR HPVs (e.g. 16, 18, 31, 33, and 45) are commonly associated with malignancies of the cervix and head and neck. HPVs 6 and 11 are classified as LR, and can cause condyloma acuminata (genital warts) (Longworth and Laimins, 2004).

The HPV life cycle is closely linked to epithelial differentiation. In normal epithelium, proliferation occurs in the basal cell layer; as cells migrate upwards they exit the cell cycle and differentiate. After virus infection, viral DNA is maintained at 50–100 copies in the basal cell layer (Hebner and Laimins, 2006; Longworth and Laimins, 2004). When the infected cells move to the differentiated suprabasal layer viral DNA is amplified and late genes are expressed resulting in mature virions. Several studies indicate that both HR and LR HPVs require the host cell to initiate cellular DNA replication (Hebner and Laimins, 2006; Longworth and Laimins, 2004; Snijders et al., 2006; zur Hausen, 2002). HPV E7 drives suprabasal cells into S-phase and causes unscheduled DNA synthesis (Flores et al., 2000; Munger et al., 2001; Collins et al., 2005); however, a recent report suggests that HPVs 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 HPVs are required for viral DNA maintenance and/or amplification (Flores et al., 2000; McLaughlin-Drubin et al., 2005; Oh et al., 2004; Zhang and Roman, unpublished data).

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 (Gage et al., 1990; Jewers et al., 1992; 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). Both HR and LR HPV E7 proteins bind pRb family members through their LXCXE binding motif (Dyson et al., 1989). *In vitro* and *in vivo* 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). 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). Casein kinase II-mediated phosphorylation of HR and LR HPV E7 is necessary for effective binding to and destabilization of p130 (Genovese et al., 2008).

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 sub-domains 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 binding to cyclin A/Cdk2 and cyclin E/Cdk2 (Classon and Dyson, 2001; Claudio et al., 2002). pRb family members each bind

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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). E2F1, E2F2, and E2F3 are almost exclusively regulated by pRb, and are referred to as activator E2Fs. p130 and p107 normally associate with E2F4 and E2F5, repressor E2Fs; p130 associates at the $G_0/G_1$ stage of the cell cycle (Dimova and Dyson, 2005; Helin et al., 1993).

p130 levels, like the other pRb family members, are regulated in response to the proliferative state of cells and are controlled by 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 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). SCF complexes are a class of ubiquitin ligase (E3) enzymes that recognize and polyubiquitylate substrates, usually in a phosphorylation-dependent manner, targeting them for degradation by the 26S proteasome (Deshaies, 1999). In contrast to pRb and p107, p130 is phosphorylated by glycogen synthase kinase 3 (GSK3) in the loop region in the B subdomain and thus stabilized in growth-arrested and terminally differentiated cells (Litovchick et al., 2004).

p130 contains three nuclear localization signals (NLSs), 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 $G_0/G_1$ 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 cytoplasm therefore provides a means of regulation (Chestukhin et al., 2002; Tedesco et al., 2002).

HPV 16 E7 has been detected in the cytoplasm and the nucleus and is known to have both cytoplasmic and nuclear targets (Nguyen et al., 2007; Smotkin and Wettstein, 1987; Smith-McCune et al., 1999; Guccione et al., 2002). In support of such observations, it has been reported that HPV 16 E7 has two NLSs and one nuclear export signal (NES) (Knapp et al., 2009). In this study HPV 16 E7, in contrast to HPV 6 E7, was shown to either retain or relocalize p130 to the cytoplasm of HFKs. Leptomycin B, a CRM1/exportin 1 inhibitor, did not have an effect on HPV 16 E7 mediated p130 localization. In addition, p130 was shown to have a shorter half-life in the nucleus of HPV 6 E7 expressing HFKs compared to HPV 16 E7 expressing HFKs or control. These results suggest that HPV 6 E7 and 16 E7 may target p130 for degradation by different mechanisms.

## Results

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

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 is phosphorylated by Cdk4/6 and is recognized by an SCF–Skp2 complex for degradation in a cell-cycle dependent manner (Tedesco et al., 2002). To ascertain whether E7-mediated degradation of p130 is dependent on cyclin/CDK activity, HFKs were infected with parental retrovirus LXSN, or retrovirus encoding HPV 16 E7 or HPV 6 E7. Infected cells were grown in keratinocyte serum-free medium (C-K-SFM) supplemented with human recombinant epidermal growth factor and bovine pituitary extract. Cells were then 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) or 50 μM flavopiridol (an inhibitor of Cdk 1, 2, 4, 6, and 7) (Sedlacek, 2001). Whole cell extracts were harvested and Western analysis performed. 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 (Fig. 1). 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 p130 was lost only upon treatment with flavopiridol, but not 3-ATA nor roscovitine (Fig. 1). The steady-state level of p130 in HPV expressing cells 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.

### *p130 localizes to the cytoplasm in keratinocytes 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. Sub-cellular 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 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 (Fig. 2).

The distribution of p130 in HFKs expressing LXSN, HPV 6 E7 or HPV 16 E7 was also determined by immunofluorescence. Localization of p130 was mostly nuclear in HFKs transduced with LXSN and HPV 6 E7.

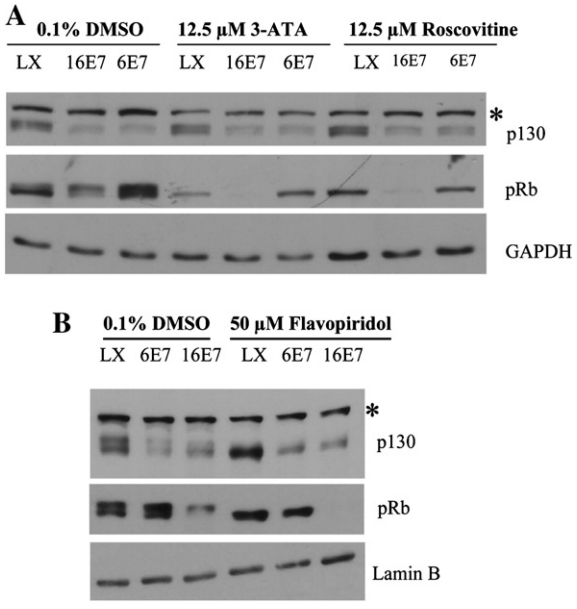


**Fig. 1.** Effect of Cdk inhibitors on p130 stability. HFKs were infected with parental retrovirus LXSN (LX) or retrovirus encoding HPV 16 E7 (16 E7) or HPV 6 E7 (6E7) and grown in C-K-SFM. Cells were then treated with 12.5 μM 3-ATA, 12.5 μM roscovitine, 50 μM flavopiridol or DMSO (vehicle) as a negative control. Whole cell extracts were harvested using EBC buffer and Western analysis performed using mouse monoclonal antibodies to p130, pRb, GAPDH and lamin B. *, unknown protein cross-reacting with anti-p130.