The Role of the Ubiquitin Proteasome Pathway in Papillomavirus Pathogenicity

Thesis

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The Role of the Ubiquitin Proteasome Pathway in Papillomavirus Pathogenicity

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This thesis is submitted for the degree of Doctor of Philosophy in the faculty of Life Sciences of the Open University, UK

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Abstract

To establish an infection in the host cell, Papillomaviruses (PVs) encode two major oncoproteins, E6 and E7, which interact with numerous cellular proteins and interfere with many cellular pathways. We use HPV E6 and RhPV-1 E7 oncoproteins as tools to investigate some of the most important characteristics of the high-risk HPV E6 proteins: their association with the ubiquitin pathway and ability to direct proteasomal degradation of PDZ domain-containing proteins. We show that E6 protein levels are dependent on the E6AP ubiquitin ligase: in its absence, E6 is degraded in a proteasome dependent manner. A proteomic analysis for HPV-18 E6 interacting partners showed that HPV-18 E6 interacts with EDD, another HECT domain ubiquitin ligase. EDD does not direct the degradation of p53 or PDZ domain-containing substrates, but appears to regulate E6AP levels, with consequent effects on E6 protein levels and its p53 targeting. These studies demonstrate a complex interplay between E6, EDD and E6AP for regulating E6's degradation of its substrate proteins. Rhesus papillomavirus 1 (RhPV-1) is a high-risk mucosal papillomavirus, but its E6 protein has no PDZ-binding motif. However, we show a remarkable evolutionary conservation, with the PDZ-binding motif present on the RhPV-1 E7 protein instead. Furthermore, this directs the binding of RhPV-1 E7 to Par3, a PDZ domain-containing protein controlling the polarity regulation pathway also controlled by hDlg and hScrib, the PDZ domain-containing targets of HPV-18 and HPV-16 E6. RhPV-1 E7 degrades Par3 analogously to HPV E6's degradation of its PDZ substrates, and also appears to interact with EDD. These studies demonstrate that PDZ domain-containing cell polarity regulators and critical components of the ubiquitin proteasome pathway are common targets of evolutionarily diverse oncogenic mucosal papillomaviruses.

This suggests that these pathways represent essential steps in the viral life cycles and in these viruses' ability to induce malignancy.

Introduction

Human papillomaviruses (HPVs) and Cancer

Cancer

Cancer is defined as a genetic disease caused by mutations in various genes. Mutations in tumor suppressors, oncogenes and DNA stability genes (mismatch repair, nucleotide-excision and base-repair genes) destabilize their normal functions, resulting in deregulated cell growth and eventually in cancer (Vogelstein and Kinzler, 2004). Interestingly, cancer can also occur through epigenetic inheritance which is defined as cellular information other than the DNA sequence itself, that is heritable during cell division. The three main epigenetic inheritances are DNA methylation, genomic imprinting and histone modification, where abnormalities in all of these have been identified in cancers (reviewed in Feinberg and Tycko, 2004). The numbers of new cancer cases are rapidly increasing each year. At present, approximately 10 million new cancer cases are reported yearly and 6 million of them (12% of worldwide deaths) will not survive (www.who.int/cancer/en). In the near future the numbers are expected to increase even more and it is predicted that by 2020 there will be 15 million new cancer cases each year (Frankish, 2003). More than 100 different cancer types and tumor subtypes have been reported. It is very interesting to note that, even though these cancer types may be distinct and affect different tissues and organs, the physiological alterations in the cells which cause the cancers follow the same pathways. In order to be established and promote its malignancy each cancer cell type needs to attain

immortalized independent cell growth, to induce continuous angiogenesis, to bypass apoptosis and, ultimately, to metastasize (reviewed in Hanahan and Weinberg, 2000). This also implies that the same molecular signaling pathways are disrupted in cancers that have evolved due to changes in the genome, as well as in cancers that are caused by infectious agents, which can be of viral or non-viral origin. A good example of this would be the p53 and pRb pathways which have been identified as being major targets of the DNA tumor viruses (Klein, 2002; Münger and Howley, 2002; zur Hausen, 2001).

HPVs and Cervical Cancer

As mentioned above, cancers can be caused by a number of different infectious agents. Currently, it is estimated that approximately 20% of all global cancer cases are caused by infections (zur Hausen, 2008) and that 70% of these (15% of cancers worldwide) are caused by viruses, accounting for almost 1.5 million cases with 900 000 deaths each year (Butel, 2000). Among these are Hepatitis B and C viruses (HBV and HCV) and Epstein Barr virus (EBV). However, arguably the most abundant are Human papillomaviruses (HPVs), accounting for approximately 5% of the world cancer burden (Parkin and Bray, 2006). Besides being involved in cervical cancer, HPVs have been linked to cancers of external genitalia, anus, mouth and oro-pharynx (Parkin and Bray, 2006). Cervical cancer is the second most common cancer among women worldwide, with an estimated 493 000 new incidences and about 274 000 deaths annually, of which approximately 83% occur in women in developing countries (Parkin and Bray, 2006). Persistent infection with certain HPV types is the most important factor for cervical cancer development (Castellsague, 2008; zur Hausen, 1996).

HPVs infect either mucosal or cutaneous epithelia and induce hyperproliferative lesions which can be manifested either on the genital tract, or in the form of nongenital tumors, such as benign skin warts, non-melanoma skin cancer and various head and neck cancers (Gillison and Shah, 2003; Harwood *et al.*, 1999, Herrero *et al.*, 2003). Those HPV types which are associated with mucosal malignancies are referred to as the high-risk types and they include HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58 and -59 (Bouvard *et al.*, 2009; de Villiers, 1994; Laimins, 1993; zur Hausen and de Villiers, 1994). HPV types such as HPV -6 and -11 induce benign warts which are not likely to progress to malignancy and they are referred to as low-risk types. It is important to mention that although approximately 99% of cervical cancers contain high-risk HPVs (Walboomers *et al.*, 1999) not every infected woman will develop the disease. The initial phase of the infection usually results in low-grade lesions, termed dysplasias or cervical intraepithelial neoplasia grade I. Lesions at this stage are more differentiated than later lesions and can be eliminated by the immune system. The average duration of agenital HPV infection has been estimated to be less than a year (Hopfl *et al.*, 2000; Jenson *et al.*, 1991). However, individuals with persistent high-risk HPV infections are likely to develop severe dysplasia which can ultimately progress to cervical cancer (Hildesheim *et al.*, 1994; zur Hausen, 1999).

During long term persistent high-risk HPV infection the viral DNA can often become randomly integrated into the host cellular chromosomes. Although the episomal HPVs can induce tumors, approximately 70% of the metastatic cells have integrated HPV sequences, which underlines the significance of the DNA integration for the later stages of the disease development (reviewed in McMurray *et al.*, 2001). It is important to bear in mind that this phenomenon is not a part of the

natural viral life cycle, it can be rather looked at as an unwanted incident through which the virus loses its ability to replicate. This integration event frequently results in large deletions of the viral DNA, followed by de-regulated expression of the viral oncoproteins E6 and E7 (Baker *et al.*, 1987; Schwarz *et al.*, 1985; Yee *et al.*, 1985). This condition of de-regulated E6 and E7 expression is then a driving force for further cancer development (Jeon *et al.*, 1995). Figure 1 shows a schematic depicting some of the major events during HPV induced malignancy. In the early stages of the infection the virus causes LSIL (low-grade squamous intraepithelial lesions) which results from a modification of the normal differentiation in the lower third of the epithelium. This kind of lesion represents a normal productive viral infection and may remain unchanged for several weeks to months, may be neutralized by the immune system or may progress to severe dysplasia or HSIL (high-grade squamous intraepithelial lesions). Severe dysplastic lesions may remain as HSIL or advance to invade below the basement membrane, leading to local invasion (reviewed in McMurray *et al.*, 2001).

Two major high-risk HPV oncoproteins, E6 and E7, induce malignant progression by targeting various cellular proteins involved in the regulation of apoptosis and cell cycle, causing immortalization and, eventually, cellular transformation (Figure 1) (Mantovani and Banks, 2001; Münger *et al.*, 2001). E7 appears to be largely responsible for driving cell proliferation in the early stages of malignant progression by targeting pRb and the related "pocket proteins", p107 nad p130 (Dyson *et* al., 1989; Imai *et al.*, 1991; reviewed in Münger *et al.*, 2004), while E6 enhances cell survival by targeting p53 (reviewed in Mantovani and Banks, 2001; Scheffner *et al.*, 1993) and is believed to contribute more towards the later, more malignant stages of the disease (Riley *et al.*,

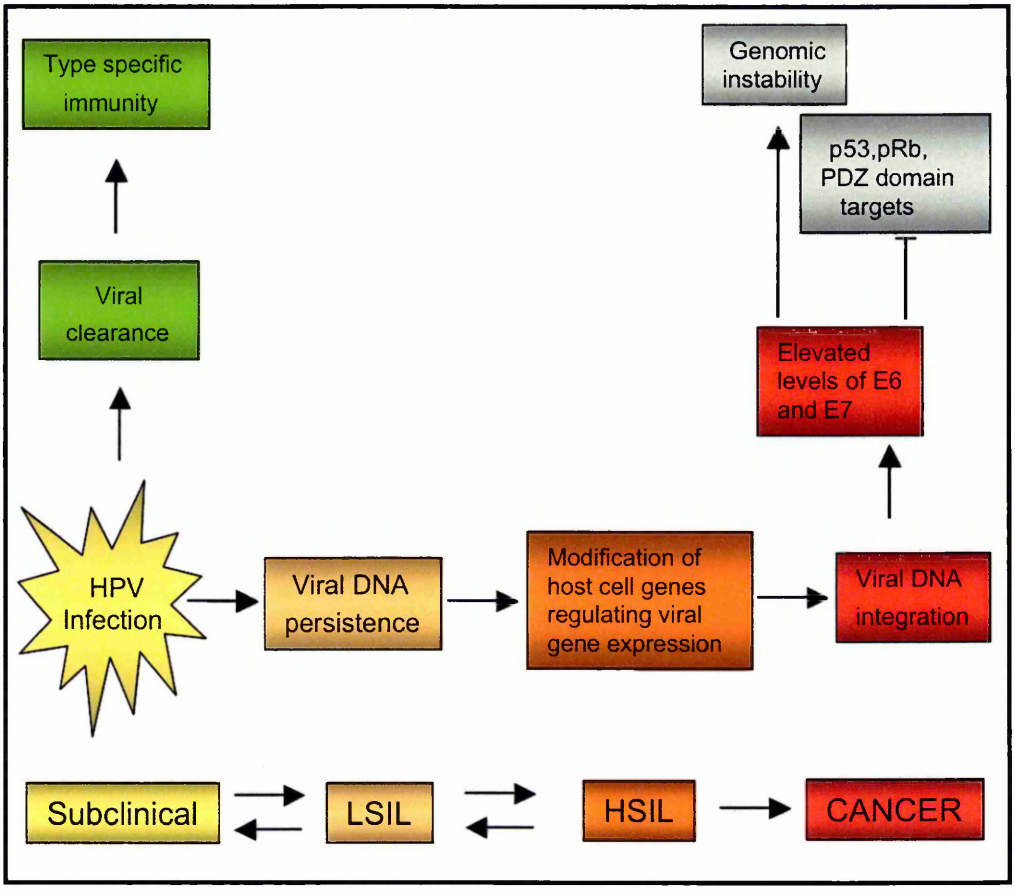


Figure 1. HPV and Cancer.
Malignant progression is closely related to the viral DNA integration. During the integration process E1, E2, E4, E5 ORFs are lost and expression of E6/E7 is upregulated. E6/E7 target cellular proteins such as p53/pRB and PDZ domain-containing proteins, which promotes cellular transformation. HSIL (high-grade squamous intraepithelial lesion); LSIL (low-grade squamous intraepithelial lesion).

2003, Song *et al.*, 2000). Interestingly, although HPV-negative cervical cancers are exceptionally rare, a few cases have been reported. HPV-negative cell lines derived from those patients were found to have p53 and pRb mutations, highlighting the requirement for inactivation of those cellular pathways in order for malignant progression to occur (Scheffner *et al.*, 1991). Furthermore, tissue culture and animal model experiments showed the necessity of E6 and E7 activities for cellular transformation and tumor development (Hawley-Nelson *et al.*, 1989; Matlashewski *et al.*, 1987; Riley *et al.*, 2003). In addition, in HPV-positive cells, inhibition of the expression E6 and E7 by the viral transcriptional regulator E2, or by siRNA and ribozyme approaches, results in an inhibition of cell growth and the induction of growth arrest and highlighting the importance of continued E6/E7 expression for maintenance of the transformed phenotype (Alvarez-Salas *et al.*, 1998; Goodwin and DiMaio, 2000; Yoshinouchi *et al.*, 2003). It is important to keep in mind that, besides p53 and pRb, E6 and E7 interfere with many other cellular activities, the disruption of which also contributes to malignant progression. Some of these pathways are summarized in Table 1 and will be discussed in more detail below.

Table 1. Various cellular activities regulated by E5, E6 and E7 oncoproteins

Viral Oncoprotein	Pathways affected	Reference
E6	Inhibition of apoptosis	Scheffner et al., 1990 Thomas and Banks, 1998
	Cell immortalization	Band et al., 1991
	Telomerase activation	Klingelhutz et al., 1996
	Cell junctions, cell adhesion and signaling	Lee et al., 1997 Huibregtse and Nakagawa, 2000
	Inteference with the immune system	Patel et al., 1999
E7	Unregulated cell growth	Arroyo et al., 1993
	Centrosome amplification	Duensing et al., 2001
	Chromosomal instability	Reznikoff et al., 1996
E5	Modulation of growth factor receptor signaling	Straight et al., 1993

The first evidence of E6 being an oncogene came from studies which demonstrated that E6 expression can lead to hyperproliferation of cells, loss of epithelial cell differentiation, and tumor formation. Early experiments reporting E6 transforming capacity were assays which showed that E6 can efficiently cooperate with an activated ras oncogene in the transformation of primary rodent cells (Pim *et al.*, 1994; Storey and Banks, 1993), while independently it can immortalize primary human mammary epithelial cells at late passage (Band *et al.*, 1991; Wazer *et al.*, 1995). In the case of primary human keratinocytes, the natural target cell of the virus, E6 only displays transforming capacity in the presence of E7, where both viral oncoproteins are required for cell immortalization (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989; Watanabe *et al.*, 1989). A particularly interesting feature of all these assays is that the ability to immortalize different cell types is exclusively restricted to high-risk HPV E6 and E7 proteins, while low-risk types have little or no immortalizing activity (Hawley-Nelson *et al.*, 1989; Schlegel *et al.*, 1988). It is also important to point out that immortalized keratinocytes will not initially cause tumor formation in nude mice. Only after long term passage or in cooperation with other activated oncogenes, do these immortalized keratinocytes become fully transformed (DiPaolo *et al.*, 1989; Hurlin *et al.*, 1991). This observation reflects the process of HPV-induced malignancy *in vivo,* where the multi-step process between the initial infection and cancer development takes many years.

As already mentioned E6 and E7 cause cellular immortalization and transformation primarily by targeting their major substrates p53 and pRb. However, each of the viral oncoproteins needs to interact with other proteins besides p53 and pRb in order to achieve optimal effect in cellular transformation. A good example of this came from tissue

culture experiments which have shown that the PDZ binding capacity of E6 is important for its ability to induce cellular transformation. Kiyono and colleagues showed that HPV-16 E6 had PDZ-binding potential and that an intact PDZ binding motif was important for the ability of HPV-16 E6 to induce morphological transformation in rat 3Y1 cells (Kiyono *et al.*, 1997). The study showed that the motif was also necessary for the ability of HPV-16 E6 to confer tumorigenicity upon a mouse fibroblast cell line. Interestingly, the HPV-31 whole-genome studies show that E6 PDZ target binding does not seem to appear to be required for the immortalization activity of the virus, but it is required for increased cell proliferation and episomal maintenance (Lee and Laimins, 2004). However, in a different keratinocyte-transformation assay it was found that the HPV-16 E6 PDZ-binding motif was required for the induction of anchorage-independent growth in cooperation with an activated ras oncoprotein, both in murine and human tonsillar keratinocyte cells (Spanos *et al.*, 2008a; Spanos *et al.*, 2008b).

Transgenic mouse models have proved to be extremely useful in assaying the relative contributions of E6 and E7 to malignant progression. Initial studies in transgenic mice have shown that animals expressing the oncogenes developed neuroepithelial tumors, and at 10 months of age 71% were dead from brain tumors (Arbeit *et al.*, 1993); while in transgenic mice where E6/E7 expression was targeted to the ocular lens, the animals developed impaired lens fiber cell differentiation accompanied by increased cell proliferation (Arbeit *et al.*, 1993). In addition, when lens cells from those mice were placed in tissue culture they became immortalized and developed a tumorgenic phenotype after continuous passage (Griep *et al.*, 1993). Further studies used a model which more accurately represents the process of HPV-induced malignancy. This model is based on the transgenic mice

containing hK14HPV16 E6/E7 transgenes, in which expression of the oncoproteins is directed from the human keratin 14 (K14) promoter, a promoter which is restricted in its activity to the *stratum basale*. Use of this promoter restricts E6/E7 expression to the cell type thought to be the precursor for subsequent HPV-induced malignancy. Mice expressing the entire HPV16 early region under the K14 promoter showed hyperplasia, papillomatosis, and dysplasia at multiple epidermal and squamous mucosal sites (Arbeit *et al.*, 1994). Moreover, both E6 and E7 were found to be able to induce epithelial hyperplasia and skin tumors when individually expressed (Herber *et al.*, 1996; Song *et al.*, 1999), but interestingly, tumors caused by E7 were benign and highly differentiated, whereas those tumors caused by E6 had a more malignant phenotype (Simonson *et al.*, 2005; Song *et al.*, 2000). In contrast, in transgenic mouse models of cervical cancer it was found that E7 increased proliferation and centrosome copy number and induced progression of multifocal microinvasive cervical cancers, while E6 elevated centrosome copy number and eliminated detectable p53 protein, but did not produce neoplasia or cancer. Importantly, the combination of both oncoproteins resulted in increased centrosome numbers and large, extensively invasive, cancers (Riley *et al.*, 2003). Similar observations were seen in models of head and neck squamous cell cancers (HNSCC). In these E7 was shown to be the major transforming oncoprotein, whereas E6 appears to be more likely to play a secondary role in contributing to the later stages of malignancy (Strati and Lambert, 2007). In addition, in studies using co-carcinogen assays on transgenic mice treated with specific carcinogens known to affect different stages of tumor formation, E7 was found to be a strong driver of tumor progression, while E6 contributed weakly to this stage of malignancy. However, E6 contributed more strongly during tumor

development, enhancing the malignant conversion of benign tumors (Song *et al.*, 2000).

These transgenic mouse models also showed that the cooperative effects of E6 and E7 are increased in the presence of estrogen, which plays a crucial role not only in the genesis of cervical cancer but also in its persistence and continued development (Brake and Lambert, 2005; Shai *et al.*, 2008). These studies also support the hypothesis that both oncoproteins need to cooperate in order for malignant progression to occur and that each protein is responsible for different functions during that process. These include both p53- and pRb-dependent and independent mechanisms (Balsitis *et al.*, 2006; Nguyen *et al.*, 2002; Shai *et al.*, 2007b).

In the case of E7 transgenic mice, studies that compared the effects of abolishing pRb expression in the epidermis with the effects of E7 expression in the same tissue in young animals showed few differences. However, when both E7 was expressed and Rb was deleted in the same tissue, increased hyperplasia and dysplasia were observed. These studies have shown that pRb inactivation is largely responsible for E7's phenotypes at an early age, but also that pRb-independent activities of E7 are detectable *in vivo* (Balsitis *et al.*, 2003). Further studies done in knock-in mice expressing 16 E7 and a mutant form of pRb (pRb(ΔLXCXE) showed that pRb inactivation was necessary, but not sufficient, for E7 to induce DNA synthesis and to overcome differentiation-dependent cell cycle withdrawal and DNA damage-induced cell cycle arrest (Balsitis *et al.*, 2006). Expression patterns of the E2F-responsive genes Mcm7 and cyclin E indicated a necessity for E7 to interact with other substrates besides pRb for its optimal transforming activity. Interestingly, E7-induced p21 upregulation was

also shown to be pRb independent (Balsitis *et al.*, 2005; Balsitis *et al.*, 2006).

In the case of E6, mutational studies in transgenic mouse models have shown that p53-null mice do not develop epidermal hyperproliferation, nor do they respond to tumor promoters as the K14 E6 mice do, demonstrating the existence of p53-independent activities of E6 (Song *et al.*, 1999). It was also shown that transgenic mice expressing a mutant of E6, $E6^{I128T}$, which is defective for binding at least a subset of the α-helix partners, including E6AP, lacked the ability to alter the radiation-induced block to DNA synthesis and to promote the formation of benign skin tumors in cooperation with chemical carcinogens. In addition, these mice showed reduced levels of skin hyperplasia, fever spontaneous skin tumors, and lower tumor progression activity, in comparison with the wild type K14 E6 mice (Nguyen *et al.*, 2002). Further studies dissecting the E6 degradatory pathways were carried out in an E6AP-null mouse background. Interestingly, in those mice it was found that E6, in the absence of E6AP, retains the ability to induce epithelial hyperplasia, to abrogate DNA damage response and to inhibit the induction of p53 protein following exposure to ionizing radiation (Shai *et al.*, 2007a). In addition, mice expressing an E6 (Δ146-151) PDZ deletion mutant in stratified squamous epithelia, but which were still able to degrade p53, failed to display epithelial hyperplasia, but retained the ability to contribute to the progression stage of malignancy (Nguyen *et al.*, 2003a; Simonson *et al.*, 2005). This observation was supported by similar experiments in the mouse lens epithelium where the intact PDZ binding motif of E6 was responsible for increased proliferation and defects in cellular adhesion and differentiation (Nguyen *et al.*, 2003b).

Perhaps the most relevant animal model for studying PV and cervical cancers are Rhesus macaques (*Maccaca mulatta*), as they are the only species except humans in which the naturally occuring mucosal PVs cause cervical neoplasia (Wood *et al.*, 2007). Rhesus papillomavirus type 1 (RhPV-1) is closely related to HPV-16 (Ostrow *et al.*, 1990): its sexual transmission and disease development resemble high-risk HPV infection in all major characteristics. However, unlike HPV-16 and HPV-18 E6, the RhPV-1 E6 protein has no PDZ-binding motif. Interestingly the virus does express a PDZ-binding motif, but on the carboxy terminus of E7 rather than E6, as is invariably found in the high-risk mucosal HPVs (Tomaić *et al.*, 2009; This thesis). The motif (A-S-R-V) corresponds to the canonical class I PDZ binding consensus sequence (X-T/S-X-L/V) (Figure 6A and 6B). This observation suggests that a PDZ-binding motif on at least one of the oncoproteins is required by high-risk PVs, such as RhPV-1, to allow the virus to interfere with those PDZ-containing proteins whose inactivation is necessary to establish an infection in the host cell.

E5 is the third oncogene encoded by the high-risk HPV types, and its transforming capacity is manifested largely through modulation of growth factor receptor regulation; for example EGFR phosphorylation and turnover (Straight *et al.*, 1993). Its attribution as an oncogene comes from tissue culture studies showing that HPV-16 E5 transforms murine fibroblasts and keratinocytes (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Straight *et al.*, 1993), enhances the immortalization capacity of E6/E7 (Stoppler *et al.*, 1996), and, in cooperation with E7, stimulates the proliferation of human and mouse primary cells (Bouvard *et al.*, 1994; Valle and Banks, 1995). In addition, recent studies in transgenic mice showed that HPV-16 E5 alters the growth and differentiation of stratified epithelia and induces epithelial tumors at a high frequency

(Genther Williams *et al.*, 2005; Maufort *et al.*, 2007). However, E5 is frequently lost during the development of cervical cancers, suggesting that it is not required for the later stages of malignant progression. Nonetheless, the above cellular and animal models would suggest a role for E5 in the early stages of malignant development in human cancers.

The Virus

HPV Genome

Papillomaviruses (PVs) belong to a group of small non-enveloped DNA tumor viruses with approximately 55 nm diameter virions, which fully replicate and assemble in the nucleus of the infected cell. Today more than one hundred different PV types have been characterized, infecting various animals from birds to mammals (Zheng and Baker, 2006). All HPVs have a double-stranded circular DNA genome which is associated with cellular histones to form chromatin-like structures (Favre *et al.*, 1997). As shown in Figure 2 the genomes of all HPV types contain, on average, eight major open reading frames (ORFs) and these are expressed from polycistronic mRNAs transcribed from the same DNA strand. Regulatory sequences required for viral replication and transcription are concentrated in a noncoding region, termed the upstream regulatory region (URR) or long control region (LCR). Transcription factor binding sites in this region include those for TFIID binding to canonical TATA boxes, and additional binding sites include those for Sp-1 and AP-1, NF-1, TEF-1, TEF-2, Oct-1, AP-2, KRF-1, and YY1, as well as for glucocorticoid responsive elements (Butz and Hoppe-Seyler, 1993; Chang and Laimins, 2000; del Mar Pena and Laimins, 2001; Gloss *et al.*, 1987; Ishiji *et al.*, 1992; Kyo *et al.*, 1997). In high-risk

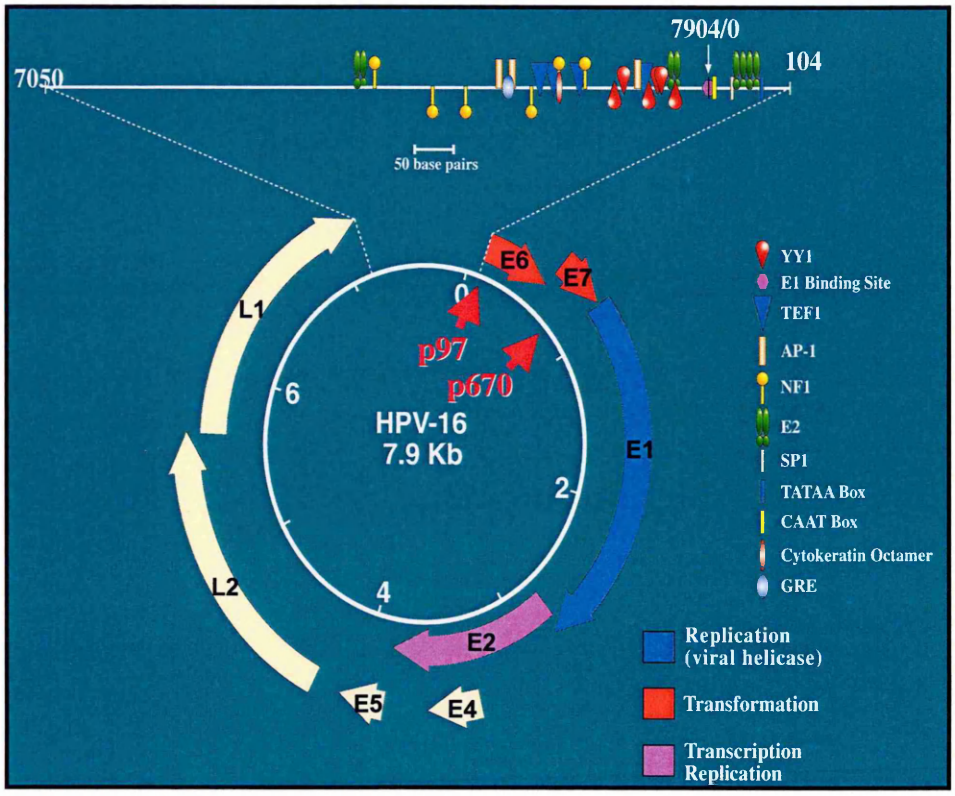


Figure 2. HPV-16 Genome

The early coding regions (indicated with letter E and corresponding numbers), the late coding regions (indicated with letter L and corresponding numbers) and Long Control Region (LCR) (7050-104) are shown. The early (p97) and the late (p670) promoter are indicated by arrows. Proteins involved in different viral functions are indicated in blue, red and violet colors. In the enlarged LCR numerous viral binding sites are shown.

HPVs transcription is initiated at two major promoters termed, the *early* and the *late*. The *early* promoter, which initiates upstream of the E6 open reading frame, is responsible for the expression of the early (E) proteins. This promoter is expressed prior to reproductive replication. In HPV -16 and -31 this promoter is termed p97, while in HPV -18 it is referred to as p105 (reviewed in Longworth and Laimins, 2004). Immediately prior to the initiation of virion synthesis, the other major promoter, the *late* promoter (L), is activated. This promoter is responsible for expression of the late (L) proteins and it has several initiation sites that map to sequences within the E7 ORF (Grassmann *et al.*, 1996; Ozbun and Meyers, 1998).

HPV Life Cycle

The life cycle of HPV is linked to the differentiation program of the infected keratinocytes, with mature virion production being restricted to the differentiated suprabasal cells. The initial HPV infection is speculated to occur through micro-ruptures in the epithelium, resulting in infection of undifferentiated stem cells or transiently-amplifiying cells located in the lower layers of stratified epithelium (Pyeon *et al.*, 2009; Stubenrauch and Laimins, 1999). However, the mechanism of viral entry is still not completely understood. Interestingly, besides their natural target keratinocytes, HPVs can infect monolayers of various cell types, and the infection is thought to be mediated through a number of host cell molecules which could potentially serve as receptors (Barnard and McMillan, 1999; Evander *et al.*, 1997). Furthermore, recent studies have also reported cell cycle progression through mitosis to be crucial for HPV infection to take place (Pyeon *et al.*, 2009). Only the basal cell compartment of the epithelium contains cells progressing through the

cell cycle, and therefore this could be one of the reasons why HPVs can establish their infection only in these cells (Pyeon *et al.*, 2009). Infection of the cells in the basal layer is continued by the activation of viral gene expression which results in the production of approximately 20 to 100 episomal copies of the viral DNA per cell. These episomes are stably maintained in the undifferentiated basal layer during the viral infection (De Geest *et al.*, 1993; Sterling *et al.*, 1990).

E1 and E2, proteins involved in viral replication, are among the first viral proteins to be expressed (Longworth and Laimins, 2004). These two proteins form a complex which then binds to the sequences at the viral origin of replication in order to recruit cellular DNA polymerase (Conger *et al.*, 1999; Frattini *et al.*, 1997). The E1, a 70 kD nuclear protein, encoded by the largest ORF, is very well conserved among the PVs especially in the C-terminal half of the protein (Danos *et al.*, 1983). The protein acts as a helicase by mediating the separation of the viral DNA strands ahead of the replication fork (Hughes and Romanos, 1993). However, E1 by itself has only weak DNA-binding activity which is enhanced by the viral E2 protein (Dixon *et al.*, 2000; Sedman *et al.*, 1997). HPV-31 studies have suggested that the primary positive role of E2 in the viral life cycle is its function as a replication factor since a modest reduction in late gene expression was observed in the mutant lines which retained replication activities, but were transactivation defective. (Stubenrauch *et al.*, 1998). It is also important to point out that intact E1 and E2 ORFs were found to be required for stable maintenance of the BPV1 extrachromosomal DNA in infected cells (Sarver *et al.*, 1984). Loss of E1 and E2 could eventually lead to viral DNA integration, and multiple studies have shown that the viral DNA integration, which leads to uncontrolled expression of E6 and E7 proteins, represents one of the initial stages in the series of events

leading to malignant development associated with papillomavirus infections (Jeon *et al.*, 1995; Song *et al.*, 2000). In addition to the replication of viral DNA, E2 is also involved in transcriptional regulation. It is a site-specific DNA binding protein with its binding site positioned next to cellular transcription factor binding sites which regulate the early promoter (Stubenrauch *et al.*, 1998). High levels of E2 repress the early promoter by blocking the binding of cellular transcription factors, conversely, low levels of E2 activate the early promoter (Steger and Corbach, 1997). This ability allows E2 to have an indirect inhibitory effect on cell proliferation through its effect on the expression on E6 and E7 (Francis *et al.*, 2000).

Normal squamous epithelial cells grow as stratified epithelium, with those in the basal layers dividing as stem cells or transient-amplifiying cells. After division, one of the daughter cells migrates towards the upper layers and begins to undergo terminal differentiation while the other remains in the basal layer as a slow-cycling, self-renewing population (Figure 3) (Watt, 1998). In HPV-free epithelia, when cells leave the basal layer they exit the cell cycle and during this process of terminal differentiation they lose their nuclei, but only in the upper layers. Therefore, in order to establish an infection HPVs need to redirect this process so that viral DNA can be replicated. The virus accomplishes this mainly through the viral oncoproteins, E7 and E6, which are expressed from the early stages of infection onwards. E7 targets pRb family proteins to assure cell cycle progression, whilst E6 prevents growth arrest and apopototic response by targeting p53 (Dyson *et al.*, 1989; Scheffner *et al.*, 1990). Additionally, E7 promotes cell cycle progression and transcription by abolishing the activities of histone deacetylases (Brehm *et al.*, 1999) and the cyclin-dependent kinase inhibitors p21 and p27 (Funk *et al.*, 1997; Jones *et al.*, 1997; Zerfass-

Thome *et al.*, 1996). Furthermore, raft culture studies have shown that mutations in the CKII phosphorylation site on HPV-18 E7 reduce its ability to drive S-phase entry (Chien *et al.*, 2000). Infected cells divide in the basal layer of the epithelium, and as they divide the viral genomes are segregated in the daughter cells. As shown in Figure 3, some of those cells then start to migrate from the basal layer and begin to differentiate. Due to the activity of E7, infected cells remain in the cell cycle after they leave the basal layer (Chen *et al.*, 1995), they reenter the S phase in a highly differentiated cell population, and they initiate expression of cellular replication factors necessary for viral replication. Cells in this phase continue to retain nuclei. Moreover, the inactivation of p53/pRb pathways by E6 and E7 is necessary for the maintenance of the episomal viral copies in this differentiated cell population (Park and Androphy, 2002; Thomas *et al.*, 1999). In addition, recent studies have also shown that, in the case of HPV-31, the thickening of the basal epithelial layer and the presence of nuclei throughout the suprabasal layers, which are normally seen in HPV infections, are lost upon the introduction of PDZ-binding defective mutants of E6 (Lee and Laimins, 2004). Furthermore, the growth rate of the mutant E6 virus-infected cells is reduced in comparison with the wild type virus-infected cells and, interestingly, the number of episomal DNA copies decreases and the frequency of viral genome integration events increases (Lee and Laimins, 2004).

The other early proteins expressed by HPVs are E4 and E5. E4 localizes in the cytoplasm and it is a highly insoluble protein, in part due to its association with keratins (Doorbar *et al.*, 1991). It is probably involved in regulating late viral functions, although additional studies need to be performed for its functions to be completely understood (Peh *et al.*, 2004). E4 can actually be considered to be primarily a late protein, as it

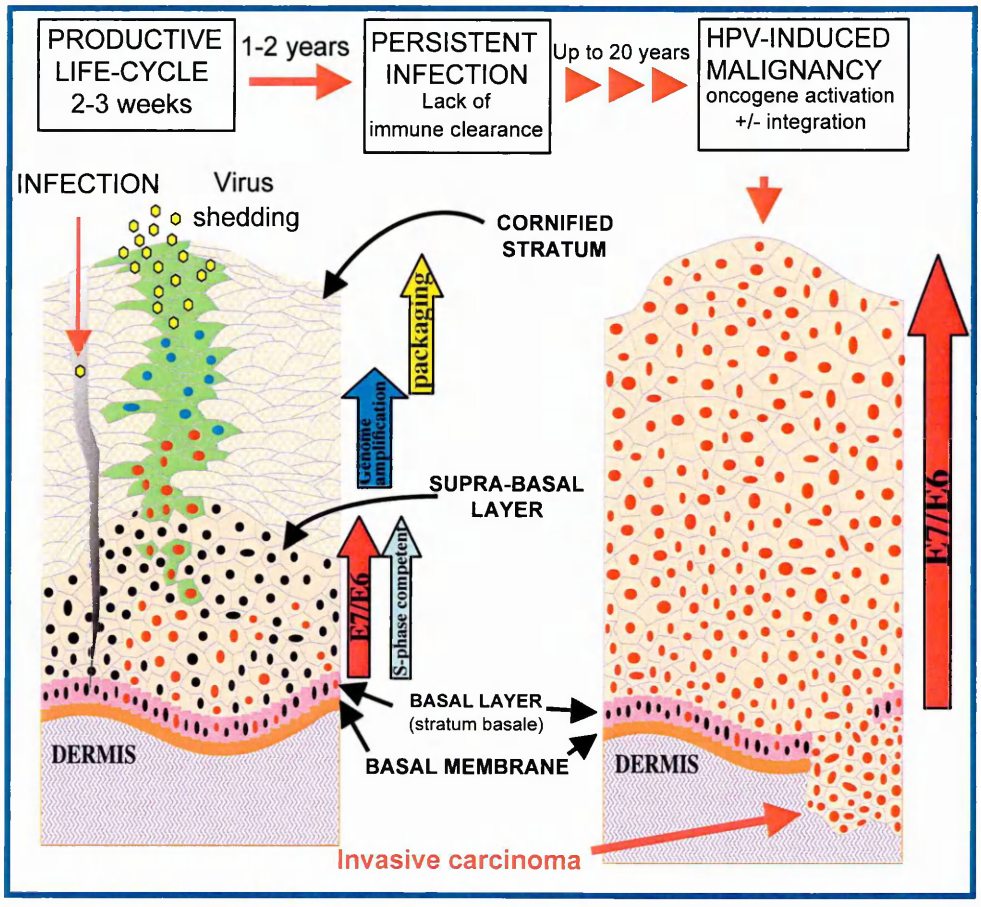


Figure 3. From HPV life cycle to malignancy.
The left-hand panel shows the normal viral life cycle in infected epithelium. E6 and E7 expression promotes S-phase induction which leads to viral genome amplification and finally to the synthesis and shedding of new viral particles. In contrast, persistent infection over a long period can lead to viral DNA integration which forms the basis for malignant progression. This is characterized by a loss of differentiation, no viral replication, and upregulated E6 and E7 expression. Adapted from Doorbar (2006).

is expressed after the activation of the differentiation-dependent promoter upon differentiation of infected keratinocytes (Chow *et al.*, 1987; Nasseri *et al.*, 1987), and it has been shown to cause collapse of the cytokeratin matrix which might contribute to virion release (Doorbar *et al.*, 1991). E4 is also involved in induction of a G2 arrest which is associated with cytoplasmic retention of active Cdk1/CyclinB1 complexes; this could play a role in creating an environment suitable for viral DNA replication (Davy *et al.*, 2006). The significance of E4 in the viral life cycle was demonstrated by studies showing that viruses lacking E4 were significantly weakened in vegetative replication and late gene expression, which could ultimately result in decreased virion production (Peh *et al.*, 2004; Wilson *et al.*, 2005).

The E5 protein is highly hydrophobic and interacts with the 16-kDa subunit of the vacuolar H^+-ATPase proton pump responsible for acidifying cellular organelles such as the Golgi apparatus (Conrad *et al.*, 1993). In addition it interacts with the endoplasmic reticulum and nuclear membrane in host cells, and it is thought that it contributes to the early stages of infection during neoplastic proliferation and the productive stage of the viral life cycle, as shown in HPV-16 and -31 studies (Genther *et al.*, 2003; Goldstein *et al.*, 1992; Fehrmann *et al.*, 2003; Valle and Banks, 1995). HPV-31 E5 mutant genomes showed a dramatic reduction in colony-forming ability following methylcellulose-induced differentiation (Fehrmann *et al.*, 2003). Furthermore, HPV-16 and -31 E5 mutated genomes showed decreased abilities to amplify viral genomes and activate late gene expression (Genther *et al.*, 2003; Fehrmann *et al.*, 2003). Additionally, several reports have shown epidermal growth factor (EGFR) to be a crucial component in the activities of E5. In mouse fibroblasts and in the presence of EGF, E5 was found to increase cellular proliferation (Leechanachai *et al.*, 1992).

E5 can also stimulate viral gene expression through AP-1 and NF-1 (Chen *et al.*, 1996), since numerous binding sites of AP-1 and NF-1 are found in the regulatory region of HPV-16 DNA (Burkhardt *et al.*, 1987).

The viral capsid proteins L1 and L2 are produced late in infection, after viral genome amplification, and spontaneously form icosahedral capsids *in vitro*. L1 protein is directed to the nucleus by its C-terminal nuclear localization signal (Zhou *et al.*, 1991) and the virus assembly occurs in the nucleus (Zhou *et al.*, 1993). L1 was also shown to be required for incorporation of DNA into papillomavirus capsids (Touze *et al.*, 2000), and its physical interaction with DNA is essential for DNA inclusion into virion-like particles (VLPs) (Schäfer *et al.*, 2002). After virion formation, mature viruses are released from the epithelial surface as shown in Figure 3 (Peh *et al.*, 2002). L1, the major viral capsid protein, can spontaneously assemble into VLPs (Kirnbauer *et al.*, 1992), while L2, the minor capsid protein, is responsible for proper encapsidation of the viral genomes (Roden *et al.*, 1996). This ability of L1 protein to assemble into VLPs provides the basis for vaccine development against HPVs (Kirnbauer *et al.*, 1992).

HPV Oncoprotein Function

Not every individual infected by high-risk HPV types will develop cervical cancer. Only a small proportion of the infected population whose immune system fails to clear the infection, and which then remains for years, will be likely to develop cervical cancer. In these instances, viral DNA often gets integrated into the host genome, resulting in the loss of E1, E2, E4, and E5 sequences, followed by uncontrolled expression of E6 and E7 oncoproteins leading to the

disruption of normal viral life cycle and malignant development as an accidental side-effect (reviewed in Doorbar, 2005). Many proteins have been identified as interacting partners of E6 and E7, however, to date, the biological consequences of the interactions have been correlated directly with only a limited number of the identified targets. Therefore, in the following section I will focus mostly on the E6/E7 oncoproteins and their interacting partners with which biological consequences have been correlated.

HPV E7 Protein

HPV E7 is 98 amino acids in length, and contains a zinc-binding domain in the C-terminus of the protein, whose structural integrity is essential for the activity of E7 (McIntyre *et al.*, 1993; Rawls *et al.*, 1990). The protein is divided into three conserved domains termed CD1, CD2 and CD3. CD1 and CD2 correspond to small parts of conserved regions 1 and 2 (CR1 and CR2) of Adenovirus E1a, while CD2 is homologous to SV40 large T antigen. Although the overall integrity of the protein is crucial for E7's optimal interaction with cellular partners, the majority of the characterized activities of E7 are mediated through the CD2 and CD3 domains (Patrick *et al.*, 1994).

E7 is post-transcriptionally modified by the proteasome and by phosphorylation. It interacts with the Skp-Cullin-F box (SCF) ubiquitin ligase complex, which results in increased ubiquitination of the protein (Oh *et al.*, 2004). It has also been shown that Casein Kinase II (CKII) phosphorylates E7 (Barbosa *et al.*, 1990; Firzlaff *et al.*, 1989) on its N-terminal domain, which is crucial for its transformational capacity. Further studies have identified an additional phosphorylation site

located on the C-terminal half of the protein; this appears to be phosphorylated primarily during S phase, although the kinase involved in this phosphorylation event still needs to be identified (Massimi and Banks, 2000).

Conserved Domains of E7

The first 20 amino acids of the N-terminus, also referred to as the CD1 domain, are very important for E7's transforming capacity (Banks *et al.*, 1990; Brokaw *et al.*, 1994). Mutations introduced in this domain show that it is required for E7's ability to induce S-phase progression and cell transformation (Demers *et al.*, 1996). The most important known binding partners of E7 reported to interact with this domain include p600 (Huh *et al.*, 2005) and p300/CBP-associated factor (P/CAF) (Huang and McCance, 2002). p600 is crucial for anchorage-independent cell growth in both HPV-negative and HPV-positive cells, while P/CAF is involved in transcriptional activation of NF-κB family members. Interaction between E7 and p600 is thought to be required for E7-mediated cell transformation (DeMasi *et al.*, 2005), however its exact role in E7's activities still need to be identified. However, the consequence of the interaction of E7 with P/CAF seems to more clear-out: E7 expression has been observed to down-regulate the P/CAF-mediated activation of the NF-κB family members, which occurs during viral infection, resulting in escape from the immune response (Huang and McCance, 2002).

Amino acid residues from 20 to 38 of E7, also referred to as the CD2 domain, contain the CKII phosphorylation site and the LXCXE binding motif involved in binding of the pocket proteins such as pRb. The CKII

phospho-acceptor site is important for E7's transforming capacity (Barbosa *et al.*, 1990; Firzlaff *et al.*, 1991) and for its ability to drive S-phase progression (Chien *et al.*, 2000), while the interaction with the pocket proteins has been implicated as one of the major functions of E7, and will be discussed in more detail below.

Amino acid residues 38-98 comprise the CD3 region located, on the C-terminus of the protein, and contain four highly conserved cysteine residues. This region of E7 is involved in interactions with several binding partners: it interacts with p21 and p27, inhibitors of CDKs, which E7 sequesters in the cytoplasm as a necessary step in inducing cell cycle progression and overcoming DNA damage-induced cell cycle arrest, although other regions of E7 were also shown to be important for interactions with these proteins (Jones *et al.*, 1997; Helt and Galloway, 2001). E7 also induces protein kinase B (PKB) phosphorylation of p21, and thus abolishes its nuclear activities (Westbrook *et al.*, 2002). This domain also interacts with the Mi2ß component of the histone deacetylase (HDAC) complex (Brehm *et al.*, 1999). E7 inhibits the activity of the HDAC complex, which can lead to upregulation of the E2F gene through acetylation of the E2F promoter (Zhang *et al.*, 2004), resulting in cell cycle progression. Furthermore, this region of E7 was also shown to be involved in interaction with TBP (Massimi *et al.*, 1997), with TBP associated factors (Mazzarelli *et al.*, 1995), with M2 pyruvate kinase (Zwerschke *et al.*, 1999), with AP-1 transcription factor family members, such as c-Jun (Antinore *et al.*, 1996), and DNA methyltransferase (Burgers *et al.*, 2007). Importantly, through its C-terminus, E7 interacts with the hTid-1 protein, a member of the DnaJ-family of chaperones. The large tumor antigens of polyomaviruses, including SV40, also encode functional J-domains that are important for viral replication, as well as for cellular transformation. Therefore the

ability of E7 to interact with a cellular DnaJ protein suggests that these two viral oncoproteins may target common regulatory pathways through J-domains (Schilling *et al.*, 1998). In HPV-positive cell lines, E7 localizes in the cytoplasm and the nucleus (Oh *et al.*, 2004; Sato *et al.*, 1989), while in overexpression assays (Guccione *et al.*, 2002) and in epithelial raft cultures (Middleton *et al.*, 2003) it is reported to be predominantly nuclear. Since no NLS has so far been identified in E7, the mechanism of its nuclear localization still needs to be elucidated. In addition to its major role in promoting cell cycle progression, the interactions of E7 with the cellular proteins mentioned above also suggests that E7 has a crucial role in destabilizing of transcriptional complexes and in chromatin remodeling, consequently affecting cellular proliferation.

E7 and Pocket Proteins

The pRb proteins play a crucial role in cell cycle regulation, promoting the transition from G1 to S phase. Under normal circumstances, pRb is unphosphorylated in the early G1 phase and becomes progressively more phosphorylated towards the S phase. The unphosphorylated form of pRb binds to the E2F transcriptional factors and represses transcription from promoters containing E2F sites (Dyson *et al.*, 1989). E2F family members are responsible for transcriptional regulation, in a cell-cycle dependent manner, of many genes involved in DNA synthesis (Slansky and Farnham, 1996). In HPV infected cells the unphosphorylated form of pRb is targeted by E7 through its LXCXE motif (Imai *et al.*, 1991) and subjected to proteasome-mediated degradation (Boyer *et al.*, 1996). The disruption of the pRb-E2F complex results in a release of free E2F, further resulting in E2F-induced

transcription leading to upregulation of CDK2 and cyclins A and E. This activity of E7 is considered to be crucial for driving cell cycle progression in differentiating epithelial cells, thus establishing an environment suitable for viral DNA replication.

Importantly, in addition to the pRb interaction, E7 also binds to the other pocket proteins p107 and p130. These proteins have been shown to have well-established roles in numerous tissue types for the regulation of cellular proliferation, differentiation and apoptosis through interaction with different molecules (reviewed in Morris and Dyson, 2001). They inhibit E2F-mediated transcription and negatively regulate the transition from G0 through G1 into the S phase of the cell cycle (Davies *et al.*, 1993). p107 is mostly expressed in proliferating cells and inhibits E2F4 (Ginsberg *et al.*, 1994), while p130 is predominantly found in non-proliferating cells and inhibits E2F5 activity (Hijmans *et al.*, 1995). The same LXCXE motif of the CD2 domain of E7 required for pRb destabilization is also required for down-regulating p107 and p130 (Helt and Galloway, 2001). This again highlights the importance of E7's interaction with the pocket proteins for its optimal ability to drive the cell cycle progression.

E7 and Cell Signaling

E7 is involved in the regulation of several signaling pathways. It has been shown that E7 interferes with the response to the insulin-like growth factor (IGF-1) signaling pathway by binding to IGF-binding protein-3 (IGEBP-3), a protein which can block proliferation in response to IGF-1. Thus, by interfering with IGEBP-3, E7 prevents IGEBP-3's pro-apoptotic properties in response to IGF-1 (Mannhardt *et al.*, 2000).

Additionally, studies have shown E7 upregulating Protein kinase B (PKB) or Akt activity, a protein associated with cellular proliferation and survival (Menges *et al.*, 2006). After being phosphorylated Akt promotes phosphorylation of its downstream targets such as BAD, p21, and BRCA-1, resulting in their nuclear export (Hanada *et al.*, 2004). Interestingly, it has been shown that E7 interacts with the Akt signaling pathway, interacting with PP2A subunits and inhibiting their interaction with Akt, thereby maintaining Akt signaling by inhibiting its phosphorylation (Pim *et al.*, 2005). Other reports suggest a link between inactivation of pRb by E7 and the up-regulation of Akt activity during cervical cancer progression (Menges *et al.*, 2006).

Furthermore, recent studies have reported E7 interacting with Steroid Receptor Coactivator (SRC-1), which is involved in hormone-dependent gene expression (Baldwin *et al.*, 2006). In the presence of E7, SRC-1 is also retained in the cytoplasm and this causes down-regulation of SRC-1 transcriptional activation, which prevents it from interacting with p300 and P/CAF (Baldwin *et al.*, 2006). These observations could provide better understanding of the molecular mechanisms by which steroid hormones act as cofactors in the induction and progression of cervical neoplasia.

E7 and Centrosomal Abnormalities

Centrosomes are the major microtubule organizing centers in the majority of animal and human cells (Bornens, 2002). The single centrosome duplication occurs exactly once before mitosis takes place, through mechanisms that are still not fully understood. Under normal circumstances, each of the two centrioles that make up a G1 phase

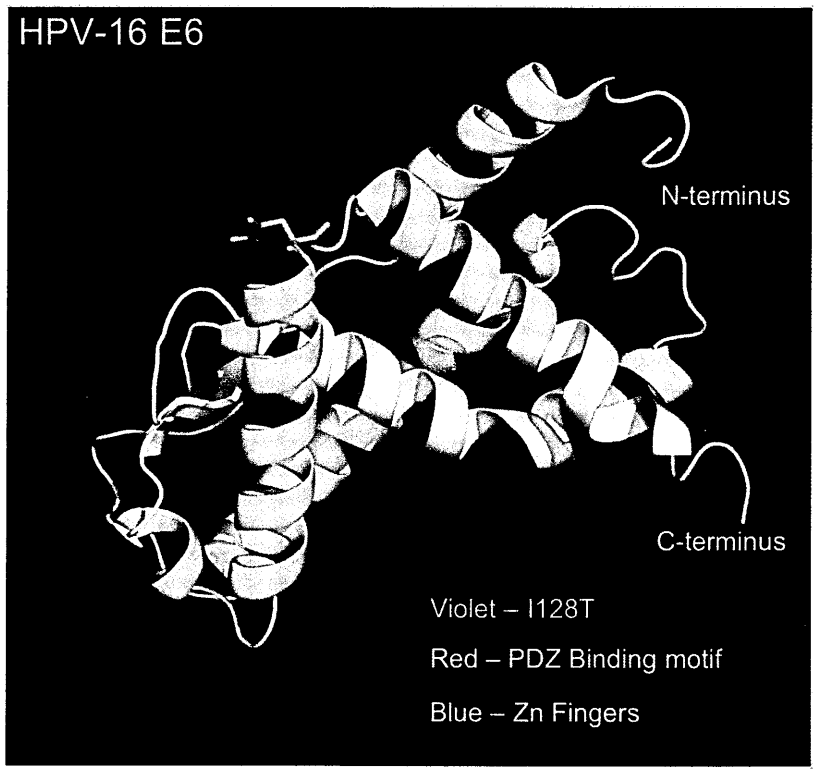
centrosome functions as a template for the formation of exactly one newly synthesized daughter centriole (Sluder and Nordberg, 2004). In order to prevent aberrant centrosome numbers, multipolar mitoses and chromosomal instability (Nigg, 2002; Salisbury *et al.*, 1999), cells go through a single round of centriole duplication per cell division (Tsou and Stearns, 2006). In contrast, tumor cells frequently have abnormal centrosome numbers (Lingle *et al.*, 1998). It is thought that aberrant centrosome numbers can arise through cell division failure or thorugh a genuine disruption of the centriole duplication cycle itself (Duensing, 2005). Additionally, many oncogenic stimuli have been related to induction of abnormal centrosome and centriole numbers *in vitro*, however not much is known about the precise mechanism.

As mentioned previously, both HPV E6 and E7 oncoproteins can independently cause centrosomal abnormalities when stably expressed in cell lines and in mouse models (Duensing and Münger, 2002; Riley *et al.*, 2003). However, in overexpression systems in primary human cells and tumor cell lines only E7 has been found to rapidly stimulate increased centrosomal numbers suggesting its direct effect in this process (Duensing *et al.*, 2001). It is possible that the effects of E6 on the centrosomal abnormalities might not be direct, but may occur as a consequence of interfering with the p53 pathway (Shinmura *et al.*, 2007). Interestingly, in cells expressing HPV16-E7, centrosomal abnormalities occur even before progression to malignancy (Duensing *et al.*, 2001). The mechanism by which E7 causes this still needs to be elucidated, however studies have shown that it is pRb independent (Duensing and Münger, 2003), implying the possibility of a direct effect of E7 on centrosome overduplication. More recent studies have suggested that one possible mechanism for E7 inducing centrosomal abnormalities could be by its inducing CDK activities where it was

shown that cyclin/CDK2 activity is critically involved in the abnormal centrosome duplication induced by HPV-16 E7 oncoprotein expression (Duensing *et al.*, 2004). In addition, it was also reported that continuing RNA pol II-mediated gene transcription is required for HPV-16 E7 induced centriole overduplication, whereas those activities may be not required for normal centriole duplication and cell cycle progression (Duensing *et al.*, 2007).

HPV E6 Protein

The HPV E6 protein is approximately 150 amino acids in length and has two zinc fingers characterized by the CXXC motif (Figure 4) (Barbosa and Wettstein, 1987; Cole and Danos, 1987). Furthermore, Cole and Danos speculate that E6 and E7 may have originated by duplication of a 33 amino acid unit containing a double cysteine, which displays a structure compatible with nucleic acid binding activity (Cole and Danos, 1987). These CXXC motifs are strictly conserved in all characterized E6 proteins and their integrity is indispensable for E6's normal function (Kanda *et al.*, 1991; Sherman and Schlegel, 1996). E6 is a relatively short protein and is difficult to isolate in a native, soluble form. The protein has a high content of α-helical and ß-sheet secondary structures and these features, together with the high cysteine content, contribute to making E6 unstable when attempting to crystallize, and insoluble after purification (Nomine *et al.*, 2001). A model for structure of HPV 16 E6 was finally obtained using an E6 mutant in which six non-conserved cysteines were replaced with serine, in a series of NMR studies (Nomine *et al.*, 2006). This E6 mutant was soluble and retained some E6 protein functions, such p53 degradation and E6AP binding. Using this mutant protein the NMR structure of the C-terminal half was obtained and,



α- Helical partners: E6AP, ERC-55(E6BP), IRF3, paxillin and tuberin

PDZ binding partners: hDlg1, hDlg4, hScrib, MAGI-1, -2, -3, MUPP1, PTPN3

Unidentified binding motif: p53, Bak, p300/CBP, hADA3, NFX1, Gps2, FADD and procaspase 8

Figure 4. The E6 protein.
Cartoon representing the HPV-16 E6 protein. The characteristic Zn-binding domains are shown in blue, the I128T amino-acid that mediates E6AP binding is shown in violet and C-terminal PDZ binding motif is shown in red. α-helical partners, PDZ binding partners and proteins binding to yet unidentified binding motifs are indicated.

based on this, a prediction for the N-terminal half was also generated. This model of the three-dimensional structure of E6 allows for the visualization of the potential parts of the protein involved in its interactions with its cellular partners. Some of them are depicted in Figure 4.

Extensive studies over the years have identified conserved E6 binding motifs, thus defining a group of proteins which are bound by E6 through similar domains. One example of such are the PDZ domain-containing proteins, which are targeted by the high-risk E6 proteins through their C-terminal PDZ binding motifs (see later for more details). Interestingly, even though PDZ domain-containing proteins have a high degree of homology, their interaction with E6 is very specific. For instance, E6 specifically binds to a single PDZ domain on hDlg and MAGI-1 (Kiyono *et al.*, 1997; Thomas *et al.*, 2001), even though these proteins have 3 and 6 PDZ domains, respectively.

Another conserved site binds to the α-helical E6 binding domain that is found in several targets of E6. These include most notably E6AP, but it is also present to a lesser or greater extent on hMcm7, E6BP/ERC-55, IRF-3, tuberin and paxillin (Baleja *et al.*, 2006; Chen *et al.*, 1995; Cooper *et al.*, 2007; Elston *et al.*, 1998; Ronco *et al.*, 1998). Interestingly, several other binding partners of E6, including FADD, Gps2, hADA3 and procaspase 8 (Degenhardt and Silverstein, 2001; Filippova *et al.*, 2004; Filippova *et al.*, 2007; Kumar *et al.*, 2002), lack the α-helical motif and PDZ binding domains, suggesting the existence of one or more additional binding motifs on the E6 oncoprotein.

Detecting endogenously expressed E6 has been a long-standing problem, due to its poor immunogenicity and apparently low levels of expression.

The initial reports characterized HPV-18 E6 protein as being a nuclear and membrane-associated protein with a bi-modal half-life of 30 minutes and 4 hours in transformed cells (Androphy *et al.*, 1987). In addition, E6 has also been reported to be found in the cytoplasm (Chen *et al.*, 1995; Liang *et al.*, 1993), as well as in a generally diffused pattern throughout the cell (Guccione *et al.*, 2002), suggesting a widespread localization and propensity to interact with protein partners in multiple cellular compartments.

Ubiquitin Ligases and p53 as Interacting Partners of E6

Ubiquitylation is a very specific and structured multistep enzymatic process achieved by the formation of an isopeptide bond between the C-terminal Gly76 carboxyl group of ubiquitin and the ε-amino group of an internal Lys residue of the substrate (reviewed in Bernassola *et al.*, 2008). The Ubiquitin activating enzyme (E1) activates the carboxyl group of ubiquitin in an ATP-dependent manner to form a high-energy thioester bond with an active Cys group of the E1 enzyme. Activated ubiquitin is then transferred to a specific Cys residue of one of the family of E2 ubiquitin-conjugating enzymes (E2s) through a similar thioester bond. The E3 ubiquitin ligases (E3s) play a crucial role in the ubiquitin conjugating cascade by recruiting ubiquitin-conjugated E2s, recognizing specific substrates and facilitating or directly catalyzing ubiquitin transfer to either the Lys residues (in the majority of cases). E3 ligases can also function in the N-end rule pathway. The pathway is a ubiquitin-dependent system where E3 ligases called N-recognins recognize type-1 (basic) and type-2 (bulky hydrophobic) N-terminal residues as part of N-degrons (Tasaki *et al.*, 2005; reviewed in Varshavsky, 1996). E3s can modify protein substrates by either adding

mono- or poly-ubiquitin chains (Hoeller *et al.*, 2007). The fate of ubiquitylated proteins is determined by the nature of the ubiquitin chains and the type of isopeptide linkage forming the polyubiquitin chain. For example, mono-ubiquitination of intracellular substrates at one or multiple lysine residues of transmembrane proteins, influences their stability, protein-protein recognition, trafficking and intracellular localization. Lys63 is one of the most characterized lysine residue whose mono- and poly-ubiqutination was shown to be involved in cellular trafficking processes such as endocytosis and vesicular sorting (reviewed in Rotin and Kumar, 2009). In contrast, when the tagging occurs through Lys48-linked polyubiquitin chains, proteins are generally labeled for 26S proteasome-mediated recognition and degradation (reviewed in Bernassola *et al.*, 2008). Besides Lys48 and Lys63, the formation of mono- and polyubiqutin chains can occur through Lys6, Lys11 and Lys29/33. These polyubiquitin chains regulate protein degradation in addition to many other cellular proteolysis-independent activities, such as DNA damage tolerance, inflammatory response, the endocytic pathway, and ribosomal protein synthesis, while nonproteolytic Lys6 and Lys11 polyubiquitin chain accumulation has been linked to neurodegenerative disorders (reviewed in Bernassola *et al.*, 2008). Interestingly, one study suggested a possibility of tagging through Cys-linked polyubiquitin chains. Ubiqutination factor Ubc7 was auto-ubiquitinated through the catalytic cysteine, and this chain functioned as a degradation signal (Ravid and Hochstrasser, 2007). Some examples of the different ubiquitylation pathways regularly encountered are depicted in Figure 5 (A Single ligase, B Multiple ligases, C E6 ligase redirection).

According to the sequence homology of their E2-binding domains, E3s are grouped into three subfamilies: the homologous to E6AP carboxyl

terminus (HECT) domain containing E3s, RING (Really Interesting New Gene) finger domain-containing E3s, and the U-box E3s (reviewed in Bernassola *et al.*, 2008). HECT E3 ubiquitin ligases are among the most widely abundant ubiquitin ligases. They have been identified across the evolutionary spectrum from yeast to humans and they range in size from approximately 80 kD to more than 500 kD. They are characterized by the HECT (homologous to E6AP C-teminus) domain, a C-terminal region of approximately 350 amino acids with significant similarity to E6AP (Huibregtse *et al.*, 1995; Scheffner *et al.*, 1995). Good examples of HECT ubiquitin ligases are HERC1, HERC2, HERC5, Nedd4-1, Nedd4-2, Smurf1 and 2, Itch, E6AP, EDD, and HETCH9, and they have been reported to be involved in various diseases. For example, EDD was found to be overexpressed in ovarian and breast cancers; E6AP is involved in Angleman's syndrome and cervical cancer; Itch was found to be associated with severe immunological diseases, including lung and stomach inflammation and hyperplasia of lymphoid and hematopoietic cells; Smurfs amplification and overexpression were found associated with pancreatic and esophageal squamous cell carcinomas; while Nedd4-1 and Nedd4-2 were found linked to Liddle's syndrome (reviewed in Scheffner and Staub, 2007).

Under normal circumstances in the absence of HPV, the p53 tumor suppressor is regulated by the RING finger domain containing ubiquitin ligase Mdm2 (Honda *et al.*, 1997). However under stress conditions, for example upon DNA damage or viral infection this regulated turn over is abolished and p53 is both stabilized and activated due to a series of phosphorylation events (reviewed in Ashcroft and Vousden, 1999). Importantly, p53 can be also stabilized in phosphorylation independent fashion. Tumor suppressor $p14^{ARF}$ binds direcly to Mdm2 in a distinct region from the p53 binding domain and inhibits the turnover of p53

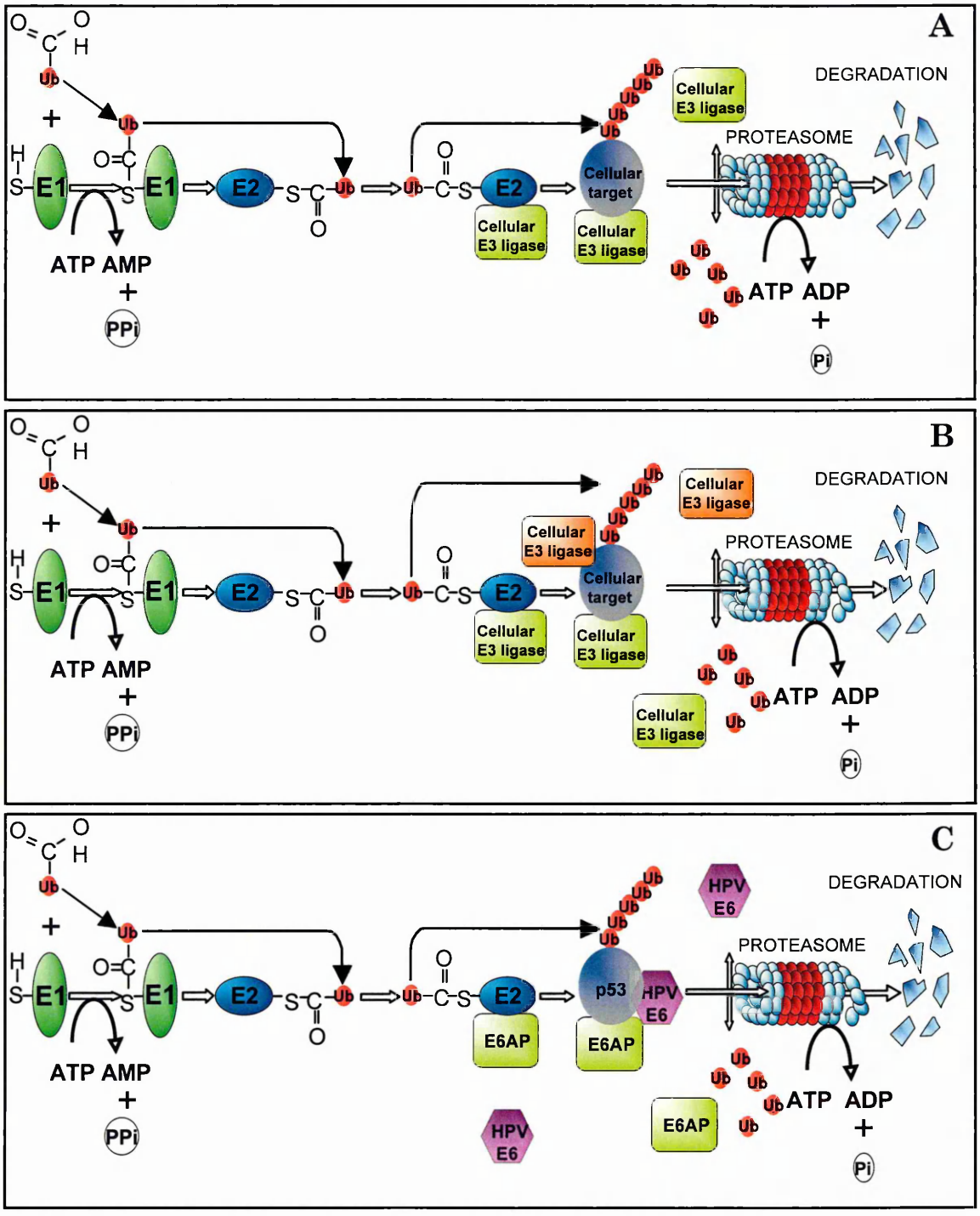


Figure 5. Ubiquitin degradatory pathways

A. Single ligase protein ubiquitination - single ligase is responsible for target protein ubiquitination which then results in proteasomal degradation of the target protein. B. Multiple ligase protein ubiquitination - more than one ubiquitin ligase is responsible for target protein ubiquitination which then results in proteasomal degradation of the target protein. C. E6 ligase redirection - E6AP ubiquitin ligase is redirected by HPV E6 to p53 targeting which leads to p53 ubiquitination and proteasomal degradation.

without preventing Mdm2 binding. Rather, $p14^{ARF}$ inhbits the ubiquitin ligase activity of Mdm2 and sequesters it into the nucleolus, thus preventing nuclear export of the Mdm2/p53 complex which is necessary for p53 degradation (reviewed in Ashcroft and Vousden, 1999). Kinases such as JNK, CKI, ATM, ATR, DNAPK have be found to be responsible for p53 N-terminal phosphorylation *in* vitro. Furthermore, following DNA damage, endogenous p53 has been found to be phosphorylated at serines 15, 20, 33, and 37. There is also evidence that ATR and ATM can phosphorylate serine 15 *in vivo* (reviewed in Ashcroft and Vousden, 1999). Most importantly, phosphorylation of p53 on residues 15, 20, 33 and 37 renders it unable to bind Mdm2 and thereby this contributes to its stabilization. In contrast, in HPV-positive cancer cells the Mdm2 pathway is completely inactive and p53 degradation is completely dependent upon E6 (Hengstermann *et al.*, 2001). This suggests that if E6 degradation of p53 occurs under conditions of DNA damage this in turn could lead to accumulation of genomic mutations which, in turn, contribute towards malignant progression (Foster *et al.*, 1994; Kessis *et al.*, 1993). Other DNA tumor viruses also use different mechanisms to inactivate p53, since it represents a major obstacle to viral replication, activated by the unscheduled viral induction of DNA replication. Normally, this would result in cell cycle arrest or apoptosis of the infected cells, (el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Lowe *et al.*, 1994) and to overcome this DNA tumor viruses encode proteins which, in different, ways functionally inactivate p53 activity. As noted above, high-risk HPV E6 protein uses proteasome-mediated degradation as the major strategy for eliminating p53 (Scheffner *et al.*, 1990). Adenovirus E4orf6 in combination with E1B-55K will also inactivate p53 degradation in a manner analogous to that of HPV E6 (Querido *et al.*, 2001), whilst SV40 LT prevents transactivation of p53 target genes through its association with its DNA binding domain (Ruppert and

Stillman, 1993). Ad E1B-55K by itself abolishes the same function by binding to the transactivation domain of p53, while the HBV X protein sequesters p53 in the cytoplasm (reviewed in Mantovani and Banks, 2001).

As shown in Figure 5, E6 highjacks a cellular E3 ligase, in this particular case E6AP, to degrade p53 (Scheffner *et al.*, 1993). E6AP has a large N-terminal region which it uses for substrate recognition, while ubiquitination of the targets is catalyzed via a cys residue with its conserved C-terminal HECT domain (Schwarz *et al.*, 1998). High-risk HPV E6 binds to E6AP within its N-terminal substrate recognition domain (Huibregtse *et al.*, 1993b), and after the stable complex E6-E6AP is formed the association with p53 occurs. This interaction between E6 and E6AP results in a redirection of the substrate specificity of E6AP towards p53 (Huibregtse *et al.*, 1993a) and several reports have confirmed the importance of this interaction for p53 degradation (Scheffner *et al.*, 1993; Talis *et al.*, 1998). When E6AP was neutralized by either the use of antisense oligonucleotides (Beer-Romero *et al.*, 1997), siRNA to E6AP (Kelley *et al.*, 2005) or catalytically inactive dominant negative mutants (Talis *et al.*, 1998), increased levels of p53 in HPV positive cells, but not in HPV negative cells, were detected. This suggested that E6AP plays a crucial role in E6 directed degradation of p53 *in vivo* and that it does not associate with p53 in HPV negative cells. Furthermore, it was suggested that the global transcriptional effects of E6 upon the cell are largely dependent on the presence of E6AP (Kelley *et al.*, 2005), suggesting indirectly, that many of E6's activities are mediated through its association with E6AP.

The efficiency with which E6 degrades p53 varies among different HPV E6 proteins and is dependent on the strength of interaction between p53

and E6. Both high- and low-risk HPV types bind to the C-terminus of p53, but not all of those interactions result in p53 degradation. High-risk E6 proteins bind p53 more strongly and the binding is enhanced by the presence of E6AP, which results in p53 degradation (Li and Coffino, 1996). For example HPV-16 E6 binds p53 much more strongly than HPV-18 E6, which results in more efficient degradation of p53 by HPV-16 E6 than by 18 E6. The low-risk HPV-11 E6 has a very weak binding capacity for E6AP *in vitro* (Huibregtse *et al.*, 1993a) and degrades p53 poorly *in vivo* (Storey *et al.*, 1998). However, new studies suggest that HPV-11 E6 can interact quite strongly with E6AP *in vivo*, suggesting that low risk HPV E6 proteins can connect directly to the proteasome (Brimer *et al.*, 2007). Why they still remain weak at targeting p53 for degradation turnover remains to be determined.

There are numerous reports showing that E6 can use other pathways to abolish p53 activities. Both high- and low-risk HPV E6 proteins are capable of neutralizing p53-mediated transcriptional repression *in vivo* (Lechner *et al.*, 1992) and this is likely to occur through binding to the p53 C-terminus (Li and Coffino, 1996). Interestingly, studies have shown that the capacity of the high-risk E6 proteins to prevent transactivation of p53 target genes does not only rely on p53 destabilization, since E6 mutants incapable of degradation still possess the ability to abolish transcriptional activation by p53 *in vivo* (Pim *et al.*, 1994). Moreover, it was shown that E6-interacting regions of p300 are necessary for E6 to inhibit p53-dependent chromatin transcription and that E6-mediated repression of p53-dependent activation correlates with inhibition of acetylation on p53 and nucleosomal core histones, without altering p53 and p300 recruitment to chromatin. This process is E6AP- independent and shows a unique mechanism of E6 repression of p53 which does not involve proteasomal degradation of p53 (Thomas

and Chiang, 2005). In addition, several more recent studies have reported that E6 is able to target p53 along with other substrates, such as PDZ domain-containing proteins, in an E6AP-independent fashion in tissue culture and in mouse model studies. Taken together, these studies suggest other, as yet unidentified mechanisms, by which E6 can target proteins to the proteolytic machinery (Camus *et al.*, 2007; Grm and Banks 2004; Massimi *et al.*, 2008; Pim *et al.*, 2000; Shai *et al.*, 2007b; Storrs and Silverstein, 2007).

A number of studies have reported variable amounts of p53 protein during viral infection and in HPV-induced cervical lesions, implying that not all p53 is degraded either during viral infection or in induction of malignancy (Cooper *et al.*, 1993; Lie *et al.*, 1999; Mantovani and Banks, 1999). One possible explanation for these observations came from a study showing that E6 preferentially targets Thr155 phosphorylated forms of p53. CNS kinase was shown to be involved in Thr155 p53 phosphorylation (Bech-Otschir *et al.*, 2001). The function of this kinase is not precisely clear, however the purified complex from human blood cells was shown to posses the kinase activity that phosphorylates transcriptional regulators such as c-Jun, IκBα and p105 (Seeger *et al.*, 1998). *In* vitro and *in* vivo experiments showed that the Thr155 site mutated to Val155 was sufficient to stabilize p53 against E6-dependent degradation (Bech-Otschir *et al.*, 2001). This suggests that variable amounts of p53 protein levels are detected in HPV infected and transformed cell owing to the fact that certain phosphorylated forms of p53 are less prone to be targeted by E6.

Another possible pathway for regulating E6 activity with respect to p53 or some other cellular targets relies on the E6 spliced forms, termed E6*. They are exclusively expressed by the high-risk HPVs through

alternative splicing of E6 mRNA, thus producing several shorter forms of E6: E6*I-IV as in the case with HPV-16 E6 or just E6*I as in the case with HPV-18 E6 (Schneider-Gadicke and Schwarz, 1986). Interestingly, E6*I from HPV-18 E6 protein was reported to interact with both the full-length E6 and with E6AP, thereby blocking the degradation of p53 during viral infection (Pim *et al.*, 1997). This phenomenon could assist E6 in the delicate control of its activity with respect to p53 and other targets during viral infection. Interestingly, it was shown that although p53 specifically inhibits HPV amplificational DNA replication *in vivo*, it has no effect on episomal maintenance, which occurs in synchrony with the cell cycle (Lepik *et al.*, 1998). Therefore, in order to obtain a proper productive infection, viral DNA amplification needs to be under control and it is reasonable to suggest that the activity of E6* could be responsible for ensuring the presence of a limited amount of p53 at the replication sites, where it could both prevent overreplication of the viral genome and, potentially, assist DNA synthesis by means of its proofreading ability.

E6 and Apoptosis

Although one of the major anti-apoptotic activities of E6 is the inactivation of p53, a number of other cellular pro-apoptotic targets of E6 have been described. One of those targets is Bak, which is normally localized in the outer mitochondrial membrane (Karbowski *et al.*, 2006). After UV exposure it is activated and stabilized independently of p53 (Jackson *et al.*, 2000). It is thought that multimerized Bak forms pores in the mitochondrial membrane that allow release of cytochrome c and other pro-apoptotic factors into the cytoplasm (Hacker and Weber, 2007). In addition, Bak is highly expressed in the upper epithelial

layers (Krajewski *et al.*, 1996), suggesting it may play a role in terminal differentiation. HPV E6 binds to Bak and induces its proteasomal degradation which is catalyzed by E6AP, thereby leading to a reduction in apoptosis (Jackson and Storey, 2000; Thomas and Banks, 1998; Thomas and Banks, 1999). Degradation of Bak with a low-risk type HPV, such as HPV-11 E6, is less effective and this correlates with a weaker anti-apoptotic activity of the low-risk mucosal HPV types (Thomas and Banks, 1999). Cutaneous HPV E6 proteins are also able to abolish p53-dependent and independent apoptosis in response to UV-induced DNA damage (Jackson and Storey, 2000), and cutaneous HPV E6 proteins also inhibit Bak activities by driving its proteasomal degradation. Bak protein levels are undetectable in HPV-positive skin cancers in comparison with HPV-negative cancers which express it (Jackson *et al.*, 2000). Thus, Bak is one of the few highly conserved targets of cutaneous and mucosal HPV E6 proteins.

An anti-apoptotic protein Survivin, was recently identified as an indirect target of HPV E6, which strongly upregulates Survivin promoter activity, resulting in the suppression of apoptosis (Borbely *et al.*, 2006). Interestingly, recent studies also demonstrated that E6 is able to inhibit apoptosis in a PDZ-dependent manner (James *et al.*, 2006), however the actual PDZ domain-containing substrate for this activity still remains to be identified. Furthermore, E6 has been reported to interact with other components of the host apoptotic machinery such as the tumor necrosis factor receptor 1 (TNF R1) (Filippova *et al.*, 2002), the adaptor molecule Fas-associated death domain (FADD) (Filippova *et al.*, 2004), and procaspase 8 (Filippova *et al.*, 2007). In the case of procaspase 8 and FADD, E6 binds to their death effector domains (DEDs) and it mediates the accelerated degradation of both proteins. Through these interactions E6 prevents

these proteins from transmitting receptor-induced apoptotic signals, thereby contributing to HPV-infected and transformed cells' escape from apoptosis.

E6, Transcription and DNA Replication

As noted above for Survivin, E6 proteins from both high- and low-risk types also modulate transcription from many host and viral genes (Desaintes *et al.*, 1992; Etscheid *et al.*, 1994; Sedman *et al.*, 1991; Veldman *et al.*, 2001). An understanding of the mechanisms by which E6 performs this function has come from several studies. Perhaps the most important of those is the ability of E6 to interact with the p300/CBP co-activators (Patel *et al.*, 1999; Zimmermann *et al.*, 1999). These proteins are crucial in regulating expression of many genes involved in the regulation of cell cycle, differentiation and the immune response. In line with a central role for p300/CBP in the life cycle of many different viruses, several other DNA tumor viruses have also been shown to interact with p300/CBP (reviewed in Goodman and Smolik, 2000), highlighting its central role in regulating cellular homeostasis. As is the case with many other E6 targets, high-risk HPVs such as HPV-16 E6 was reported to bind strongly to p300/CBP whereas a low-risk HPV-6 E6 bound weakly (Patel *et al.*, 1999). It was also shown that HPV-16 E6 inhibits the intrinsic transcriptional activity of p300/CBP on both p53 and NF-κ-B responsive promoter elements. In the case of p53 this is partly due to an inhibition of p300-induced acetylation of p53 (Thomas and Chiang, 2005). It has also been shown that HPV-16 E6 targets hAda3, a component of the histone acetyltransferase complex, which is itself a p53 transcriptional coactivator (Kumar *et al.*, 2002). The HPV 16 E6 Y54D mutant which is able to target hAda3 for

degradation, but not p53, was able to protect mammary epithelial cells (MECs) from p14ARF-induced senescence, a function associated with p53 acetylation and which requires hAda3 (Sekarić *et al.*, 2007). Furthermore, recent studies showed that E6-targeted degradation of hAda3 is E6AP-dependent in HPV-transformed cell lines (Hu *et al.*, 2009). Certainly, by binding and inactivating transcriptional effectors such as NF-κ-B and c-myc, E6 would promote escape of HPV-infected cells both from the immune system and from the induction of apoptosis (Filippova *et al.*, 2002; Gross-Mesilaty *et al.*, 1998). Moreover, p300/CBP has an effect on the expression of numerous cellular promoters, including those regulating differentiation (Bannister and Kouzarides, 1995; Goodman and Smolik, 2000). However, with the most likely exception of p53, it remains to be determined which are the truly relevant other target genes of the E6/p300/CBP complex.

In addition to its association with transcription, HPV E6 is also involved in the deregulation of the cellular DNA replication machinery. Normal somatic cells finish their replicative life span by reaching the state of senescence, which occurs due to the major shortening of telomere DNA and therefore most normal cells do not have telomerase activity. For neoplastic cells to activate telomerase they first need to overcome senescence check-point mechanisms. After this is achieved, telomerase can be activated, which then forms the basis for indefinite propagation. In senescent cells the levels of $p16^{ink}$ are upregulated, and its activation has been reported to be an early step during the induction of senescence of human epithelial cells (Kiyono *et al.*, 1998). Furthermore, activated telomerase is found in cervical carcinomas and in a certain number of high-grade cervical lesions associated with high-risk HPVs (Snijders *et al.*, 1998). It was shown that HPV-16 E6 can induce telomerase activity in primary epithelial cells through a p53-independent mechanism

(Klingelhutz *et al.*, 1996; Veldman *et al.*, 2001). This is thought to occur by E6-mediated transcriptional activation of the gene encoding the telomerase catalytic subunit, hTERT, while the minimal promoter region involved in induction by E6 was found to require an intact E box and Myc (Gewin and Galloway, 2001; Veldman *et al.*, 2001; Veldman *et al.*, 2003). Interestingly, there are contradictory reports about the activation of the hTERT promoter. Originally, it was reported that hTERT activation was dependent on the ability of E6 to interact with E6AP and that the activation was dependent upon Myc binding sites in the promoter (Gewin and Galloway, 2001; Liu *et al.*, 2005), while more recent studies show that binding of HPV-16 E6 to E6AP is not required for activation of hTERT (Sekarić *et al.*, 2008), and that an interaction with c-myc itself is more relevant. In addition, it was also shown that E6/E6AP dependent degradation of the transcriptional repressor NFX1-91 that binds to the hTERT promoter dissociates the mSin3A/HDAC complex from the hTERT promoter and induces hTERT transcription (Xu *et* al., 2008). Thus, although the mechanism of activation is controversial, hTERT activation by E6 would appear to be a critical aspect in HPV-induced malignancy, although its role in a normal viral life cycle remains to be determined.

E6 in Epithelial Organization and Differentiation

It was reported that HPV-16 E6 interacts with E6BP/ERC-55 (Chen *et al.*, 1995), a putative calcium-binding protein localized in the endoplasmic reticulum (ER) (Weis *et al.*, 1994). E6BP was shown to form a complex with both E6 and E6AP *in vivo*, but interestingly the complex formation did not result in proteasomal degradation of E6BP (Chen *et al.*, 1995). Since epithelial differentiation occurs in response to

Ca^{2+}-mediated signaling, it could be reasoned that E6 targeting of E6BP likely contributes to E6's ability to interfere with terminal differentiation.

Attachment to the extracellular matrix is responsible for the regulation of various cellular processes ranging from cell morphology to proliferation and migration. Therefore, preservation of proper epithelial architecture is necessary for a cell to regulate its growth appropriately. Normal cell division takes place when cells respond to proliferation signals, and terminates when the signal is removed or when proper tissue size is reached. In order to overcome limitations in cell proliferation, high-risk E6 proteins interact with, and subsequently inactivate, various proteins that are associated with epithelial organization. The proteins that belong to this category and which are targets of HPV E6 are Paxillin (Tong and Howley, 1997), zyxin (Degenhardt and Silverstein, 2001), and fibulin-1 (Du *et al.*, 2002).

Paxillin is involved in mediating signaling from the plasma membrane to focal adhesions and to the actin cytoskeleton (Turner, 2000). HPV-16 has been shown to bind paxillin, and this interaction correlates with E6 transforming capacity, although it does not lead to paxillin degradation (Tong and Howley, 1997). Zyxin is a focal adhesion molecule responsible for connecting the ECM to the cytoskeleton, for regulating cell proliferation and differentiation, and for transmitting signals from the sites of cell adhesion to the nucleus and for organizing actin (Degenhardt and Silverstein, 2001). Binding of E6 to zyxin impairs its ability to maintain proper cellular structure, which could also contribute to E6 induced transformation. Finally, interactions between E6 and fibulin-1 have also been reported to support-cellular transformation and

tumor invasion by further contributing to the destabilization of the extra cellular matrix (Du *et al.*, 2002).

E6 and PDZ Domain-containing Proteins as Interacting Partners

Analysis of the amino acid sequence from high-risk HPV E6 proteins shows a remarkable conservation of amino-acids at the extreme carboxy termini of the proteins. As shown in Figure 6 all of those E6 proteins have a class I PDZ (PSD95/Dlg/ZO-1)-binding motif [x-T/S-x-L/V (Songyang *et al.*, 1997)] at their carboxy termini. Interestingly, none of the so-called low-risk types have this motif and it can be argued that this represents a possible marker of the malignant potential of mucosal HPVs.

PDZ domains are approximately 90 amino acid residues in length and serve to mediate protein-protein interactions (reviewed in Javier, 2008). They are named after the first three PDZ domain-containing proteins identified: the postsynaptic density protein PSD95/SAP90, the *Drosophila* septate junction protein Discs-large, and the epithelial tight junction protein ZO-1. Proteins which have PDZ domains are referred to as PDZ domain-containing proteins and can be divided into three general groups: 1. PDZ-only proteins, 2. membrane associated guanylate kinases (MAGUKs) and 3. PDZ proteins with other protein domains (reviewed in van Ham and Hendriks, 2003). PDZ containing proteins typically function as scaffolds to assemble receptors and cytosolic factors into supramolecular signaling complexes and to localize them at specialized membrane regions of cell-cell contact, such as adherens junctions and tight junctions (reviewed in Javier, 2008).

Numerous studies have shown that both HPV-16 and -18 use their PDZ-binding motifs to interact with a number of different PDZ domain-containing cellular proteins. Table 2 shows some of the major PDZ domain-containing targets of E6 that have so far been identified. Many of these are involved in the regulation of epithelial cell polarity, implicating the significance of interaction with this pathway for both viral replication and for virus-driven malignant progression.

Table 2. Identified PDZ-containg targets of Human papillomavirus oncoproteins

Protein	Function	Effect on target protein	Reference
Dlg	Cell polarity/tumor suppressor		Gardiol et al. 1999
Scribble	Cell polarity/tumor suppressor	Ubiquitination and proteasomal degradation	Nakagawa and Huibregtse 2000
MAGI-1 MAGI-2 MAGI-3	Cell polarity/tumor suppressor PTEN localization to membrane		Glaunsinger et al. 2000 Thomas et al. 2002
MUPP1	Signaling complex scaffold		Lee et al. 2000
PATJ	Tight Junctions formation and integrity	Ubiquitination and proteasomal degradation plus E6*-directed degradation	Latorre et al. 2005 Storrs and Silverstein 2007
PSD95	Signaling complex scaffold	Ubiquitination and proteasomal degradation	Handa et al. 2007
PTPH1/ PTPH3	Protein tyrosine phosphatase		Jing et al. 2007 Töpffer et al. 2007
PTPN13	Non-receptor phosphatase	?	Spanos et al. 2008a
TIP-1	Downregulator of PDZ interactions	?	Hampson et al. 2005
TiP-2/GIPC	TGFß signaling	Ubiquitination and proteasomal degradation	Favre-Bonvin et al. 2005
CAL	Intracellular trafficking		Jeong et al. 2007

The specificity of PDZ binding is a very important factor in E6 PDZ target recognition. As can be seen in Table 2 there is a variety of proteins that have been reported as being HPV E6 targets, and Figure 6 shows that different high-risk HPV E6 proteins have distinct PDZ binding motifs, suggesting that different E6 proteins could preferentially target some PDZ domain-containing proteins over others. Studies which show that HPV-18 E6 exhibits stronger binding than HPV-16 E6 to Dlg1 and MAGI-1 support this hypothesis (Thomas *et al.*, 2001). Furthermore, substituting the last amino-acid residue of the

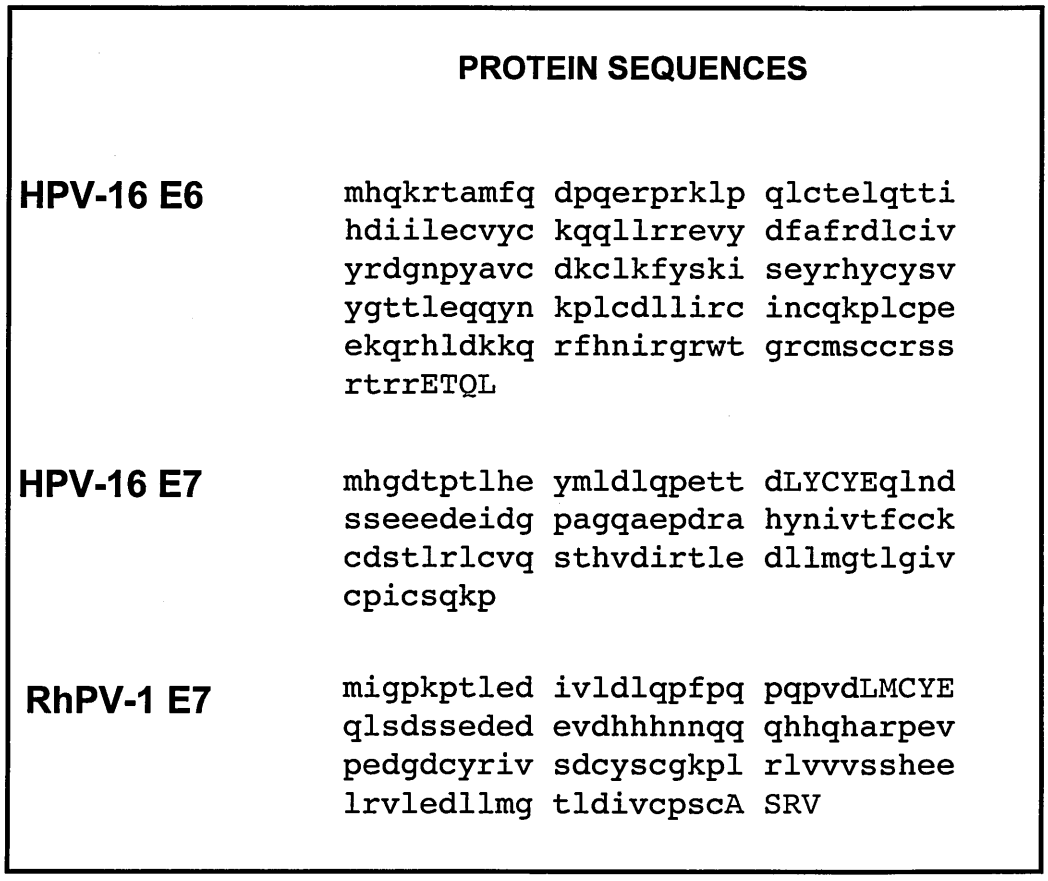


Figure 6. Mucosal PV types and PDZ binding motifs. A. Protein sequences of HPV-16 E6, HPV-16 E7, and RhPV-1 E7 are shown. pRb (blue) and PDZ binding motifs (red) are indicated.

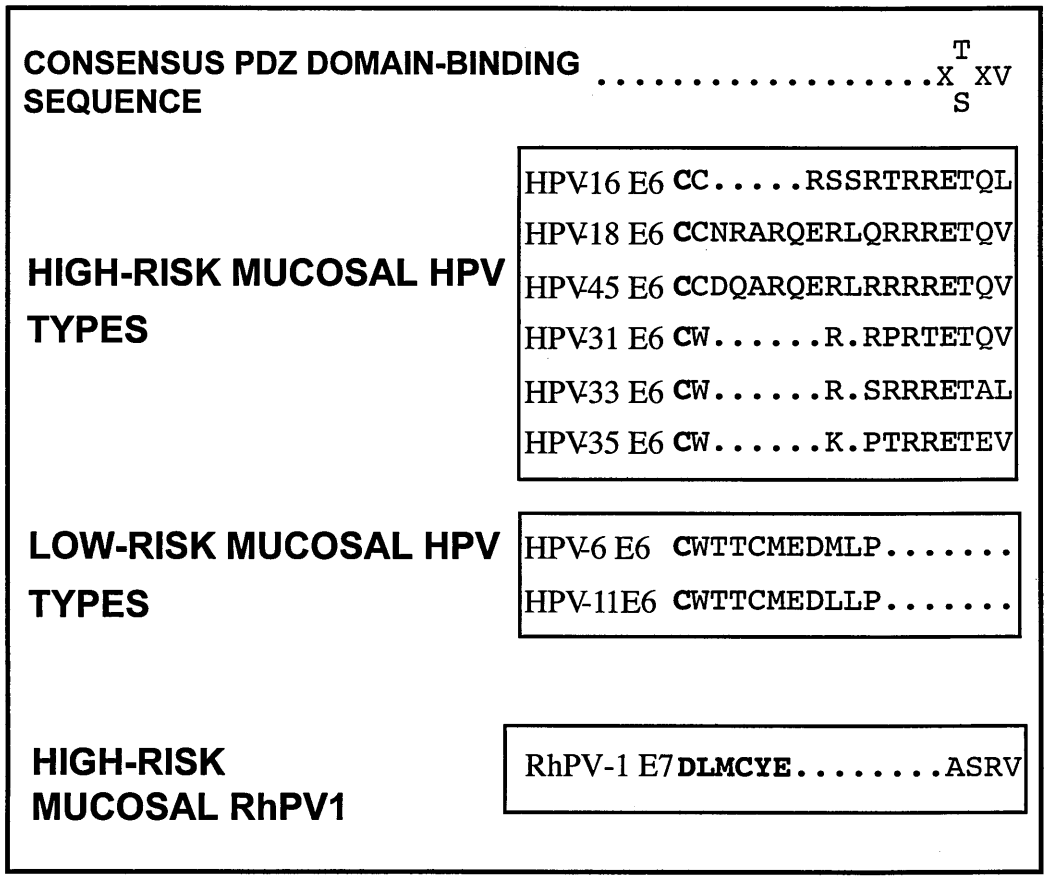


Figure 6. Mucosal PV types and PDZ binding motifs. (cont.) B. The carboxy-terminal PDZ-binding motif is common to high-risk mucosal papillomavirus (PV) proteins. The PDZ-binding motif (X-T/S-X-V/L) is absent from low-risk mucosal HPV type E6s and from RhPV-1 E6. It is found at the carboxy-terminus of high-risk HPV E6s and RhPV-1 E7.

HPV-16 E6 protein with the equivalent amino-acid residue of HPV-18 E6 switches this phenotype (Thomas *et al.*, 2001). In contrast, HPV-16 binds hScrib more strongly than HPV-18 E6 and this is again dependent upon the last carboxy terminal amino-acid residue (Thomas *et al.*, 2005). This demonstrates that the exact sequence of the E6 PDZ-binding motif is crucial for substrate recognition. Additional support for these observations came from studies which revealed the crystal structures of MAGI-1 PDZ1 and the Dlg1 PDZ2 and PDZ3, demonstrating that a peptide homologous to the HPV-18 E6 carboxy-terminus forms different strength bonds with the different PDZ domains of these proteins (Liu et al 2007; Zhang *et al.*, 2007). Interestingly, as mentioned earlier in the text, Rhesus papillomavirus 1 E7 protein has a canonical class I PDZ binding motif which is significantly different from the PDZ binding motif in HPV-16 and -18 (Figure 6). The difference in the binding motif of E7 also has an impact on its preferred PDZ domain-containing targets, and this will be discussed in more detail in the Results section.

The Regulation of Cell Polarity

Epithelial cells are polarized along their apicobasal axis and this is controlled by the action of three cell polarity regulating protein complexes; the Crumbs complex (constituted by Crumbs (Crb), Pals (Sdt in *Drosophila*) and PATJ), the Par complex (constituted by Par3 (Bazooka in *Drosophila*), Par6 and aPKC) and the Scribble polarity complex (constituted by Scribble (Scrib), Dlg and Hugl-1(Lgl in *Drosophila*)) (Figure 7) (Humbert *et al.*, 2006). The Crumbs and Par complexes are localized to the subapical region and the Scribble polarity complex is localized to the basolateral region and adherens junctions region. There are mutual antagonistic interactions between these

polarity complexes, which are necessary for restricting the activity of each complex to specific cortical domains, and for the positioning of the adherens junctions (constituted by E-cadherin, α-catenin and ß-catenin). In *Drosophila* embryonic epithelial studies, it was shown that the Par complex is required first for the establishment of apicobasal cell polarity, and it acts to repress the activity of the Scribble polarity module and promote the activity of the Crumbs complex (Humbert *et al.*, 2008). In addition, Lgl inhibits aPKC activity, and aPKC phosphorylates Lgl excluding it from the apical cortex. The Scribble polarity complex also plays a role in negative regulation of cell proliferation by inhibiting the expression of the cell cycle regulator, Cyclin E, and in promoting apoptosis by blocking expression of the apoptosis inhibitor, DIAP1 (Humbert *et al.*, 2008).

Although Dlg and Scribble are the most well-characterized PDZ domain-containing targets of HPV E6, a number of other cell polarity regulations have been reported as being E6 targets. These include MAGI-1, -2, and -3 located at regions of cell-cell contact, and MUPP1, located at subapical tight junctions (Thomas *et al.*, 2008). Both HPV-16 and -18 have also been reported to promote degradation of PATJ, a component of the Crumbs complex (Latorre *et al.*, 2005; Storrs and Silverstein, 2007), further highlighting the necessity of a continued viral assault on these polarity regulators during the viral life cycle. Additionally, these reports support the previously discussed ability of E6 to affect the stability of entire cellular complexes by targeting only one component of the complex and causing just minor alterations in protein concentrations which are sufficient to destabilize the entire complex. For example, even slight alterations in balances between the proteins of the Scrib, PAR and Crumbs complexes can have quite serious effects on the proper functioning, not only of each complex, but also of

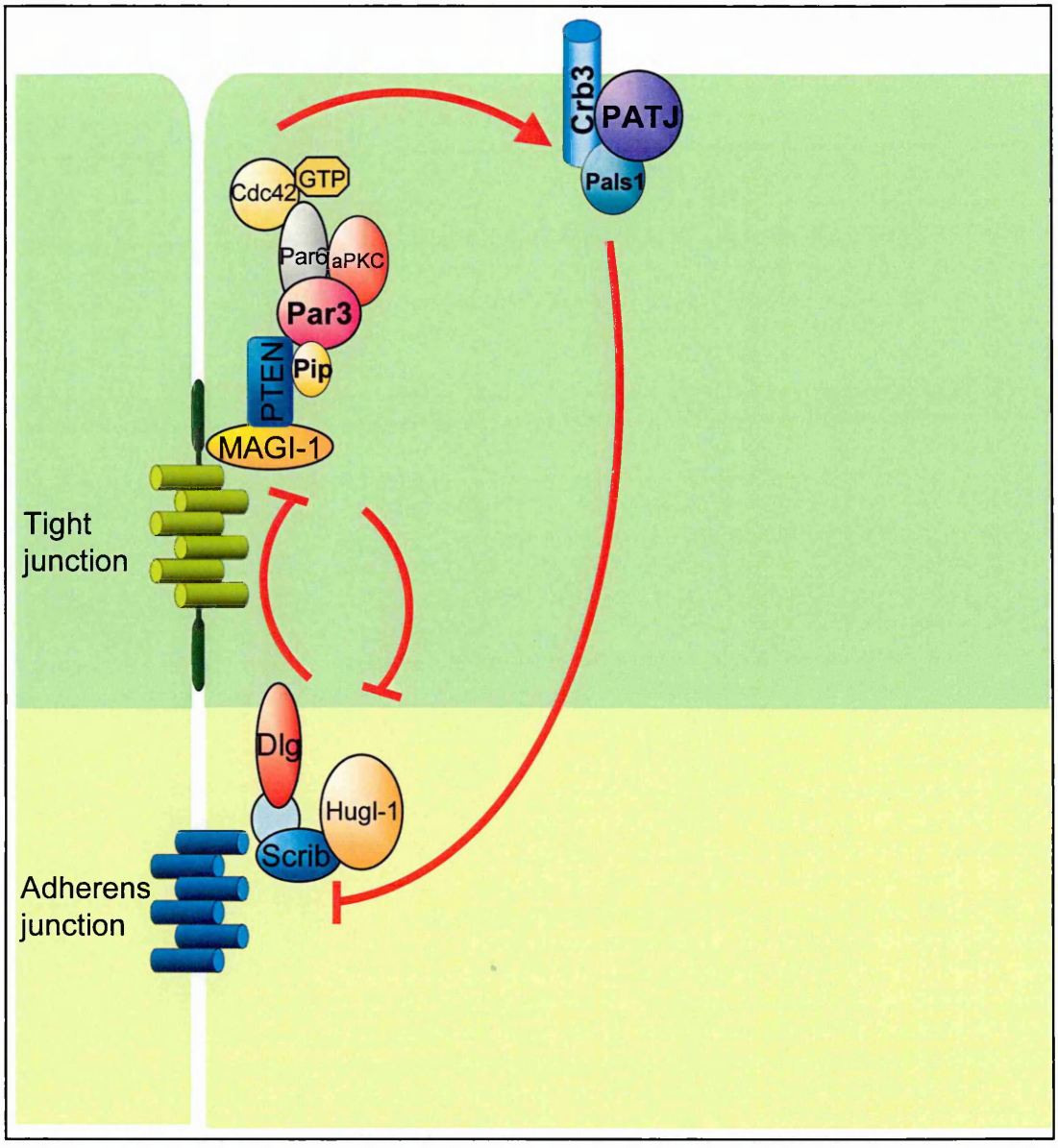


Figure 7. Cell polarity regulators
The Crumbs complex, the Par complex and the Scribble complex are the main three cell polarity regulating complexes. There are mutual antagonistic interactions between these polarity complexes, which are essential for restricting the activity of each complex to specific cortical domains and to control the positioning of the adherens junctions. The Par complex acts to repress the activity of the Scribble polarity module and to promote the activity of the Crumbs complex. Hugl-1 inhibits aPKC activity, and aPKC phosphorylates and excludes Hugl-1 from the apical cortex.

the 'polarity super-complex', that embraces them all. Furthermore, this suggests that this ability of E6 to target various substrates of the polarity complex could trigger crucial events during different stages of the viral life cycle, in addition to potentially driving malignant progression.

Thesis Aim

The aim of this thesis is to understand how HPV E6 is regulated through interaction with the proteasome pathway, and to further characterize the relevance of PDZ binding for HPV oncogenicity. The first part of the thesis focuses on HPV E6 interaction with ubiquitin ligases and the proteasome degradatory pathways. Many studies have suggested that some of E6's biochemical activities were E6AP independent, and consequently I was interested in investigating further E6's association with E6AP, and determining the consequences of this interaction for both E6 function and stability. At the same time I also pursued a series of studies to investigate whether E6 could associate with other components of the ubiquitin proteasome pathway. In the second part of the thesis I performed a series of studies on the Rhesus Papillomavirus 1 E7 (RhPV-1 E7) protein since, unlike high-risk mucosal HPVs, the PDZ binding motif is found on E7 as opposed to E6. The aim of these being to verify that this was a bona fide PDZ binding motif and to identify which cellular PDZ domain-containing cellular proteins were the substrates of RhPV-1 E7.

Results

PART I: Ubiquitin Ligase Regulation of HPV-18 E6

E6 Protein Stability is E6AP-dependent in HeLa Cells

Many of the previous studies investigating the respective roles of E6 and E6AP have involved either overexpression systems or, in cases where endogenous E6 was analysed, conditions in which the level of E6 protein expression was not determined. The question of their interdependence seemed particularly relevant considering the apparent overwhelming importance of E6AP for E6's global effects on cellular transcription (Kelley *et al.*, 2005). Therefore in order to further clarify the role of E6AP in the function of E6, we first investigated the levels of E6 expression in HPV-positive cells, in the presence and absence of E6AP. To do this, HPV-18-containing HeLa cells were transfected with siRNAs directed against either E6AP, HPV-18 E6, or Luciferase for control, and the levels of both proteins were then analysed by Western blot analysis. The proteasome inhibitor CBZ was also added to the cells 2.5 h prior to harvesting, to determine whether any changes in the pattern of protein expression were proteasome-dependent. Cells were harvested 72 h post-transfection, and the total cellular lysates were subjected to Western blot analysis using anti-E6AP, anti-Tubulin, and anti-18E6 antibodies. The results obtained are shown in Figure 8. As can be seen, the reduction of E6 levels by siRNA results in a marked upregulation in the level of E6AP expression, and this is consistent with previous observations showing that E6 induces the auto-ubiquitination and degradation of E6AP (Kao *et al.*, 2000). Most strikingly however, ablation of E6AP expression also results in a dramatic reduction in E6

protein, to levels that are even lower than those obtained using the E6 siRNA. In addition, inclusion of the proteasome inhibitor CBZ prior to harvest rescued the expression of E6, indicating that its decreased level in the cell in the absence of E6AP is proteasome-mediated. In order to reduce the possibility of off-target effects of the E6AP siRNA, we repeated the analysis using a different siRNA from another supplier (SCBT lane), and an identical reduction in the level of E6 expression was obtained. These results demonstrate that E6 steady-state levels are strongly dependent upon the presence of E6AP, and that when E6AP is not present E6 is down-regulated in a proteasome-dependent manner.

E6AP Silencing does not Affect E6 RNA Transcripts Levels

To further investigate the effects of E6AP depletion upon E6 expression levels, we proceeded to determine whether this was in any way related to the level of E6 gene transcription. To do this, HeLa cells were transfected with siRNA luciferase or siRNA E6AP. After 72 h the cells were harvested and total RNA was extracted and processed, with and without reverse transcriptase. The cDNAs were then amplified using HPV-18 E6 flanking primers and the results obtained are shown in Figure 9. Two PCR products were obtained, the upper corresponding to full length E6 and the lower corresponding to the shorter, alternatively spliced, E6* transcript. The relative difference in amounts being a reflection of higher levels of E6* mRNA than of full length E6 mRNA in HeLa cells (Schwarz *et al.*, 1985). However, it can be seen that the presence or absence of E6AP has minimal effects upon the level of HPV-18 E6 gene expression, as determined by this semi-quantitative assay. These results demonstrate that the decrease in the levels of E6 protein that is observed when E6AP is depleted is not due to any major changes

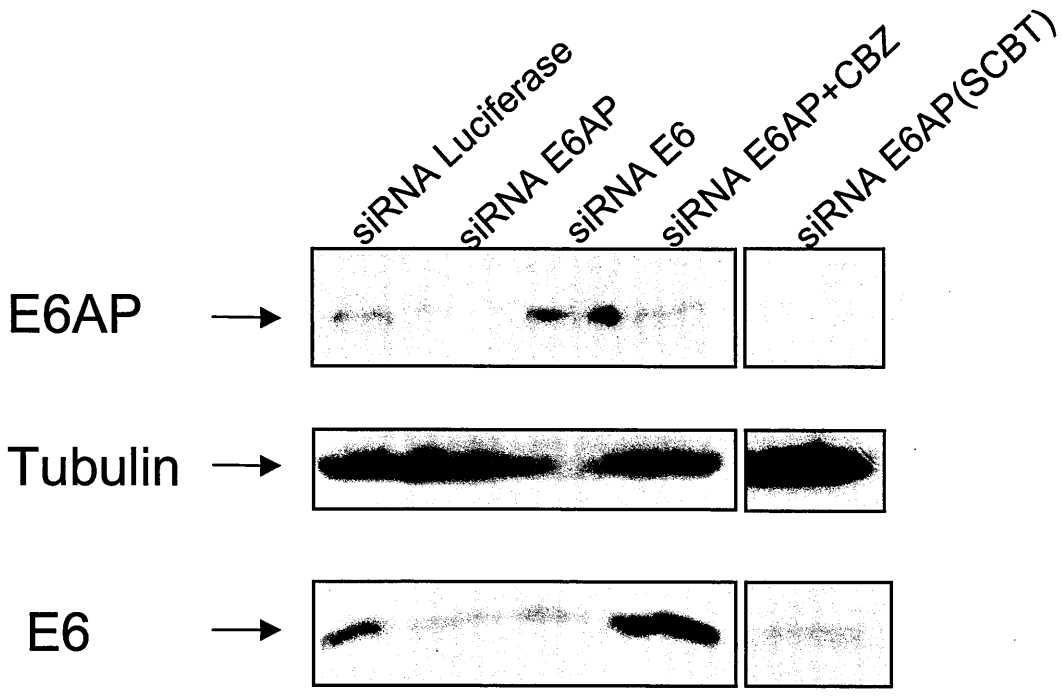


Figure 8. HPV E6 protein stability in HPV positive HeLa cells is E6AP-dependent. HeLa cells were transfected with siRNA Luciferase, or siRNA E6AP, or siRNA 18E6/E7. After 72 h cells were incubated with or without CBZ (Z-Leu-Leu-al/Sigma) for 2.5 h to block the proteasome, with DMSO treatment as control. The cells were then harvested and the protein levels were detected using western blotting with anti-E6AP antibody, anti-Tubulin antibody to monitor protein loading, and anti-18E6 antibody; followed by HRP-coupled anti-mouse antibody and ECL detection. Note that the siRNA E6AP (SCBT) track is from the same experiment and western blot. Arrows indicate the positions of the E6AP, Tubulin, and E6 proteins.

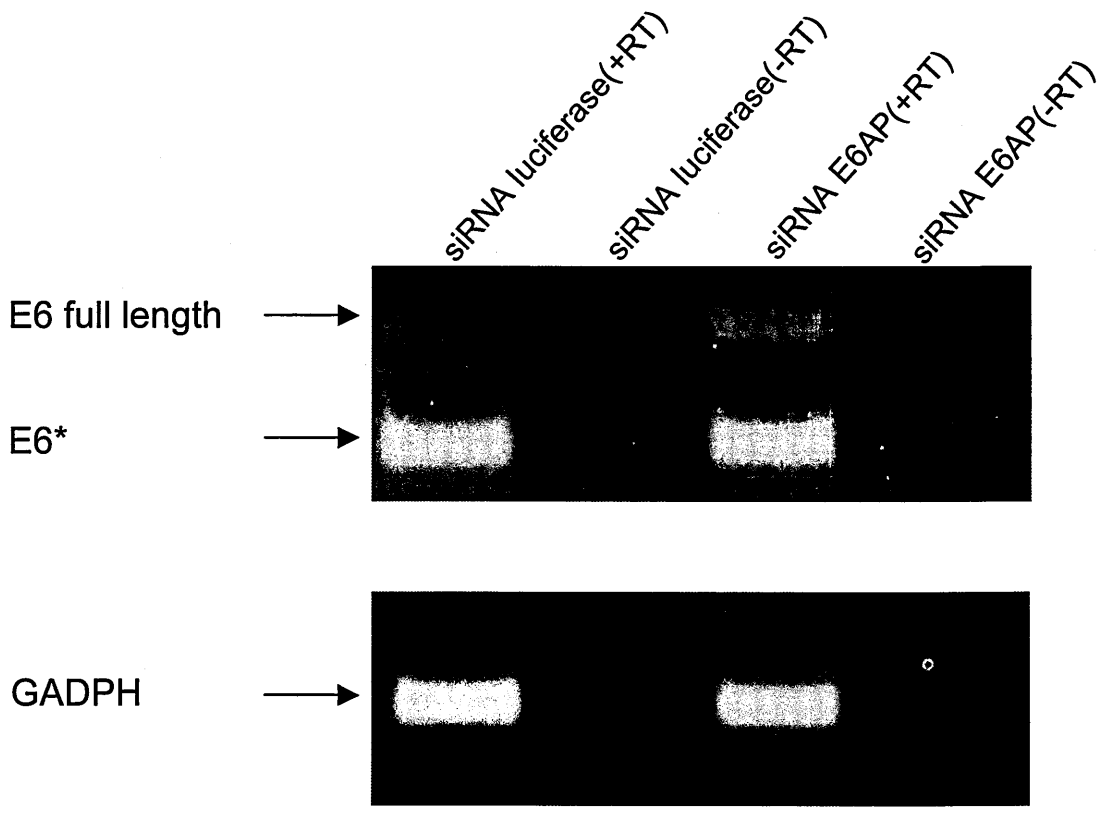


Figure 9. E6 RNA transcripts are not affected by E6AP silencing. HeLa cells were transfected with siRNA Luciferase or siRNA E6AP. After 72 h cells were harvested and total RNA was extracted. Purified RNAs were then annealed with random decamers and cDNAs generated with Reverse Transcriptase; to control for plasmid DNA carry over, a parallel set of random decamer-annealed samples were incubated without RT (-RT). The cDNAs generated from the RT step, and their control samples, were amplified using HPV-18 E6 flanking oligos. Arrows indicate the position of full length E6, alternatively spliced E6* and the GADPH loading control.

in the levels of HPV gene expression, but rather is mostly due to a decrease at the protein level.

E6AP Regulates E6 Protein Turnover

Having shown that E6AP depletion reduces the steady-state levels of E6, we then wanted to analyse the effects upon E6 protein turnover. Assays were performed as above in HeLa cells, using siRNA Luciferase as a negative control and siRNA against E6AP. 48 hours post-transfection the cells were treated with Cycloheximide for different times in order to determine whether E6AP had any effect on E6 protein half-life. The levels of E6 protein were then assessed by Western blot analysis using the anti-18 E6 monoclonal antibody and the results obtained are shown in Figure 10A, with the quantitation from multiple assays shown in Figures 10B and 10C. As can be seen, when cells were treated with the control siRNA the E6 protein levels remained relatively unchanged from time-point 0 to 60 minutes, and they started to decrease at the 120 minute time-point, similar to what has been reported previously on endogenously expressed protein (Androphy *et al.*, 1987, Grossman *et al.*, 1989). In contrast, when E6AP is depleted from the cells the E6 levels show a significant decrease by the 30 minute time-point, and the protein is completely absent by the 120 minute time-point. As expected E6AP protein levels did not change significantly in the control cells, since previous studies have reported that E6AP has a half-life of 25 hrs in HPV-negative cells and that in HeLas the half-life is reduced to 7 hrs due its increased turnover regulated by E6 (Kao *et al.*, 2000). These results show that silencing E6AP in HPV-positive HeLa cells results in a dramatic increase in E6 protein turnover.

Both Wild Type E6AP and Catalytically Inactive Mutant E6AP (C->A) Up-regulate E6 Protein Stability

Since most studies have focused on HPV-16 E6 in the context of the its association with E6AP (Kao *et al.*, 2000; Kelley *et al.*, 2005), we were obviously interested in determining whether HPV-16 E6 was similarly affected by loss of E6AP. Unfortunately, my attempts to detect endogenous HPV-16 E6 in CaSKi cells by Western blotting were unsuccessful. Therefore, as an alternative approach, we reasoned that under conditions of E6 ectopic expression, co-transfection of exogenous E6AP should also result in an increase in the levels of E6 protein expression. To investigate this, HEK 293 cells were transfected with HPV-16 and HPV-18 E6 expression plasmids, together with FLAG-tagged wild type E6AP and a catalytically inactive E6AP mutant, in order to determine whether E6AP enzymatic activity was also required for E6 stabilisation. The expression levels of E6AP and E6 were then determined by western blotting. The results, in Figure 11, show a number of interesting points. Firstly, HPV-16 E6 appears to direct the degradation of E6AP more strongly than HPV-18 E6, and this is in part dependent upon E6AP catalytic activity. These results are in agreement with previous studies (Kao *et al.*, 2000) and also support data suggesting that HPV-16 binds much more strongly to E6AP than HPV-18 E6 does (Huibregtse *et al.*, 1993b). Most importantly however, both the wild type and mutant E6AP significantly increase the levels of both HPV-16 and HPV-18 E6 expression. These results demonstrate that E6AP contributes directly to the increased stability of HPV-16 and HPV-18 E6 and, further, that this is at least in part independent of E6AP's catalytic activity.

A

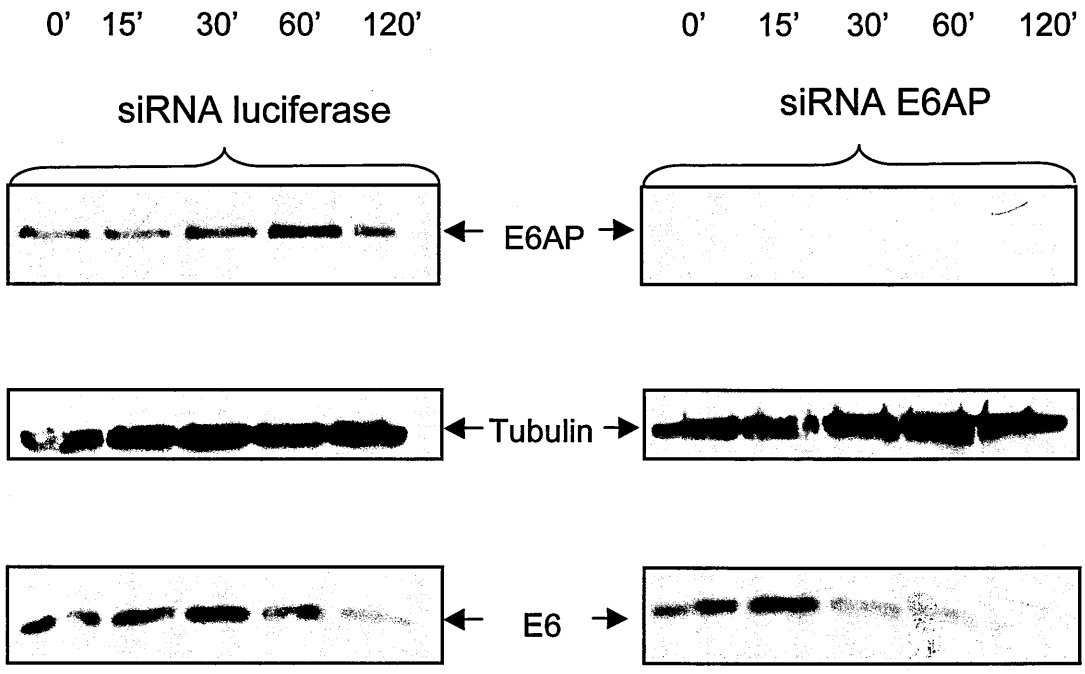


Figure 10. E6 protein turnover is regulated by E6AP. A. HeLa cells were transfected with siRNA Luciferase or siRNA E6AP. 48 h post transfection and prior to harvesting cells were treated with Cycloheximide for 5 different time points (0', 15', 30', 60', and 120'). Protein levels were detected using western blotting with anti-E6AP antibody, anti-Tubulin antibody to monitor protein loading, and anti-18E6 antibody; followed by HRP-coupled anti-mouse antibody and ECL detection. Arrows indicate the positions of the E6AP, Tubulin, and E6 proteins.

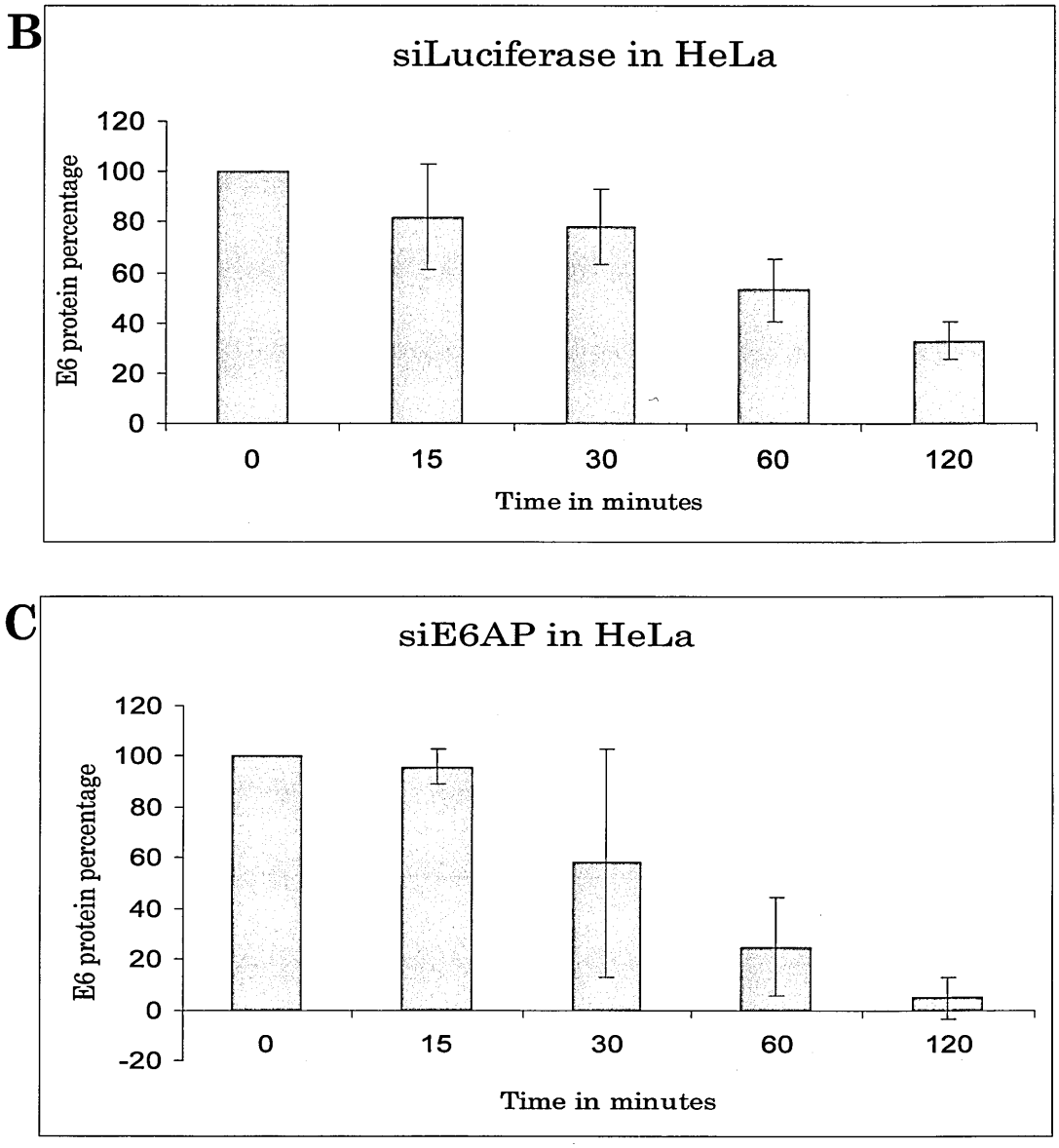


Figure 10. E6 protein turnover is regulated by E6AP. (cont.) The collated results from 3 independent experiments to measure E6 protein turnover in cells treated with siRNA Luciferase and siRNA E6AP are shown in panels B and C respectively. Band intensities were determined using the OptiQuant quantification program. E6 levels were normalized to 100% at time 0. Standard deviations are also shown.

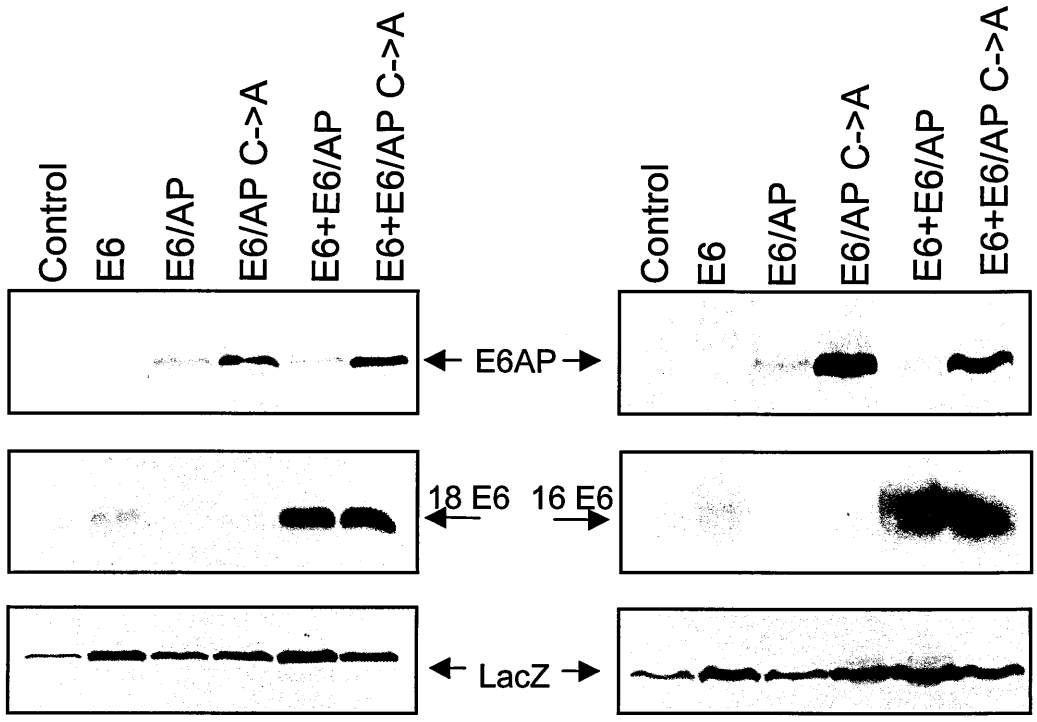


Figure 11. Stabilisation of E6 by E6AP. HEK 293 cells were transfected with plasmids expressing HPV-16 and HPV-18 E6, together, as indicated, with FLAG-tagged wild type and a catalytically inactive mutant of E6AP (C->A) plus a LacZ expression plasmid. After 24hrs the cells were harvested and the levels of E6AP monitored using anti-FLAG antibody, and HPV-16 and HPV-18 E6 were monitored using the respective anti-E6 monoclonal antibodies. Blots were stripped and re-probed for LacZ to control for transfection efficiency. Westerns blots were developed using appropriate HRP conjugated secondary antibodies and ECL detection.

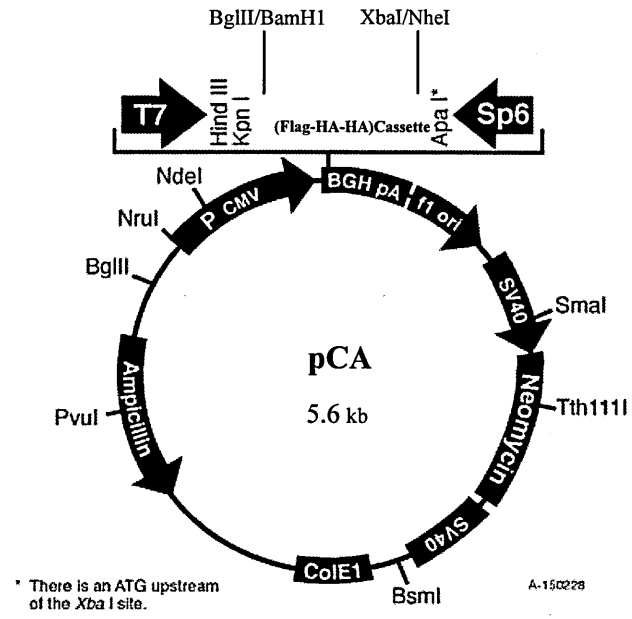
Identification of HPV-18 E6 Interacting Partners

Based on the above studies, it is clear that association with E6AP is critical for regulating E6 stability, it also demonstrates that E6 most likely interacts with components of the proteasome system in the absence of E6AP. In addition, many studies have raised the possibility that E6 can interact with other components of the proteasome pathway to target certain substrates for proteasome-mediated degradation, including p53 and the PDZ-containing substrates (Camus *et al.*, 2007; Grm and Banks, 2004; Massimi *et al.*, 2008; Shai *et al.*, 2007b; Storrs and Silverstein, 2007), which in some cases appears to be E6AP independent. Therefore, we were interested to investigate whether we could identify other, as yet unidentified, components of the proteasome degradatory pathways, with which E6 might interact. To do this we decided to take a proteomic approach: overexpressing HA-tagged E6, and immunoprecipitating it, then performing a mass spectrometry analysis of the total protein complex. Therefore, I first generated the pCA vector based on pCDNA3, with a cassette insert containing two HA tags and one FLAG tag, such that the FLAG and HA tags were cloned into the multiple cloning site of pCDNA3 between the BglII and XbaI sites (Figure 12A). This was then used as the basis for cloning HPV-18 E6 wild type and E6* (Pim *et al.*, 1994) into the tagged expression vector. These proteins were cloned in the EcoRI and HpaI cloning sites of the cassette so that the tags were positioned on the N-terminal part of the E6 proteins, as shown with HPV-18 E6 in Figure 12B. A splicing defective mutant, E6SM, was also constructed from pCA 18 E6, such that only full length E6 would be expressed. To verify that these constructs were functional, 293 cells were transfected with the three different expression plasmids, 24 hrs post-transfection cells were harvested and the total cellular lysates were subjected to Western blot

analysis using the anti-18 E6 monoclonal antibody. As shown in Figure 13, pCA:18 E6 expressed both the full length protein and the E6* spliced form, while the splicing defective mutant pCA:18E6SM only expressed the full length protein and pCA:18E6* expressed only the spliced form of the protein (Pim *et al.*, 2009).

The tagged E6 protein was then used as a bait for isolating additional binding partners. 293 cells were transfected with pCA:18E6 alone, or in combination with a known target, MAGI-2, in case the interaction with any component of the ubiquitin pathway might require the binding of E6 to a substrate. The pCA vector was also transfected alone as the control for nonspecific protein binding. After 24h the cells were treated with a proteasome inhibitor CBZ for a further 2.5 hrs. Cells were then extracted and immunoprecipitated on HA-conjugated agarose beads. The protein complexes were then subsequently processed by Dr. Mike Myers and subjected to mass spectrometry analysis.

A subset of the proteins identified are listed in Table 3, anything that was also identified in the control pulldown being discarded. We also only focused on those interactions that had a potential link to the proteasome regulating pathways. These results show a number of interesting features: the identified targets are categorized in the order of abundance, and proteins marked in red are those that are unique for each complex. In both samples where E6 complexes were assessed we pulled down 18 E6 protein and the known ubiquitin ligase E6AP. Along with those targets we also identified a large number of the proteasome pathway components in both samples, as shown in Table 3. In addition to E6AP, we identified several components of the 26S proteasome degradatory pathway, such as ubiquitin and various 26S proteasome subunits which are directly involved in the degradation of ubiquitinated



Cassette Sequence

BglII **Kozack** **Flag Tag**

GA TCT GAT ATC **GCC ACC** ATG GGC GGG TCA **GAC TAC AAG GAC GAT GAC GAT AAG** GGT GGA

HA Tag **HA Tag**

CAG CAA GGT GGC **TAC CCA TAC GAT GTT CCA GAT TAC GCA TAC CCA TAC GAT GTT CCA GAT**

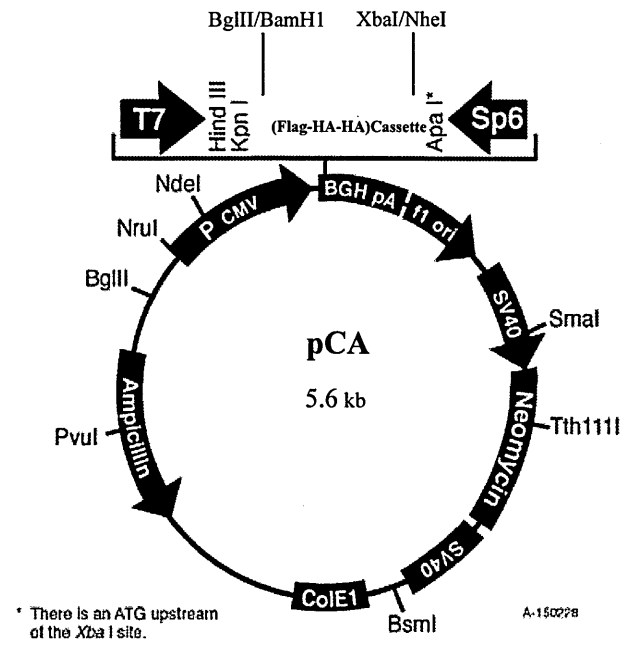
EcoRI **HpaI**

TAC GCA GTA CCT GGA TCC GAA TTC GGT ACC GCA TTT AAA TCA CGT GCG TTA ACG TCG ACT

NheI

AGCTAGC

Figure 12. pCA and pCA-18 E6 expression plasmids. A. Cassette with Flag and two HA tags was cloned into PCDNA-3 *in vivo* expression plasmid in the multiple cloning site between the BamHI and XbaI cloning sites.



Cassette Sequence

BglII **Kozack** **Flag Tag**

GA TCT GAT ATC **GCC ACC** ATG GGC GGG TCA **GAC TAC AAG GAC GAT GAC GAT AAG** GGT GGA

HA Tag **HA Tag**

CAG CAA GGT GGC **TAC CCA TAC GAT GTT CCA GAT TAC GCA TAC CCA TAC GAT GTT CCA GAT**

EcoRI **HpaI**

TAC GCA GTA CCT GGA TCC GAA TTC | —— **18 E6** —— | G TTA ACG TCG ACT

NheI

AGCTAGC

Figure12. pCA and pCA-18 E6 expression plasmids. (cont.) B. E6 was cloned into the cassette, containing Flag and two HA tags, which was previously cloned into the PCDNA-3 *in vivo* expression plasmid, between the EcoRI and HpaI cloning sites.

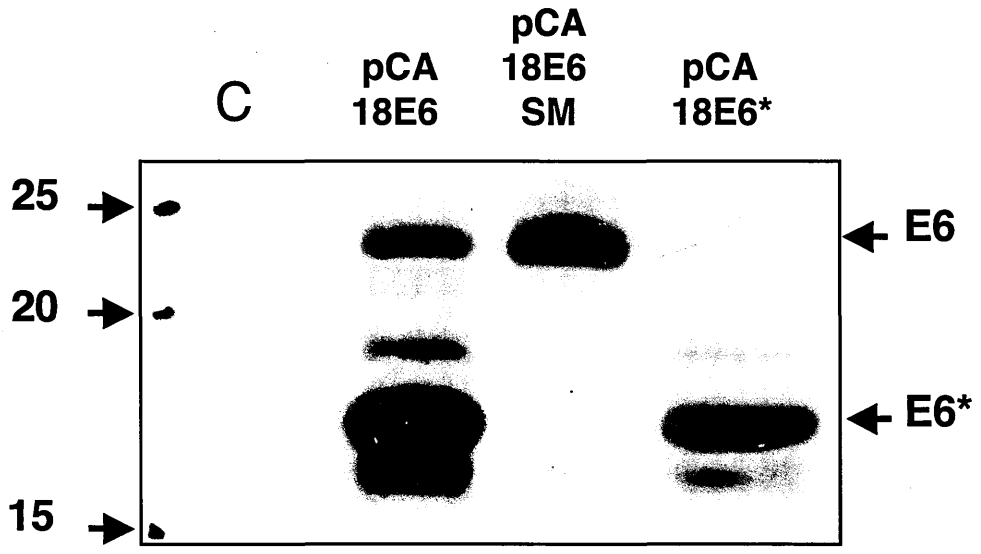


Figure 13. Western blot of pCA18E6 WT, pCA18E6 SM and pCA18E6* expressed in 293 cells. 293 cells were transfected with 5 µg of pCA:18E6 or 5 µg of pCA:18E6SM or 5 µg of pCA:18 E6* or 5 µg of pCA plasmid. 24h post transfection cells were harvested and E6 protein levels were were assessed by Western blot analysis using anti-18 E6 monoclonal mouse antibody. Control lane is labeled as C. Wild type pCA:18E6 shows two bands E6 full length protein, and E6*, while the other two lanes show only E6 full length protein (pCA:18E6SM) and E6* (pCA:E6*).

substrates. This again highlights E6's strong association with the proteasome pathway and with ubiquitin protein ligases. Furthermore, both samples also confirmed E6's interaction with PDZ-domain containing substrates. This suggests that Dlg is likely to be the preferred HPV-18 E6 PDZ-domain-containing target *in vivo* in 293 cells. In contrast, in the other sample where MAGI-2 was overexpressed together with 18 E6, only MAGI-2 was pulled down together with E6, suggesting that MAGI-2 was in excess and therefore competing out hDlg in interacting with E6 (Table 3).

Table 3. HPV-18 E6 and HPV-18 E6 + MAGI-2 Mass Spectrometry Comparison Data

	# of	
HPV-18 E6	peptides	**HPV-18 E6 + MAGI-2**
hDlg	96 104	MAGI-2
E6AP	6 14	E6AP
E6	7 8	E6
EDD1	3 5	EDD1
26S Proteasome Non-ATPase regulatory subunit 3	9 8	26S Proteasome Non-ATPase regulatory subunit 3
26S Protease regulatory subunit 8	4 5	26S Protease regulatory subunit 8
26S Proteasome Non-ATPase regulatory subunit 13	4 5	26S Protease regulatory subunit 6A
		26S Protease regulatory subunit 7
26S Proteasome Non-ATPase regulatory subunit 2	6 4	26S Protease regulatory subunit 4
	3	26S Protease regulatory subunit S10B
26S Protease regulatory subunit 4	3 2	26S Proteasome Non-ATPase regulatory subunit 13
26S Proteasome Non-ATPase regulatory subunit 4	4	
	3	26S Proteasome Non-ATPase regulatory subunit 2
26S Protease regulatory subunit 6A	2	
26S Proteasome Non-ATPase regulatory subunit 6	3 2	26S Proteasome Non-ATPase regulatory subunit 11
Ubiquitin	2 2	26S Proteasome Non-ATPase regulatory subunit 6
26S Protease regulatory subunit 7	2	
Ubiquitin-like protein 4A	1 2	Ubiquitin
	3	26S Proteasome Non-ATPase regulatory subunit 4
	1	Proteasome subunit alpha type 2

However, the most interesting feature of this mass spectrometry analysis was the identification of another ubiquitin ligase, EDD, in addition to E6AP. Marked in blue and shown in Table 3, EDD was pulled down in both complexes. EDD, a 300kD nuclear protein, is a mammalian ortholog of the *Drosophila melanogaster* "hyperplastic

discs" tumor suppressor gene (hyd) (Callaghan *et al.*, 1998) and it has critical roles in control of cell proliferation and tumorigenesis (Mansfield *et al.*, 1994). Studies have shown that EDD (-/-) mice die at mid-gestation due to failed yolk sac angiogenesis and defective placental development, leading to general failure of embryonic cell proliferation and widespread apoptosis (Saunders *et al.*, 2004). EDD has been found to be overexpressed in breast and ovarian cancers (Clancy *et al.*, 2003); while truncating mutations of the protein are commonly found in gastric cancers with micro-satellite instability (Mori *et al.*, 2002). It is also involved in DNA damage signaling, and, intriguingly, functions as an E3 HECT ubiquitin ligase (Callaghan *et al.*, 1998; Munoz *et al.*, 2007).

HPV-18 E6 Binds to EDD *in vitro*

Since EDD is a ubiquitin ligase, we were particularly interested in investigating further its potential interactions with E6 and how this might affect and/or contribute to E6 functions. We first analyzed the potential of HPV-18 E6 to interact with EDD *in vitro*. EDD was *in vitro* translated, ^{35}S radiolabelled and then incubated with GST-18 E6, GST-Dlg, GST-18 E6* or GST alone for comparison. The result of the assay in Figure 14A shows that HPV-18 E6 binds to EDD more strongly than either Dlg or the GST control. Interestingly, the binding between E6* and EDD is also very weak, suggesting that most of the interaction motif lies within the carboxy terminal half of HPV-18 E6.

We were also interested in determining if EDD binding is specific for HPV-18 E6. Therefore we used GST fused HPV-16 E6 and HPV-11 E6 as comparisons for the ability to bind EDD. EDD was *in vitro* translated, ^{35}S radiolabelled and then incubated with GST-18 E6, GST-

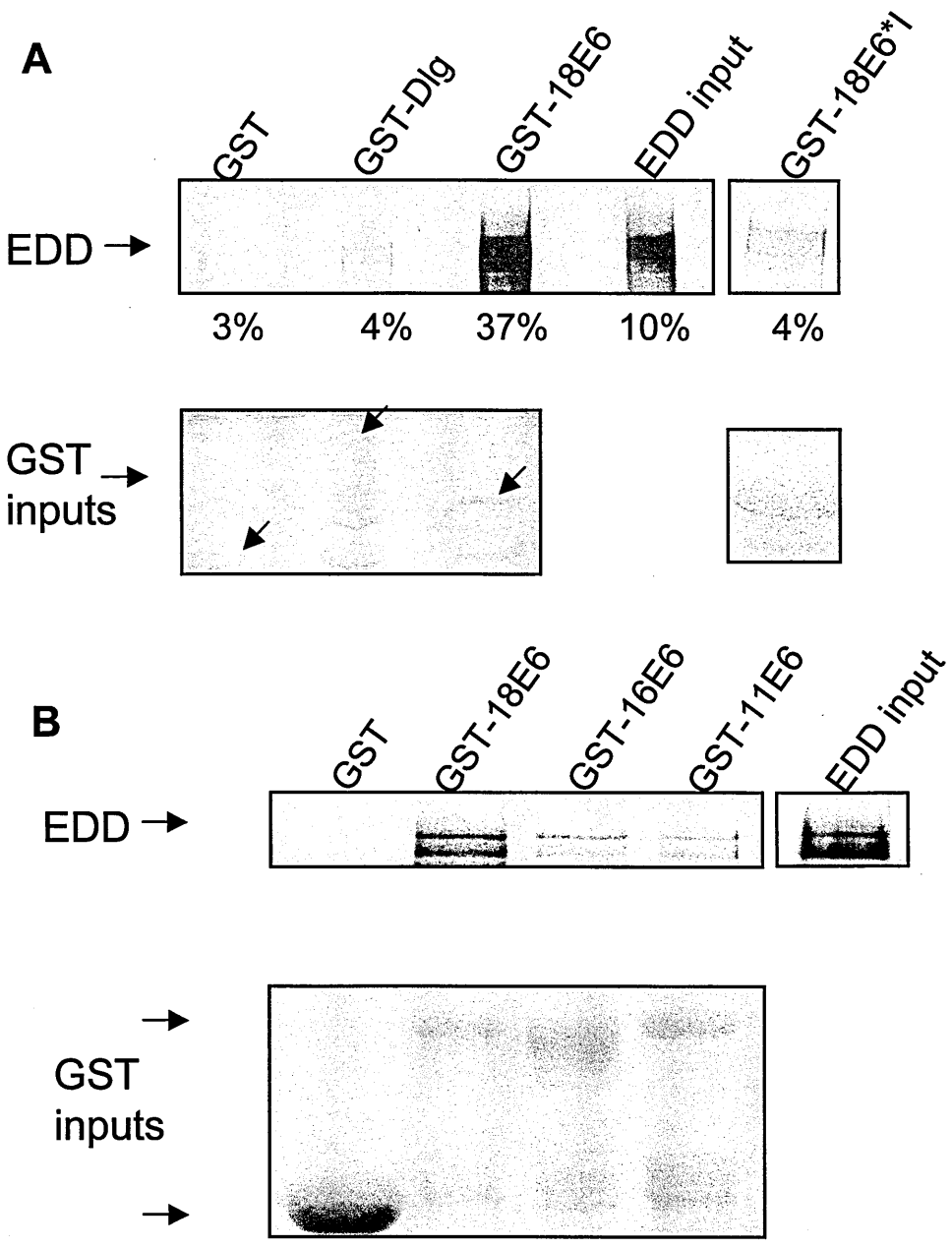


Figure 14. HPV-18 E6 protein binds to EDD *in vitro*. A. *In-vitro*-translated EDD was incubated with GST, GST-Dlg, GST-18 E6, and GST-18 E6*. Bound proteins were assessed by autoradiography and the input GST fusion proteins were visualized with Coomassie staining (lower panel). Input of EDD (10%) is also shown. PhosphoImager quantitation of EDD bound to GST- 18 E6 is 37%. B. *In-vitro*-translated EDD was incubated with GST, GST-18E6, GST-16E6, and GST-11E6. Bound proteins were assessed by autoradiography and the input GST fusion proteins were visualized with Coomassie staining (lower panel). Input of EDD (10%) is also shown.

16 E6 GST-11 E6 or GST alone for control. The result in Figure 14B shows that HPV-18 E6 binds to EDD more strongly than both HPV-16 E6 and HPV-11 E6. Interestingly, both HPV-16 E6 and HPV-11 E6 bind EDD at a similar level, demonstrating that the ability of E6 to bind EDD is not restricted to the high risk types.

HPV-18 E6 Binds to EDD *in vivo*

We then proceeded to confirm that the interaction between E6 and EDD occurrs *in vivo*. To do so, we performed pull-down assays in cells where EDD was overexpressed alone, together with HA-tagged 18 E6 or in combination with HA-tagged 18 E6 and HA-tagged MAGI-2. The results in Figure 15 confirm the interaction between EDD and 18 E6 *in vivo*. In both experiments (panels A and B) EDD was co-immunoprecipitated when overexpressed with E6. When EDD was overexpressed by itself or in combination with MAGI-2, binding to the HA-resin was very weak or absent. Interestingly, EDD was able to bind to E6 when those two proteins were expressed together, and also when both were expressed in combination with MAGI-2, suggesting that these three proteins might interact in a triple complex. The fact that E6 could interact with MAGI-2 and EDD in a complex also suggests that the binding between EDD and E6 does not adversely effect E6's ability to bind MAGI-2, further suggesting that the EDD-binding region on E6 is probably not located in the C-terminal PDZ binding domain of E6.

EDD Inhibits HPV 18 E6 Degradation of Dlg *in vitro*

As already mentioned, HPV E6 is not able to degrade targets by itself, it needs to form a complex with a cellular ubiqutin ligase, such as E6AP, and with such target proteins as p53, Bak, or various PDZ domain-containing proteins for proteasomal degradation to occur (Handa *et al.*, 2007; Kuballa *et al.*, 2007; Scheffner *et al.*, 1993; Thomas and Banks, 1998; Thomas and Banks, 1999). Since EDD is a ubiquitin ligase we were obviously interested in investigating the potential role of EDD in E6's degradatory activities. To do this E6, Dlg and EDD were translated *in vitro* and co-incubated at 30°C for 1 and 2 hrs. The level of Dlg protein remaining was then ascertained by SDS-PAGE and autoradiography. The results in Figure 16 show the levels of *in vitro* translated Dlg at time-points 0, 60 and 120 minutes. As expected, Dlg remained relatively stable over this time period. After the addition of E6 the levels of Dlg were dramatically decreased at the 60 minute time-point, while at 120 minutes time-point Dlg was almost completely degraded. Interestingly, in the presence of EDD, Dlg protein degradation was greatly reduced at both the 60 and 120 minute time-points. This observation is opposite to the outcome that one might expect if EDD was acting as an E6-dependent ubiquitin ligase to degrade its targets. The result from Figure 16 suggests that EDD somehow interferes with E6 degradatory activity.

EDD Interferes with HPV 18 E6 Degradation of Dlg, MAGI-2 and p53 *in vivo*

To investigate these effects of EDD further, we analyzed whether EDD could rescue Dlg protein levels in the presence of E6 *in vivo* in human

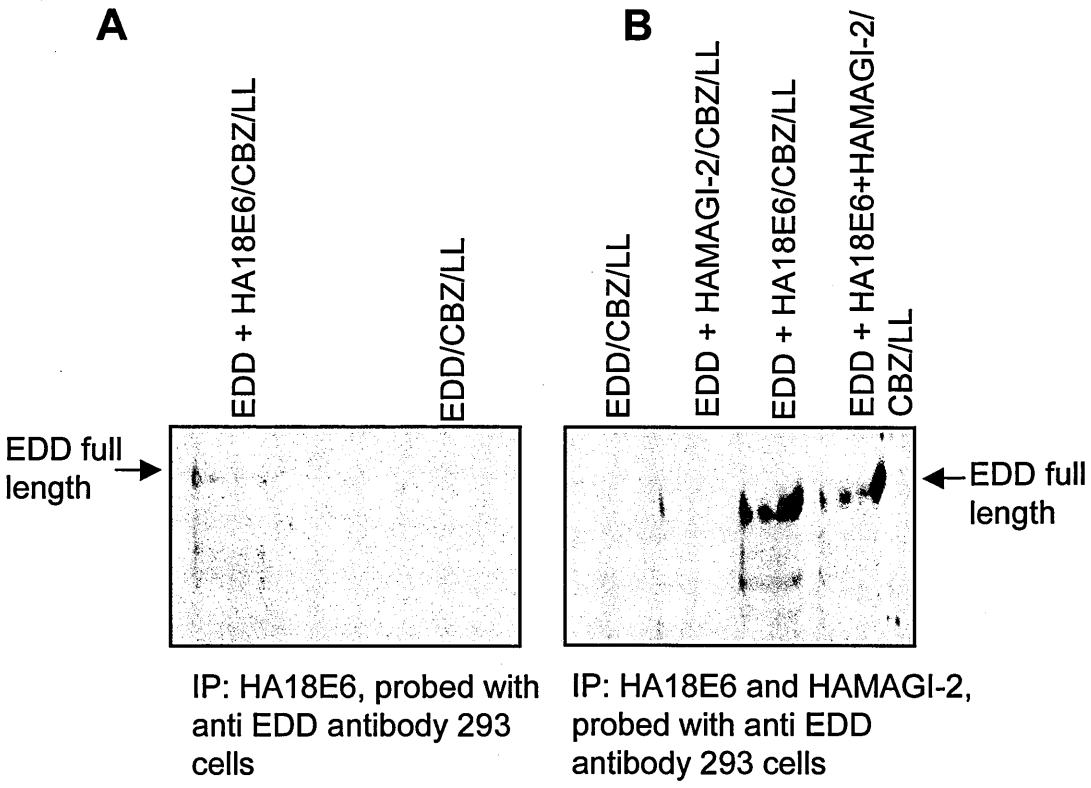


Figure 15. HPV-18 E6 protein binds to EDD1 *in vivo*. 293 cells were transfected with 10 µg of HA tagged HPV18 E6, 5 µg EDD1, or 5 µg HA-tagged MAGI-2, alone or in combination. After 24 h cells were incubated for 3h with proteasome inhibitor (CBZ) before harvesting. Soluble fractions were incubated with HA-antibody conjugated agarose beads for 2h at 4°C. Immunoprecipitated EDD1 protein was assessed by Western blot analysis using anti-EDD1 antibody (A and B). EDD1 protein was only detected in lanes which included co-transfected HA-18 E6 or combination of HA-18 E6 and HA-MAGI-2.

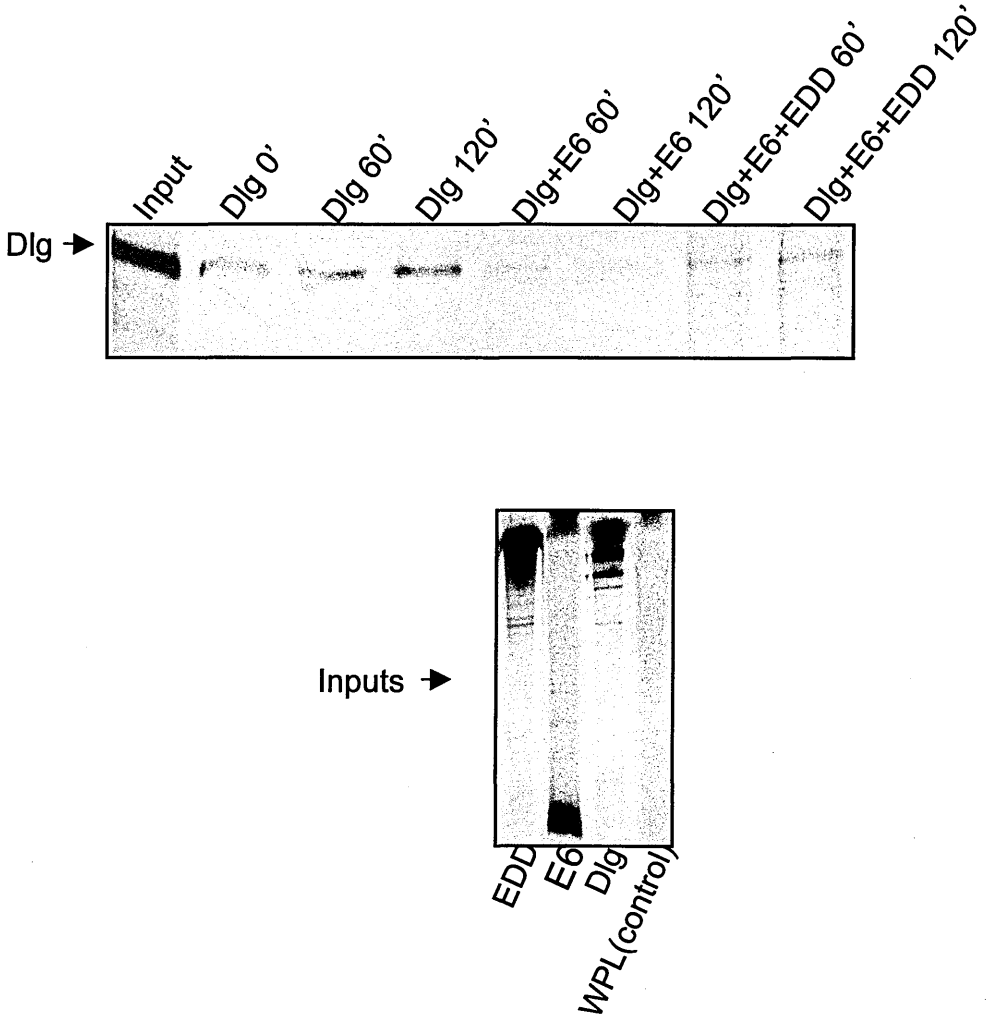


Figure 16. EDD down-regulates HPV-18 E6 degradation of Dlg *in vitro.* EDD, HPV-18 E6 and Dlg were translated, and co-incubated at 30°C for the times indicated. Residual Dlg was then detected by immunoprecipitation, SDS-PAGE and autoradiography. Inputs are also shown (lower panel). WPL refers to water prime lysate.

293 cells which were transfected with plasmids expressing Dlg alone or in combinations with HPV E6 and EDD (Figure 17A). In addition, we introduced MAGI-2 into the same experiment (Figure 17B), to determine whether the observations from Figure 16 were Dlg-specific, or whether EDD could have the same effects on other E6 PDZ domain-containing targets. Finally, we also included p53 (Figure 17C) in this experiment to investigate if EDD could have the same effect on rescuing p53 from E6-mediated degradation. 24 h post-transfection, cells were harvested in SDS lysis buffer and Dlg, MAGI-2 and p53 levels were assessed by Western blot analysis. The results in Figure 17 show degradation of Dlg, MAGI-2 and p53 in the presence of E6 as expected. Interestingly, and similar to what was seen in the Figure 16, after EDD was co-transfected with both Dlg and MAGI-2 protein levels were restored back to control levels. Interestingly, p53 protein levels were also rescued in the same way. Furthermore, when EDD and MAGI-2, Dlg or p53 were combined in the absence of E6 there were no major changes in the protein levels of these targets. These results suggest that the effects of EDD in rescuing MAGI-2, Dlg and p53 from E6-induced degradation are most likely associated with a direct effect on E6 function, rather than an effect on the target proteins per se, or on indirect effects involving general components of the proteasome pathway.

Endogenous EDD Directly Regulates HPV E6 Activity *in vivo*

To further elucidate these observations and to investigate the role of EDD in the context of HPV E6 function in cervical tumor-derived cell lines, we performed siRNA EDD silencing experiments in HPV-18-positive HeLa and HPV-16-positive CaSKi cells. Both HeLa and CaSKi

cells were transfected with siRNAs directed against either EDD, HPV-18 or HPV-16 E6, respectively, or Luciferase for comparison, and the levels of both proteins were then analysed by western blotting after 72 hrs. The results obtained are shown in Figure 18. As can be seen, the reduction of E6 levels by siRNA results in a marked upregulation in the level of p53 expression (Figure 18 A and B), and this is consistent with previous observations showing that E6 uses E6AP in targeting p53 for proteasome-mediated degradation (Kao *et al.*, 2000). Interestingly however, depletion of E6AP expression results in even greater upregulation in p53 protein levels, to levels that are even higher than those obtained using the E6 siRNA. These results reflect our observations in Figure 8, where we show that E6 levels are lower in cells treated with siRNA E6AP than in cells treated with siRNA E6 only, highlighting again that E6 protein stability is highly dependent on E6AP levels.

However, even more striking results were observed from cells treated with siRNA EDD. In both cell lines (Figure 18 A and B), cells treated with siRNA EDD show a dramatic reduction in p53 levels in comparison with the control p53 levels, suggesting that the presence of EDD is inhibiting E6 degradatory function with respect to p53. Interestingly, there were still some residual levels of EDD after siRNA treatment, suggesting that complete silencing of EDD protein levels would probably result in even greater p53 degradation. These results show that EDD is a crucial component of the E6 degradatory machinery and that it directly or indirectly has a major effect on E6's ability to direct the degradation of its main target, p53.

Effects of siRNA EDD Depletion on HPV E6 and E6AP in HeLa Cells

From the results mentioned above it is clear that EDD does not have any major direct effects on E6 targets such as the PDZ domain-containing proteins, but that it rather has a direct effect on E6 or on some component linking E6 to the proteasome pathway. Therefore, we were interested in investigating the potential effects of EDD upon the levels of both E6 and E6AP. Assays were performed as above in HeLa cells, using siRNA Luciferase as a negative control and siRNA against E6AP, siRNA against EDD, and combined siRNAs against E6AP and EDD. 72 hours post transfection cells were harvested and total cellular lysates were subjected to Western blot analysis using the anti-18 E6 antibody, an anti-E6AP antibody, and an anti-Tubulin antibody as a loading control. The results obtained are shown in Figure 19. As shown above, when E6AP was silenced in HPV-positive cells the levels of E6 were dramatically reduced, and that observation was confirmed in this experiment. However, more interesting results were observed in cells treated with siRNA EDD, where upregulation of both E6 and E6AP was observed after EDD was silenced. To exclude the possibility that EDD is the ubiquitin ligase which turns over E6 when E6AP is not present, we also included cells treated with siRNA EDD and siRNA E6AP. As can be seen from lane four, there was no significant E6 protein rescue when both ubiquitin ligases were silenced. These results demonstrate that EDD has a potential direct effect on E6AP stability, since EDD silencing leads to E6AP accumulation, which consequently results in E6 upregulation. The results also suggest that E6 protein turnover is not EDD-dependent in the absence of E6AP, which suggests several possibilities that will be discussed in more detail in the Discussion.

HPV E6, EDD and E6AP can Interact *in vitro*

In order to investigate if E6, EDD and E6AP can potentially interact, EDD and E6AP were *in vitro* translated in Wheat Germ Extract. The reasoning for using Wheat Germ system was because it does not contain E6AP which is present in the Rabbit Reticulocyte system. The *in vitro* translated proteins were incubated individually or in combination with GST-18 E6, and GST alone for the negative control. The result of the binding assay in Figure 20A shows that both proteins bind to E6. The introduction of either E6AP or EDD to the mixture does not increase the binding affinity of the proteins towards E6. Additionally, this result suggests that EDD could form a complex with E6 independently of E6AP.

Furthermore we wanted to investigate if an E6 mutant (I130T), which is significantly reduced in its ability to bind E6AP, would also have any effects on EDD binding. EDD and E6AP were *in vitro* translated, ^{35}S radiolabelled and then incubated with GST-18 E6, GST-18 E6 (I130T), and GST alone for control. The result of the assay in Figure 20B shows that HPV-18 E6 binds to EDD similarly to the HPV-18 E6 (I130T) mutant, while there is a difference between wild type HPV-18 E6 binding to E6AP in comparison to the I130T mutant. This again suggests a possibility that E6 does not require E6AP for EDD binding and that the binding sites for EDD and E6AP are distinct on E6.

To clarify this even further, and to additionally confirm that EDD can bind to E6 without E6AP, we decided to perform pull-down assays in E6AP-null cells. NIH3T3 mouse fibroblasts were used as the positive control since they are positive for E6AP. Prior to harvesting, cells were treated with the proteasome inhibitor CBZ for 2.5 hrs and then

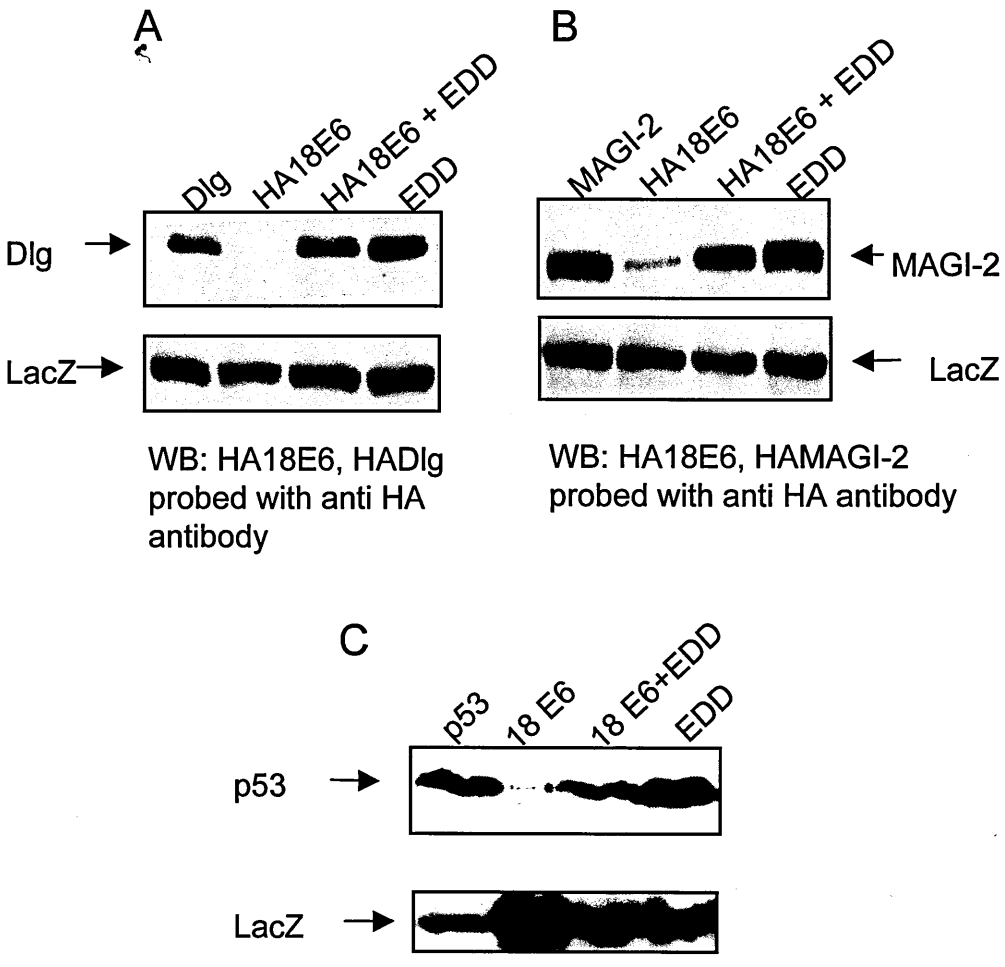


Figure 17. EDD down-regulates HPV-18 E6 degradation of Dlg, MAGI-2 and p53 *in vivo.* 293 cells were transfected with 3 µg of HA-tagged Dlg, 5 µg of HA-MAGI-2, 5 µg of EDD1, and 5 µg of HA-18 E6 (2 µg with p53), alone or in combination. After 24 h cells were harvested in SDS sample buffer and residual Dlg (A), MAGI-2 (B) and p53 (C) protein levels were assessed by Western blot analysis using anti-HA antibody. The expression of ß-galactosidase (Lac Z) was used as a control of transfection efficiency and loading (lower panels).

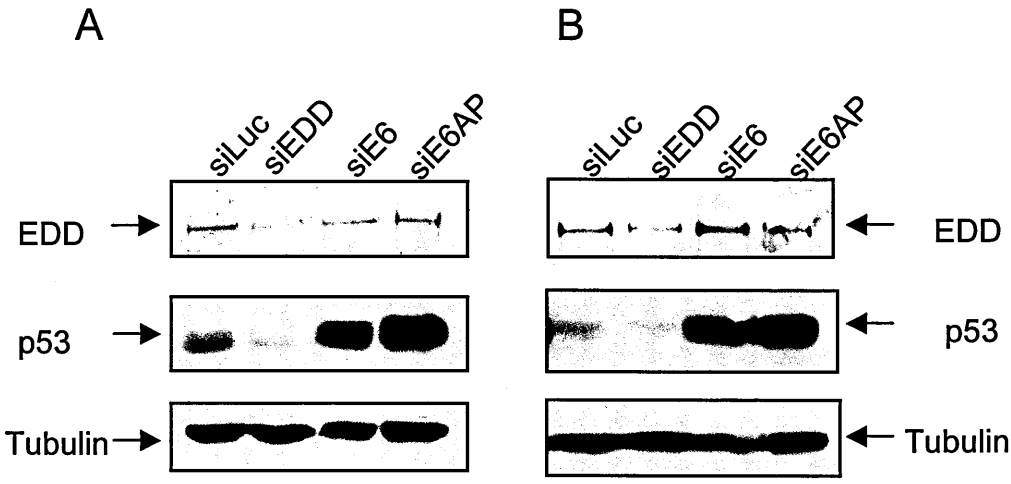


Figure 18. Effects of siRNA EDD depletion on HPV E6 substrates. HeLa cells (A) and Caski cells (B) were transfected with siRNA Luciferase, or siRNA EDD, or siRNA E6AP, or siRNA 18E6/E7, or siRNA 16E6/E7. After 72 h cells were harvested and the protein levels were detected using Western blotting with anti-EDD, anti-p53 and anti-Tubulin antibody to monitor protein loading, followed by HRP-coupled anti-mouse anti goat-antibody and ECL detection. The arrows indicate the positions of the EDD, p53 and Tubulin proteins.

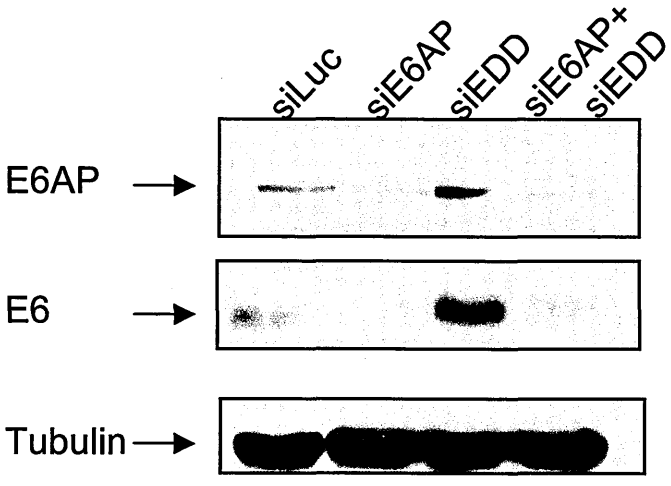


Figure 19. siRNA EDD effects on HPV E6 and E6AP in HeLa cells. HeLa cells were transfected with siRNA Luciferase, or siRNA E6AP, or siRNA EDD, alone or in combination After 72 h cells were harvested and the protein levels were detected using Western blotting with anti-E6AP antibody, anti-E6 and anti-Tubulin antibody to monitor protein loading, followed by HRP-coupled anti-mouse and ECL detection. The arrows indicate the positions of the E6AP, E6 and Tubulin proteins.

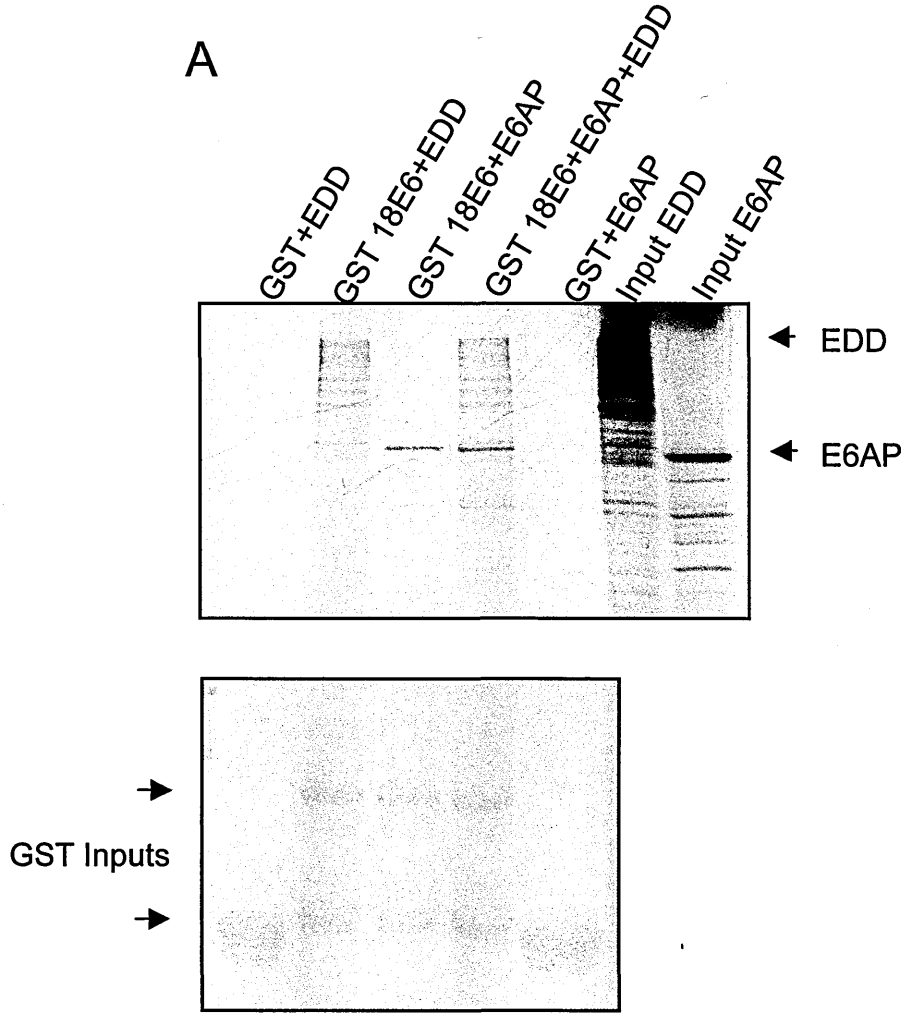


Figure 20. HPV-18 E6, EDD and E6AP interact *in vitro*. A. Wheat Germ *in-vitro*-translated EDD and E6AP were incubated with GST and GST-18 E6 alone or in combination. Bound proteins were assessed by autoradiography and the input GST fusion proteins were visualized with Coomassie staining (lower panel). Full amounts of EDD and E6AP used in the binding assays are also shown.

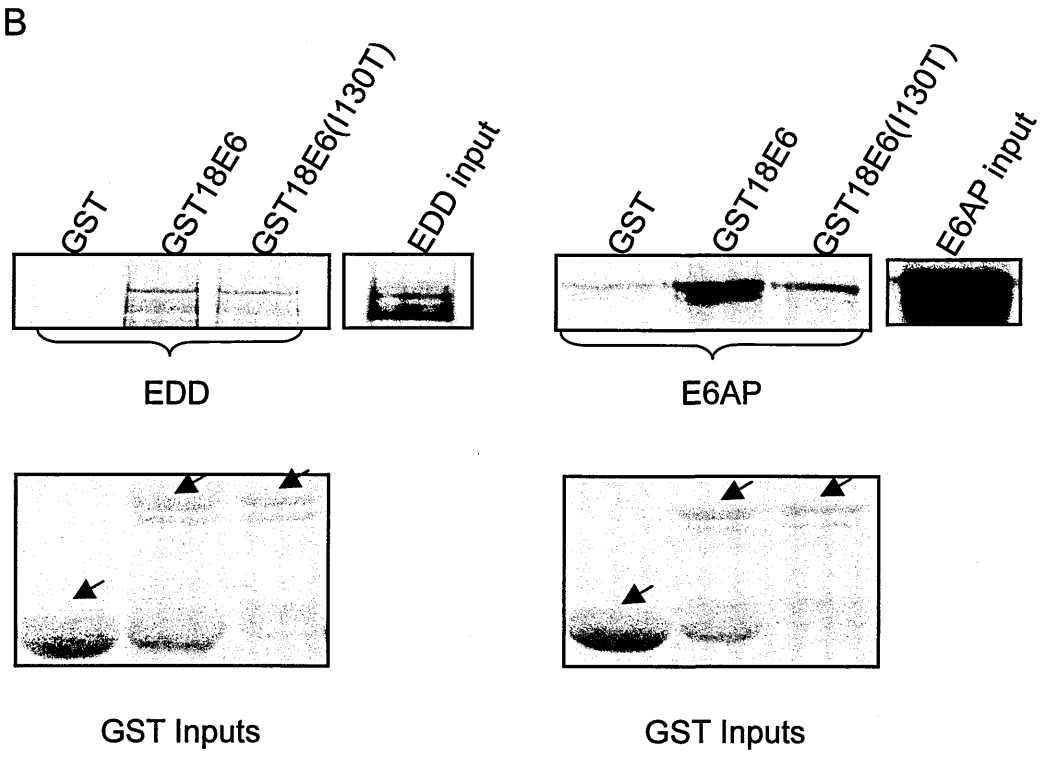


Figure 20. HPV-18 E6, EDD and E6AP interact *in vitro*. (cont.) B. *In-vitro*-translated EDD and E6AP were incubated with GST, GST-18E6 and GST-18E6 (I130T). Bound proteins were assessed by autoradiography and the input GST fusion proteins were visualized with Coomassie staining (lower panel). GST-18 E6(I130T) binding to EDD is 21% lower in comparison with GST-18 E6, while GST-18 E6(I130T) binding to E6AP is 77% lower in comparison to GST-18 E6. Arrows indicate GST fusion proteins.

incubated with GST-18 E6 or GST alone for comparison. The result of the assay in Figure 20C shows that E6 was able to bind to EDD regardless of whether E6AP was present or not. Interestingly, the result also shows that the presence of E6AP did not significantly increase the amount of EDD bound to E6, confirming the result from Figure 20A.

siRNA EDD Effects on HPV E6 Substrates in HPV-negative Cells

Since EDD has a downregulating effect on E6AP in HPV positive cells, we wanted to investigate further if EDD is also responsible for E6AP regulation in HPV negative cells and if it has any effects on p53. First, 293 cells were transfected with E6AP alone or in combination with either EDD or an EDD (C->A) catalytically inactive mutant. Cells were harvested 24 hrs post-transfection and the total lysates were analyzed by Western blot analysis using anti-FLAG and anti-ß Gal antibodies. The results are shown in Figure 21. As can be seen, E6AP protein levels are completely depleted in the presence of EDD, and in the presence of the catalytically inactive EDD (C->A) mutant E6AP levels remain unchanged. This result demonstrates that EDD is responsible for E6AP protein turnover and this process is proteasome regulated.

Furthermore, we wanted to confirm this effect on endogenous protein levels in a HPV negative background. To do this, HT1080 cells were transfected with siRNAs directed against either EDD, E6AP or Luciferase for comparison, and the levels of both proteins were then analysed by western blot analysis. Cells were harvested 72 hrs post-transfection, and the total cellular lysates were subjected to western blot analysis using anti-EDD, anti-E6AP, anti-p53 and anti-Tubulin

antibodies. The results obtained are shown in Figure 22. As can be seen, the reduction of EDD and E6AP levels by siRNA did not result in any marked changes in the level of p53 expression (Figure 22A). However, in contrast, siRNA silencing of EDD in the same cell type resulted in a dramatic upregulation of E6AP (Figure 22B). These results demonstrate that the effects of EDD on p53 downregulation are specifically associated with an HPV-positive environment and the consequent E6-regulated p53 protein turnover. Furthermore, these results also show that EDD effects on E6AP are not necessarily HPV-related, but that it also occurs in HPV-negative cells, and that EDD could therefore be a crucial factor in regulating E6AP protein levels.

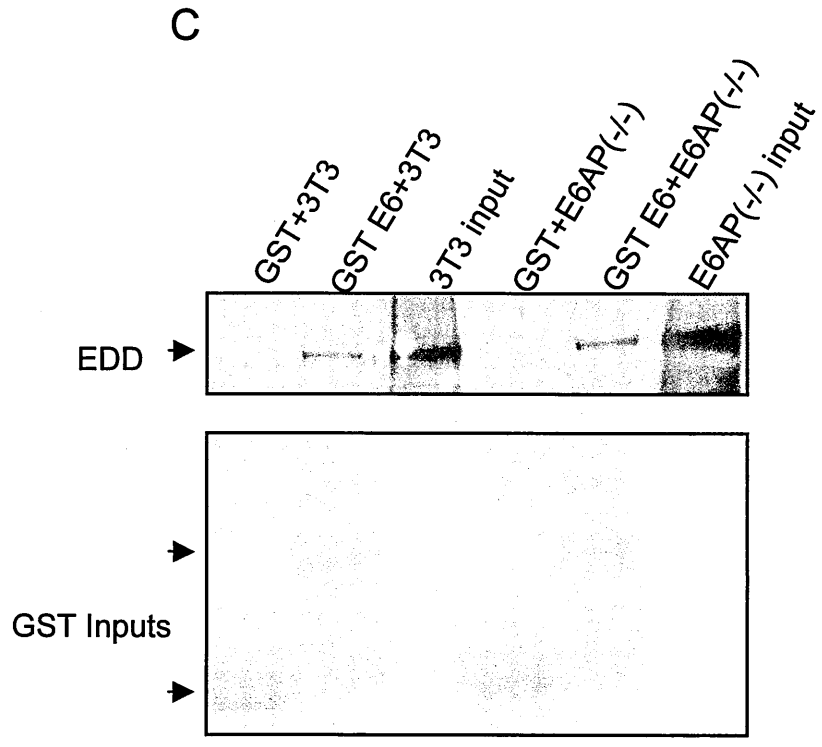


Figure 20. HPV-18 E6, EDD and E6AP interact *in vitro*. (cont.) C. E6AP(-/-) and 3T3 cells were treated with CBZ proteasome inhibitors 2.5 hrs prior to harvesting. The soluble fractions of each sample were then incubated with GST and GST-18 E6 fusion proteins for 2 hrs at 4°C. Bound proteins were assessed by Western Blot analysis using EDD antibody. The EDD inputsfrom the cells are also shown.

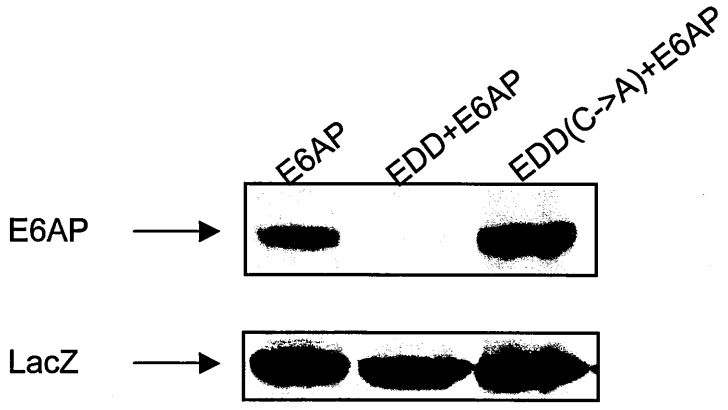


Figure 21. EDD degrades E6AP *in vivo*. 293 cells were transfected with 5 µg of FLAG-tagged E6AP, 5 µg of EDD and 5 µg catalytically inactive EDD (C->A), alone or in combination. After 24 hrs cells were harvested in SDS sample buffer and residual E6AP protein levels were assessed by Western blot analysis using anti-FLAG antibody. The expression of ß-galactosidase (Lac Z) was used as a control of transfection efficiency and loading (lower panel).

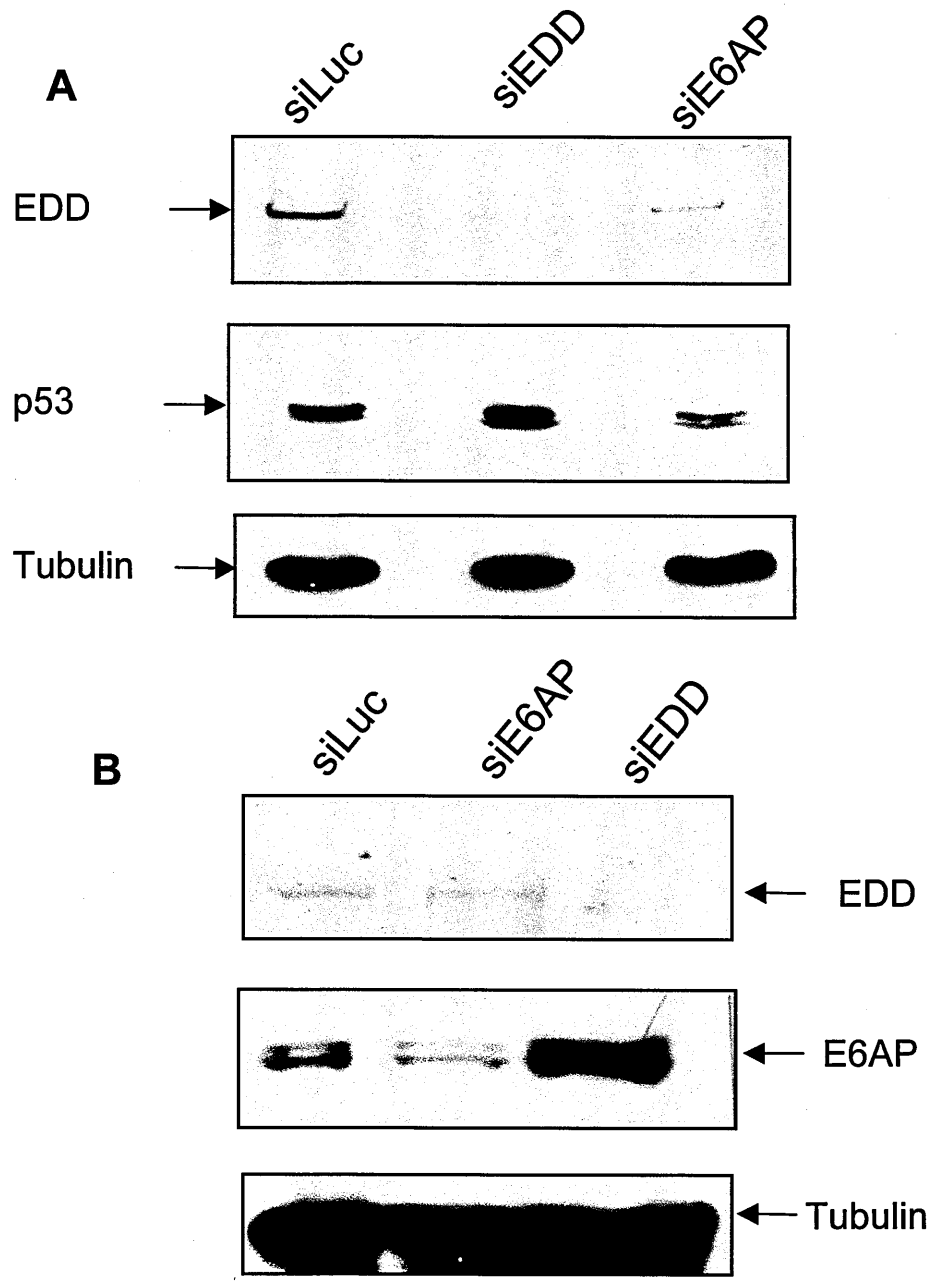


Figure 22. siRNA EDD effects on HPV E6 substrates in HPV negative cells. HT1080 (A and B) cells were transfected with siRNA Luciferase, or siRNA EDD, or siRNA E6AP. After 72 h cells were harvested and the protein levels were detected using Western blotting with anti-EDD, anti-p53, anti-E6AP and anti-Tubulin antibody to monitor protein loading, followed by HRP-coupled anti-mouse and ECL detection. The arrows indicate the positions of the EDD, E6AP and Tubulin proteins.

PART II: PDZ Binding is Conserved Between HPV and RhPV

RhPV-1 E7 has a Functional PDZ Binding Domain

As noted above, PDZ binding appears to be an important aspect of high-risk mucosal HPV types. Since RhPV-1 induction of cervical carcinoma in monkeys has been proposed as a model for HPV induced malignancy, we were interested to know whether RhPV-1 E6, like HPV-16 and HPV-18 E6, contains a PDZ binding motif. As can be seen from the amino acid sequence alignment in Figure 23, no such motif exists in RhPV-1 E6, whether it is compared with class I, as in the case of HPV-16 and HPV-18 E6, or class II and class III binding motifs. However, most intriguingly, the RhPV-1 E7 C-terminal sequence is a perfect match with a class I PDZ binding motif (Figure 23). This retains the canonical residues S/T and the C-terminal V/L, although the non-canonical residues are different from those present in HPV-16 and HPV-18 E6 proteins. To determine whether this is a true PDZ recognition motif, we investigated whether RhPV-1 E7 could bind a panel of known PDZ domain-containing targets of HPV-18 E6. The wild type RhPV-1 E7 plus carboxy terminal V->A and ΔPDZ mutants, which should destroy the PDZ binding site (Figure 23), were expressed as GST fusion proteins and incubated with *in vitro* translated, ^{35}S radiolabeled hScrib, MAGI-2 and MAGI-3. The results of the pull-down assays in Figure 24 and Figure 25 show that wild type RhPV-1 E7 can interact with MAGI-2, MAGI-3 and hScrib, albeit not as strongly as HPV-18 E6, while the RhPV-1 E7 V->A and ΔPDZ mutants show much weaker interactions. Interestingly, as seen in Figure 24, the V->A mutation did not

completely abolish the binding of RhPV-1 E7 to hScrib, suggesting that the entire PDZ binding motif is involved in binding hScrib. As seen from Figure 25, when the mutant lacking the entire PDZ-binding motif was introduced the binding to all three targets was abolished. These observations demonstrate that the C-terminus of RhPV-1 E7 is a recognition motif for three different PDZ-domain containing substrates.

Furthermore, these results also indicate that, whilst RhPV-1 E7 has PDZ binding potential, it is very different from HPV-18 E6, and this is not surprising when one considers the amino acid differences in the respective PDZ binding motifs. To investigate this further we performed pull-down assays using expression constructs of MAGI-3 that had been used previously to map the PDZ domain recognized by HPV-18 E6 (Thomas *et al.*, 2002). The results in Figure 26 confirm that HPV-18 E6 only binds to MAGI-3 PDZ1. In contrast, the binding of RhPV-1 E7 to MAGI-3 is weaker, and is not dependent upon a single domain. RhPV-1 E7 can bind both to the N-terminal portion of MAGI-3, including the PDZ 1 domain, and to the C-terminal portion of the protein, including PDZ domains 2-5, suggesting a more promiscuous form of PDZ recognition than that seen with HPV-18 E6. Interestingly, as observed before the point mutation V->A did not completely abolish the interaction highlighting again the fact that more than one amino acid in the PDZ binding motif is involved in PDZ recognition.

Par3 is a Target of RhPV-1 E7

The degeneracy in PDZ domain recognition shown by RhPV-1 E7, coupled with its weaker binding characteristics, led us to conclude that these substrates of HPV-18 E6 were unlikely to be strong substrates for

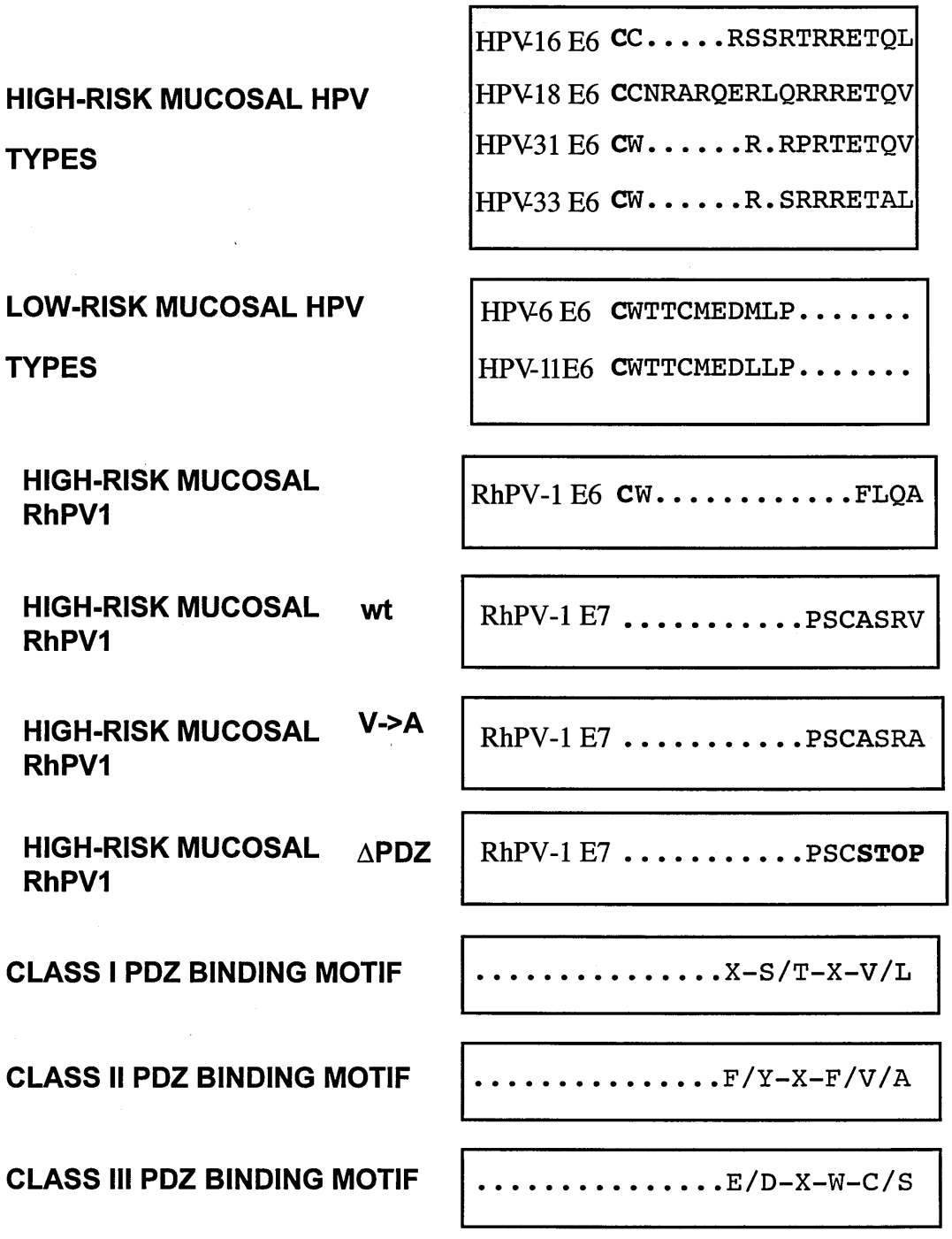


Figure 23. The C-terminal PDZ-binding motif is common to high-risk mucosal PV proteins. The PDZ binding motif (X-T/S-V/L) is absent from low risk mucosal HPV type E6s and from RhPV-1 E6. It is found at the C-terminus of high-risk HPV E6s and RhPV-1 E7. Below are the three different classes of PDZ binding motifs.

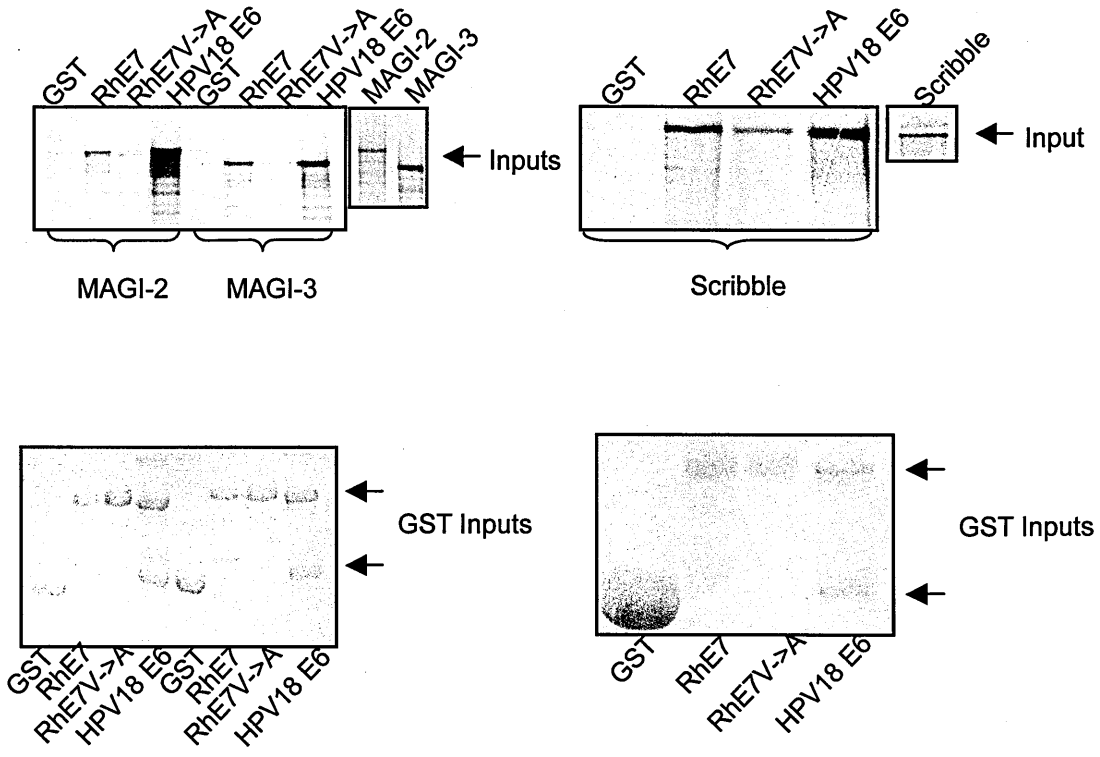


Figure 24. RhPV 1 E7 binds to MAGI-2, MAGI-3 and Scribble. *In vitro*-translated MAGI-2, MAGI-3 and Scribble were incubated with GST, GST- RhPV 1 E7, GST-RhPV 1 E7 (V->A) and GST-HPV18 E6 fusion proteins. The protein inputs are shown (MAGI-2 17%, MAGI-3 25%, Scribble 17%). Bound protein was assessed by autoradiography and input GST fusion proteins were visualized by Coomassie staining (lower panels).

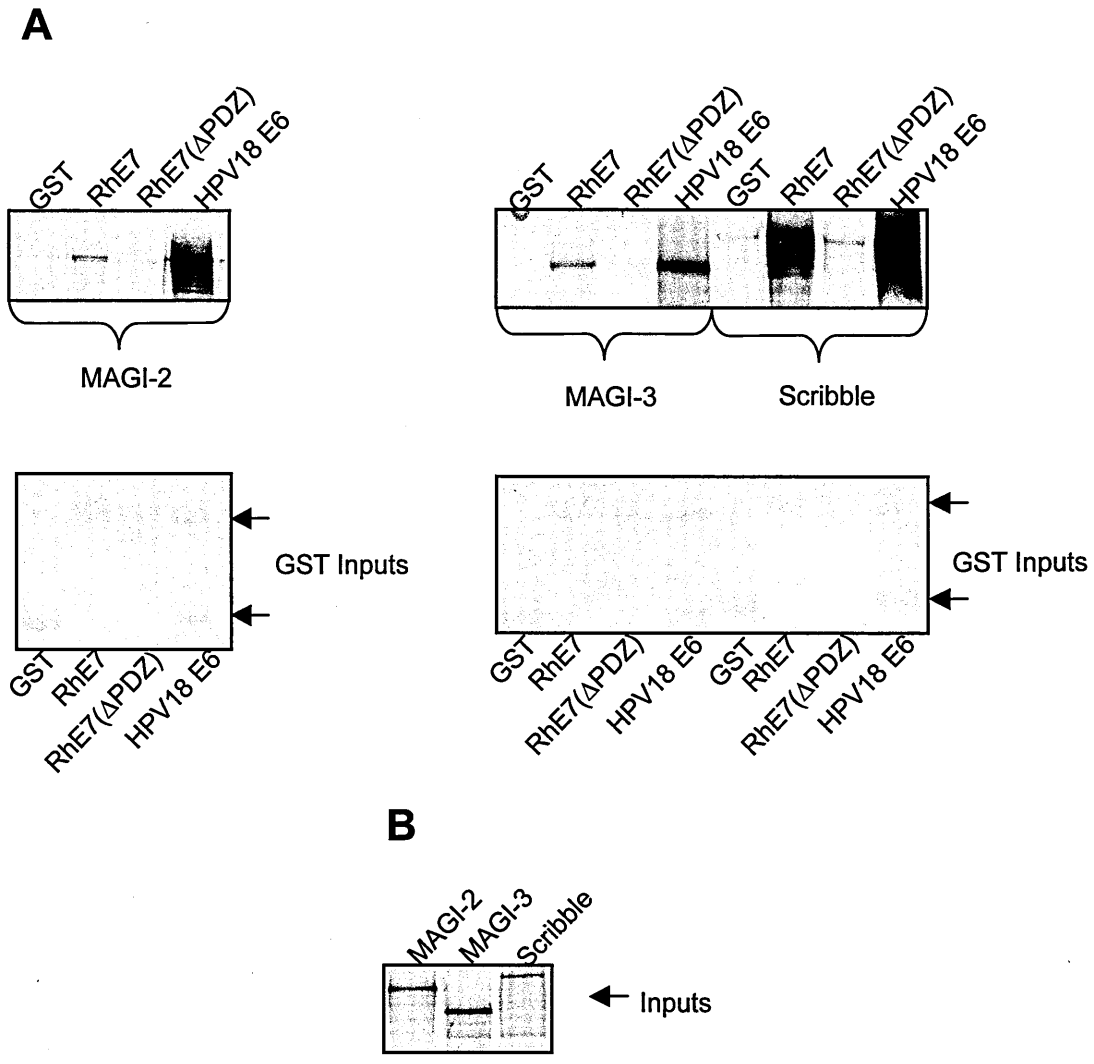


Figure 25. RhPV 1 E7 protein binds to MAGI-2, MAGI-3,and Scribble via its PDZ binding domain. A. In vitro-translated and radiolabeled MAGI-2, MAGI-3 and Scribble were incubated with GST, GST- RhPV 1 E7, GST-RhPV 1 E7 (ΔPDZ) deletion mutant, and GST-HPV 18 E6 fusion proteins. Bound proteins were assessed by autoradiography and the input GST fusion proteins were visualized by staining the gels with Coomassie blue (lower panel). B. The inputs of the in vitro-translated proteins are shown (MAGI-2 25%, MAGI-3 33%, Scribble 13%).

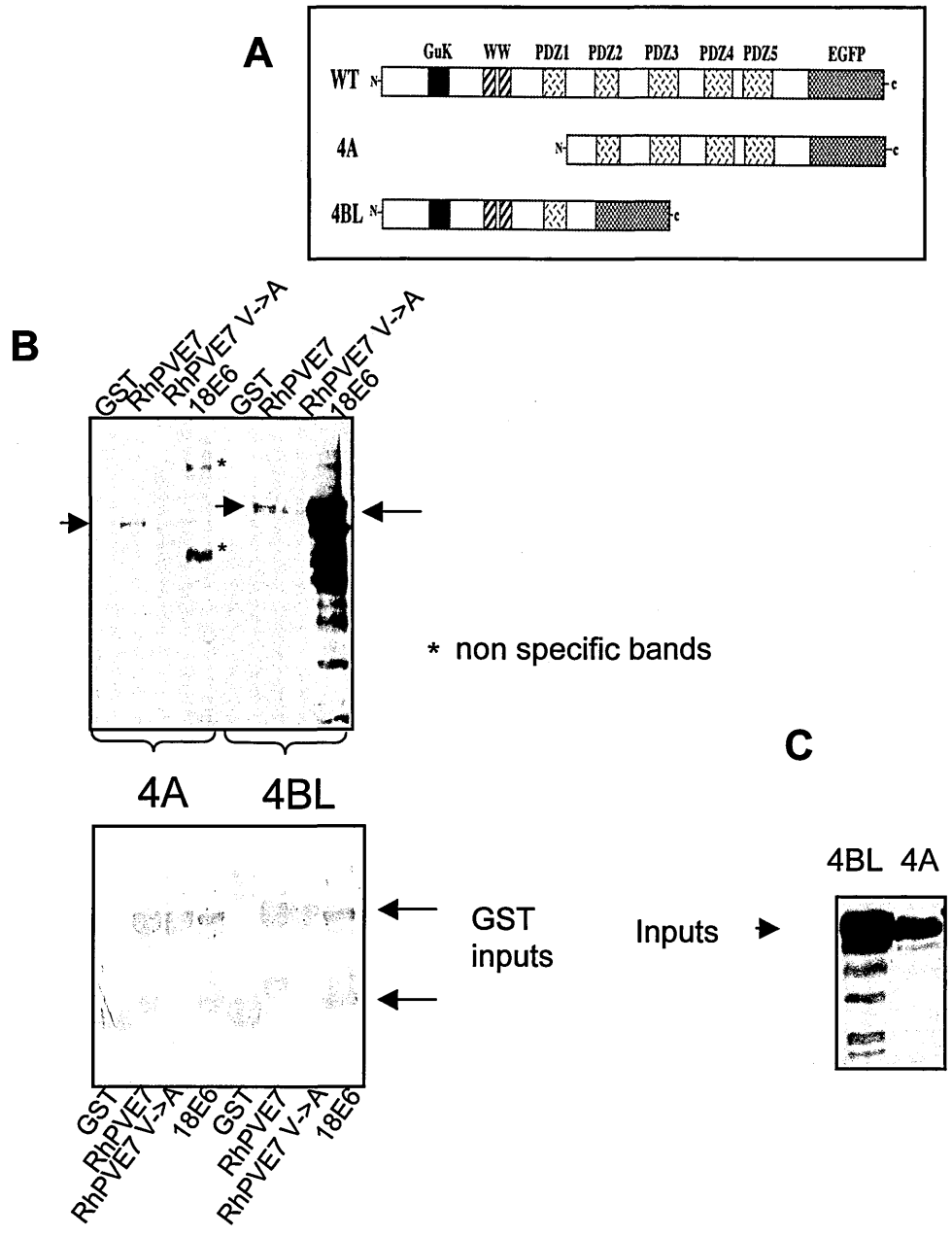


Figure 26. RhPV 1 E7 binds to MAGI-3 in a different manner from HPV 18 E6. A. Schematic diagram shows the full length MAGI-3 and two deletion mutants. B. 293 cells were transiently transfected with 5 µg of empty vector or 5 µg of plasmid encoding the truncated V5-tagged MAGI-3 proteins (4A and 4BL) as indicated. Cells were harvested 24 h post transfection. The soluble fraction of each sample was then incubated with GST, GST- RhPV 1 E7, GST-RhPV 1 E7 (V->A) mutant, and GST-HPV 18 E6 fusion proteins for 2 h at 4°C. Bound proteins were assessed by Western Blot analysis using anti-V5 antibody. The input GST fusion proteins were visualized by staining gel membranes with Ponceau red stain (lower panels). C. The inputs of the transfected MAGI-3 constructs are shown.

RhPV-1 E7 *in vivo*. To identify preferred cellular PDZ substrates of RhPV-1 E7 we decided to use a proteomic approach. To do this, human 293 cells were transfected with an N-terminally HA-tagged RhPV-1 E7 expression construct, such that the C-terminal PDZ binding motif was preserved. 24h post transfection cells were treated with CBZ proteasome inhibitor for 2.5 hrs prior to harvesting. Extracts were immunoprecipitated with anti-HA antibody and the complexes were subjected to mass-spectrometric analysis. Several potentially important targets of RhPV-1 E7 were identified in the pull-down assay. The most relevant ones are shown and categorized in the order of abundance in Table 4.

Table 4. Mass spectrometry analysis on HA-RhPV1 E7

	# of peptides	
Identified proteins specific to RhPV-1 E7	14	RhPV-1 E7
	11	EDD
	12	pRb
	6	Par3
	4	Retinoblastoma-associated protein factor 600
	1	26 S proteasome non ATP-ase regulatory subunit 3
	1	26 S protease regulatory subunit 4
	1	Proteasome subunit alpha type 4
	1	Cullin-2 (CUL-2)

293 cells were transfected with either HA-RhPV1 E7 or with the empty plasmid for the control of the unspecific binding. Beads were then subjected to mass spectrometry analysis. Identified proteins shown in the table are RhPV-1 E7 specific and some of them are known targets of HPV E7 proteins.

Targets such as pRb (Dyson *et al.*, 1991), Cullin-2 (Huh *et al.*, 2007), and Retinoblastoma-associated protein factor 600 (p600) (Huh *et al.*, 2005); (DeMasi *et al.*, 2005) which was shown to function as an N-recognin E3 ligase (Tasaki *et al.*, 2005), have been reported as some of the major interacting partners of HPV E7. Interestingly, all of those substrates were identified in this analysis. In addition to these targets 26 S protease regulatory subunit 4 was identified in this analysis, which was

previously identified as an interacting partner of HPV-16 E7 and suggested to be involved in 26 S proteasomal degrdadation of pRb (Berezutskaya and Bagchi, 1997). Therefore, this mass spectrometry analysis also shows some important conserved targets between the E7 proteins of the two species, as well as other potential targets which could be type-specific. Besides those targets, components of the proteasome pathway, including the HECT3 ubiquitin-ligase EDD were also pulled down (as discussed above). This suggests that RhPV-1 E7 could interact with components of other proteasome degradatory pathways besides Cullin-2. Interestingly, of a number of potential E7 binding partners, the only cellular PDZ-containing protein found was identified as Par3 (Table 4). This was intriguing since Par3 functions in the regulation of cell polarity upstream of the HPV-16 and HPV-18 E6 targets, hDlg and hScrib (Humbert *et al.*, 2006). Moreover, together with Par6 and aPKC it constitutes the PAR complex, which with the Crumbs and Scrib complexes controls cellular apico-basal polarity (Figure 7) (Humbert *et al.*, 2006).

RhPV-1 E7 and Par3 Bind *in vitro*

To investigate this further we analysed the potential of RhPV-1 to interact with Par3 *in vitro*. Par3 was translated then incubated with RhPV-1 GST-E7, plus HPV-18 E6 for comparison. The result of the assay in Figure 27 shows that RhPV-1 E7 binding to Par3 is much greater than that of HPV-18 E6. A single amino acid substitution in the PDZ consensus motif greatly reduces E7 binding to Par3 and this is reduced to almost background levels if the whole PDZ-binding domain is deleted. Interestingly, substitution of a single amino acid in the PDZ binding motif of RhPV-1 E7 did not completely abolish the binding,

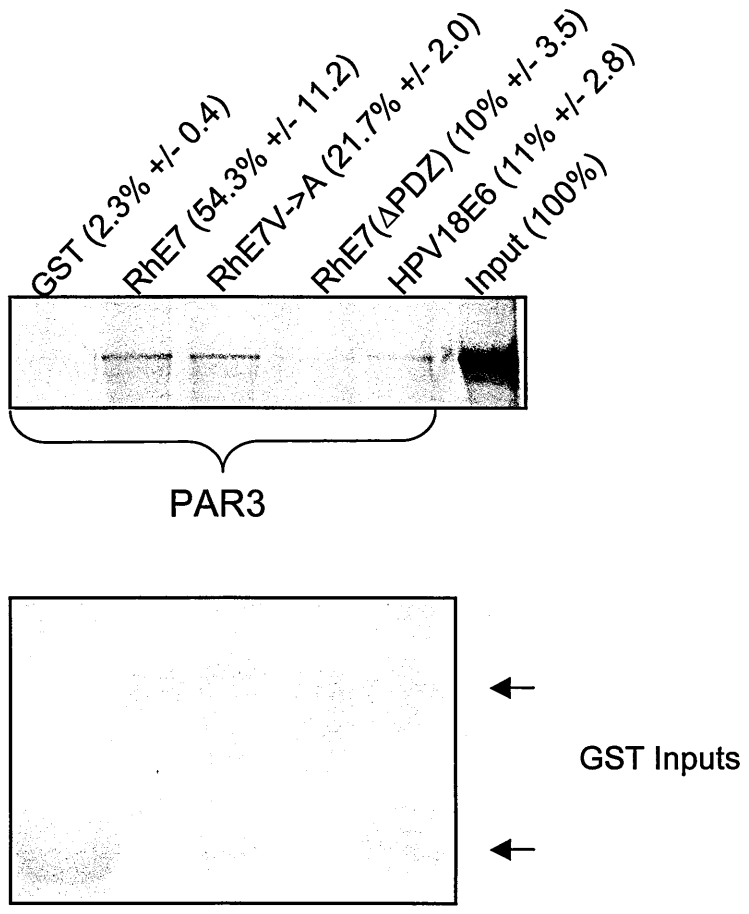


Figure 27. RhPV 1 E7 protein binds to Par3 via its PDZ binding domain. *In vitro*-translated Par3 was incubated with GST, wild type and mutant GST-RhPV 1 E7, and GST-HPV-18 E6. Bound proteins were assessed by autoradiography and the input GST fusion proteins were visualized with Coomassie staining (lower panel). Input of Par3 is also shown. The numbers above each lane show the mean percentage of Par3 bound (with standard deviations) from at least 3 independent experiments.

suggesting that other amino acids in the PDZ binding motif of the E7 protein were still able to interact with Par3. Moreover, although E7's ability to bind Par3 was almost completely abolished when its PDZ binding motif was removed, there was still a minor interaction detected between the two proteins, suggesting that additional amino acids further upstream in Rh E7 could contribute to its binding activity. Furthermore, the strong interaction of RhPV-1 E7 with Par3, compared with the very weak interaction of HPV-18 E6, also suggests that the differences in the amino acid sequence of the PDZ binding motifs in the two proteins could have a significant impact on the target preference, as was previously discussed with HPV-18 E6 and its preference in targeting Dlg over other PDZ domain-containing proteins (Table 3).

RhPV-1 E7 and EDD Bind *in vitro*

We were also intrigued by the identification of EDD as a binding partner, and wanted to determine whether this was also a specific target of RhPV-1 E7. Therefore, EDD was *in vitro* translated, ^{35}S radiolabeled, and then incubated with GST, RhPV-1 GST-E7 and HPV-16 GST-E7 for comparison. As shown in Figure 28, RhPV-1 E7 bound strongly to EDD, while HPV-16 E7 binding to EDD was very weak, suggesting that RhPV-1 E7 binding to EDD is indeed specific. These results suggest that although RhPV-1 E7 and HPV-16 E7 are closely related proteins with many conserved functions, there are still some important differences between the two proteins with respect to their interacting partners and with pathways with which they might connect.

RhPV-1 E7 Directs Par3 Degradation *in vitro*

A characteristic of the HPV E6-PDZ interaction is substrate degradation (Gardiol *et al.*, 1999; Nakagawa and Huibregtse, 2000). We wished to investigate whether RhPV-1 E7 could likewise direct the degradation of Par3. In order to investigate this E7 and Par3 were translated *in vitro* in rabbit reticulocyte lysate and co-incubated at 30°C. The level of Par3 protein remaining was ascertained by SDS-PAGE and autoradiography. The results in Figure 29 show substantial degradation of Par3 in the presence of RhPV-1 E7, the activity of which is dependent upon an intact PDZ binding motif. Reduction in Par3 protein levels was noticeable at the 60 minute time-point, and at the 120 minute time-point Par3 was almost completely degraded. This was not the case with the ΔPDZ mutant, where the Par3 protein levels were still detectable after 120 minutes. This suggests that the degradation activity is much weaker with the ΔPDZ mutant of E7, demonstrating that this is also largely PDZ dependent. However, a weak degradative activity is still observed, which is consistent with the low level of interaction still seen between the ΔPDZ mutant and Par3 (Figure 27).

RhPV-1 E7 Degrades Par3 *in vivo*

To investigate whether RhPV-1 E7 could degrade Par3 *in vivo,* human 293 cells were transfected with plasmids expressing either Par3 alone or in combination with wild type or ΔPDZ mutant RhPV-1 E7. After 24 hrs the cells were harvested, separated into soluble and insoluble fractions and Par3 levels were analysed by western blotting. The results in Figure 30 show that it the presence of wild type RhPV-1 E7 there is a reduction of Par3 levels in the insoluble fraction, part of which is due to

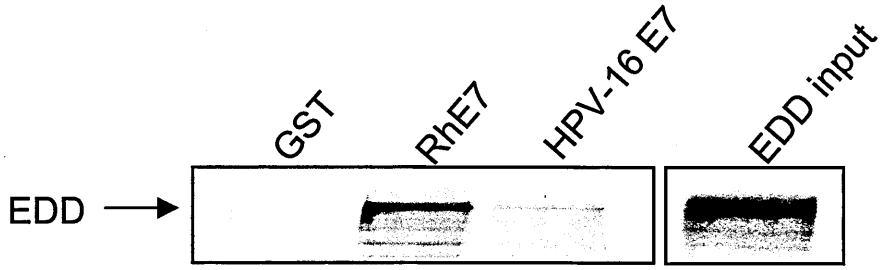


Figure 28. RhPV 1 E7 binds to EDD. *In vitro*-translated EDD was incubated with GST, GST- RhPV 1 E7, GST-RhPV 1 E7 (V->A) and GST-HPV 16 E7 fusion proteins. The protein input is shown (EDD 10%). Equal amounts of GST fusion protein were added in each sample.

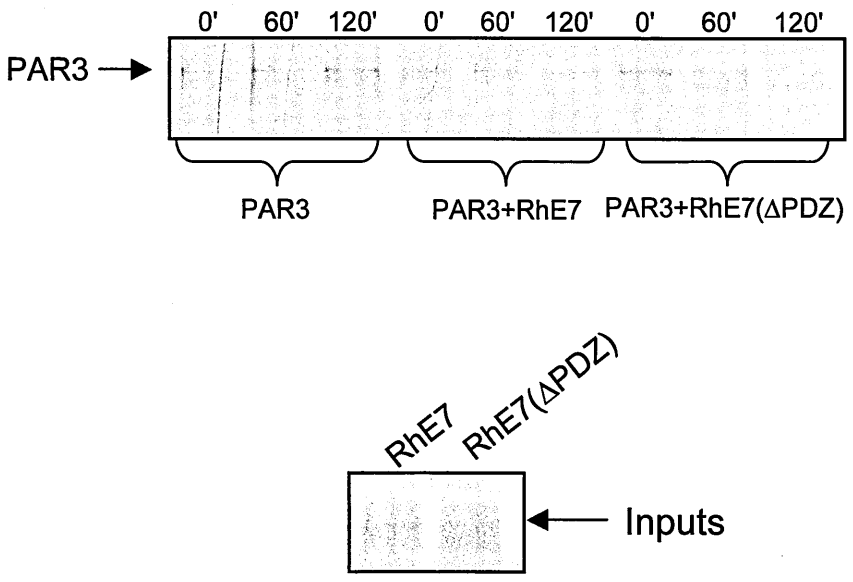


Figure 29. RhPV-1 E7 directs the degradation of Par3 *in vitro*. Par3, RhPV-1 E7 and the ΔPDZ mutant were translated, and co-incubated at 30°C for the times indicated. Residual Par3 was then detected by immunoprecipitation, SDS-PAGE and autoradiography. PhosphoImager quantitation of triplicate assays provides the mean percentage degradation by wild type E7 of 32.3% (60mins) and 64.9% (120mins) with the ΔPDZ mutant degrading Par3 by 22.9% (60mins) and 23.5% (120mins).

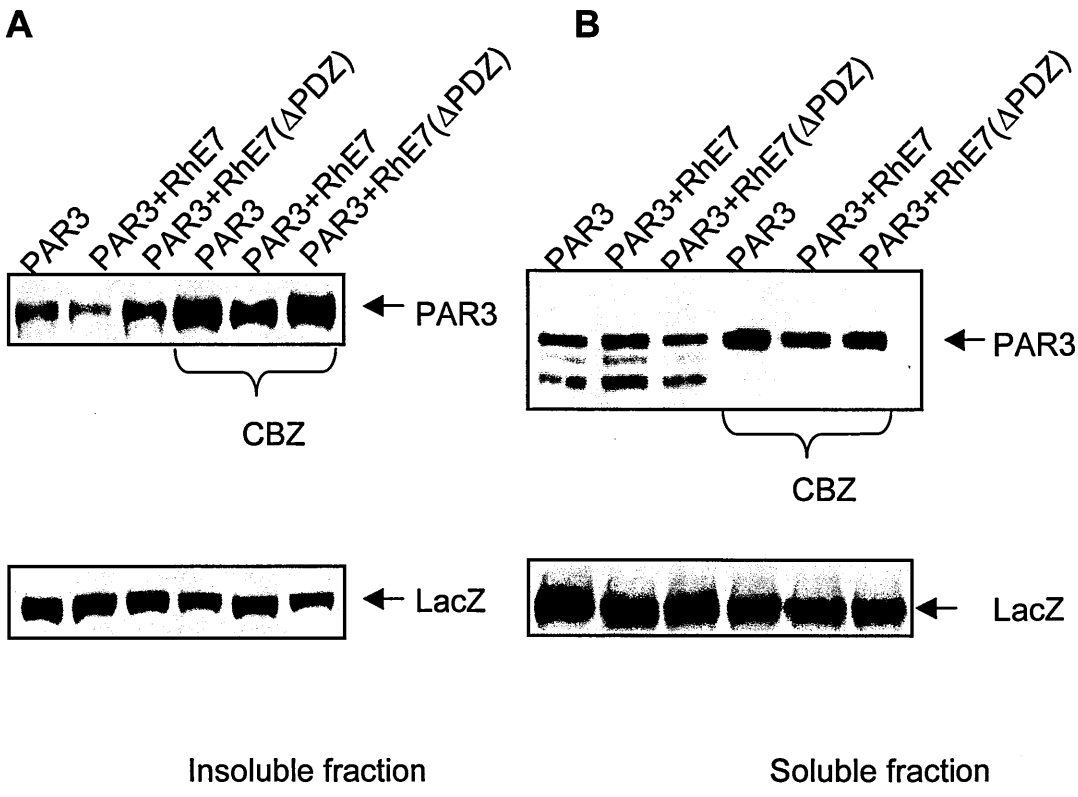


Figure 30. RhPV 1 E7 protein enhances degradation of Par3 via the proteasome pathway. 293 cells were transfected with 1 µg Myc-tagged Par3 plus pCDNA3 (-) or plasmids expressing RhPV 1 E7 and the ΔPDZ mutant as indicated. After 24h cells were incubated for 3h with or without proteasome inhibitor (CBZ) before harvesting. Residual Par3 protein levels in the soluble (A) and insoluble (B) fractions were assessed by Western blot analysis using anti-Myc antibody. The expression of β-galactosidase (Lac Z) was used as a control of transfection efficiency and loading (lower panel). Scanning of multiple assays in the absence of CBZ treatment (in relation to the LacZ control) shows a mean change in Par3 levels in the soluble fraction of a 67% (± 18.8) reduction in the presence of wild type E7 and an 11.8% (± 10.2) reduction in the presence of the ΔPDZ mutant. In the insoluble fraction the mean change in Par3 levels are a 50.3% (± 8.3) decrease with the wild type E7 and an 11% (± 10.8) increase with the ΔPDZ mutant.

an accumulation of Par3 in the soluble fraction. In contrast, Par3 is unaffected by the ΔPDZ mutant, and the protein levels of Par3 remain unchanged in the presence of the RhPV-1 E7 ΔPDZ mutant. Furthermore, the reduction in Par3 levels by RhPV-1 E7 is partly proteasome dependent, since a protein levels are restored after incubation with the proteasome inhibitor CBZ. Interestingly, in comparison with high-risk HPV E6 proteins, which only target PDZ domain-containing proteins in a proteasome dependent manner, these data suggest that, in addion to inducing proteasomal degradation of Par3, RhPV-1 E7 also needs to partially relocalize Par3 from the insoluble to the soluble fraction. Additionally, these results support previously shown *in vitro* experiments and suggest that Par3 is likely to be a biologically relevant PDZ target of RhPV-1 E7 *in vivo*.

RhPV-1 E7 Wild Type and ΔPDZ Mutant Proteins Induce pRb Degradation Equally Well

As a major target of HPV-16 E7 is the pRb tumour suppressor, we were interested to determine whether RhPV-1 E7 could likewise target pRb for degradation, since it has the pRb binding motif which is commonly found on HPV E7 proteins. Therefore, pRb (-/-) Saos-2 cells were transfected with pRb alone or in combination with either HPV-16 E7 used as a positive control, RhPV-1 E7, RhPV-1 E7 (V->A) or RhPV-1 E7 (ΔPDZ), and the levels of pRb expression were ascertained by Western blotting. The results in Figure 31 show that RhPV-1 E7 targets pRb for proteasome mediated degradation, similarly to HPV-16 E7. Furthermore, similar results were obtained with both PDZ mutant RhPV-1 E7 proteins. These results indicate that the point mutation (V-

>A) and the complete PDZ motif deletion in RhPV-1 E7 do not have any significant effects upon its ability to degrade pRb.

The RhPV-1 E7 ΔPDZ Mutant has Reduced Ability to Transform Primary BRK Cells in Comparison with the Wild Type Protein

Previous studies have shown that RhPV-1 E7 can cooperate with EJ-ras in the transformation of primary BRK cells, an activity that is probably dependent upon the LxCxE motif (Ostrow *et al.*, 1993). However we wanted to know whether the PDZ binding potential of RhPV-1 E7 could play a role in transformation. Therefore, co-transformation assays were performed in BRK primary cells using wild type E7 or the ΔPDZ mutant together with EJ-ras while HPV-16 E7 was used as a positive control. BRK cells from 9-day-old Wistar rats were transfected with 2 μg EJ-ras either alone or together with 5 μg HPV16 E7, RhPV 1 E7, RhPV 1 E7 (ΔPDZ) expression plasmids. Cells were maintained in medium containing 200 μg/ml G418 for 2 weeks and then fixed and stained. Morphologically transformed colony numbers from 4 independent experiments are shown in Table 5. The results show that wild type RhPV-1 E7 cooperates with EJ-ras somewhat more weakly than wild type HPV-16 E7. This could be attributed to either the different expression plasmids used in the experiment (pJ4Ω16 E7 and pCDNA3RhPV-1 E7), or to differences in the pRb binding motif, that of RhPV-1 E7 being LMCYE compared with the HPV-16 E7 LYCYE, although Figure 31 would suggest no major differences in their respective abilities to target pRb for degradation. In contrast, the ΔPDZ mutant shows significantly reduced levels of transforming activity in comparison with RhPV-1 E7 and HPV-16 E7 proteins, suggesting that

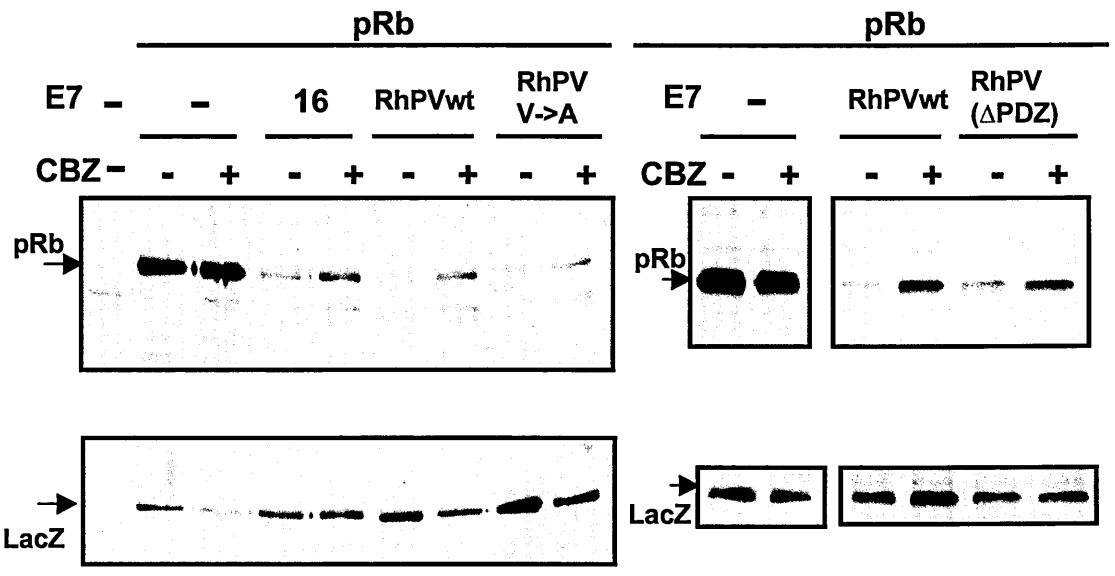


Figure 31. RhPV-1 E7 degrades pRB via the proteasome pathway. Saos-2 cells weretransfected with 3 µg pRb expression plasmid plus pCDNA3 (-) or plasmids expressing HPV-16 E7, wild type RhPV-1 E7, the V->A and ΔPDZ mutants, as indicated. After 24h cells were incubated for 3h with (+) or without (-) CBZ before harvesting. Residual pRb levels were ascertained by Western blotting using anti-pRb antibody. The expression of Lac Z was used as a control of transfection efficiency and loading (lower panel).

an intact PDZ binding motif is required for the optimal transforming activity of RhPV-1 E7.

Table 5. BRK Transformation assays.

Constructs	Experiment 1	Experiment 2	Experiment 3	Experiment 4
	Number of Transformed Colonies			
RhPV1 E7+EJ-ras	32	38	22	8
RhPV1 E7(ΔPDZ)+EJ-ras	15	18	3	0
HPV16 E7+EJ-ras	50	72	58	48
EJ-ras alone	1	2	0	2

RhPV-1 E7 and Par3 Co-localise

We were also interested in investigating if the RhPV-1 E7 and Par3 interaction would have any effect on the cellular localization of those two proteins. Par3 is primarily expressed at sites of cell contact and in the cytoplasm (Joberty *et al.*, 2000), while we would expect RhPV-1 E7 to be predominantly nuclear. To determine whether specific pools of either protein could co-localise, U2OS cells were transfected with a plasmid expressing Myc-tagged Par3, either alone or in combination with HA-tagged wild type and ΔPDZ RhPV-1 E7. After 24h the expression of each protein was assessed by immunofluorescence. The results in Figure 32A show that Par3 is largely cytoplasmic, whilst both wild type and ΔPDZ E7 show nuclear and cytoplasmic expression. Interestingly, the deletion of the PDZ binding motif of the RhPV-1 E7 protein did not have any effect on the cellular localization of the protein. Upon co-transfection with Par3, however, wild type E7 is redistributed to the cytoplasm and is mostly absent from the nucleus (Figure 32B(i) and 32B(ii)), whereas the ΔPDZ mutant localization is largely unaffected (Figure 32C(i) and 32C(ii)), and remaining predominantly nuclear after being co-transfected with Par3, suggesting that the removal of the PDZ binding motif of the Rh E7 protein significantly reduced its ability to

interact with Par3. These results demonstrate that the co-localization is pronounced when E7 and Par3 are co-expressed, and that this depends in part upon an intact PDZ motif in E7.

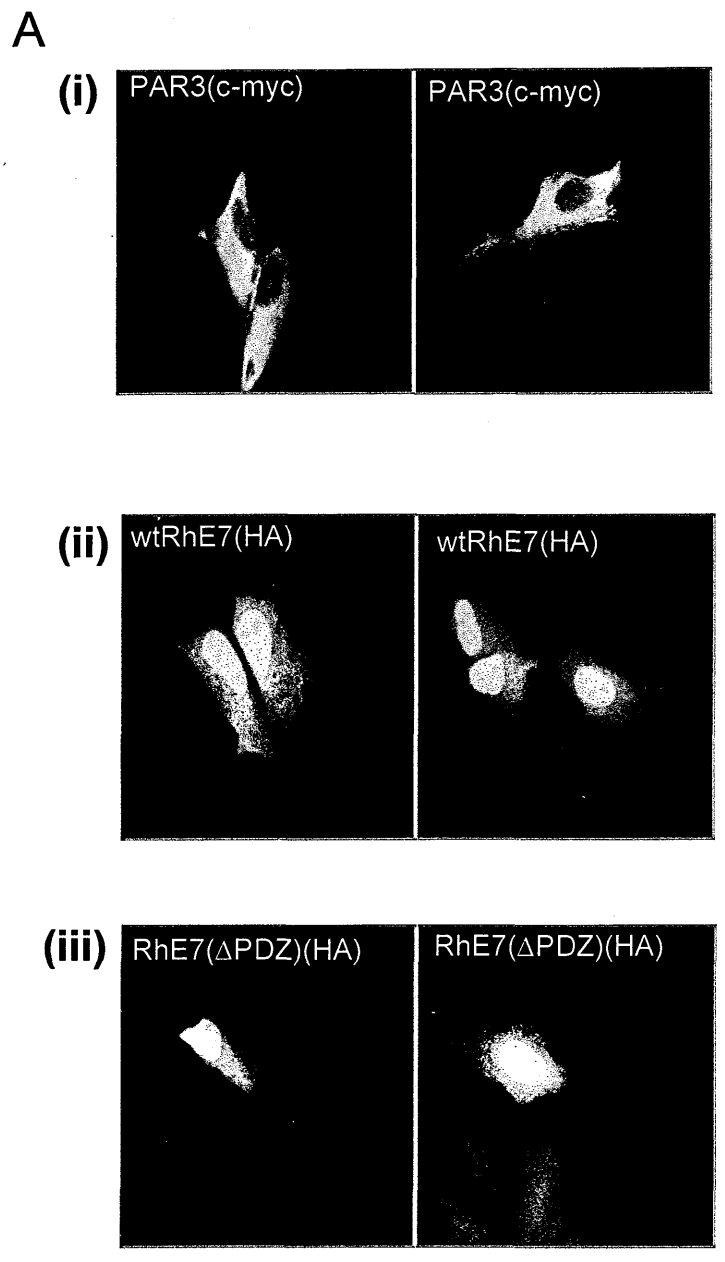


Figure 32. Co-localization of RhPV-1 E7 and Par3. U20S cells were transfected with 1 µg c-myc tagged Par3, 2 µg HA-tagged wt or mutant RhPV-1 E7, alone or in combination. After 24h, cells were fixed and probed with mouse anti-c-myc and rabbit anti-HA antibodies, followed by Rhodamine-conjugated goat-anti mouse (red, for Par3) and FITC-conjugated goat-anti rabbit (green for RhPV-1 E7) antibodies. The two fields from each slide were scanned with a Leica DMLB fluorescence microscope. Panel A: Par 3 alone (i), wt E7 alone (ii) and mutant E7 alone (iii).

B and C

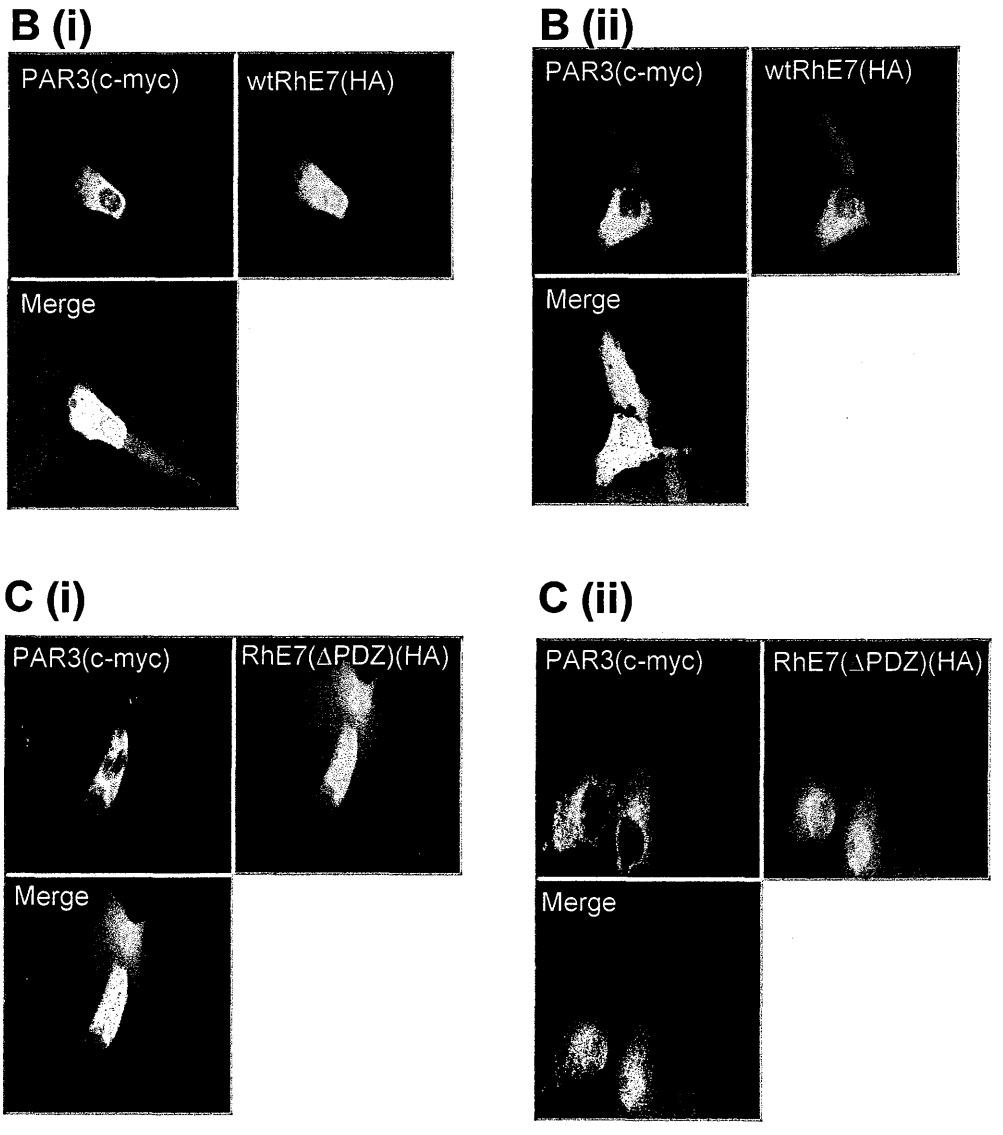


Figure 32. Co-localization of RhPV-1 E7 and Par3. (cont.) Panel B: Par3 plus wt E7 (fields i and ii). Panel C: Par3 plus mutant E7 (fields i and ii)

Discussion

PART I: Ubiquitin Ligase Regulation of HPV-18 E6

Previous studies have shown that E6AP is critical for the ability of E6 to target p53 for proteasome-mediated degradation, which is in part a consequence of E6AP's ubiquitin ligase activity (Scheffner *et al.*, 1993). However, there have been several reports showing that E6 can degrade proteins, albeit weakly, in the absence of functional E6AP, suggesting the existence of other routes by which E6 can degrade its substrate proteins (Camus *et al.*, 2007; Massimi *et al.*, 2008). Most importantly, however, it was also found that all the effects of E6 on cellular gene expression appeared to be mediated by E6AP (Kelley *et al.*, 2005). At first glance, these different studies appear irreconcilable. However, the data presented in the Results section of this thesis are perfectly consistent with all of the above reports. Thus, in the absence of E6AP, E6 levels are very low and the protein is rapidly degraded at the proteasome. Obviously, a by-product of this would be an apparently central requirement for E6AP in all of E6's biochemical activities, with loss of E6AP mimicking E6 ablation. However, in the light of the studies presented above, we can now conclude that the actual biochemical functions of E6, whilst requiring E6AP for stability, are not necessarily E6AP-dependent with respect to substrate targeting and degradation.

E6 Protein Levels are E6AP-dependent

The results presented above raise a number of interesting points. Firstly, we clearly show in both overexpression and endogenous settings

that HPV-16 and HPV-18 E6 protein levels are highly dependent on E6AP, while E6AP depletion does not have a significant effect on E6 mRNA transcript levels. This was shown in cells derived from cervical cancers, where siRNA ablation of E6AP greatly decreased the levels of HPV E6 expression. This reduction is proteasome dependent, since treatment with proteasome inhibitors rescues E6 from degradation. We also showed a clear reduction in the half life of the E6 protein from 120 to 30 minutes in the absence of E6AP. In a series of overexpression assays we also showed that both HPV-16 and HPV-18 E6 protein levels could be increased in the presence of ectopically expressed E6AP. Interestingly, the ability of E6AP to induce a stabilization of E6 was independent of its ubiquitin ligase activity since the catalytically inactive E6AP (C->A) mutant is as effective as wild type E6AP in stabilizing E6.

An important conclusion from the results is that HPV-16 and HPV-18 E6 are regulated by the proteasome independently of E6AP, suggesting that other ubiquitin ligases may be involved in regulating E6 turnover. This possibility is supported by the fact that E6 has been shown to complex with at least two other ligases, EDD (this thesis) and HERC2 (Vos *et al.*, 2009). Based on studies presented here, there does not appear to be very strong evidence that EDD is the ligase responsible for E6 degradation in the absence of E6AP. Obviously, it will be of a great interest to assess whether HERC2 possesses this ability.

How E6AP regulates E6 turnover is an important question and there are a number of possibilities. The results in this thesis suggest that E6AP somehow blocks E6 degradation; this might be through either masking E6 sites of ubiquitination or masking binding sites for other ubiquitin ligases. Alternatively, E6AP binding could also alter E6

structure, thus acting as a molecular chaperone. Prior to considering some of these possibilities in more depth, we need to consider whether E6 requires a physical association with E6AP for these effects to occur, or whether the effects of E6AP are indirect. In addition, the relative levels of expression of E6 and E6AP need to be considered. Previous studies have suggested that HPV-16 E6 E6AP binding-defective mutants (L37S and L110Q), which do not associate with E6AP *in vivo*, are expressed in cells at levels similar to wild type E6 (Sekarić *et al.*, 2008). If a simple 1:1 interaction is required for the effects of E6AP, then one would expect the protein stability of L37S and L110Q mutants to be very low in cells, since their inability to associate with E6AP would result in rapid proteasomal degradation. However, the situation could be much more complex. It is possible that the levels of E6AP are actually much lower than those of E6 in HPV-positive tumor cells, and the Western blots in this thesis would tend to support this. In addition, reports have suggested that native E6 predominantly exists in multimeric forms (Garcia-Alai *et al.*, 2007; G. Travé, personal communication), and since experiments for detecting E6 protein levels are performed under strong denaturing conditions, this could likely be the explanation for our detecting predominantly the monomeric forms of E6 (V. Tomaić, personal observation). Therefore, it is possible that a multimer of E6 is bound to only one or two molecules of E6AP. Also, one needs to bear in mind that the E6AP half-life is more than 3 times longer in HPV-positive cells than the half-life of E6 (Kao *et al.*, 2000), which would suggest that a lower number of E6AP molecules could be sufficient to interact with a higher number of E6 molecules. In a such situation, any residual binding activity displayed by mutants of E6 might actually be sufficient to allow E6 stabilization by E6AP. With these points in mind, I would like to consider the three possible mechanisms by which E6AP might stabilize E6. In the first, it could be

that E6AP is involved in masking E6 sites of ubiquitination or in inhibiting the binding of ubiquitin ligases to E6, as depicted in Figure 33A. In this model, if E6AP is not bound to E6, the site is free and gets occupied by as yet unidentified ubiquitin ligase(s), which then leads to E6 ubiquitination and its subsequent degradation at the proteasome. In the second possibility, E6AP could be acting as a molecular chaperone (Figure33B), where it is suggested that when E6 is not bound to E6AP it loses its conformation, the cell recognizes it as an unfolded protein, which is then rapidly degraded at the proteasome. This is actually a reasonable explanation for E6's rapid turn over in the absence of E6AP. It has been shown that heat shock proteins such as HSP70 and HSP40 promote the proper folding and refolding of non-native proteins. However, under circumstances when the molecular chaperone system is unable to promote the proper folding of a protein substrate to its native state, the protein substrate is selected for degradation (reviewed in Sherman and Goldberg, 2001). The protein substrate degradation in many instances is thought to be mediated by CHIP (carboxyl terminus of Hsp70-interacting protein), a co-chaperone that functions as an E3 ubiquitin ligase linking the polypeptide binding activity of HSP70 to the ubiquitin proteasome system (Rosser *et al.*, 2007; reviewed in Sherman and Goldberg 2001). In our proteomic analysis we identified HSP70 as an interacting partner of HPV-18 E6 and therefore it is possible that misfolded E6 in the absence of E6AP is degraded through the HSP70-CHIP-mediated ubiquitin proteasome system. In addition, recent studies have suggested that E6AP can interact with the substrate binding domain of HSP70 chaperones and promote the degradation of chaperone-bound substrates, such as misfolded luciferase that is bound to HSP70 (Mishra *et al.*, 2009), thereby providing evidence of a link between E6/E6AP and cellular chaperones. Finally, E6AP could be acting indirectly on a third protein which would normally target E6 for

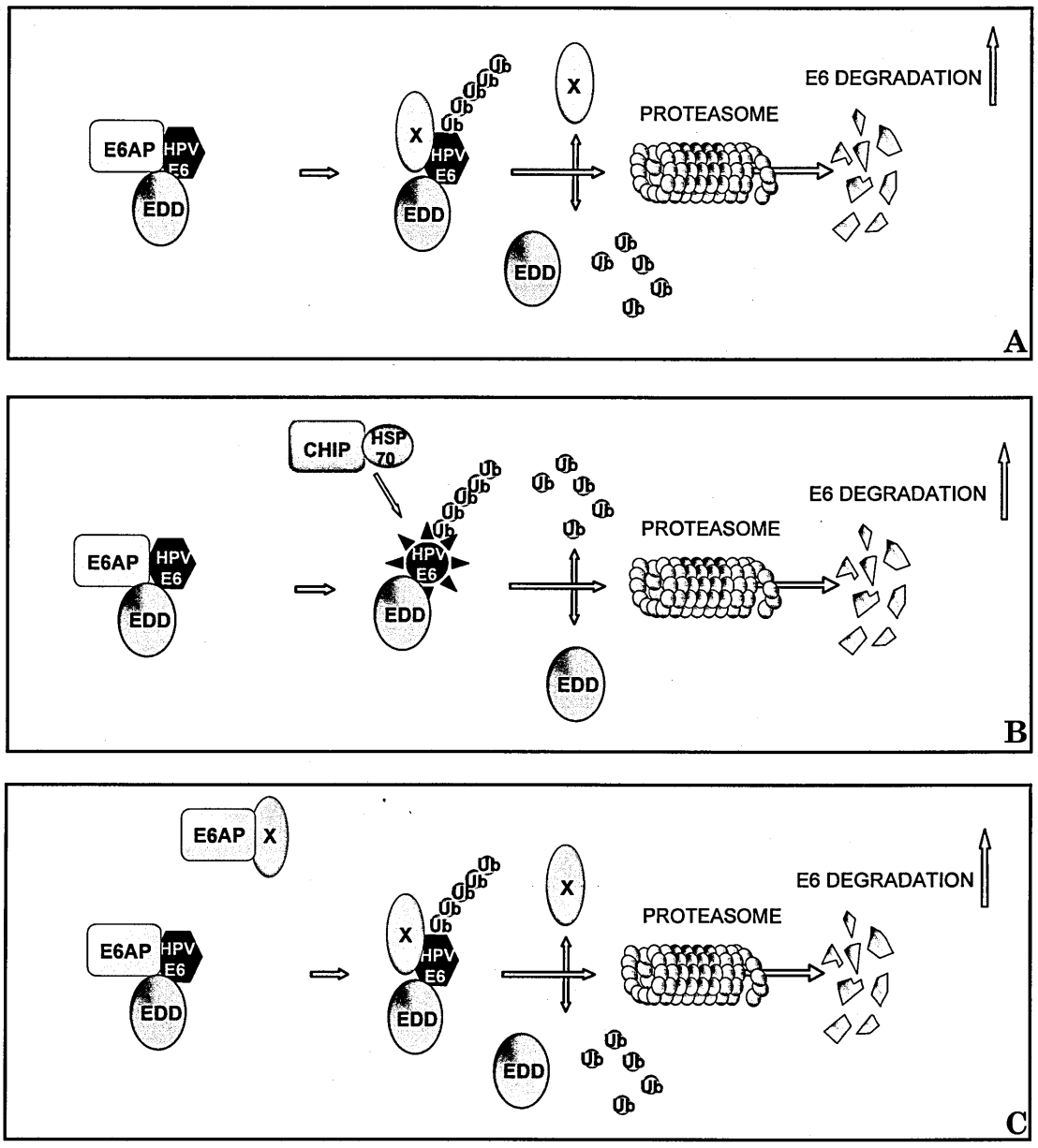


Figure 33. HPV E6 is rapidly turned over in the absence of E6AP. A schematic representation of possible E6 outcomes in absence of E6AP. A. The binding site of E6AP on E6 is occupied by another ubiquitin ligase (X) in the absence of E6AP, which results in E6 ubiquitination and proteasomal degradation. B. E6AP acts as a chaperone and in its absence E6 loses its conformation and is degraded at the proteasome, possibly via the CHIP-HSP70 complex. C. Besides being bound to E6, E6AP is also involved in regulation of another cellular ligase. Upon E6AP depletion, this ligase is released and induces the proteasomal degradation of E6.

degradation. This model is depicted in Figure 33C. Here we speculate that a certain pool of E6AP which is not bound to E6 could be interacting with other cellular proteins, such as other ubiquitin ligases. When E6AP is depleted from cells the potential ubiquitin ligase, which is regulated by E6AP, is released and consequently degrades E6. In this case this second ubiquitin ligase does not necessarily need to bind to the same site of E6 as E6AP, but the absence of E6AP leads to its upregulation to levels sufficient to efficiently degrade E6.

Obviously, the above results highlights the fact that the E6AP (C->A) mutant can still stabilize E6, showing that it does not require its ubiquitin ligase activity for this effect. Furthermore, the results also suggest that this mutant, which can act in a dominant negative fashion with respect to E6 degradation of p53 (Beer-Romero *et al.*, 1997; Talis *et al.*, 1998), may alter E6 structure or inhibit ligase recruitment, rather than just acting to destabilise E6. However, our data also suggest that wild type E6AP is likely to be more rapidly turned over than the catalytically inactive mutant C->A in an E6-negative background, an observation that could be attributed to the catalytically active site on the wild type protein, which has previously been shown to be involved in self-ubiquitination of E6AP (Kao *et al.*, 2000). Interestingly, even though wild type E6AP protein is significantly less stable than E6AP (C->A), it seems that the wild type protein is equally able to stabilize E6. This observation could be due to protein saturation, and suggests that a certain amount of E6AP, regardless of its catalytic activity, is sufficient to stabilize E6 protein to a certain level. In addition, E6AP seems to be a very stable protein, with a half-life of 25 hrs in HPV-negative cells, while in HPV-positive cells this is reduced to 7hrs due to its increased turnover regulated by E6 (Kao *et al.*, 2000). Therefore, from these data it could be argued that E6 does not only interact with E6AP to target

cellular proteins such as p53, but that it is also likely for E6 to preferentially complex with E6AP due its high stability. A short half-life of E6AP would automatically reflect on the half-life of E6 and therefore this could result in a reduced ability of E6 to interact with various cellular proteins. As was shown in Figure 10 the half-life of HPV-18 E6 is about 120 minutes and over that time period the levels of E6AP remain unchanged, providing the necessary stability for E6. In contrast, in the cells which had depleted E6AP, the E6 half-life was reduced to only 30 minutes thus making E6 a 4-fold less stable protein.

Additionally, the results presented above suggest that variations in the levels of certain E6 substrate proteins such as p53 and Dlg, frequently observed in cervical tumour tissues (Cavatorta *et al.*, 2004, Cooper *et al.*, 1993), might actually be a reflection of alterations in E6AP levels or of the ability of E6AP to bind E6. Future studies will have to be undertaken to elucidate these aspects further and clarify how E6 mediates its degradation functions in the presence and absence of E6AP.

Proteomic Analysis of HPV-18 E6

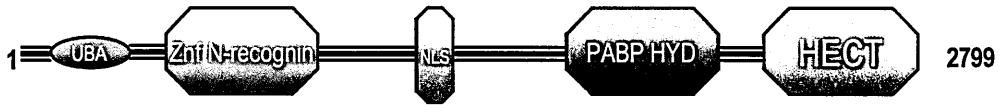
Based on the above studies, we decided to use a proteomic approach to identify new components of the proteasome pathway with which E6 might interact: both with respect to those that might be potential novel ubiquitin ligases for E6 degradation functions, and also those proteins that might be involved in regulating E6 turnover. Using this approach a number of interesting candidate binding proteins of E6 were identified. Confirmation that the assay was functional was provided by the identification of hDlg as a binding partner (Gardiol *et al.*, 1999). Interestingly, this was the only PDZ domain-containing protein of E6 to

be identified in this analysis, further suggesting that Dlg is a major target of HPV-18 E6. In contrast, in samples which contained both HPV-18 E6 and ectopically expressed MAGI-2, only MAGI-2 was identified, suggesting that overexpressed MAGI-2 out-competed endogenous Dlg for E6 binding. This observation also suggests that HPV-18 E6 is obviously capable of interacting with various PDZ domain-containing proteins, but it could be argued that under different circumstances with respect to the viral life cycle and/or cell transformation, E6 can preferentially target certain PDZ proteins over other ones.

Since the major aim of this analysis was to identify new E6 targets in the proteasome pathway, we were primarily focused on proteasome-related proteins. Again, identification of E6AP as a major interacting partner verified the integrity of the assay. Interestingly, many other components of the proteasome pathway, such as ubiquitin and 26S proteasomal subunits, were also identified. This observation highlights the fact that E6 is involved in interaction with many proteins associated with cellular degradatory activities. It is also important to mention that this assay does not rule out indirect interactions between the proteins identified in this analysis, suggesting that some of the identified proteins could be as a result of an interaction with E6AP rather that directly with E6. However, the most exciting result of this analysis was the identification of EDD, another HECT domain ubiquitin ligase. EDD is an interesting cellular protein involved in many cellular functions. In Figure 34 EDD protein domains and reported functions are depicted. EDD was originally reported to play a critical role in coordinating the balance between cell cycle progression and differentiation (Henderson *et al.*, 2002), while more recent studies have shown that amplification and overexpression of EDD is frequently found in several cancers, including

those of ovary and breast, while truncating mutations are also found in gastric and colon cancer (Clancy *et al.*, 2003; Mori *et al.*, 2002). In addition, EDD was shown to be a crucial factor during development, where EDD-deficient embryos in mice had delayed growth and development, failed yolk sac and vascular development, together with defective chorioallantoic fusion, leading to a general failure of embryonic cell proliferation and widespread apoptosis (Saunders *et al.*, 2004). However, although EDD is implicated in various diseases, there is still not much known about the biochemical activities of the protein and only a few EDD interacting partners have been reported so far. Importantly, EDD was shown to be an E3 HECT-domain ubiquitin ligase (Callaghan *et al.*, 1998) and more recently it was also shown that EDD can operate as an N-recognin in the N-end rule degradatory pathway (Tasaki *et al.*, 2005). Binding assays have shown that EDD was captured by type 1 (Arg), but not by type 2 (Phe) X-peptide beads, suggesting that EDD can bind to type 1, but not to type 2 N-degrons (Tasaki *et al.*, 2005). However, this is only a proposed model for EDD function based on peptide recognition, and whether the actual substrate proteins are targeted in this way still needs to be elucidated.

Some of the major reported interacting partners of EDD include Paip2, one of the poly(A)-binding proteins (PABP), which was reported to be targeted by EDD for proteasomal degradation, suggesting EDD involvement in mRNA metabolism (Yoshida *et al.*, 2006). CIB1/KIP (calcium- and integrin-binding protein), a protein that interacts with a number of DNA damage response proteins, including the catalytic subunit of the DNA-dependent protein kinase (Wu and Lieber, 1997) and polo-like kinases PLK1 and PLK3 (Winkles and Alberts, 2005), has been shown to be a binding partner of EDD (Henderson *et al.*, 2002). In addition, Topoisomerase II-binding protein (TopBP1), a protein



EDD

* HECT domain 2432-2799 aa
* PABP HYD 2375-2452 aa
* ZN Finger domain 1177-1244 aa
* NLS (nuclear localization sequence) 1402-1602 aa
* UBA (ubiquitin assoc. domain) 188-225 aa

- mammalian ortholog of the *Drosophila melanogaster hyperplastic discs* tumor suppressor gene (*hyd)*
- critical role in cell proliferation during development
- nuclear protein
- E3 ubiquitin protein ligase
- DNA damage signaling
- carcinogenesis
- protein kinase DYRK2 scaffold for EDD

Figure 34. EDD protein sub-domains and functions. A schematic representation of EDD protein is shown. EDD sub-domains and reported functions are indicated.

associated with DNA damage response and cell cycle regulation, is also targeted by EDD for proteasome degradation, further suggesting EDD involvement in the DNA damage response (Honda *et al.*, 2002). Furthermore, EDD was also reported to be required for optimal CHK2 Thr68 phosphorylation and kinase activity and for cell survival after DNA damage (Henderson *et al.*, 2006). More recent studies have suggested that EDD can form a ligase complex with DDB1 (DNA-damage binding protein 1) and VPRBP (VPR-binding protein), known as the EDVP (EDD, DDB1, VPRBP) complex. This is dependent on a protein kinase, DYRK2, for its formation, and for the subsequent phosphorylation, ubiquitylation and degradation of their substrates (Maddika and Chen, 2009), which are involved in regulating mitotic progression.

It is clear that the majority of EDD interacting partners are involved in DNA damage signaling, which suggests that EDD could play an important role in regulation of that cellular pathway (Henderson *et al.*, 2002; Henderson *et al.*, 2006; Honda *et al.*, 2002). Therefore, it is not surprising that EDD is targeted by E6, since it has been shown that E6 is involved in inducing DNA damage and genomic instability (reviewed in Duensing and Münger, 2004). It is possible that E6 interacts with EDD and alters its cellular functions in order to have an additional effect on eliminating the DNA damage response. Improper functioning of those mechanisms could result in DNA damage accumulation, which if not repaired, might further contribute to the virus on its way to establish an optimal infection in the cell. Indeed it has been shown that E6 can affect DNA damage response pathways independently of p53 (Shin *et al.*, 2006; Song *et al.*, 1998). Moreover, a recent report has suggested that HPV-16 E6 targets a protein or proteins other than p53 to deregulate the activity of CHK1 in carcinogen-damaged cells (Chen *et*

al., 2009). Whether some of these activities are mediated through its association with EDD remains to be determined. In addition, it was also shown that activation of CHK2 is mediated by ATM, which directly phosphorylates the Thr68 of this protein kinase, leading to its dimerization (Matsuoka *et al.*, 1998). Furthermore, ATM was also shown to be involved in p53 Ser15 phosphorylation *in vivo* (reviewed in Ashcroft and Vousden, 1999). As discussed above EDD was shown to be required for optimal CHK2 phosphorylation (Henderson *et al.*, 2006), however it seems that the effect of EDD is specific only to CHK2, since downstream substrates of ATM, such as p53, are unaffected in irradiated HPV-negative MCF-7 cells upon EDD depletion (Henderson *et al.*, 2006). Therefore, it would be of a great interest to investigate these interactions in more detail in an HPV-positive environment and to observe whether E6 has any effects on CHK2 activity (Henderson *et al.*, 2006).

EDD Down-regulates HPV-18 E6 Mediated Degradation of p53 and PDZ domain-containing substrates

Having found in the proteomic analysis that EDD is a potential target of E6, it raised the obvious possibility that this ligase might be involved in some of E6's degradatory activities. We first verified the interaction between E6 and EDD *in vitro* and *in vivo* in a number of binding assays and confirmed that EDD is a strong binding partner of HPV-18 E6. Interestingly, we also found that HPV-11 E6 and HPV-16 E6 could also interact with EDD, but the strength of association seems much weaker than that seen with HPV-18 E6. Whether this has any reflection on the apparently high pathology of HPV-18 E6 (Burger *et al.*, 1996; Walker *et al.*, 1989) remains to be determined. In addition, *in vivo* co-

immunoprecipitation assays we verified the E6's interaction with both EDD and MAGI-2 when co-expressed, suggesting that the binding regions for EDD and MAGI-2 on E6 are likely to be different and demonstrating that these partners of E6 do not compete for E6 interaction.

Since EDD is an ubiquitin ligase and it is likely to exist in a complex with E6 without affecting E6's ability to interact with its PDZ substrates, we performed several *in vitro* and *in vivo* degradation assays to investigate the effects of EDD on the degradation process. Interestingly, and opposite to what we expected, EDD inhibited the ability of E6 to degrade several of its PDZ substrates in both experimental settings. We also included p53 in the degradation assays to determine whether effects of EDD on E6 function was PDZ-specific or if it could be also observed with p53. Interestingly, the same effect was observed with p53, leading us to conclude that the effect of EDD on E6 degradatory activities is not only associated with PDZ domain-containing targets, but that it is likely to be a general effect on some of E6's target proteins. These observations were also verified *in vivo* on endogenous p53, where we could show that EDD depletion in both HPV-18 positive HeLa, and HPV-16 positive CaSKi cells results in increased degradation of p53. E6 and E6AP-depleted HeLa and CaSKi cells were used in parallel for the control, and depletion of both proteins resulted in upregulation of p53, as expected. At the same time however, we did not observe any corresponding increase in hScrib and hDlg protein degradation in HeLa or CaSKi cells after EDD silencing, and thus could not confirm the effects observed in the overexpression assays. The reasons for this are unclear, but E6's targeting of endogenous PDZ substrates seems to be more complex than its targeting of p53 (Massimi *et al.*, 2006; Narayan *et al.*, 2009). Indeed, in cells treated with siRNA

against E6 and E6AP, which were used as the positive controls, only marginal rescue of those proteins was observed, suggesting that other factors could be involved in their targeting. For example, it was shown that E6 preferentially targets certain phosphorylated nuclear forms of hDlg (Narayan *et al.*, 2009), suggesting that other post-translational modifications are likely to be important in E6's degradation of the PDZ substrates. To fully elucidate the particular effects of EDD on E6/PDZ targeting requires further study.

E6AP is a Cellular Target of EDD

The increased degradatory activity of E6 with respect to p53 in the absence of EDD led us to suspect that EDD could be having a direct effect either on E6 or on some of E6's interacting partners. Therefore, we also investigated the effects of EDD depletion upon the levels of E6 and E6AP in HeLa cells and found a clear increase in both in the absence of EDD. Further, we show that EDD appears to be mediating this effect largely via E6AP, since when both ligases were depleted from HeLa cells there was no apperent rescue of E6 protein levels, suggesting that there could be other ligase(s) involved in E6 turnover in the absence of E6AP. This also suggests that the effects of EDD on E6 levels are indirect, and as a result of the increase in E6AP. This scenario is summarized in Figure 35: when EDD is absent, E6AP levels are upregulated and, as a consequence, this leads to E6 protein upregulation, overall resulting in increased ubiquitination and proteasomal degradation of p53.

We were also interested in investigating whether EDD could affect E6AP levels in a HPV-negative background. In overexpression assays

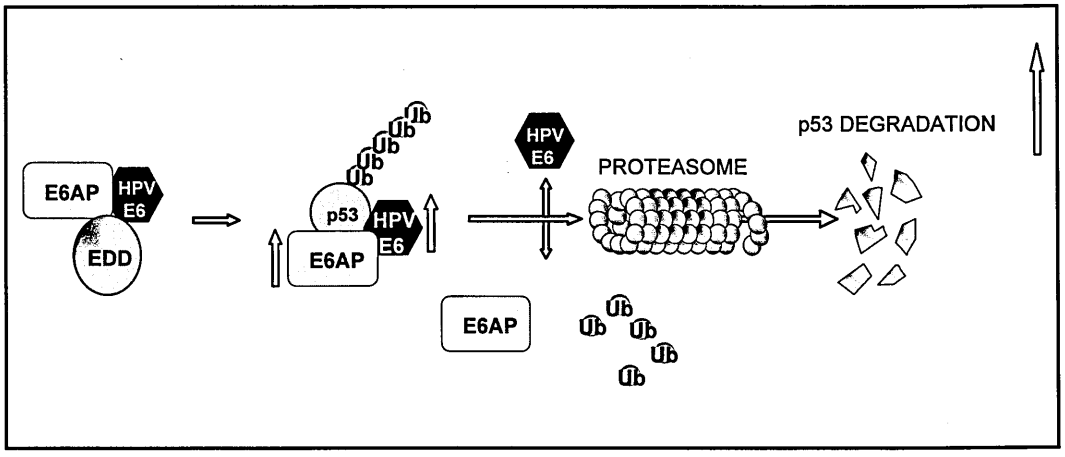


Figure 35. E6AP is upregulated in the absence of EDD. A schematic representation of E6AP protein stabilization in the absence of EDD, which consequently leads to E6 protein stabilization, overall resulting in increased ubiquitination and degradation of p53.

we show that EDD can target E6AP for proteasome-mediated degradation (Figure 21). In addition, we also show that EDD depletion in HPV-negative cells leads to a strong upregulation of E6AP levels. This suggests that E6AP is likely to be an EDD substrate regardless of the presence or absence of HPV E6. In HPV-positive cells it is possible that E6, by binding to both E6AP and EDD, alters the biochemical interaction between these proteins, possibly resulting in a decrease of the EDD-induced turnover of E6AP, and consequently increasing E6 stability. As already mentioned in the Results section of this thesis we clearly show that E6 can complex with EDD in an E6AP negative background, suggesting that E6 is likely to interact with both ligases and that they do not exclude each other from the E6 interaction. Furthermore, in *in vitro* binding experiments using an HPV-18 E6 I130T mutant, which is greatly reduced in binding to E6AP, showed that EDD binding to HPV-18 E6 and HPV-18 E6 I130T is similar, whereas E6AP binds significantly more strongly to wild type HPV-18 E6 than to the mutant. In addition, HPV-16 E6, which binds E6AP more strongly than HPV-18 E6 (Huibregtse *et al.*, 1993b), in contrast binds EDD more weakly than HPV-18 E6. Taken together this all suggest that the E6 interaction with EDD is not mediated through E6AP, and that the binding sites on E6 for those two proteins are distinct.

Since EDD appears to be involved in the regulation of various cellular processes, there is more than one possible outcome for its interaction with E6. Initial studies characterized EDD as a tumor suppressor, where less severe mutations resulted in impaired cell proliferation (Mansfield *et al.*, 1994), while more recent studies have shown that severe mutations in EDD were lethal for transgenic mice (Saunders *et al.*, 2004). Therefore, it is likely that E6 by binding to EDD could be altering some of its tumor suppressor properties and in that way may

have a direct impact on cellular proliferation which might in turn promote viral production and cellular transformation. Finally, since EDD is an ubiquitin ligase with an N-recognin potential in targeting cellular proteins, it is also possible that E6 could be redirecting EDD towards certain cellular substrates in order to drive their proteasomal degradation through this pathway. However, further analysis is needed in order to clarify this possibility, as well as to characterize potential target proteins.

Many studies have reported variable amounts of certain E6 cellular substrates, such p53 and PDZ domain-containing proteins, during viral infection and in HPV-induced cervical lesions, highlighting the fact that not all of them are degraded completely, either during viral infection or during the induction of malignancy (Cavatorta *et al.*, 2004; Cooper *et al.*, 1993; Lie *et al.*, 1999; Mantovani and Banks, 1999). The results presented above suggest that the variation in protein levels of those E6 substrates could be a reflection of fluctuations in the levels of either EDD or E6AP, which in turn affect E6 function. Further studies need to be performed to elucidate these aspects and to clarify the role of EDD in E6 degradatory functions with respect to the viral life cycle and to HPV-induced malignancy.

PART II: PDZ Binding is Conserved Between HPV and RhPV

PDZ domain recognition is a true molecular signature of E6 proteins derived from cervical cancer-causing HPVs. Only high-risk HPV E6 proteins have PDZ binding motifs on their extreme C-termini (Figure 6), through which they target various PDZ domain-containing proteins (Table 2). Rhesus Macaques are arguably the most relevant animal model for studying PV and cervical cancers. RhPV-1 oncoproteins E6 and E7 have high homology with equivalent oncoproteins to HPV-16 (Ostrow *et al.*, 1991) and the virus also causes cervical cancer in monkeys, which is phenotypically similar to HPV-16 related malignancy (Kloster *et al.*, 1988; Ostrow *et al.*, 1991). Interestingly, although RhPV-1 E6 is derived from a high-risk mucosal papillomavirus, it has no PDZ-binding motif. However, a PDZ binding motif is encoded by the virus, and we now show a remarkable evolutionary conservation of PDZ-binding activity on the RhPV-1 E7 protein instead. Intriguingly, this directs the binding of E7 to Par3, a PDZ domain-containing protein controlling the same polarity regulation pathway as that controlled by hDlg and hScrib (reviewed in Aranda *et al.*, 2008), the preferred PDZ domain-containing targets of HPV-18 and HPV-16 E6, respectively (Thomas *et al.*, 2005). These studies thus provide compelling evidence for a direct functional role for the inactivation of this pathway in the development of cervical cancer.

Targeting of PDZ Domain-containing Proteins is a Conserved Function among High-risk PV Types

HPV-16 and HPV-18 E6 degradation of a number of PDZ domain-containing targets contributes to these viruses' ability to induce malignancy (Table 2 and Figure 36). From this it is clear that for high-risk HPV types it is necessary to interact with various members of the cell polarity regulators, suggesting that these interactions could play pivotal roles during different stages of the viral life cycle, as well as in driving malignant progression. It is also clear, as discussed previously, that HPV-16 and HPV-18 preferentially target Scribble and Dlg, respectively. However, besides Dlg and Scribble other PDZ domain-containing proteins have been reported to be targeted by HPV E6 proteins, as also shown in Figure 36. Good examples of such substrates are the MAGI and PATJ proteins, which have been shown to be degraded through a proteasome dependent pathway by high-risk HPV E6 proteins (Glaunsinger *et al.*, 2000; Latorre *et al.*, 2005; Storrs and Silverstein 2007; Thomas *et al.*, 2002). This diversity of PDZ substrates which can be targeted by HPV E6 does not only suggest that there is a clear specificity in the targeting, but also that there is a constant requirement for those substrates to be destabilized to allow the viruses to establish a productive infection in the cell. In addition, it can be reasoned that during different stages of the viral life cycle certain PDZ domain-containing proteins could be preferentially targeted over other ones. Interestingly, loss of the PDZ binding domain of E6 in the context of the whole virus has a deleterious effect upon viral episome maintenance. This was first reported for HPV-31 (Lee and Laimins, 2004), but has been confirmed with HPV-16 (K. Raj, personal communication) and HPV-18 (S. Roberts, personal communication). One interesting question would be to determine whether the domain

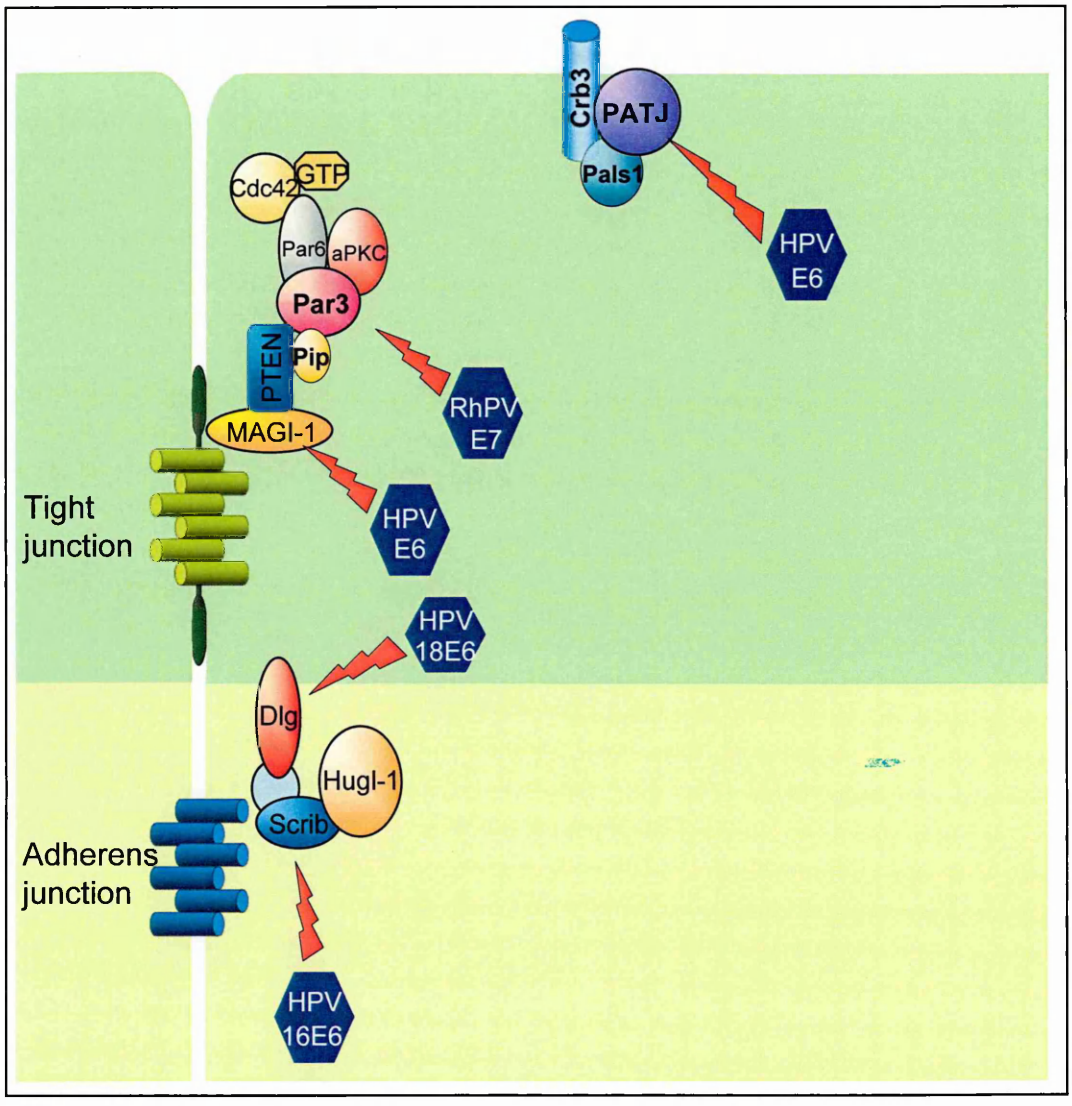


Figure 36. High-risk PVs exert a coordinated attack on cell polarity regulators. A schematic representation of different components of the cell polarity 'supercomplex' that are targeted by papillomavirus oncoproteins. HPV-16 E6 preferentially targets Scribble, while HPV-18 E6 preferentially targets Dlg. MAGI-1 and PATJ have also been identified as HPV E6 targets. Meanwhile Rhesus papillomavirus 1 E7 (RhPV-1 E7) targets Par3.

that we have identified in RhPV-1 E7 is also similarly important. How PDZ binding might contribute to this aspect of the viral life cycle is a matter of great debate. However, based on the substrates so far identified we could propose one or two possibilities. Either it reflects a need to alter polarity of cell division or, alternatively, perhaps to alter certain intracellular signaling pathways, such as MAPK, in order to efficiently maintain the viral episomes or promote cellular proliferation. Further analysis is required to identify which of these possibilities are true.

The lack of a PDZ-recognition motif on RhPV-1 E6 was a concern since this virus is a high-risk mucosal PV type and induces equivalent malignancies in its natural host. However, the finding that the RhPV-1 E7 protein has such a motif resolves this issue. In several assays we show that the carboxy-terminal four amino acids of the RhPV-1 E7 protein form a PDZ-recognition site. Using a single point mutation or deletion of the entire motif weakened PDZ recognition. However, the ability of RhPV-1 E7 to bind PDZ domains was different from that of HPV-18 E6, with respect both to the precise PDZ domains recognized as well as in the strength of the interactions. This is not surprising when one considers that the actual PDZ-binding motifs are very different, even though the consensus sequence is conserved, and this would be expected to alter substrate recognition (Zhang *et al.*, 2007). *In vivo* studies, which were also supported by structural studies, have shown that for the degradation of Scribble the L/V difference at the -1 position in the PDZ binding motifs of E6 appears to be critical, which makes Scribble more susceptible to degradation by HPV-16 than by HPV-18 (Thomas *et al.*, 2002; Zhang *et al.*, 2007). Furthermore, crystallographic studies have also revealed that a Q at the -2 position of the HPV E6 proteins, which is highly conserved, is in part responsible for E6 binding

to MAGI-1 and Dlg. Furthermore, T rather than S at the position -3 has also been shown highly correlated to, necessary for efficient Dlg binding, while E at the position -4 contributes to Dlg and MAGI-1 binding (Zhang *et al.*, 2007). Interestingly, in addition to the critical roles played by each of the four amino acids in the PDZ binding motif of HPV E6 proteins in substrate recognition, it was shown that amino acids positioned further upstream of the PDZ recognition motif could also significantly contribute to the binding affinity for different proteins. Highly conserved R at the positions -5 and -6 have been reported to be involved in MAGI-1 binding, while R at the position -5 contributes to E6 ability to interact with and degrade Dlg (Thomas *et al.*, 2008; Zhang *et al.*, 2007). As can be seen from Table 5, there are major differences between the extreme carboxy termini of high-risk HPV E6 and RhPV-1 E7 proteins. Of particular importance would appear to be the S/T difference, which has been shown previously to be not functionally equivalent (Thomas *et al.*, 2008). In addition, there are significant differences at the -2 position with an R in RhPV-1 E7 compared with Q in HPV E6, but perhaps most critically, 3 major changes at -4, -5 and -6 positions with A, C and S in Rhesus compared with E, R and L in HPV. Considering the obvious importance of these residues in PDZ recognition it is therefore not surprising that RhPV-1 E7 preferentially targets PDZ substrates that are different from those that are targeted by HPV-18 and HPV-16 E6. In this case Par3 appears to be the preferred substrate.

Table 6. Extreme C-terminal amino acid sequences of HPV-18 E6 and RhPV-1 E7 oncoproteins

Nucleotide position	-6	-5	-4	-3	-2	-1
HPV-18 E6	R	R	E	T	Q	V
HPV-16 E6	R	R	E	T	Q	L
RhPV-1 E7	S	C	A	S	R	V

PDZ Binding Motif of RhPV-1 E7 Plays an Important Role in Cellular Transformation

A role for this motif in one of the biological activities of RhPV-1 E7 was shown by its requirement for optimal co-transforming activity in primary cells. Previous studies had shown that RhPV-1 E7 can cooperate with EJ-ras to transform primary BRK cells (Ostrow *et al.*, 1993), which is expected since RhPV-1 E7 retains the pRb recognition site (Ostrow *et al.*, 1991). Interestingly, the pRb binding motif of RhPV-1 E7 (LMCYE) slightly differs from HPV-16 E7 (LYCYE). Although this could partially account for the difference in the numbers of transformed colonies between the wild type forms of these two proteins, the overexpression assays suggest that both proteins are equally effective in targeting human pRb for degradation (Figure 29). However, the most intriguing finding from this assay was that the RhPV-1 ΔPDZ E7 mutant has a significantly weaker co-transforming activity than the wild type RhPV-1 E7 protein, suggesting that the ability to target one or more PDZ domain-containing substrates might contribute to the ability of RhPV-1 E7 to bring about cell transformation. Interestingly, it was reported that in human cells expressing oncogenic ras, loss of Scribble or

related polarity regulators, Dlg and Lgl, is sufficient to induce tumorigenic growth and promote spontaneous invasion and metastasis (Dow *et al.*, 2008). In addition, it was also shown that deregulation of Scribble in combination with overexpressed myc oncogene promotes mammary tumorigenesis in mice and human breast cancer (Zhan *et al.*, 2008). These observations might provide explanations for the difference in numbers of the transformed colonies between RhPV-1 E7 and RhPV-1 ΔPDZ E7 proteins. The mutant protein was unable to target Par3 and consequently destabilize the polarity complexes, which resulted in greatly reduced transforming capacity of the protein in combination with activated ras. Furthermore, in the binding assays we show that RhPV-1 E7 had some potential to interact with Scribble, although we did not observe any protein degradation. This would suggest that besides targeting Par3 RhPV-1 E7 might also interact with Scribble, and the loss of binding to Par3 or Scribble with the ΔPDZ mutant would explain reduced co-transforming activity with EJ-ras. These results also support previous studies which have shown that the PDZ binding motif on HPV E6 proteins plays critical roles in cellular transformation, cell proliferation, and in induction of anchorage-independent growth in tissue culture (Kiyono *et al.*, 1997; Lee and Laimins, 2004; Spanos *et al.*, 2008a; Spanos *et al.*, 2008b). Moreover, transgenic mice expressing an E6 (Δ146-151) PDZ deletion mutant failed to display epithelial hyperplasia (Nguyen *et al.*, 2003a; Nguyen *et al.*, 2003b). This further suggests that RhPV-1 is likely to interfere with certain cellular pathways or complexes known to be targeted by high-risk HPVs, but in the case of PDZ substrates this occurs through E7 rather than E6.

Proteomic Analysis of RhPV-1 E7

Because of the major differences between RhPV-1 E7 and HPV-18 E6 in binding known PDZ substrates, we attempted to identify the other, potentially preferred, PDZ-containing substrates of RhPV-1 E7 using a proteomic approach. The most exciting cellular target identified was the PDZ domain-containing protein, Par3. Besides Par3, a number of other cellular targets were identified which had previously been reported for HPV-16 E7. pRb is known to be one of the main targets of HPV E7 (Dyson *et al.*, 1991) and since RhPV-1 and HPV-16 are closely related viruses it was likely that RhPV-1 E7 would interact with human pRb, and this was confirmed by the proteomic analysis. This also suggests that the one amino acid difference (M->Y at position 2) between the RhPV-1 E7 and HPV-16 E7 pRb binding motifs does not seem to have a significant effect upon the ability of RhPV-1 E7 to interact with human pRb. This was further confirmed by *in vivo* degradation assays which showed that RhPV-1 E7 and HPV-16 E7 direct the degradation of human pRb to similar levels.

p600, a protein involved in regulation of anchorage-independent growth, was found to be another intriguing target of RhPV-1 E7. Previous studies have shown p600 to be an important cellular target of both HPV-16 and BPV-1 E7 which was required for some of the transforming activities of E7, shown to be pRb independent (Huh *et al.*, 2005; DeMasi *et al.*, 2005). Therefore the identification of p600 as an interacting partner of RhPV-1 E7 in this proteomic analysis would suggest that p600 is a very important target of PV E7 proteins and that the interaction is conserved among many different PV types. Interestingly, both the HPV E7 and BPV E7 proteomic analyses were performed on C-terminally tagged E7 proteins since it was previously shown that N-

terminally tagged E7 is functionally defective (Gonzalez *et al.*, 2001). This loss of function might reflect loss of p600 interaction, since it was also shown that N-terminally tagged HPV E7 was incapable of interacting with p600 (Huh *et al.*, 2005). However, in our mass spectrometry analysis we used N-terminally tagged RhPV-1 E7 because our primary objective was to look for PDZ domain-containing substrates and therefore had to leave the C-terminus untagged. Unlike HPV E7 and BPV E7, our N-terminally HA tagged RhPV-1 E7 protein was still able to interact with p600 in this analysis, suggesting that RhPV-1 E7 may interact with p600 in a manner different to that seen with HPV E7 and BPV E7. Alternatively, this might indicate that the RhPV-1 E7 association with p600 is not direct, but is rather mediated through another cellular protein. Further studies will be required to clarify these aspects.

Besides p600 this proteomic analysis also identified other important components of the ubiquitin proteasome pathway, consistent with the ability of RhPV-1 E7 to target proteins for proteasome-mediated degradation. These were Cullin-2 and EDD, toghether with a number of proteasome subunits. Cullin-2 was previously shown to complex with HPV E7 and pRb, leading to poly-ubiquitination of pRb *in vivo*, resulting in aberrant degradation of pRb in HPV-16 E7-expressing cells (Huh *et al.*, 2007). Therefore, it seems likely that RhPV-1 E7, at least in part, interacts with this ubiquitin ligase in order to target pRb. I was particularly interested in the results demonstrating that EDD was a potential binding partner of RhPV-1 E7, especially as I have shown that this is potentially important in HPV E6 activity. Thus, it would appear that RhPV-1 E7 has two functions which are normally found in HPV E6: EDD binding and PDZ binding. The EDD binding to RhPV-1 E7 was confirmed by *in vitro* binding assays where we show that EDD binds

much more strongly to RhPV-1 E7 than to HPV-16 E7, suggesting that its interaction with RhPV-1 E7 is specific. As already discussed, EDD was also shown to act as an N-recognin ubiquitin ligase (Tasaki *et al.*, 2005). This would suggest that the RhPV-1 E7 interaction with EDD could result in redirection of EDD towards a pool of cellular substrates which are then targeted in a proteasome dependent manner. Again, further studies are required to characterize this interaction in more detail.

Interestingly, based on the protein sequence comparisons EDD has a potential pRb binding site (LCCND) and four potential LXXLL binding motifs. The LXXLL binding site is found on several interacting partners of E6 that are referred to as α-helical partners (Baleja *et al.*, 2006; Chen *et al.*, 1995), with the best characterized protein that belongs to this group being E6AP (Baleja *et al.*, 2006). Therefore it can be speculated that EDD could interact with E6 through one of its conserved LXXLL motifs in a similar way to E6AP. In contrast, the interaction of EDD and RhPV-1 E7 could be partially direct and partially indirect through EDD's potential association with pRb.

Par3 is a Target of RhPV-1 E7 both *in vitro* and *in vivo*

From the standpoint of searching for potentially relevant PDZ domain substrates of RhPV-1 E7, identification of Par3 in the proteomic analysis was the most important finding. We verified the interaction *in vitro* in a number of basic protein-protein interaction assays and confirmed that the interaction required an intact PDZ binding motif of RhPV-1 E7. Par3 has 3 PDZ binding domains, and unfortunately we have not been able to access clones to verify which is the exact recognition domain. However, the interaction appears to be very strong,

and most importantly, results in some degradation of the Par3 protein. We were particularly surprised that we could detect this in an *in vitro* assay, suggesting that this is really very efficient. The mechanism by which RhPV-1 E7 degrades Par3 is currently unknown, however the fact that this activity is readily detectable in an *in vitro* assay suggests that it is quite different from that used for the degradation of pRb, which is extremely difficult to recapitulate *in vitro* (V. Tomaić, personal observation).

An important concern was whether E7 could be found in similar cellular locations to those expected for Par3 (Joberty *et al.*, 2000) imunofluorescent analysis showed that RhPV-1 E7 is in both nuclear and cytoplasmic compartments, while Par3 is mainly cytoplasmic. Interestingly, when HA-tagged RhPV-1 E7 is co-expressed with Par3 there is a clear co-localization of both proteins, with an apparent recruitment of E7 from the nucleus into the cytoplasm. This re-localization absolutely depends upon an intact PDZ-recognition motif, further supporting its role in Par3 recognition. It is notable that these studies were done using HA-tagged E7, where no degradation of Par3 was seen, and this is an important similarity with HPV-16 E7, which also requires a free N-terminal region to direct substrate proteins for proteasome-mediated degradation (Reinstein *et al.*, 2000; Gonzalez *et al.*, 2001).

The most intriguing finding of this study is that RhPV-1 E7 targets Par3, a protein that provides anchorage to assemble the Par complex at the apical-lateral border by binding Par6 and recruiting Par6-associated proteins (reviewed in Aranda *et al.*, 2008). The regulation of cell polarity and directional cell migration involves three different protein complexes: the Crumbs complex, comprising Crumbs and Stardust; the

Par complex, comprising Par3, Par6, Cdc42 and aPKC; and the Scribble complex, comprising Dlg, Scrib and Lgl. Each component of these complexes is essential for the proper functioning of the whole (Humbert *et al.*, 2006). The three complexes spatially segregate and are functionally antagonistic, restricting each other's precise cellular localization in different ways, depending on the cellular context (Humbert *et al.*, 2006) (Figure 7).

Interestingly, in the case of the cancer-causing mucosal HPV types, HPV-16 E6 would appear to preferentially target hScrib, whereas HPV-18 E6 targets hDlg, and thereby inducing alterations in cell migration and proliferation control (Nakagawa and Huibregtse, 2000; Watson *et al.*, 2003). RhPV-1, which causes the same cancer, also targets cell polarity and proliferation albeit through a component of a different complex, Par3, but via a conserved PDZ-recognition motif (Figure 36). This evolutionarily conserved assault on polarity control is a very powerful argument for a critical role of the Crumbs/Par/Scrib complexes in the life cycle of mucosal HPVs and it also is compelling evidence for a functional relevance in the induction of cervical cancer.

In addition to their role in cell polarity maintenance, members of the Par complex have been implicated in various human and mouse malignancies such as ovarian, head and neck, and breast cancers. For example, hyperactivation or mislocalization of aPKC kinase was shown to have an impact on tumor growth, motility and proliferation in cell lines, suggesting that improper control of aPKC by normal polarized activity of the Par complex may lead to its pro-oncogenic activities (reviewed in Aranda *et al.*, 2008). Furthermore, overexpressed Par3 was shown to inhibit TGF-ß-induced loss of E-cadherin and epithelial-to-mesenchymal transition (EMT), suggesting that TGF-ß alters the Par

complex from one that is responsible for polarity control to one that promotes transformation (reviewed in Aranda *et al.*, 2008). The RhPV-1 targeting of the PAR complex through its interaction with Par3 may have two consequences. By degrading Par3, or by re-localising it from insoluble to soluble fractions, RhPV-1 E7 might de-stabilize Par complexes, allowing mislocalization of aPKC, thus increasing cell proliferation. In the context of the natural viral life cycle this might promote viral production, while in the context of transformation this might increase the oncogenic potential of the Par complex.

Future Directions

The results of this thesis define important aspects of the control of HPV E6 protein stability and its association with the proteasome pathway. Furthermore, evolutionary they also demonstrate important conserved activities between the two high-risk mucosal papillomavirus types in targeting cell polarity regulators through their oncoproteins, HPV E6 and RhPV-1 E7. To expand on these findings in more detail and to better understand how these oncoproteins' functions contribute to the viral life cycle and to PV-induced malignancy, there are a number of aspects which will need further investigation.

Further clarification of the role played by E6AP in E6 stability is something that needs to be investigated for a better understanding of the mechanisms regulating E6 protein stability. Firstly, it should be established whether E6 protein stability is directly or indirectly dependent on its association with E6AP. E6AP binding-defective mutants of E6 (L37S and L110Q) (Sekarić et *et al.*, 2008) will be used for these experiments and they should help to clarify this aspect. In addition, we will investigate whether E6AP functions as a molecular chaperone in providing conformational stability for E6. This will also include investigation of HSP70 and CHIP ligase involvement in E6 protein turnover in the absence of E6AP. We are also planning to perform a proteomic analysis of E6AP and to search for other ubiquitin ligases which may complex with E6AP and which may also be involved in E6 turnover in the absence of E6AP. Other obvious experiments will include investigation into whether the HERC2 ubiquitin ligase (Vos *et al.*, 2009) is involved in E6 protein turnover in the absence of E6AP. We could also expand our search and look for other ubiquitin ligases which

might be involved in this process. This could be done using HeLa cells in a high-throughput analysis, where siE6AP would be co-expressed with an siRNA library to the known human ubiquitin ligases, the positive readout being rescue of E6 expression when cells are lacking E6AP and one other ligase.

In the proteomic analyses EDD was identified as an interacting partner of both HPV E6 and RhPV-1 E7 proteins, suggesting that this is a common interacting protein of the two viruses, but although associated with different oncoproteins. It will be of a great interest to characterize the EDD association with HPV E6 and RhPV-1 E7 proteins in more detail in order to better understand any potentially conserved functions between the two viruses. From the data presented in this thesis, it is likely that EDD has an indirect effect both on E6 stability and its ability to degrade p53 through its regulation of E6AP. However, since EDD is involved in many other cellular processes, such as protein turnover (Honda *et al.*, 2002), carcinogenesis (Clancy *et al.*, 2003; Mori *et al.*, 2002), and DNA damage response (Henderson *et al.*, 2006) it is likely that both HPV E6 and RhPV-1 E7 complex with EDD in order to interfere with some of its other functions. Therefore, since DNA damage appears to be a common theme, studies should be carried out to determine if the abilities of E6 and RhPV-1 E7 to induce DNA damage, or to perturb DNA damage response pathways is in any way linked to their ability to bind EDD. For example, does their ability to interact with EDD have any effects on CHK2 phosphorylation?

Furthermore, since EDD is a HECT domain ubiquitin ligase with an N-recognin ligase potential (Callaghan *et al.*, 1998; Tasaki *et al.*, 2005), we also plan to search for potential cellular substrates whose degradation is E6 and RhPV-1 E7-mediated through association with EDD. This will

be particularly important with respect to RhPV-1 E7 targeting of Par3. Finally, from the data presented in this thesis it seems possible that EDD could function as a natural ubiquitin ligase of E6AP, and therefore we are planning to characterize the association of those two ligases in more depth, which could be an important step for better understanding the signaling pathways disrupted in the Angleman's syndrome.

Materials and Methods

Cells and transfection. U2OS (human osteosarcoma, p53+/+ pRb+/+), 293 (human embryonic kidney), SAOS-2 (human osteosarcoma p53-/- pRb-/-), Baby Rat Kidney (BRK), HT1080 (fibrosarcoma), NIH3T3 (mouse fibroblasts), E6AP (-/-) (mouse epithelial kidney cells), HeLa (HPV-18 positive) and CaSKi (HPV-16 positive) cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (100U/ml) and glutamine 292µg/ml. Transfection was carried out using calcium phosphate precipitation as described previously (Matlashewski *et al.*, 1987) or using Lipofectamine2000 (Invitrogen) according the manufacturer's protocol.

Plasmids.

pCA: The pCA plasmid was created by inserting a cassette containing 2 x HA and 1 x FLAG epitopes derived from the AdTrack vector (kindly provided by Patricio Meneses) into the multiple cloning site of pCDNA3 between BamHI and XbaI restriction sites as shown in Figure 12A.

HPV E6: Wild type HPV-18 E6 was amplified by PCR using the following primers to produce N-terminally tagged E6: forward primer 5' CAAGACAGTATTGGAACTTACAGAAGTATTTGAATTT; reverse primer 5' TAGTAAGTTAACTTATACTTGTGTTTCTCTGCGTCG followed by digestion using ECoRI and HpaI restriction enzymes into the pCA plasmid (Figure 12B). E6* (Pim *et al.*, 1997) was amplified by PCR using the same primers as for the wild type E6 followed by digestion using the same pair of enzymes as for the wild type protein

and cloned into pCA. Splicing defective mutant E6SM was constructed from pCA 18 E6 using the Gene Tailor Mutagenesis kit (Invitrogen) according to the manufacturer's instructions, where G was replaced with A at the nucleotide position 233. Untagged HPV-18 E6 and HPV-16 E6 pCDNA-3 expression plasmids have been described previously (Gardiol *et al.*, 1999; Pim *et al.*, 1994). The GST-fusion proteins HPV-18 E6 and HPV-18 E6* have been described previously (Pim *et al.*,1997; Thomas *et al.*, 1996).

RhPV-1 E7: RhPV-1 E7 was amplified from RhPV-1 genomic DNA (kindly provided by Michele Ozbun) by PCR to produce untagged and N-terminally tagged E7: The following primers were used for producing the N-terminally tagged E7: forward primer 5' TACATGAATTCATGATTGGGCCTAAACCT; reverse primer 5' ATGAAGTTAACTTACACTCTGCTGGCACA followed by digestion using ECoRI and HpaI restriction enzymes into pCA plasmid. To generate untagged (pCDNA3) E7 and GST fusion proteins, RhPV-1 E7 was amplified from RhPV-1 genomic DNA by PCR using the following primers: forward primer 5' TACATGGATCCATGATTGGGCCTAAACCT; reverse primer 5' AGTAAGAATTCTTACACTCTGCTGGCACA followed by digestion using BamHI and ECoRI restriction enzymes into pCDNA3 and GST plasmids. The point mutant (V->A) in the PDZ binding motif and the complete PDZ deletion mutant (ΔPDZ) were generated using the Gene Tailor Mutagenesis kit (Invitrogen) according to the manufacturer's instructions.

Additional *in vitro* expression plasmids. For bacterial expression of GST-tagged proteins the following constructs were used: GST-Dlg (Gardiol *et al.*, 2002). For *in vitro* transcription translation: hScrib,

MAGI-2 and MAGI-3 (Thomas *et al.*, 2002); Dlg (Gardiol *et al.*, 2002); E6AP (Huibregtse *et al.*, 1993a); and EDD (Clancy *et al.*, 2003).

Additional *in vivo* expression plasmids. The following *in vivo* expression plasmids used have been described previously: EJ-ras expression plasmid pEJ6.6 (Matlashewski *et al.*, 1987); the wild type and mutant E6AP (C->A) expression plasmids were kindly provided by Scott Vande Pol (Brimer *et al.*, 2007); FLAG-p53 was kindly provided by Georgine Faulkner; HA-Dlg (Gardiol *et al.*, 2002); V-5 tagged MAGI-3 constructs and HA-MAGI-2 (Thomas *et al.*, 2002); pJ4Ω16 E7 (Storey *et al.*, 1988); the pRb expression plasmid was kindly provided by Giannino Del Sal; the EDD expression plasmid was kindly provided by Collin Watts; the Myc-tagged Par3 expression plasmid was kindly provided by Ian Macara

Antibodies. Mouse monoclonal antibodies against HPV-18 E6 (1:1000/ [N-terminus #399]) and mouse monoclonal anti-16E6 antibody (1:1000/ [N-terminus #74 and C-terminus #813]) were generated and generously provided by the Arbor Vita Corporation.

The following commercial antibodies were used at the dilutions indicated in parenthesizes: anti-HA monoclonal antibody 12CA5 (Roche; WB 1:100, IF 1:100); anti-ß-galactiosidase (Promega, WB 1:5000); anti-FLAG mouse monoclonal antibody M2 (Sigma, WB 1:5000); mouse anti-p53 DO-1 (Santa Cruz 1:1000); mouse anti-pRb (Santa Cruz; WB 1:500), mouse anti-γ-tubulin (Sigma; WB 1:5000); mouse anti-E6AP (BD Transduction Labs; 1:500 WB); goat anti-EDD M-19 (Santa Cruz; WB 1:200); mouse anti-Dlg SAP97 (Santa Cruz; WB 1:200); goat anti-

hScrib (Santa Cruz; WB 1:200); mouse anti-V5 (Sigma; WB 1:5000); mouse anti-c-myc (Santa Cruz, WB 1:1000).

Appropriate secondary antibodies conjugated to HRP were purchased from DAKO and used for Western blotting at a dilution of 1:1000. In the case of immunofluorescence staining, secondary antibodies conjugated to either fluorescein or rhodamine were purchased from Molecular Probes and used at a concentration of 1:700.

BRK Transformation assays. BRK cells from 9-day-old Wistar rats were transfected with 2 µg EJ-ras either alone or together with 5 µg HPV16 E7, 5 µg RhPV 1 E7 and 5 µg RhPV 1 E7 (ΔPDZ) expression plasmids. Cells were maintained in medium containing 200 µg/ml G418 for 2 weeks and then fixed and stained with Giemsa-Blue (Diagnostica Merk) and then morphologically transformed colonies were counted. .

Fusion protein purification and *in vitro* binding assays. GST-tagged fusion proteins were expressed and purified as described previously (Thomas *et al.*, 1996). Briefly, 40 ml of an overnight culture of *E.Coli* strain DH5-α previously transformed with the appropriate expression plasmids were inoculated in Luria Broth (LB) containing ampicillin and grown at 37°C up to an OD of 0.6 at 395 nm. Recombinant protein expression was induced for 3 hrs with 1nM isopropyl-ß-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (PBS, 1% Triton X-100, 100 U/ml DNAse, protease inhibitors cocktail I, Calbiochem) and the lysates were then cleared from cell debris by centrifugation. The GST-fusion proteins were then incubated for 2hr

with glutathione-conjugated agarose beads. The purity of all fusion proteins was determined by SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining.

In vitro transcription translation was performed in either rabbit reticulocyte lysate or wheat germ extract using the Promega TNT system and were radiolabelled with [^{35}S] cysteine or [^{35}S] methionine (Amersham). Equal amounts of *in vitro*-translated proteins were added to GST fusion proteins bound to glutathione resin and incubated for 1 hr at 4°C. After extensive washing with PBS containing 0.25% NP-40, or as otherwise indicated, the bound proteins were analysed by SDS-PAGE and autoradiography.

Binding assays were quantified using a PhosphoImager (Packard) and the percentage binding with respect to inputs was calculated.

GST pull downs using cellular extracts were performed by incubating GST-fusion proteins immobilized on resin with cells extracted in E1A buffer for 1 hr at 4°C on a rotating wheel. The resin was then washed extensively with the extraction buffer and bound proteins were detected using SDS-PAGE and Western blotting using the appropriate antibodies.

Immunoprecipitation and Western blotting. Total cellular extracts were prepared by directly lysing cells from 6 cm^2 or 10 cm^2 dishes in SDS lysis buffer. To obtain the soluble and insoluble fractions separately, cells were lysed in E1A extraction buffer (25mM HEPES pH 7.0, 0.1% NP-40, 150mM NaCl, plus protease inhibitor cocktail set I; Calbiochem). After incubation on ice for 20 min lysates were cleared by

centrifugation at 13000 rpm for 10 min. The supernatant (soluble fraction) and the pellet (insoluble fraction) were analyzed by SDS-PAGE and Western blotting. For Western blotting, either 0.45 or 0.22 µm nitrocellulose membrane (Schleider & Schuell) were used and membranes were blocked for 1hr at 37°C in PBS+10% milk followed by the incubation with the appropriate primary antibody diluted in PBS 10% milk/0.5%Tween 20 for 2 hrs. For E6 antibodies the protocol included 5% milk and 2% BSA in 1xTBS for blocking and primary E6 antibodies were diluted in 2.5% milk and 1% BSA in 1xTBS/0.1% Tween 20. After several washings with either PBS 0.5% Tween 20 or TBS 0.1% Tween 20, secondary antibodies conjugated with HRP (DAKO) were diluted in either 10%milk PBS/0.5% Tween 20 or 2.5%milk 1%BSA TBS/0.1% Tween 20 and incubated for 1hr. Blots were developed using either Amersham ECL, or ECL+ in the case of 16 E6, according to the manufacturer's instructions.

For co-immunoprecipitations and mass spectrometry pull-down experiments, 293 cells were transfected with the appropriate plasmids. 24 hrs post transfection, either E1A (250 mM NaCl, 0.1% NP_{40}, 50 mM Hepes pH 7.0) or mass spectrometry lysis buffer (50 mM Hepes pH 7.4 (at 4°C), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.25% NP_{40}) extractions were performed and the soluble fraction was incubated with anti-HA beads (Sigma) to pull down either E6 or RhPV-1 E7 for 2-3 hrs on a rotating wheel at 4°C. The beads were then extensively washed in the extraction buffer depending on the experiment type, dried and sent to Mike Myers for proteomic analysis.

Sample preparation for mass spectrometry. The proteins were then eluted directly from the affinity beads using 50 ng of sequencing

grade trypsin (Promega) in 20 mM diammonium phosphate pH 8.0, for 6 hrs at 37°C. The supernatant was removed from the beads and the cysteines were reduced and alkylated by boiling for 2 min in the presence of 10 mM Tris(2-carboxyethyl)phosphine (Pierce, Milan, Italy) followed by incubating with 20 mM acetaminophen (Sigma) for 1 h at 37°C. The reactions were stopped by the addition of acetic acid (Sigma) to 0.1%. The resulting mixture was desalted using C18 Ziptips (Millipore, Milan, Italy) and lyophylized to dryness.

Mass spectrometry. Nanobore columns were constructed using Picofrit columns (NewObjective, Woburn, MA, USA) packed with 15 cm of 1.8 mm Zorbax XDB C18 particles using a homemade high-pressure column loader. The desalted samples were injected onto the nanobore column in buffer A (10% methanol/0.1% formic acid) and the column was developed with a discontinuous gradient and sprayed directly into the orifice of an LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA). A cycle of one full scan (400–1700 m/z) followed by eight data-dependent MS/MS scans at 25% normalized collision energy was performed throughout the LC separation. RAW files from the LTQ were converted to mzXML files by READW (version 1.6) and searched against the Ensembl human protein database and the NCBInr Viral database using the Global Proteome Machine interfaced to the X!Tandem algorithm (version 2006.06.01.2).

Half-life experiments. 48 hrs or 72 hrs post transfection, cells were treated for different time points as indicated with cycloheximide (50µg/ml in DMSO) to block protein synthesis. DMSO treated cells were used as the control. Total cellular extracts were then analyzed by

Western blot and the intensity of the bands on the X-ray film was measured using Optiquant program. The standard deviation was calculated from three independent assays.

RT-PCR. Total RNA was isolated from HeLa cells 72 hrs after transfection with either siRNA against Luciferase (control) or siRNA against E6AP using TRI reagent (Sigma) according to the manufacturer's instructions. A total of 1 µg of RNA was subjected to reverse transcriptase (RT) using RETROscript system (Ambion). No reverse transcriptase control was also added for assaying contamination with DNA. PCR was performed with 20 cycles and an annealing temperature of 55°C and 58°C for GAPDH. PCR primers for E6 (Pim et al., 1997) have been described previously. GAPDH primers were as follows: forward 5' FCCATCACCATCTTCCAGGAG; reverse 5' GGATGATGTTCTGGAGAGCC.

Immunofluorescence and Microscopy. Cells were stained and fixed for immunofluorescence as described previously (Grm *et al.*, 2005). Briefly, cells were fixed with 3.7% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Primary antibodies were incubated for 2 hr at 37°C, followed by extensive washing with PBS and incubation for 30 min at 37°C with secondary anti-rabbit or anti-mouse conjugated with fluorescein- or rhodamine (Molecular probes). Cells were then subjected to extensive washing with H_2O and mounted.

Slides were analysed using a Leica DMLB fluorescence microscope equipped with a Leica photo camera (A01M871016) and the data were collected utilizing the 100X objective oil immersion lens.

***In vivo* degradation assays.** 293 cells (for FLAG-p53; HA-Dlg, HA-MAGI-2; FLAG-E6AP, myc-Par3) and SAOS-2 cells (for p53 degradation) were transfected with 2µg of the constructs noted above, along with 0.5 µg of LacZ. Additionally, the following plasmids were also included: pCDNA3 18 E6 (3µg); pCA 18 E6 (3µg); EDD1 (5µg). 24 hrs post-transfection, or as otherwise indicated, the cells were harvested and analyzed by Western blotting.

***In vitro* degradation assays.** Proteins were transcribed and translated *in vitro* in rabbit reticulocyte lysate using the Promega TNT system according to the manufacturer's instructions. The HPV-18 E6 and RhPV-1 E7 proteins were radiolabelled with [^{35}S]-cysteine while the Dlg, Par3 and EDD1 were radiolabelled with [^{35}S]-methionine. Degradation assays were performed as previously described (Thomas *et al.*, 2001). Briefly, radiolabelled proteins were mixed and incubated for the indicated times at 30°C. Volumes were adjusted using water-primed lysate. The remaining Dlg or Par3 proteins were immunoprecipitated with anti-Dlg polyclonal rabbit serum (Gardiol *et al.*, 1999) or anti-myc mouse monoclonal antibody (Santa Cruz), respectively, and analyzed by SDS–PAGE and autoradiography.

Inhibitors. The following inhibitors were dissolved in DMSO and used at indicated concentrations: proteasome inhibitor Z-leu-leu-leu-al (CBZ; Sigma) (50µM); Calpain Inhibitor (LLnL; Sigma) (50 µM); proteasome inhibitor Epoxomycin (Sigma) (25 µM); protease inhibitors Cocktail Set I (Calbiochem).

siRNA experiments. HPV-positive HeLa and CaSKi cells and HPV-negative HT1080 cells were seeded in 6 cm^2 dishes and transfected using Lipofectamine2000 (Invitrogen) with the following siRNAs: siRNA against luciferase (Dharmacon) as the control; siRNA against 18 E6/E7 (5' CAUUUACCAGCCCGACGAG) (custom ordered from Dharmacon); siRNA against 16 E6/E7 (5' UUAAAUGACAGCUCAGAGG) (custom ordered from Dharmacon); siRNA against E6AP (Dharmacon and Santa Cruz); siRNA against EDD (Santa Cruz).

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