THE ROLE OF THE PSD95/Dlg/ZO-1 (PDZ) BINDING MOTIF OF HUMAN PAPILLOMAVIRUS TYPE 18 E6 ONCOPROTEIN IN THE VIRUS LIFE CYCLE

By

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ABSTRACT

A PSD95/Dlg/ZO-1(PDZ)-binding motif (PBM) in the E6 protein of high-risk, cancer-causing human papillomaviruses (HPV) targets a subset of cellular PDZ domain-containing proteins involved in diverse regulatory processes including cell polarity and proliferation, for proteasome-mediated degradation. Interaction with this select group of PDZ domain-containing proteins is negatively regulated by cAMP-dependent protein kinase (PKA) mediated phosphorylation of the E6 PBM. This thesis has sought to address the hypothesis that the PBM of E6 plays an important role within the HPV life cycle. This study has shown that deletion of the E6 PBM from HPV18 genomes affects the morphology and growth of viral episome-containing human keratinocytes and furthermore links E6 PBM function to viral episome replication (maintenance replication and differentiation-dependent amplification). Loss of negative regulation of the E6 PBM by mutation of the PKA recognition motif was associated with increased cell growth and indeed the growth of wildtype HPV18 genome-containing cells responded to changes in PKA signalling. Constitutive E6 PBM function was also associated with invasion of cells suggesting that malignant progression of HPV-infected cells may be linked to changes in PKA signalling. Modulation of the E6 PBM function in the viral genome-containing cells was associated with a change in protein levels of the PDZ domain-containing protein discs large (hDlg) and changes in the non receptor protein phosphastase PTPN13 specific species.

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ABBREVIATIONS

β-ME	Beta-mercaptoethanol
AA	Amino acids
AJ	Adherens Junctions
AJC	Apical Junctional Complex
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ATM	Ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
ATP	Adenine triphosphate
BPV	Bovine papillomavirus
BrdU	5-bromo-2-deoxyuridine
BS	Binding site
BSA	Bovine serum albumin
cAMP	Cyclin adenosine monophosphate
cDNA	Complementary DNA
CDC20	Cell divison cycle protein 20
CDK	Cyclin-dependent protein kinase
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
DABCO	1,4-diazabicyclo{2,2,2} octane
DAPI	4',6'-diamino-2-phenylindole
Dlg	*Drosophila* Discs large
DMEM	Dulbeccos modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri-phosphate
E6-AP	E6-associated protein
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FBS	Foetal bovine serum
FK	Forskolin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gy	Gray
H89	Dihydrochloride
H	Hour
H and E	Haematoxylin and Eosin
HBV	Hepatitis B virus
HCV	Hepatitiss C viruss

HFK	Human foreskin keratinocytes
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hScrib	Scribble
Hug-1	Lethal giant larvae
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
Kb	Kilobase
kDa	Kilodalton
KGM	Keratinocyte growth medium
KSHV	Kaposi's sarcoma herpes virus
L	Litre
LB	Luria-Bertani
LCR	Long control region
LRE	Late regulatory element
LRR	Localisation regulatory region
M	Mitosis
MAb	Monoclonal antibody
mA	Milli-amp
MAGI	Membrane associated guanylate kinase homology with an inverted domain
MAPK	Mitogen-activated protein kinase
MC	Methycellulose
MCV	Merket cell polyomavirus
Min	Minute
ND10	Nuclear domain 10
NHERF-1	Na^+/H^+ exchange regulatory factor
OC	Open circular
ORF	Open reading frame
Ori	Origin of replication
P	Passage
PAGE	Polyacrylamide gel electrophoresis
Pals1	Pals associated with lin-7
PATJ	Pals-1 associated tight junction protein
PBM	PDZ-Binding domain motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor receptor
PDZ	PSD-95/discs large/ZO1
PI	Propidium Iodide
PKA	Protein kinase A
PKA-RM	Protein kinase A recognition motif
PML	Promyelocytic leukaemia protein
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNase	Ribonuclease
SC	Supercoiled
SCC	Squamous cell carcinoma
SDS	Sodium Dodecyl sulphate
Sec	Second

SRPK	Serine-argenine protein kinase
SCC	Saline sodium citrate
SV40	Simian virus 40
TAg	Large T Antign
TE	Tris-EDTA
TEMED	N,N,M',N'' tetrtamethylethylene-diamine
TBE	Tris/Borate/EDTA
TBS-T	Tris-buffered saline – Tween20
TIP-2	Tax interacting protein
TJ	Tight Junctions
TNF	Tumour necrosis factor
TNS	Trypsin-neutralising solution
TRADD	TNFR1-associsated death domain
TRAIL	TNF-related apoptosis-inducing ligand
URR	Upstream regulatory region
UTR	Untranslated region
UV	Ultraviolet
V	Volt
VLP	Virus-like particle
vol/vol	Volume per volume
wt/vol	Weight per volume

CHAPTER 1 GENERAL INTRODUCTION

1.1 Infectious agents and cancer

The link between human cancers with viral origin was made nearly half a century ago with the discovery of the association between Epstein-Barr virus (EBV) and Burkitts Lymphoma in 1965 (Javier & Butel, 2008). Since then it has become apparent that a number of viruses play significant roles in the multistage development of human cancers. Approximately 15-20% of cancers are associated with viral infections (zur Hausen, 2001; Parkin, 2006). Oncogenic viruses can contribute to different steps of the carcinogenic process, and the association of a virus with a given cancer can be anywhere from 15 to 100% (Parkin, 2006).

Human tumour viruses belong to a number of different families, including the RNA virus families *Retroviridae* and *Flaviviridae* and the DNA virus families *Hepadnaviridae*, *Herpesviridae* and *Papillomaviridae*. These viruses have been associated with the development of a range of cancers including human papillomavirus (HPV) with anogenital cancers and head and neck cancers; hepatitis B virus (HBV) and hepatitis C virus (HCV) with hepatocellular carcinoma; human T-cell leukaemia virus (HTLV-I) with adult T-cell leukaemia and Kaposi's sarcoma herpes virus (KSHV), also referred to as human herpes virus type 8 (HHV-8), associated with Kaposi's sarcoma and Castelmans disease (McLaughlin-Drubin & Munger, 2008). More recently, a human polyomavirus has been linked to the development of Merkel cell polyomavirus (MCV); a rare but aggressive form of skin cancer (Shuda *et al*., 2008). Besides viruses, other pathogens have been identified as oncogenic agents. These include the bacterium *Helicobacter pylori* which are associated with gastric cancer and the parasite *Schistosoma hematobium* which has been associated with bladder cancer (zur Hausen, 2009).

Even though human oncogenic viruses belong to different virus families and utilise different strategies to cause cancer, these viruses do share a common trait of chronicity. Each of these viruses are capable of establishing a long-term latent or chronic infection in humans, increasing exposure time to viral oncogenic pressures and the risk of developing cancer. DNA viruses such as HPV, EBV, HBV and KSHV usually persist by either integrating into the host genome or remaining episomally. Whilst these viruses act directly to promote carcinogenesis, for other viruses such as the human immunodeficiency virus (HIV) the mechanism of action is indirect. By inducing immunosuppression, HIV acts to permit secondary infections with other viruses including HHV-8 (Feller *et al*., 2007), HPV (Palefsky, 2009) and EBV (Tran *et al*., 2008), which can subsequently result in associated cancers.

The study of oncogenesis is difficult because it is a slow and uncertain process in a living organism, and when transformed tissue is found, oncogenesis has already occurred and the process remains unobserved (McLaughlin-Drubin & Munger, 2008). It is further complicated by the enormous diversity in forms of cancer and oncogenic mechanisms available. The use of human Adenoviruses (Ads) has somewhat enhanced our understanding of this process. Adenoviruses lack an association with human cancers, however they have the ability to transform rodent cells (Sawada *et al*., 1988) and a subset of the viruses can induce tumours in experimentally infected rodents (Graham *et al*., 1984). As such, the use of these viruses serves as excellent models for better understanding the process of tumourogeneisis.

Oncogenic viruses have given us an insight into cancer biology as well as cell biology through their transforming properties and served as experimental models to investigate the discovery of oncogenes and tumour suppressors as well as processes that govern cellular pathways (McLaughlin-Drubin & Munger, 2008; Carrillo-Infante *et al*., 2007). The revelation that a subset of human cancers are caused by viruses presents a means by which the burden of cancer may be reduced and viruses are now attractive targets on which to base

preventative therapeutics. It is proposed that future research is likely to uncover further links between human cancers and infectious agents and this will further increase our understanding of carcinogenesis (zur Hausen, 2009).

1.2 Human papillomaviruses and cancer

The papillomaviruses of the family *Papovaviridae* are viruses with many distinctive properties. They are small double stranded DNA viruses, which replicate vegetatively in the nucleus of epithelial cells such as the keratinocytes of the skin. At present more than 120 distinct HPV types have been characterised based on DNA sequence analysis (Bernard *et al*., 2010). Variations in restriction enzyme digest pattern have led to further division into different subtypes. The two main HPV genera are the Alpha and the Beta papillomaviruses, with approximately 90% of currently characterised HPVs belonging to one or other of these groups (Doorbar, 2006). Differences in the regulatory sequences and the coding potential within the viral genome are likely to explain the significant differences that are apparent in the biology of different papillomaviruses (Doorbar, 2005). Alpha papillomaviruses constitute the mucosal or genital species and can be further classified according to their oncogenic potential, into low-risk and high-risk subtypes dependent upon their propensity to cause cancer. Within the group of muscoal HPVs, "high-risk" types are distinguished from "low risk" types depending upon the risk of an infected individual to develop carcinogenic lesions. Low risk HPV types, of which HPV6 and 11 predominate, are associated with the formation of benign hyperproliferative lesions including condyloma acuminata, which clinically manifests as genital warts and rarely progress to malignancy (Longworth & Laimins, 2004).

The association between HPV and human cancer was first proposed more than 30 years ago by Harald zur Hausen (zur Hausen *et al*., 1975; zur Hausen, 1976). Since then, HPV genotypes have been shown to account for more than 99.7% of cervical cancers (Walboomers

et al., 1999). Twelve HPV types have been classified as high-risk, and infections with these subtypes of HPV, have a higher propensity to progress to cervical carcinoma (Bouvard *et al*., 2009), with HPV16 and 18 being the most common types. The most common member of the high-risk group is HPV16 which accounts for more than 60% of all cervical cancers.

A meta-analysis of HPV type specific prevalence data worldwide from 1990-2010 showed that infection with HPV types 16 and 18 accounted for between 70 and 76% of all global cervical cancer infections, with prevalence rates of HPV16 and HPV18 at 57% and 16% respectively (Li *et al*., 2011). Whilst HPV16 is the predominant HPV type of squamous cell cervical carcinomas, HPV18 predominates within adenocarcinomas (Bulk *et al*., 2006).

Cervical cancer is the second most common cancer for women worldwide, accounting for 274,000 deaths per annum (World Health Organisation, 2008). A meta-analysis of 194 studies from 1995 to 2009 with more than 1 million women with normal cytological findings found a global prevalence of 11.7% and the highest HPV prevalence rates in Sub-Saharan Africa (24.0%), Eastern Europe (21.4%), and Latin America (16.1%) (Bruni *et al*., 2010).

Cancers of the uterine cervix are classified into two major histological forms, dependent upon the cell type from which the cancer originated, with squamous cell carcinoma (SSC) constituting around 80-85% of these malignancies, whilst adenocarcinomas, derived from adenomatous glandular cells are more infrequent, occurring in only 15-20% of cases (Green *et al*., 2003).

In addition, papillomaviruses are also able to infect exo-genital and oral epithelial cells and cause lesions ranging in severity from benign to malignant. It is estimated that 40% of penile, vulva, and vaginal cancers are attributed to HPV infection whilst 90% of anal cancers are predicted to be a result of infections with this virus (Parkin & Bray, 2006). Infection with HPV has also been suggested to be an aetiological factor for oral and oro-pharyngeal cancer (Gillson & Shah, 2003). The incidence of the overall HPV prevalence in oro-pharyngeal

cancers has increased over the past 10 years (Chaturvedi *et al*., 2011; Mehanna *et al*., 2011), rising from 40.5% before 2000 to 72.2% between 2005 and 2009, whilst HPV prevalence at non-oropharyngeal sites has not increased during the same time frame (Mehanna *et al* 2011; Chaturvedi *et al*., 2011).

HPVs that infect the gential tract are sexually transmitted and it is estimated that around two thirds of individuals who have sexual relations with an infected partner will become infected (Bekkers *et al*., 2004). However the majority of these infections are sub clinical and infections cleared (Singer *et al*., 1995). Whilst the majority of infections are transient, persistent infection with a high-risk HPV type is the greatest risk factor for cervical cancer (zur Hausen, 2006). Additional risk factors include multiple sexual partners, use of the contraceptive pill, co infection with human immunodeficiency virus (HIV) and cigarette smoking (Kjellberg *et al*., 2000). Whilst the exact mechanisms are unknown, these cofactors are proposed to influence progression to carcinogenesis through suppression of the immune system, resulting in increased viral persistence, or through elevating levels of cellular DNA damage.

1.3 Preventative and Therapeutic strategies

Disparities exist in mortality rates of cervical cancer worldwide, and a significant burden is imparted upon the developing world, with 85% of deaths occurring within low income countries (World Health Organisation, 2008). Within developed countries the incidence and mortality rates of cervical squamous cell carcinoma have significantly reduced by as much as 80% over the past four decades, due largely to the widespread implementation of effective screening programmes which enable detection and early treatment of pre-cancerous changes within the cervix (Parkin & Bray, 2006).

Whilst development of low grade lesions is frequent within young women, development of cervical cancer is more prevalent within older women, with an average age of around 45 years for diagnosis of cervical cancer (Schiffman *et al*., 2007). Often progression from the initial infection to malignancy can take many years and even decades. Therefore the use of screening programmes can be effective at detection of pre-cancerous lesions (Bekkers *et al*., 2004).

The Papanicolaou test or Pap smear is used routinely to screen women for the detection of abnormal changes to the cervical epithelium resulting from a high risk HPV infection, which may have the propensity to progress to invasive cervical cancer (Bekkers *et al*., 2004). Although the use of Pap tests has reduced both the incidence and the associated mortality of cervical cancer by over 70% (Safaeian & Solomon, 2007), many women still go untreated because they do not routinely undergo Pap test screening. An analysis of the CDCs 1998 National Health Interview Survey of more than 100,000 individuals revealed that only 83% of women aged 40 to 64 years reported Pap testing in the previous 3 years (Selvin & Brett, 2003). Screening rates are lower among women who are uninsured, younger, poorer, and less educated, probably contributing to the higher cervical cancer mortality in minority groups such as African Americans (Jemal *et al*., 2007). Even among women who undergo screening,

approximately 40% of those with abnormal Pap test results fail to return for additional testing (Kupets & Paszat, 2011; Felix *et al*., 2009). These statistics show that although cervical cancer screening is effective, many individuals are still not receiving preventive measures. For this reason, an HPV vaccine has become another important means for physicians to help reduce the risk of cervical cancer and other diseases associated with the HPV virus.

The ability of the HPV to self-assemble virus like particles (VLPs) by the synthesis and assembly of its major L1 capsid protein formed the basis of the prophylactic vaccine currently in use. Early experimental studies showed immunogenicity and efficacy with L1 VLP vaccines in three animal models: the dog (Suzich *et al*., 1995), cow (Kirnbauer *et al*., 1996) and rabbit (Breitburd *et al*., 1995). In these studies, immunisation with L1 VLPs induced circulating neutralising antibody to the L1 capsid protein and the animals were completely resistant to challenge with large amounts of the virus.

To date, two prophylactic vaccines have been granted licences by the European Union, the bivalent Cervarix® vaccine (GlaxoSmithKline) and the quadravalent Gardasil® vaccine (Merck). Currently, the HPV quadrivalent recombinant vaccine is approved for the prevention of HPV types 6, 11, 16, and 18. The vaccine requires 3 doses, with the second and third doses at 1 to 2 months and then 6 months, respectively. The Gardasil® vaccine is approved for use in boys, girls, men, and women between the ages of 9 and 26 years (CDC, 2010). A second type of vaccine, the HPV bivalent Cervarix® recombinant vaccine, is approved by the US Food and Drug Administration (FDA) for females age 10 to 25 years to prevent HPV types 16 and 18 (CDC, 2010). Vaccination is currently recommended for all girls and women aged 9 to 26 years whether or not they have ever tested positive for any HPV infection. The Gardasil® vaccine is beneficial for at least 5 years, and studies have been carried out to determine overall long-term efficacy (Harper, 2007). Results from vaccine studies for the Gardasil® and Cervarix® vaccines have shown that both vaccines are highly

effective (FUTURE II, 2007; Paavonen *et al*., 2007; Hendrix, 2008). In a study of 5455 women, in which 2723 women received the Gardasil® vaccine, 98% of the women who were given the vaccine were protected against precancers (cervical intraepithelial neoplasia [CIN] 2 or 3, adenocarcinoma) cause by HPV for at least 3 years (CDC, 2010).

It is important to note that these vaccines are preventative. Women already infected with these HPV types are not protected and vaccination must therefore be received prior to the onset of sexual activity (Kahn, 2009). In the UK, a Cervairx® immunisation programme has been approved for use in girls aged 12-13 since September 2008. Although some evidence of cross protection of HPV types has been observed, there is a continual need for cervical screening alongside the programme of vaccination, incurring significant costs. Furthermore, the high cost of the vaccine and its cold chain delivery, in addition to poor healthcare infrastructure, may prevent implementation of vaccination programmes within developing countries where the disease burden is greatest (Agosti & Goldie, 2007). As such, work has begun on finding new alternatives to the L1 VLP vaccine currently in use. One approach currently under development is the use of the minor capsid protein L2 for immunotherapy. Protection studies in naïve cattle and rabbits and in-vitro neutralisation assays suggest that unlike L1 vaccines, L2 vaccines might provide broad spectrum protection (Gambhira *et al*., 2006; Gambhira *et al*., 2007a; Gambhira *et al*., 2007b). The challenge now for this potential vaccine is to produce neutralising antibody titres at levels comparable to L1 vaccines (Roden *et al*., 2000).

Recent studies have identified synthetic long peptides as a potential therapeutic vaccine against HPV. Vaccination studies with synthetic long peptides spanning the complete HPV16 E6 and E7 proteins have been shown to induce an HPV16 specific $CD4^+$ and $CD8^+$ t-cell response in 100% of patients with advanced vulvar intraepithelial neoplasia (Kenter *et al*.,

2009). This work provides the first evidence of a therapeutic vaccine against HPV for which there is currently none.

1.4 Genome Organisation

The HPV virus is a small non-enveloped virus (~55nm in diameter) with an icosahedral capsid (Longworth & Laimins, 2004). Between papillomaviruses the overall genomic organisation is highly conserved. Viral genomes are composed of ~8 kb of double-stranded circular DNA which can be divided into three main regions: a long coding region (LCR) and early (E) and late (L) regions (Figure 1.1). The LCR, sometimes referred to as the upstream regulatory region (URR) is a non-coding region which contains transcriptional, post-transcriptional and replicative cis-regulatory elements (Graham, 2008; Thierry, 2009). Located within this region is the viral origin of replication (ori), the early promoter and overlapping binding sites for transcriptional activators and repressors (Thierry, 2009). The viral genome is composed of eight open reading frames (ORF) which encode for proteins which can be categorised into early and late proteins according to their expression during the virus life cycle (Longworth & Laimins, 2004). The early region of the viral genome consists of genes designated E1-E7 which are responsible for transcription, viral genome replication and transformation of the host cell, whilst the late region contains the L1 and L2 genes which encode the structural capsid protein. The L1 protein is the primary structural element, with infectious virions containing 360 copies of the protein organised into 72 capsomeres (Modis *et al*., 2002).

1.4.1 Regulation of viral transcription during the HPV life cycle

Viral genes are transcribed from a single DNA strand into polycistronic mRNA from two main promoters: an early and late promoter, with usage dependent upon the differentiation state of the cell which divides the viral life cycle into early and late stages (Kalantari & Bernard, 2006). Aside from these two main promoters, further promoters are also likely to be utilised, and may play important roles in the virus life cycle (Ozbun & Myers, 1998; Milligan *et al.*, 2007). A process of alternative splicing mechanisms are known to regulate the combination of proteins expressed (Longworth & Laimins, 2004).

1.4.2 Regulation of transcription from the early promoter

During the early phase of the HPV life cycle the viral DNA is transcribed from the early promoter referred to as P_{97} in HPV type 16, P_{99} in HPV type 31 and P_{105} in HPV18 (Francis *et al.*, 2000; Wang *et al.*, 2011), lying immediately upstream of the E6 ORF within the LCR. Transcripts terminate at polyadenylation sites within E5 and L1 (Stubenrauch & Laimins, 1999). Polycistronic transcripts encoding the E1, E2, E6 and E7 proteins are derived from the early viral promoter with coding potential for the E5 and E1^E4 proteins (Figure 1.1). However these latter proteins are not detectable within undifferentiated cells and are likely to be expressed at low levels (Longworth & Laimins, 2004). HPV transcription is regulated by cis-responsive elements within the LCR and involves the E1 and E2 viral proteins in addition to a number of cellular factors (Longworth & Laimins, 2004). The LCR contains the binding sites for a number of cellular transcription factors including SP1 (Longworth & Laimins, 2004) (Figure 1.1). The E2 protein is well characterised as a viral transcription factor and has been shown to function as both a transcriptional activator and repressor dependent upon the levels of this protein within the cell (Bouvard *et al.*, 1994). The E2 protein has four binding sites (E2BS 1-4) which have a conserved arrangement between HPV types (Kalantari & Bernard, 2006). At low levels E2 binds only to the E2BS4, for which the protein has the

highest affinity and activates transcription (Steger & Corbach, 1997). However, as the levels of E2 accumulate within the cell as a result of enhanced transcription, the other E2BS become occupied by E2 such as E2BS1 and E2BS2 (Steger & Corbach, 1997). HPV transcription is therefore regulated by a negative feedback loop whereby levels of transcription are regulated by the abundance of E2 within the cell (Steger & Corbach, 1997). As such, regulation of HPV transcription from the early promoter acts to modulate expression of the early proteins.

1.4.3 Regulation of transcription from the late promoter

Within the stratum spinosum of the epithelium, viral transcription is induced from the late promoter sequence located within the E7 ORF, designated P_{742} in HPV31, P_{670} in HPV16 and P_{811} (Graham, 2006; Wang *et al*., 2011). Utilisation of this promoter is dependent upon differentiation-specific cellular factors thereby limiting the expression of late transcripts to the terminally differentiating layers of the epithelium (Longworth & Laimins, 2004). Late viral transcripts from the differentiation-dependent promoters of HPV16, 31 and 18 terminate at polyadenylation sites at the end of the L1 ORF and encode for the capsid proteins L1 and L2 in addition to the E1, E2, E1^E4 and E5 proteins (Graham, 2006; Wang *et al*., 2011).

Late gene expression is also regulated post-transcriptionally due to the presence of cis-acting RNA elements within late viral transcripts (Graham, 2006). A late regulatory element (LRE), overlapping with the 3' end of the L1 gene, represses late gene expression within undifferentiated cells, ensuring that the viral capsid proteins are not expressed until the cells reach the upper layers of the epithelium (Cumming *et al*., 2008).

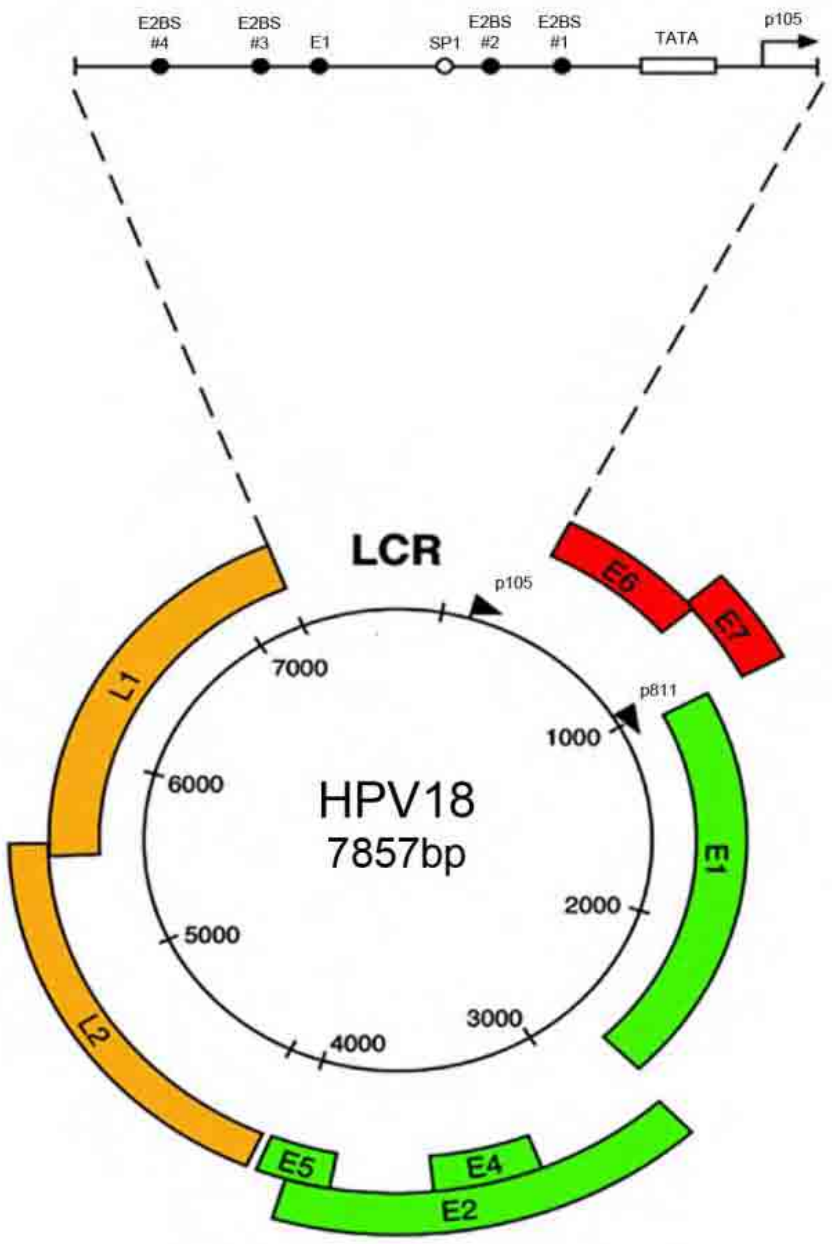

Figure 1.1 Genomic organisaton of HPV18. The genome can be divivded into three regions. The early region, late region and the long control region (LCR). The early region contains the open reading frames from E6,E7,E1,E2,E4 and E5 whilst the late region contains the L1 and L2 ORFs. The P^{105} early promoter lies upstream of the E6 ORF whillst the differntiation inducuble late promoter P^{811} lies within the E7 ORF. Adapted from Doorbar 2006.

1.5 Virus Life Cycle

The life cycle of HPV is closely linked to the differentiation status of the host epithelium. Infection by papillomaviruses requires that virus particles gain access to the epithelial basal layer and enter the dividing basal cells. HPV infects a primitive basal keratinocyte via microabrasion of the mucosal epithelium (Figure 1.2). Following infection, HPV genomes are established as extrachromosomal elements or episomes (Moody & Laimins, 2010). The receptor for entry of the virus into cells is currently unknown; however heparin sulphate has been shown to mediate the initial attachment of virions to cells (Shafti-Kermat *et al*., 2003). It is thought that α-6 integrin may play a secondary role in the attachment and efficient entry of this virus into the cell (McMillan *et al*., 1999). Other candidate receptors for HPV have been suggested such as laminin-5 which is able to mediate binding to the extracellular matrix (Culp *et al*., 2006).

Following attachment, the virus is internalised. The mode of internalisation is not conserved among HPV types with HPV16 infecting cells through clathrin-dependent endocytosis (Day *et al*., 2003) and HPV31 by caveolae (Bousarghin *et al*., 2003). Following internalisation, the viral capsid disassembles within the endosome compartment and N-terminal cleavage of L2 by furin-a proprotein convertase, necessary for translocation of the capsid protein into the cytoplasm (Richards *et al*., 2006). L2 mediates relocation of the viral genome from the endosomal compartment into the nucleus, where the L2-chaperoned viral genome accumulates at ND10 bodies (Day *et al*., 2004).

Replication of HPV DNA requires the E1 and E2 replication proteins, however due to the limited coding capacity of the HPV genome, the virus is also dependent upon the host cellular replication machinery for viral replication (Chow & Broker, 2006). During early stages of the virus life cycle transcription from the early promoter results in expression of the early proteins: E6, E7, E1 and E2 (Longworth & Laimins, 2004). The E2 protein plays several

roles during productive infection and in basal cells; expression of E2 is required for the initiation of viral DNA replication and genome segregation. HPVs do not encode polymerases or any other enzymes necessary for viral replication, therefore in addition to the E1 and E2 proteins, the virus must also utilise the host cell replication proteins to mediate viral DNA synthesis (Chow & Broker, 2006). The viral genomes are replicated in synchrony with cellular DNA replication. The E2 protein is responsible for anchoring the viral proteins to mitotic chromosomes through the Brd4 protein (You *et al*., 2004) or directly attaching to repetitive ribosomal sequences within the viral DNA as is in the case of HPV8 (Poddar *et al*., 2009). It is speculated that the immediate early events of virus growth involve an amplification of virus copy number from 1-10 copies/cell to 50-100 episomes/cell. The next phase of virus growth is one of plasmid maintenance in which the virus and the cell replicate in tandem and there is no amplification of virus copy number. This occurs in the suprabasal layers of the stratified epithelium.

The infected cell then enters the differentiating compartment of the epithelium (Figure 1.2). HPV replication occurs in cells which have committed to terminal differentiation and have exited from the cell cycle. To overcome this, the proliferative capacity of these HPV-infected cells is uncoupled from differentiation and is controlled by a number of cellular factors, including members of the Retinoblastoma (Rb) family, consisting of p105, p107 and p130 (Dyson *et al*., 1989). The inactivation of these targets results in the release and activation of E2F transcription factors that drive expression of S phase genes. Different HPV types have been shown to display varying degrees of affinity for pRB and a correlation with oncogenic potential has been proposed, with high risk E7 proteins possessing a significantly greater affinity for pRB than E7 proteins of low risk types (Munger *et al*., 1989). Whilst E7 proteins of low risk HPV types are able to associate with pRB, this interaction is not sufficient to enable activation of E2F responsive genes (Longworth & Laimins, 2004).As a result of

unscheduled DNA replication cellular apoptosis is triggered within these cells, which is counteracted by the E6 oncoprotein through degradation of the host tumour suppressor protein p53, preventing cell growth inhibition in undifferentiated and differentiated cells (Scheffner *et al*., 1990). The high-risk E5 protein cooperates with E6 and E7 to promote hyperproliferation of infected cells and is likely to facilitate malignant progression (DiMaio & Mattoon, 2001).

The productive phase of the virus life cycle is dependent upon cellular differentiation, commencing within differentiating cells of the epithelium. For high risk HPV types including 16 this occurs within the spinous layers of the epithelium (Peh *et al*., 2004; Breitburd *et al*., 1987). As previously mentioned, during the productive phase of the virus life cycle transcription occurs from the differentiation-dependent late promoter-P_{811} located within the E7 ORF (Wang *et al*., 2011). This promoter drives the expression of E1, E2, E4 and E5 proteins as well as the late proteins L1 and L2 (Wang *et al*., 2011). Virus gene expression is markedly upregulated in these upper layers, especially the E4 protein, which is expressed abundantly in replication competent suprabasal cells. Viral DNA amplification generates thousands of viral genomes (Cheng *et al*., 2005). Recent work from the Chow lab has suggested that HPV is capable of amplifying its genome in the G_2M phase of the cell cycle since viral DNA was able to initiate amplification in G_2 arrested cells (Wang *et al*., 2009). Moreover, they suggest that E7 alone is sufficient to induce this G2 arrest required for viral DNA amplification, following S-phase re-entry, by inducing the inhibitory phosphorylation of cdc2 and cdc25 (Banerjee *et al*., 2011), a method shared by other viruses including the vpr protein of HIV type 1 and the p30 protein of HTLV-1 (Datta *et al*., 2007; Kino *et al*., 2005).

The late phase L1 and L2 proteins encapsulate newly synthesised viral genome which is then followed by the shedding of the virus from the uppermost layers of the epithelium via the natural sloughing of the skin (Figure 1.2).

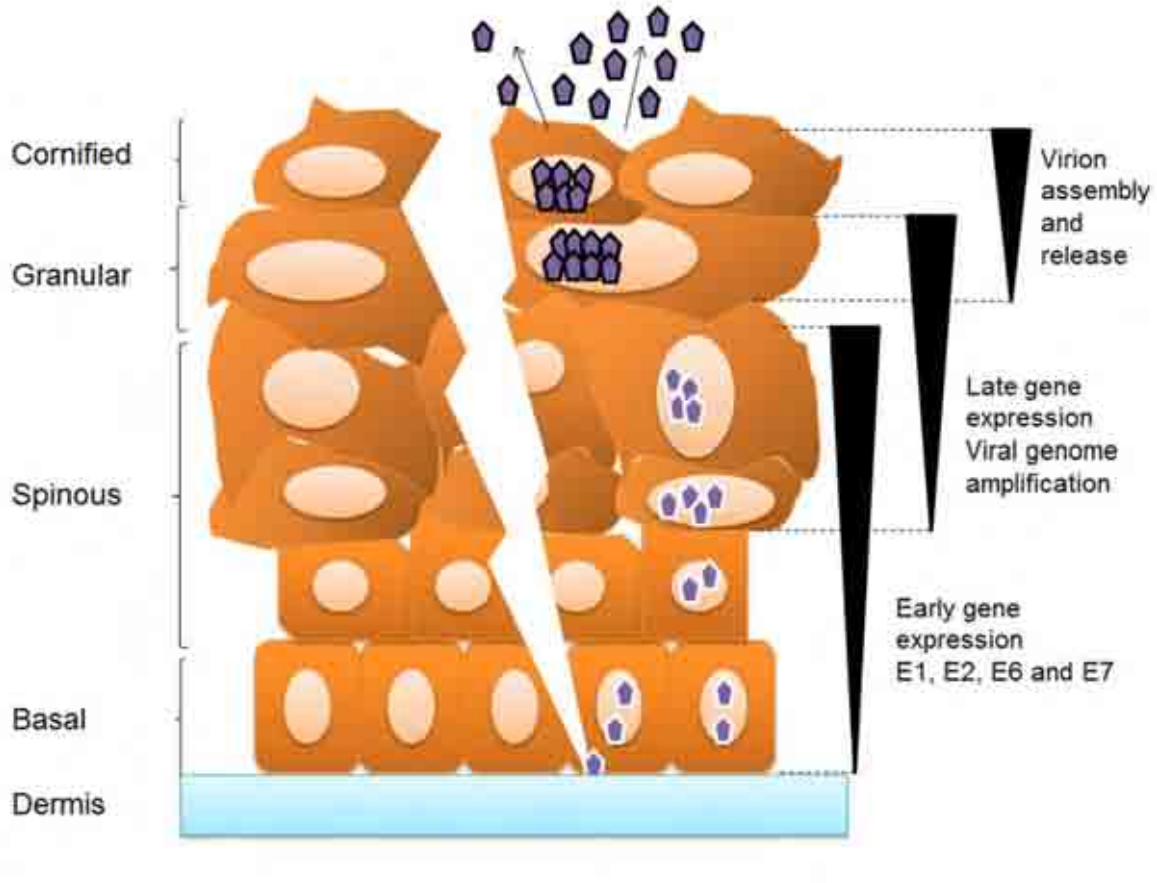

Figure 1.2 The HPV life cycle. Key stages of the virus life cycle are shown in relation to what stage of keratinocyte differentiation they occur. The virus enters through micro abrasions in the surface of the skin and infects the basal layer of the epithelium. Genomes are established in the basal cell layer with a copy number of around 50 genomes per cell. After cell division, one daughter cell migrates from the basal cell layer and undergoes differentiation. Differentiation within the spinous layers of the cell leads to the production of thousands of copies of the genome per cell. The virus is packages by the L1 and L2 capsid proteins and shed from the upper most layers of the epithelium.

1.6 HPV Proteins

1.6.1 E1 and E2 proteins

Among the first of the viral proteins to be expressed are the replication factors E1 and E2. Both E1 and E2 have been shown to be important for the replication of a number of HPV types (Ustav & Stenlund, 1991). These proteins are thought to form a complex which binds to sequences at the viral origin of replication and recruits polymerases and proteins to mediate replication (Conger *et al*., 1999). As well as binding to specific sequences at the origin of replication, both E1 and E2 proteins bind to each other (Frattini & Laimins, 2004a; Sun *et al*., 1996).

E1 is highly conserved amongst papillomaviruses and has been shown to play an important role in papillomavirus replication as an origin (ori) recognition protein initiator (Wilson *et al*., 2002; Chow & Broker, 2006). E1 has a weak affinity for a consensus motif (AACNAT) repeated six times within the viral origin of replication (Chen & Stenlund, 2001). The E1 protein contains two regions of DNA binding; a DNA binding domain (DBD) and non specific binding of the helicase domain (Stenlund, 2003).

The E1 protein also exhibits helicase activity, permitting the separation of the viral DNA strands ahead of the replication complex (Hughes & Romanos, 1993). In addition, it recruits a number of cellular replication proteins including the DNA polymerase α-primase (Park *et al*., 1994) and the replication protein A (RPA) which prevents the reassociation of dsDNA by binding to ssDNA (Loo & Melendy, 2004). It is also able to associate with a number of cellular Hsps (heat-shock proteins) in particular Hsp40 and Hsp70 and this contributes to the formation of E1 di-hexamers (Lee *et al*., 1999).

The E2 protein, of 40-45 kDa, is integral to the regulation of HPV replication and transcription (Morgan & Donaldson, 2006). E2 is the primary ori binding HPV protein and consists of three main domains: an N-terminal transactivation (TA) domain of approximately

200 amino acids., a central flexible hinge region and a C-terminal dimerisation and DNA binding domain (DBD) approximately 90 amino acids (Chow & Broker, 2006). The hinge region of E2 overlaps with the E4 ORF and is a highly divergent region of the protein, with variable sequence and length between E2 proteins of different HPV types, and is thought to provide a flexible linker between the two other domains. E2 is a site specific DNA binding protein that helps to recruit E1 to the origin but also plays a role in regulating viral transcription from the early promoter. Binding sites for E2 are located adjacent to sites for cellular transcription factors that activate the early promoter (Stubenrauch *et al*., 1998). Within dividing basal cells, E2 protein acts to ensure equal segregation of the viral genomes by tethering the viral genomes to the mitotic spindle during cell division (Sekhar *et al*., 2010). At the basal layer of the epithelium, cell division results in one daughter cell remaining in the basal layer and one daughter cell migrating up to the suprabasal layers to begin the differentiating process. E2 ensures that viral episomes are distributed into both new daughter cells and therefore maintains a source of HPV DNA for persistent infection. To achieve this, E2 either interacts directly with the chromatin or indirectly through cellular factors that tether the viral genome to the cellular chromatin (Skiadopoulos & McBride, 1998; Zheng *et al*., 2005).

During a natural infection, E1 is expressed at low levels and as mentioned previously, requires the presence of E2 in order to be efficiently targeted to its binding sites. E2 associates with E1 primarily through its N-terminus and binds to DNA as a dimer through its C terminus (Moscufo *et al*., 1999). The initial E1:E2 origin complex is thought to be inactive for replication initiation and is proposed to serve as a template for formation of an active replication complex. Whilst E1 and E2 proteins are required for HPV replication, only the E1 protein is required for elongation (Chow & Broker, 2006).

The episomal replication of HPV in keratinocytes has been shown to be controlled by the levels of E1 and E2 proteins, since increasing the expression of either results in an increase in the copy number of HPV genome per cell (Frattini & Laimins, 1994b). The link between replication and transcription provides a mechanism by which the virus can limit the timing and duration of genome amplification. This suggests that HPV transcription is therefore regulated by a negative feedback loop whereby levels of transcription are regulated by the abundance of E2 within the cell (Steger & Corbach, 1997). The E2 protein has also been shown to regulate the viral early promoter- p97 in HPV16, p105 in HPV18 and p99 in HPV31, with high levels of E2 acting to downregulate the expression of E6 and E7 in experimental systems (Hines *et al*., 1998). E2 binds to the papillomavirus oncoproteins E6 and E7, leading to the modulation of their functions (Gammoh *et al*., 2006; Grm *et al*., 2005) In BPV, E1 negatively regulates the activation of the major early promoter by E2 (Le Moal *et al*., 1994) and this mechanism may be responsible for the suppression of transformation by E1 (Schiller *et al*., 1989). High-risk, but not low-risk HPV E2 proteins can induce themselves growth arrest and apoptotic cell death in several HPV-negative carcinoma cell lines. Apoptosis can occur via a p53-dependent as well as independent pathways (Demeret *et al*., 2003; Parish *et al*., 2006). E2 binds to the cellular protein p53. Expression of p53 can inhibit papillomavirus DNA replication and alter the transcriptional activity of E2 (Frattini *et al*., 1997; Lepik *et al*., 1998)

Cells expressing E1 in the absence of other viral gene products exhibit perturbations of the cell cycle, resulting in decreased duration of the G1 phase and increased S and G2 phase durations (Belyavskyi *et al*., 1996). These cell cycle effects are coincident with changes in histone H1 kinase activity and the abundance and timing of appearance of cyclin D1 (Belyavskyi *et al*., 1996). Thus, in addition to the viral oncoproteins, E1 may also participate

directly in altering the host cell growth program which is an essential component of the papillomavirus life cycle.

The fidelity of cellular replication is controlled by a number of pathways which block the propagation of damaged DNA (Sancer *et al*., 2004; Harper & Elledge, 2007). These are controlled by the ATM (ataxia-telangiectasia mutated) and the ATR (ATM and Rad3-related) kinases (Harper & Elledge, 2007). ATM mediates the cellular response to double strand breaks (Lee & Paull, 2007) whilst ATR controls the response to UV damage as well as stalled replication forks (Cimprich & Cortez, 2008). Both kinases control the G_1, S and G_2 cell cycle checkpoints necessary for viral replication upon differentiation. Recent work from the McBride lab has shown that E1 and E2 are able to activate the DNA damage response pathways by phosphorylating a number of proteins of the pathway including Chk2 and H2AX (Sakakibara *et al*., 2011). This work substantiates an earlier study by Moody and colleagues who showed that HPV31 genomes were able to activate the ATM DNA damage response pathways by phosphoryltaing CHK2, BRCA1 and NBS1. Furthermore, activation of CHK2 was required to induce caspase 3 and caspase 7 activation, required for viral genome amplification (Moody & Laimins, 2009). More recent studies by Fradet-Turcotte and colleagues have shown that nuclear export of E1 prevents S phase arrest and activation of a DNA damage response. Moreover, complex formation of HPV E1 with E2 was associated with the prevention of undifferentiated cells from undergoing a DNA damage response (Fradet-Turcotte *et al*., 2011).

1.6.2 L1 and L2 Proteins

The HPV genome is surrounded by an icosahedral capsid-55nm in diameter and composed of two structural proteins, the major (L1) and minor (L2) capsid proteins respectively. L1 proteins are highly conserved to form 72 five fold capsomers with each composed of five L1 major proteins, with a hole in the centre, where L2 is thought to associate (Holmgreen *et al*., 2005). It is believed that L2 localises with L1 through a hydrophobic region near the C-terminus of the protein (Finnen *et al*., 2003).

Although L1 can spontaneously self-assemble to form an icosahedral VLP, L2 plays an integral role in assembly of virions and is required for efficient encapsidation of viral DNA (Holmgreen *et al*., 2005). The assembly of infectious virions in the upper epithelial layers is thought to require the E2 protein in addition to the capsid proteins L1 and L2 (Day *et al*., 1998) and it has been suggested that E2 may improve the efficiency of viral encapsidation. Activation of the differentiation dependent late promoter results in production of viral transcripts from which late proteins L1 and L2 are transcribed. Capsid proteins accumulate after the onset of genome amplification with L2 expression preceding the expression of L1 (Florin *et al*., 2002).

Capsids have evolved to fulfill a number of roles, critical to the establishment of infection. For non enveloped viruses such as HPV, the capsid proteins act to coat the nucleic acid and also provide the initial interaction site of the viral particle with the host cell (Horvath *et al*., 2010). The capsid proteins relocate from the cytoplasm to the nucleus and assemble into icosahedral capsids in which the viral genomes are packaged (Conway & Myers, 2009). Further studies have identified roles for the capsid proteins in the endosomal escape of virions (Campos & Ozbun, 2009) and the nuclear transport of the HPV genome (Campos & Ozbun, 2009; Florin *et al*., 2006).

The L1 and L2 capsid proteins are also implicated in the process of cellular entry, uncoating and delivery of viral genomes to the nucleus. Although cell surface interactions predominantly depend on the major capsid protein L1, it seems likely that the secondary cell surface receptor is L1-specific, although it is possible that L2 may contribute to surface interactions (Sapp & Bienkowska-Hoba, 2009).

L2 localises to the nucleus via a nuclear localisation signal, situated at its N and C termini, and once there it associates with promyelocytic leukaemia (PML) bodies (Florin *et al*., 2004). The PML is the structural component of ND10 bodies. ND10 bodies are distinct subnuclear structures which have been proposed as transient deposition sites for nuclear proteins as well as having implications in the control of transcription (Doucas, 2000), cell growth and apoptosis (Gottifredi & Prives, 2001). Moreover, ND10 bodies have been proposed as sites of papillomavirus replication (Swindle *et al*., 1999). Studies have shown that the HPV E1 and E2 proteins associate and accumulate in these ND10 bodies (Swindle *et al*., 1999) as well as the L2 protein (Day *et al*., 1998). The L2 association is thought to result in ND10 body reorganisation, followed by the recruitment of preformed L1 capsomeres and the assembly of L1 and L2 protein into an icosahedral virion (Florin *et al*., 2004). L2 is also required for efficient packaging of the genome (Stauffer *et al*., 1998) and enhancing virus infectivity (Roden *et al*., 2001) as loss of L2 in the context of HPV31 resulted in a 100-fold reduction in the packaging efficiency and virus infectivity compared with wild type virus (Holmgreen *et al*., 2005).

1.6.3 E5 Protein

E5 is a hydrophobic membrane-bound protein, approximately 83 amino acids in length, which associates with the Golgi apparatus, endoplasmic reticulum and perinuclear membrane. Although the definitive functions of this protein remain unresolved, accumulating evidences have suggested that E5 oncoprotein may also contribute to cervical carcinogenesis through modulating cellular signalling pathways in addition to augmenting the immortalization potential of E6 and E7.

The HPV E5 proteins have little homology to the BPV counterparts; however their mode of action is similar. In BPVs, E5 encodes the primary transforming activity and acts by associating with the platelet derived growth factor (PDGF) receptor (Schneider-Gaadickle & Schwarz, 1986). In addition, overexpression of the HPV E5 protein increases the phosphorylation of the epidermal growth factor, EGF receptor as well as inhibiting its degradation, suggesting that HPV E5 action involves binding to the epidermal growth factor (EGF) and that this binding is similar to that seen by BPV and the PDGF (Conrad *et al*., 1993; Rodriguez *et al*., 2000). In addition, E5 expression results in increased EGFR signalling and activation of the MAP kinase (MAPK) pathway, which augments the activities of E6 and E7, resulting in uncontrolled proliferation (Crusis *et al*., 1997). E5 also contributes to the actions of E6 and E7 by modulating the transit of signalling proteins through the endoplasmic reticulum (ER) as well as interacting with factors such as the B cell receptor-associated protein 31 (BAP31) and the vacuolar H^+ -ATPase in endosomes (Rodriguez *et al*., 2000; Regan & Laimins 2008).

Knock out studies revealed that loss of E5 within the context of the complete HPV31 genome resulted in impaired activation of the late viral protein functions in differentiating cells, suggesting that its primary activity is in differentiating cells (Fehrmann *et al*., 2003). A similar E5 knock out study in HPV16 NIKS cells suggested that E5 may have a subtle role in

the productive stages of the virus life (Genther *et al*., 2003). Further evidence to support E5 as a potential oncogene comes from transgenic mouse studies. Data from these studies indicate that high level expression of HPV16 E5 in the skin induces epithelial hyperproliferation that results in tumour formation (Maufort *et al*., 2007). Furthermore, in oestrogen-treated mice, expression of E5 alone can induce cervical cancers (Maufort *et al*., 2010).

Moreover, it has been suggested that E5 may play a role in viral immune evasion by down regulating the major histocompatibility complex (MHC) and preventing the transport of HLA class 1 molecules to the cell surface, by retaining the complex in the Golgi apparatus, preventing clearance of the infected cells by the immune response (Ashrafi *et al*., 2006).

Multiple mechanisms, including activation of EGFR or inflammatory cell signaling pathways and immune regulation have been implicated in malignant transformation of HPV by E5. Therefore, targeting E5 may be a rational approach for chemoprevention and treatment of cervical cancer, and understanding its oncogenic processes may help the design of novel therapeutic strategies.

1.6.4 E4 Protein

E4 is the most highly expressed of all the HPV proteins. The E4 open reading frame (ORF) is translated from spliced transcripts as a fusion with the first 5 amino acids of E1 to generate E1^E4 fusion proteins (Chow *et al*., 1987). Even though the E4 ORF lies in the early region of the genome, E4 is known to accumulate in the cell at the time of viral genome amplification and its loss has been shown to disrupt late events in a number of experimental systems (Wilson *et al*., 2005; Nakahara *et al*., 2005; Wilson *et al*., 2007; Peh *et al*., 2004).

E1^E4 is first expressed within the upper layers of the epithelium, with expression coincident with the onset of vegetative viral genome amplification, suggesting a role for this protein in initiation of the productive phase of the HPV life cycle (Peh *et al*., 2002). The continued

presence of E1^E4 in cells within the uppermost layers of the epithelium in which virion assembly occurs, also implies a function for this protein during the later stages of virus production and egress (Peh *et al*., 2002). Although the exact function E1^E4 is still to be elucidated, there is growing evidence to suggest that this protein may have multiple roles at different stages of the virus life cycle; with E4 function being regulated through a combination of proteolytic cleavage and phosphorylation. This protein is thought to be involved in a number of cellular processes including ND10 reorganisation, G_2 arrest and chromosomal re-replication, keratin association and late gene expression.

A sequence alignment of E1^E4 proteins reveals little sequence homology between HPV types, and variation in the size of E1^E4 proteins. The E4 ORF lies within the hinge region of the E2 ORF, which has been shown to link the two major functional domains of the E2 protein and is an extremely divergent region of this protein (Zhu, 1999). Whilst there are considerable differences between E1^E4 proteins of different HPV types, particularly within the central region of the protein, certain domains show a greater degree of conservation. Sequences within the amino and carboxy termini of E1^E4 are important in mediating biological and biophysical characteristics of this protein. The N terminus of E1^E4 proteins contain a (LLXLL) sequence motif, which has been shown to be required for the association between E4 and the keratin cytoskeleton (Roberts *et al*., 1994) and also binding to mitochondria (Raj *et al*., 2004) (Figure 1.3). E1^E4 proteins from a number of different subtypes have been shown to associate with these keratin filaments. The function of the keratin filaments is to maintain cell structure and protect the epithelial tissues from stress. Whilst E1^E4 proteins from high risk, low-risk and benign viruses are able to co-localise with these filaments, not all induce their collapse in transient transfection assays (Doorbar *et al*., 1991). The association perhaps permits the egress and escape of virus particles during the later stages of the virus life cycle (Doorbar *et al*., 1991). More recent studies have shown that

the cellular cysteine protease calpain is capable of cleaving the 16E1^E4 protein to generate species that lack the N terminus (Khan *et al*., 2011). These C-terminal fragments are able to multimerize and form amyloid-like fibers. This can lead to accumulation of 16E1^E4 and disruption of the normal dynamics of the keratin networks (Khan *et al*., 2011).

The E1^E4 protein also contains a number of other biologically functional regions such as the proline rich region near the C terminus which has been linked to the ability of E1^E4 to interfere with G2-M progression of the cell cycle (Davy *et al*., 2002; Knight *et al*., 2004) (Figure 1.3). Moreover, sequences at the C terminus of E1^E4 also mediate interactions between itself (Wang *et al*., 2004; Roberts *et al*., 1997) and a cellular RNA helicase (Doorbar *et al*., 2000) (Figure 1.3).

1.6.4.1 E4 and the cell cycle

Several studies have now found that E1^E4 expression in cultured cells can influence the cell cycle. The G2/M checkpoint of the cell cycle ensures that the DNA is replicated correctly, prior to entry into mitosis. This prevents the proliferation of damaged cells. The ability of E1^E4 to cause cell cycle arrest in G_2 and to antagonise E7-mediated cell proliferation is a common feature of E1^E4 proteins from a number of HPV types including HPV1, 11, 16 and 18 (Knight *et al*., 2004; Davy *et al*., 2002; Nakahara *et al*., 2002).

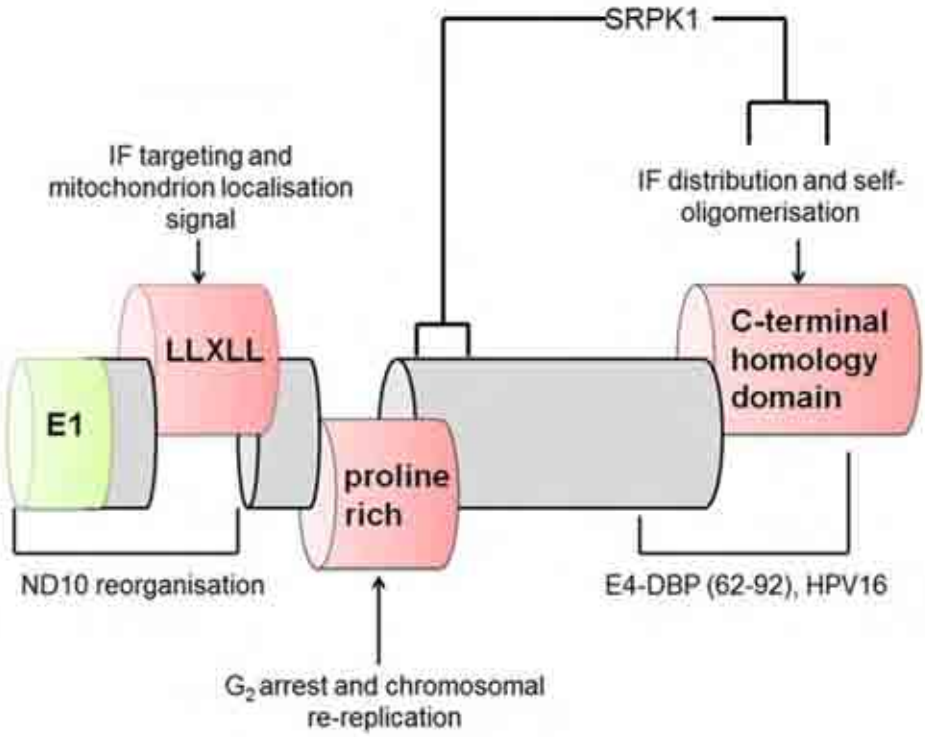

Figure 1.3 The E4 protein. A schematic representation of the E1^E4 protein in which the known biological functional regions are highlighted as well its interaction with cellular targets. Adapted from Roberts, 2006.

Several studies have identified that different E1^E4 proteins are able to target different mechanisms of the G2/M transition to cause cell cycle arrest (Knight *et al*., 2006; Davy *et al*., 2006). Interestingly, the G_2 arrest function of HPV1 E1^E4 is mediated by a truncated 16 kDa E1^E4 polypeptide species, lacking the extreme N terminus of the E1^E4 protein and not by the full length E1^E4 protein (Knight *et al*., 2004). Moreover, coexpression of the full length and truncated E1^E4 isoforms have a far greater deleterious effect on keratinocyte cell growth than the individual polypeptides themselves (Knight *et al*., 2004).

In addition to the ability of E1^E4 to induce a G2 arrest, the viral protein has also been shown to inhibit entry into S phase. Co-expression of both the full length HPV1 E1^E4 protein and an N-terminally truncated E1^E4 species within keratinocytes prevented entry of these cells into S phase (Knight *et al*., 2004). It is not clear what role E1^E4 induced cell cycle arrest plays in the productive life cycle, however it is believed to counteract the effects of E7 which acts to push these cells into S phase (Flores *et al*., 2000). Furthermore, the HPV1 E1^E4 protein has been shown to inhibit cellular DNA replication by blocking the recruitment of cellular licensing factors onto chromatin (Roberts *et al*., 2008). It is believed that this is a mechanism by which the virus produces an environment which is suitable for viral DNA replication without competition from the host.

1.6.4.2 E4 and Late Gene Expression

A number of studies have identified E1^E4 actions that might indicate a role for E1^E4 in regulating gene expression during the later stages of the virus life cycle. Full length HPV16 E1^E4 has been shown to bind via its C-terminus to a RNA helicase, E4-DEAD-box protein (E4-DBP) (Doorbar *et al*, 2000) (Figure 1.3). This interaction between E4-DBP and E1^E4 has been shown to inhibit the RNA-independent ATPase activity of E4-DBP activity. Moreover, the E1^E4 protein of both high risk and low risk types (HPV16, HPV18 and HPV1) have been shown to associate with the serine-argenine (SR)-specific protein kinase

(SRPK1) (Bell *et al*., 2007) (Figure 1.3). In the context of HPV1, this interaction results in a sequestration of E1^E4 to inclusion bodies with subsequent phosphorylation of the E1^E4 protein. To date, phosphorylation of the E1^E4 protein of high risk types has not yet been observed. SRPK1 is known to phosphorylate SR proteins, many of which have an intimate role in regulating RNA splicing and it is speculated that association of SRPK1 with E1^E4 may represent a mechanism of regulating viral late gene expression (Bell *et al*., 2007).

1.6.4.3 E4 and the virus life cycle

Whilst E1^E4 proteins are not detected within undifferentiated cells, there is evidence to suggest that the E1^E4 protein contributes to the early stages of the virus life cycle. A study by Wilson and colleagues showed that HFK cell lines containing HPV18 genomes exhibited slower growth rates than an E1^E4 deletion mutant, suggesting that E1^E4 may have a negative effect on cellular proliferation (Wilson *et al*., 2007). E1^E4 has also been shown to be important for the replication of viral genomes during the early stages of the virus life cycle. In an HPV16 background, the ability of viral genomes to replicate following transfection into immortalised keratinocyte NIKS cells was investigated. An E1^E4 mutant, expressing only the first 9 aa of the protein affected the ability of the virus to replicate extrachromosomally, suggesting that the E1^E4 protein may play an important role during viral genome replication (Nakahara *et al*., 2005). Moreover, a leucine rich motif within HPV16 E1^E4 was shown to be important for this replication (Nakahara *et al*., 2005). This function of E1^E4 appears to be subtype specific, as the same effect was not seen in E1^E4 proteins of other HPV subtypes including HPV11, HPV18 and HPV31 (Wilson *et al*., 2005; Fang *et al*., 2006; Wilson *et al*., 2007; Nakahara *et al*., 2005).

The E1^E4 protein of HPV16, HPV18 and HPV31 have also been shown to be involved in the late stages of the virus life cycle, including in the differentiation dependent amplification of viral genomes. E1^E4 proteins of HPV18 and HPV31 in which the E4 ORF had been

severely truncated had a reduced ability to undergo viral genome amplification upon differentiation (Wilson *et al*., 2005; Wilson *et al*., 2007). Whilst suprabasal DNA synthesis was reduced within HPV31 E1^E4 mutant organotypic raft cultures (Wilson *et al*., 2005), consistent with observations seen in HPV16 (Nakahara *et al*., 2005) this function was not shown in HPV18, indicating that this functions may differ between different HPV types (Wilson *et al*., 2007). As previously discussed, the G_2 arrest function of HPV E1^E4 is a conserved feature among different HPV types (Knight *et al*., 2006; Davy *et al*., 2006). A recent analysis of the contribution of this function to viral DNA amplification showed that it is not required for viral genome amplification or capsid protein induction (Kinght *et al*., 2011).

1.7 E6 and E7 Proteins

1.7.1 Transforming abilities of E6 and E7

Similar to the oncoproteins of other DNA viruses, the E6 and E7 proteins of HPV govern the rate of cell division as well as cell cycle and differentiation patterns. As previously mentioned, during epithelial differentiation the P_{97} of HPV16 and the P_{105} of HPV18 directs the expression of E6 and E7 genes necessary for S phase entry (Francis *et al*., 2000; Wang *et al*., 2011). Further expression of E6 is achieved through splice donor sites in some HPV types, which give rise to truncated forms of E6, named E6* (Shirasawa *et al*., 1994). While some papillomavirus types, such as HPV16, seem to have splicing patterns that allow the expression of up to four E6* species, dependent upon the position of the downstream splice acceptors, HPV18 appears to transcribe only one mRNA species that is capable of expressing E6* (Pim *et al*., 2009). The polypeptide product of this transcript shares the first 44 amino acids with full-length E6 before the first splice donor site; thereafter, it has 13 unique amino acids that are derived from E6 intronic sequences (Pim *et al*., 2009).

The primary transforming activity of high risk HPVs is provided by the E6 and E7 oncoproteins. The HPV E7 gene encodes for the major transforming function of high risk HPV types and expression of E7 is necessary and sufficient for immortalization of human keratinocytes whilst E6 contributes towards immortalization within these cells. These two proteins act together in the development of HPV-induced cancers, with the action of one protein complementing that of the other.

The expression of high risk E7 proteins by themselves can immortalise human keratinocytes at a low frequency, but E6 has no such activity (Tommasino & Crawford, 1995). The combination of both E6 and E7 proteins are highly efficient at immortalising most types of primary cells (Hawley-Nelson *et al*., 1989; Munger *et al*., 1989). Immortalised keratinocytes are not fully transformed and conversion to malignancy is dependent upon factors in addition to HPV. Co-transfection assays of primary baby rat kidney epithelial cells with HPV DNA with the activated *ras* oncogene demonstrated that high risk HPV types but not low risk types are able to cooperate with *ras* to transform primary cells (Storey *et al*., 1988). In addition, transgenic mice that express high risk E6 and E7 in epithelial cells develop squamous carcinomas when treated with low doses of oestrogen (Arbeit *et al*., 1996). In this mouse model, E7 alone is sufficient to induce high grade cervical dysplasia and invasive cervical malignancies. The addition of E6 results in larger and more extensive cervical cancers, indicating the cooperative activity of the E6 and E7 proteins in promoting tumourigenesis (Riley *et al*., 2003). Furthermore, the growth of keratinocytes expressing both E6 and E7 in organotypic raft cultures results in changes similar to those seen in high-grade squamous intraepithelial lesion *in vivo* (McCance *et al*., 1988) which further supports this argument.

In the productive phase of the HPV life cycle, E6 and E7 promote the proliferation of undifferentiated and differentiated suprabasal cells as well as protect the cells from apoptosis. This leads to the accumulation of DNA damage and mutations that can result in

transformation and the development of cancers. To understand how the cooperative actions of these proteins lead to cancer, it is useful to examine the multiple pathways targeted by these proteins.

1.7.2 E7 Protein

E7 is an 18 kDa nuclear phosphoprotein of approximately 100 aa in length, with functional similarities to the E1A adenovirus protein and the SV40 large T antigen (TAg) (Howley & Lowry, 2009). The E7 gene product from the high risk HPV types functions to deregulate normal cell cycle controls and increase DNA synthesis without affecting the differentiation programme of the host keratinocyte. It achieves this by interacting with a variety of cell regulatory proteins.

1.7.2.1 E7 and Rb

E7 proteins do not possess any intrinsic enzymatic or DNA-binding activities but function by binding to several cellular factors. The best characterised of these interactions is with the Rb tumour suppressor and also with the related family members p107 and p130. E7 interacts with Rb family members through a conserved LXCXE motif that is present in its amino-terminus of high risk E7 proteins (Munger *et al*., 1989). As previously mentioned, the Rb family of "pocket" proteins includes the prototypical Rb as well as p107 and p130, and these proteins are differentially expressed throughout the cell cycle (Berezutskaya *et al*., 1997; Classon & Dyson, 2001). Whilst Rb is constitutively expressed throughout the cell cycle, p107 is expressed predominantly during S phase, and p130 predominates at G_0 (Classon & Dyson, 2001). The Rb family of proteins controls the G1-S transition by regulating the activity of the E2F family of transcription factors (Dyson, 1998). The E2F family is a group of transcription factors of which there are at least 8 family members. Some of the family members act as transcriptional activators, whilst others act as transcriptional repressors. E2F-

E2F #1-5 has binding domains for pocket proteins whilst E2F #6-8 lack these residues and therefore regulate gene expression independently of Rb family members (Lammens *et al*., 2009). Binding of Rb occurs through one of three conserved regions contained on the E7 protein (termed CR1-CR3) (Lammens *et al*., 2009).

Regulation of the G1/S checkpoint ensures that environmental conditions are favourable and that the cellular DNA is undamaged prior to commitment of the cell to DNA replication. Stimulation of cells to divide by extracellular signals induces transcription of cyclin D which forms a complex with the cyclin dependent kinases (CDK) -4 or -6 catalytic subunits which then phosphorylate downstream target proteins of the retinoblastoma pathway (Sherr & McCromick, 2002). During G_1-S progression, these cyclin kinase complexes hypo-phosphorylate Rb, resulting in the release of pRb from E2F transcription complexes and the transcription of genes involved in DNA synthesis such as cyclin A and cyclin E, promoting early S phase entry and DNA synthesis (Zerfass *et al*.. 1995) (Figure 1.4). In addition, high risk E7 is able to bind to another E2F family group member-E2F1 and induce E2F1 driven transcription (Hwang *et al*., 2002). Thus E7 is able to activate the host cells DNA replication machinery for the purposes of viral DNA replication. Different HPV types have been shown to display varying affinities for pRb. High risk E7 proteins possess significantly greater affinity for pRb than low risk types. Whilst E7 proteins of low risk HPV types are able to associate with pRB, this interaction is not sufficient to enable activation of E2F responsive genes (Longworth & Laimins, 2004). These differences correlate with the oncogenic potential of these different HPV E7 subtypes (Munger *et al*., 1989).

In addition to binding pRb, E7 mediates its degradation through the ubiquitin proteasome pathway, which is dependent upon the presence of calpain (Boyer *et al*., 1996; Jones *et al*., 1997). E7 is thought to associate with a cullin-2 ubiquitin ligase complex, which contributes to the degradation of the Rb protein (Huh *et al*., 2007).

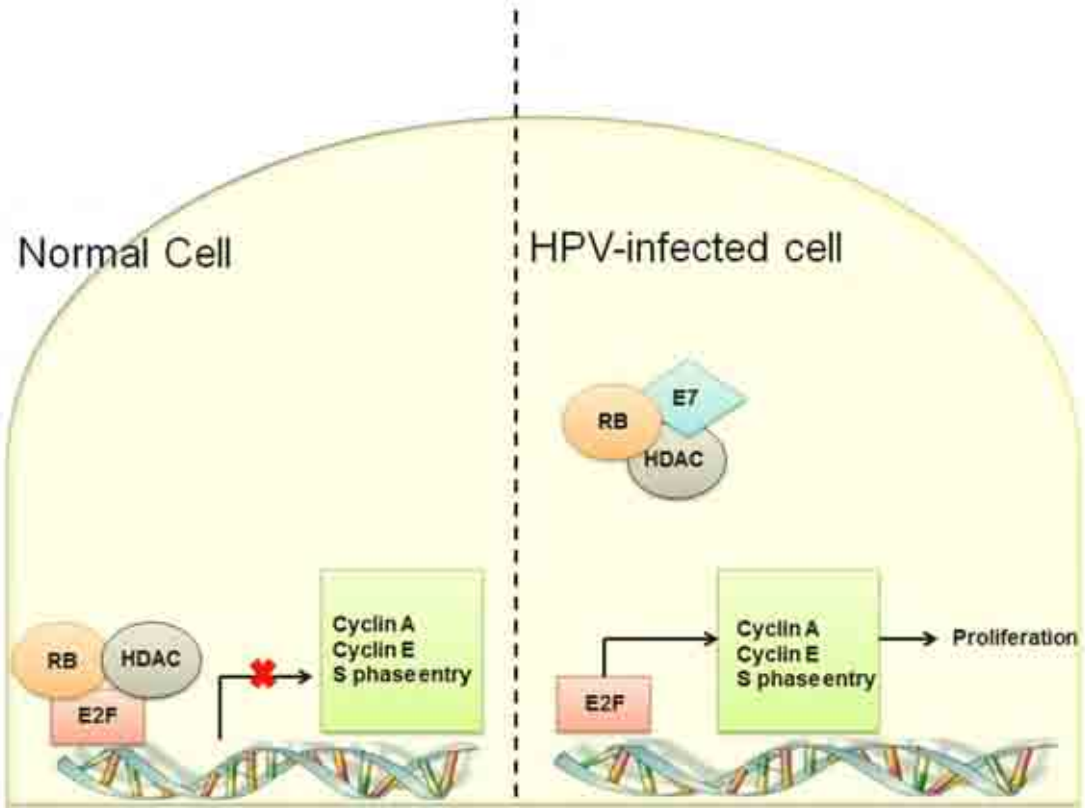

Figure 1.4 The human papillomavirus E7 Rb pathway. High risk human papillomaviruses E7 proteins induce proliferation in the cell through inhibition of Rb as well as constitutive activation of E2-F responsive genes. E7 also affects cellular gene expression through an interaction with histone deacetylase (HDAC's). The inactivation of these targets results in the release and activation of E2F transcription factors that drive expression of S phase genes such as cyclin A and cyclin E. Adapted from Moody and Laimins 2010.

1.7.2.2 E7 and cyclins

Besides targeting the pocket proteins, E7 can alter the cell cycle via additional mechanisms. The cyclin dependent kinase (CDK) inhibitors p21 and p27 are important regulators of growth arrest during epithelial differentiation and p21 is thought to act as a tumour suppressor in cervical carcinogenesis (Shin *et al*., 2009). The main target of p21 and p27 in human keratinocytes is CDK2, which is important for G_1-S phase entry and progression through its association with cyclin E and cyclin A (Desphande *et al*., 2005). E7 proteins have many strategies to maintain high CDK2 activity. The carboxy-termini of high risk E7 proteins bind p21 and p27, neutralising the inhibitory effects of cyclin E and cyclin A activities (Jones *et al*., 1997; Funk *et al*., 1997; Zerfass-Thome *et al*., 1996) allowing CDK2 activity to remain high.

The ability of high risk E7 to inactivate p21 has been shown to contribute towards carcinogenesis (Shin *et al*., 2009). Expression of mutants of E7 which are unable to inactivate p21, results in a marked reduction of E7 within mice, compared with control mice (Shin *et al*., 2009). Low risk E7 proteins are also able to bind to p21, however with a reduced affinity and decreased ability to abrogate the negative effects of p21 (Demers *et al*., 1994). High risk E7 has been shown to increase the levels of the CDC25 phosphatase, which promotes the induction of tyrosine dephosphorylation of CDK2, promoting its activation which is another method by which E7 maintain high CDK2 activity (Nguyen *et al*., 2002; Bloomberg & Hoffman, 1999).

1.7.2.3 E7 and HDACs

In normal cells, Rb represses the transcription of E2F dependent promoters by directly binding to the E2F transactivation domain and recruiting various chromatin modifiers such as histone deacetylases (HDACs) (Harbour & Dean, 2000). High risk E7 proteins can bind to HDACs through sequences distinct from those with which they bind Rb, and they can target

HDACs to repress transcription (Longworth & Laimins, 2004). This E7-Rb-HDAC interaction is essential for viral episome maintenance as well as maintaining an S phase environment (Longworth & Laimins, 2004; Longworth *et al*., 2005). Moreover, HDACs can directly deacetylate E2F factors, resulting in loss of their function (Marks *et al*., 2001). E7 also affects the expression of S phase genes by directly interacting with E2F factors (Hwang *et al*., 2002). E7 binds to E2F6 which acts as transcriptional repressor by recruiting polycomb group (PcG) complexes. This E7/E2F6 interaction is thought to prevent repression by E2F6/PcG complexes, creating an S phase environment which is condusive to virus replication (Hwang *et al*., 2002).

1.7.2.4 Further functions of E7

The E7 protein has also been shown to have a number of other diverse functions. HPV16 E7 can act as a transcription factor by binding to the TATA box binding protein (TBP) and inhibit the binding of this protein to DNA (Massimi *et al*., 1996). Phosphorylation of E7 by Caesin Kinase II (CKII) modulates this E7 inhibitory function. Interestingly, phosphorylation of the CKII motif of E7 has shown to enhance the ability of E7 protein to promote degradation of the pocket protein p130 in differentiated keratinocytes; pushing cells into S phase (Genovese *et al*., 2008).

Most HPV associated malignancies have numerous chromosomal imbalances, including gains or losses of whole chromosomes (zur Hausen, 1999). High risk HPV E7 protein has been shown to induce centrosome amplification, which correlates with cell division errors and occurs before the detection of genomic instability (Duensing *et al*., 2001). E7 mediated centrosome amplification is dependent on high levels of CDK2 activity (Duensing *et al*., 2006), linking this function to the degradation of Rb family members. E7 can also induce genomic instability through induction of DNA damage and activation of the ataxia ATM pathway. High risk E7 proteins have been shown to activate the ATM pathway in

undifferentiated and differentiated keratinocytes (Moody & Laimins, 2009). An important aspect of the ATM pathway is the induction of cell cycle checkpoints. E7 can abrogate these checkpoints to promote mitotic entry (Moody & Laimins, 2009). It can also degrade claspin (Spardy *et al*., 2009) a regulator of the DNA damage signalling pathway. Activation of DNA damage pathways by E7 is necessary for virus replication and malignant progression (Moody & Laimins, 2009). HPV has also been linked to the Fanconi anaemia (FA) pathway (Kutler *et al*., 2003) which promotes DNA repair in response to replication stress. HPV16 E7 normally activates the FA pathway, however for cells deficient for a normal FA pathway, the presence of E7 leads to increased chromosomal instability (Spardy *et al*., 2007).

1.7.3 E6 Protein

The E6 proteins of both low and high risk types are approximately 150 aa in length and share functional similarity with the adenovirus E1B and SV40 Tag viral oncoproteins (Thomas *et al*., 2006). The major structural characteristic of E6 is the presence of two zinc fingers. At the base of each of these fingers are two motifs containing zinc binding Cys-X-X-Cys domains which are conserved in all E6 HPV types (Howie *et al*., 2009)(Figure 1.5). E6 has been shown to interact with a number of different proteins which mediate a number of cellular processes including the apoptotic pathway, chromosomal stability, epithelial organisation, differentiation, cell-cell adhesion, polarity and proliferation (Table 1.1). Some of these interactions will be discussed below.

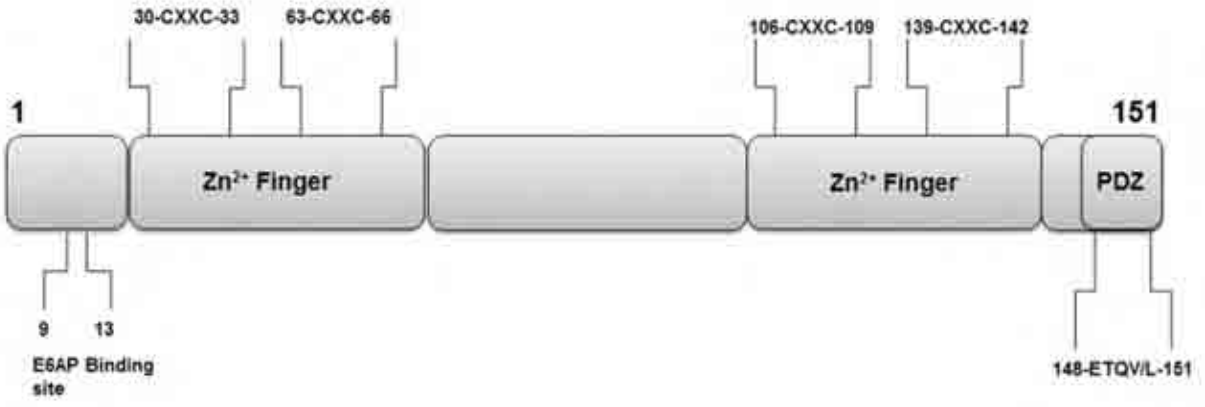

Figure 1.5 E6 Protein. Schematic representation of the E6 protein. The E6 proteins contain two zinc finger regions as medicated. E6 also contains at the C-terminus a consensus PDZ binding motif (E-T-Q-V/L). The E6AP binding region is also shown.

Function	Proteins which are bound by E6
E3 Ubiquitin ligase	E6AP
Mediators of apoptosis	Bak p53 C-myc Procaspase-8 FADD TNRF1
Transcriptional regulators	CBP/p300 E6TP1 hADA3 Gps2 Tuberin
Mediators of immune regulation	IRF-3
Mediators of chromosomal stability	MCM7
Mediators of epithelial differentiation and organisation	E6-BP Fibulin-1 Paxillin Zyxin
Mediators of cell polarity, cell-cell adhesion and proliferation *	PDZ proteins

Table 1.1 Protein partners of HPV E6 protein. Table of known functions of E6 as well as the cellular proteins which is known to bind. Mediators of cell polarity has been highlighted* as this group is expanded later in this study.

1.7.3.1 E6 and p53

A major consequence of the targeting of the Rb-E2F and other cell cycle regulators by E7 is an increase in the levels of the tumour suppressor protein p53, which impairs growth and increases the susceptibility of E7 expressing cells to apoptosis (Eichten *et al*., 2004). The role of p53 is to ensure the integrity of the cellular genome, preventing cell division after DNA damage or delaying it until the damage has been repaired. Alternatively if the replication of damaged DNA has occurred or is too large, p53 can trigger apoptosis of the cell (Steele *et al*., 1998; Lane, 1984).

High risk E6 proteins use several mechanisms to interfere with p53 function. The E6 proteins of high risk HPV types, recruit the cellular E3 ubiquitin ligase E6-associated protein (E6AP) to a trimeric complex with p53 (Huibregtse *et al*., 1991), which leads to the subsequent ubiquitylation and degradation of p53 (Scheffner *et al*., 1990) (Figure 1.6). E6 proteins of high risk and low risk types are also able to bind directly to p53 and block transcription by interfering with its DNA-binding activity (Lechner & Laimins, 1994) (Figure 1.5). Interestingly, low risk E6 proteins are also able to form complexes with E6AP (Brimer *et al*., 2007); however this union does not result in the degradation of p53 (Li & Coffion, 1996) suggesting that other cellular factors may be targets for the low risk E6-E6AP complex.

Another mechanism by which E6 proteins are able to interfere with p53 binding is through the binding of two related histone acetyltransferases p300 and CREB binding protein (CBP). The binding of these transcriptional co-activators, inhibits the ability of these proteins to acetylate p53 and therefore increase the stability of the E6 protein (Patel *et al*., 1999; Zimmermann *et al*., 1999). E6 proteins have also been shown to bind to the histone acetyltransferase ADA3; however in contrast to p300 and CBP, E6 inactivates ADA3 by targeting it for degradation (Kumar *et al*., 2002).

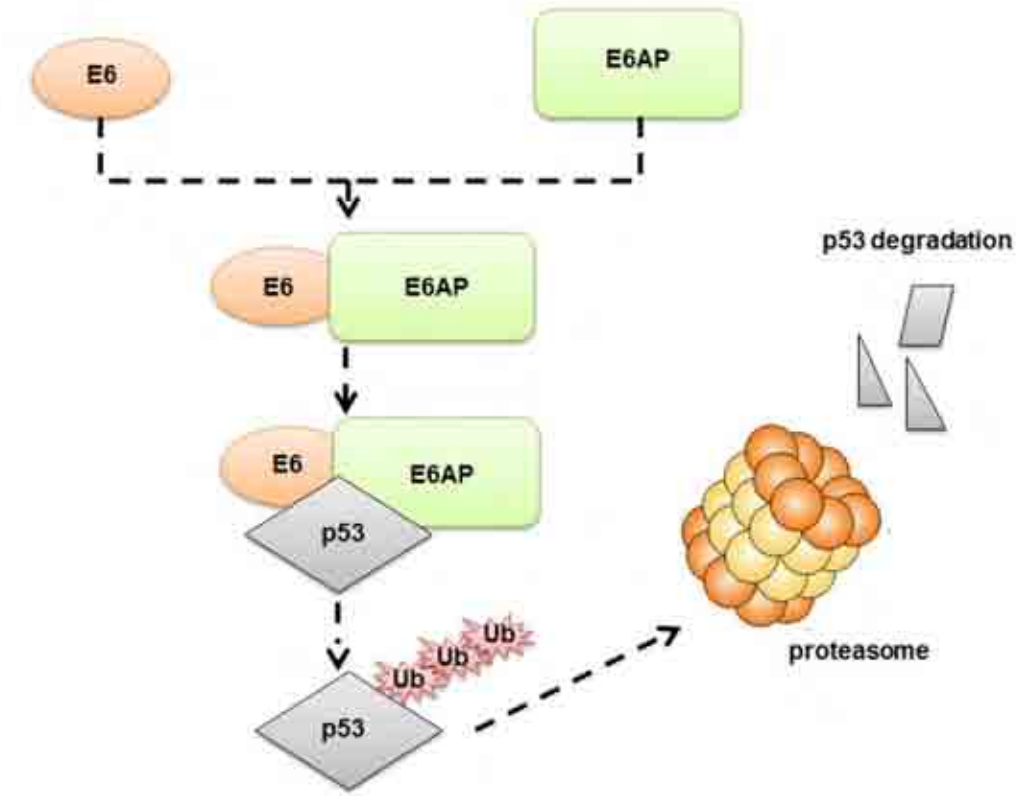

Figure 1.6 E6 and the p53 pathway. E6 targets p53 for degradation via the ubiquitin proteasome pathway. The E6 protein associates with the ubiquitin ligase E6-AP. This complex then binds p53 leading to its subsequent ubiquitination. Adapted from Gittoni, 2009.

The ability of E6 to degrade p53 has consequences for tumour development as the abrogation of p53 function allows genetic mutations to accumulate that would normally have otherwise been repaired. Interestingly, the E6 protein does not bind or degrade the p53 homologues- p63 and p73, suggesting that these proteins are not required for HPV-mediated transformation (Marin *et al*., 1998; Roth & Dobbelstein, 1999).

1.7.3.2 p53 independent inhibition of apoptosis

The E6 proteins of high risk and low risk types have been shown to interfere with the apoptotic pathway independently of p53, through its association with Bak, a pro-apoptotic protein, and member of the Bcl-2 family of proteins (Thomas & Banks 1998; Thomas & Banks 1999). Bak is an important regulator of apoptosis. In response to cellular stress, Bak forms pores in the mitochondrial membrane, where it resides, resulting in the release of cytochrome c from mitochondria and activation of the apoptotic cascade, ultimately leading to death of the cell (Howie *et al*., 2009). Analogously to its effect on p53, the E6 proteins are able to degrade Bak, via the ubiquitin mediated pathway, thereby blocking the induction of apoptosis (Thomas & Banks 1998; Thomas & Banks 1999). In normal epithelia, Bak protein is highly expressed in the upper layers during differentiation. It is possible that the degradation of this protein by the HPV E6 protein may be necessary for the progression of the virus life cycle.

1.7.3.3 Resistance to cytokines

Papillomaviruses have also evolved mechanisms to evade apoptosis mediated by the immune system (Howie *et al*., 2009). In response to virus entry, a cell produces a number of inflammatory mediators such as tumour necrosis factor (TNF) – which is a potent inhibitor of keratinocyte proliferation (Basile *et al*., 2001). Inflammatory cytokines can activate the extrinsic pathway through the TNF receptor TNFR1, FAS and the TNF-related apoptosis-

inducing ligand (TRAIL) receptors (Howie *et al*., 2009). High risk E6 proteins block apoptosis induced by TNF by directly binding to TNFR1, which inhibits the formation of a death inducing signalling complex and induction of apoptotic signals (Filippova *et al*., 2002). In addition to its interaction with TNFR1, E6 is also able to interact with the FAS-associated protein with death domain (FADD) and caspase 8 to block cell death in response to FAS and TRAIL (Garnett *et al*., 2006; Fillippova *et al*., 2004).

1.7.3.4 E6 and Inhibition of Anoikis

Another major apoptotic pathway targeted by the HPV E6 protein is anoikis, which is associated with anchorage independent growth (Chiarugi & Giannoni, 2008). Integrins interact with the extracellular matrix (ECM) and regulate signal transduction through the focal adhesion kinase (FAK). This interaction results in the activation of FAK and its downstream target paxillin which leads to cytoskeletal reorganisation and formation of focal adhesions. The bovine papillomavirus (BPV) E6 protein has been shown to bind to paxillin and this interaction correlates with its transforming function (Tong & Howley, 1997; Vande Pol *et al*., 1998). Moreover, HPV16 E6 has also been shown to bind to paxillin; however it is still unclear how this interaction contributes to pathogenesis (Tong & Howley, 1997; Vande Pol *et al*., 1998). Fibulin 1 is another target of HPV16 E6 (Du *et al*., 2002) which is involved in transformation and tumour invasion. Taken together, the ability of HPV E6 proteins to interact with these proteins, coupled with FAK activation, promote the resistance to anoikis and allow HPV transformed cells to proliferate in the absence of the ECM.

1.7.3.5 Activation of Telomerase

Another major function of the high risk E6 proteins that is important for immortalisation is their ability to activate the expression of the catalytic subunit of telomerase, hTERT (Klingelhutz *et al*., 1996). p53 independent mechanisms have been described for the

observed TERT induction by E6 – direct transcriptional activation of the hTERT promoter by an E6/c-Myc complex (Veldman *et al*., 2002) and indirect induction through the E6AP dependent degradation of a natural repressor of the hTERT promoter, NFX1-91 (Gewin & Galloway, 2001; Gewin *et al*., 2004).

1.7.4 E6 and PDZ proteins

The targeting of p53 by E6 is the most extensively studied function of E6; however recent studies have identified p53 independent interactions which are important for the immortalisation of human cells. E6 mutants deficient for degradation of p53 can still immortalise human mammary epithelial cells (Kiyono *et al*., 1998), suggesting that interactions with other cellular factors are necessary for immortalisation. Among the important p53-independent targets are those which contain a PDZ (PSD-95/Dlg/ZO-1) domain-so called as these were the first three proteins in which this domain was identified. These PDZ proteins have been shown to associate with high risk E6 proteins (Thomas *et al*., 2008).

The C-terminal domain of high risk E6 proteins is highly conserved and contains a PDZ-binding motif (PBM) (E-T-Q-V/L) (Figure 1.5) which resembles a class 1 PBM which mediates specific interactions with a number of PDZ substrates (Table 1.2). PDZ containing proteins are primarily associated with the establishment and maintenance of cell polarity in epithelial cells; however they also provide scaffolding for protein folding and have cell signalling and tumour suppressor activity (Craven & Bredt, 1998; Gomperts, 1996). The main PDZ targets are shown in Table 1.2, along with their putative and known functions (Table 1.2). Through interactions with these proteins, E6 is capable of affecting the formation of the three major complexes required for establishing cell polarity, attachment and signal transduction in cells – the Scribble, PAR and CRUMBS complexes- which are discussed in Chapter 5.

PDZ Protein	Summary of Known/Putative functions
hDlg	Tumour suppressor APC interaction and inhibition of cell cycle progression Adherens junction formation Tight junction protein recruitment
hScrib	Adherens junction formation Tumour suppressor via APC interaction* Crumbs complex localisation
PSD95	Signalling complex scaffold
MAGI-1	Localises PTEN to tight junctions
MAGI-2	Stabilises PTEN Enhances PTEN tumour suppressor activity
MAGI-3	Enhances PTEN tumour suppressor activity
PATJ	Cell polarity maintenance
MUPP-1	Signal transduction* Tight junction localisation*
TIP-1	Induction of cell proliferation and motility Induction of cell differentiation Down regulation of PDZ interactions
TIP-2/GIPC	Cell signalling TGF-β signal transduction Proliferation inhibition
CAL	Vesicle trafficking of cell membrane proteins
PTPN13	Tumour suppressor Inhibits anchorage independent growth
PTPN3	Growth inhibition
PAR3	Tumour suppressor activity
NHERF-1	Cell signalling Inducer of EMT

Table 1.2 E6 PDZ binding partners. The binding partners of the E6 PDZ binding motif along with their known functions. Those displayed with an (*) are thought to be putative functions of the protein.

PDZ proteins can be classified into further sub-families based on the domain structure of their proteins. PDZ proteins such as hDlg, MAGI-1, MAGI-2 and MAGI-3 belong to the membrane-associated guanylate kinase homologue (MAGUK) family of proteins. Members of this PDZ family are characterised by a core arrangement of three distinct protein-protein interaction domains. Each protein contains a guanylate kinase homologous (GUK) domain, a Src homology domain (SH3) and repetitive PDZ domains (Frese *et al*., 2006) (Figure 1.7). MUPP1 and PATJ are members of a multi-PDZ domain family containing 13 and 10 PDZ domain respectively as well as an amino-terminal L27 domain (Javier, 2008). The leucine rich and PDZ domain (LAP) family of PDZ proteins such as Scribble are characterised by four PDZ domains and a leucine rich domain (Javier, 2008) (Figure 1.7). The final major family group belongs to members of the tyrosine phosphatase family. Members of this family include PTPN13-characrtersied by a PTP domain, FERM domain and varying PDZ domains (Javier, 2008) (Figure 1.7).

PDZ domains consist of a stretch of 80-90 amino acids which are contacted by four amino acids at the extreme carboxy terminus of high risk HPV E6 proteins (Ponting & Phillips, 1995). The sequence (E-T-Q-V/L) permits the binding of E6 with PDZ proteins. The E6 proteins of high risk types have a T and the -3 position and V/L at the -1 position which is characteristic for binding PDZ proteins (Flores *et al*., 2006). The E6 proteins from different HPV types have different affinities for different PDZ domains. Whilst HPV16 E6 binds to hDlg by PDZ domain 2 (Kiyono *et al*., 1997), the E6 protein from HPV18 binds to hDlg via all three of its PDZ domains (Gardiol *et al*., 1999). Structural studies have revealed that the interaction between E6 and its substrates occurs within the substrate groove of PDZ molecules between βB strand and the αβ helix (Zhang *et al*., 2007) with the main chain carboxyl group of the E6 peptide anchored within the motif of the PDZ protein (Zhang *et al*., 2007).

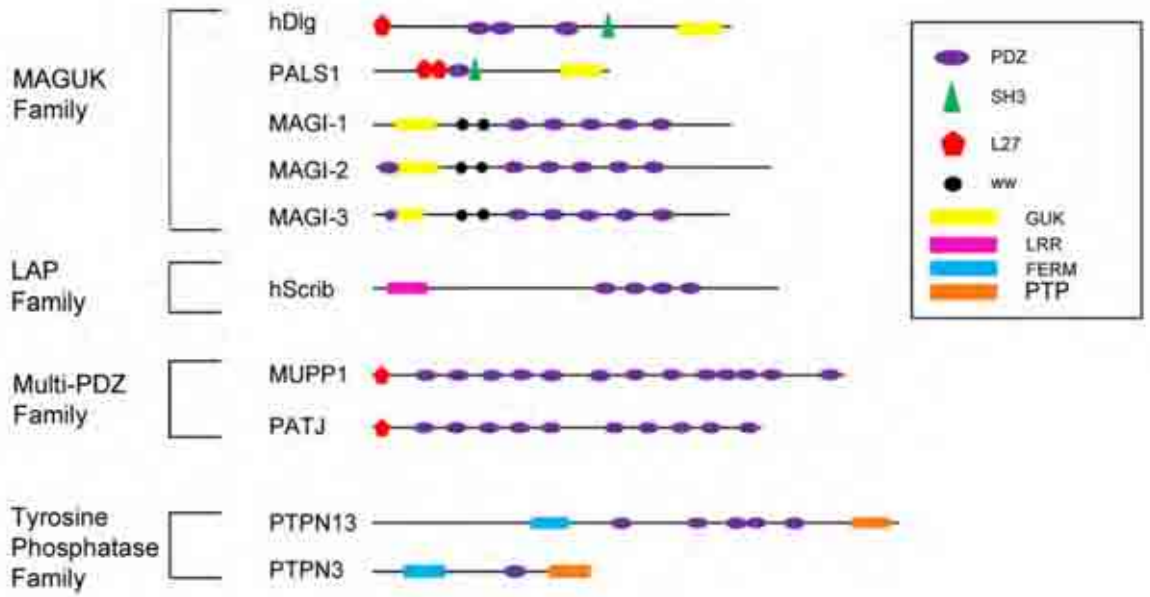

Figure 1.7 PDZ proteins and their structural subunits. Domain structures of the MAGUK, LAP, Multi-PDZ and Tyrosine phosphatase family of PDZ proteins. Adapted from Javier and Rice, 2011.

The importance of an E6-PDZ interaction has been confirmed by Kiyono and colleagues who showed that E6 proteins which lack the PBM retain the ability to degrade p53 and immortalise human mammary epithelial cells (Kiyono *et al*., 1998) but are unable to transform rodent fibroblast cell lines (Kiyono *et al*., 1997). In additional mice studies by Nguyen and others, the ability of E6 to increase tumour size and frequency in the cervix was dependent upon the ability of E6 to bind to its PDZ domain containing substrates (Nguyen *et al*., 2003; Shai *et al*., 2007; Shai *et al*., 2008). In a tissue culture background the role of the PDZ binding motif is less clear. In the context of the complete HPV31 genome, the E6 PBM was not required for the immortalisation of human keratinocytes; however it was required for cell proliferation (Lee & Laimins, 2004). Furthermore, in cooperation with the *ras* oncoprotein, the E6 PBM was shown to be required for anchorage independent growth of human tonsillar keratinocytes (Spanos *et al*., 2008a; Spanos *et al*., 2008b). Induction of epithelial to mesenchymal transition (EMT) is a hallmark of cancer development. Several studies have shown that both HPV16 and HPV18 can induce EMT-like changes in human keratinocytes (Watson *et al*., 2003; Spanos *et al*., 2008a). Interestingly, loss of the PBM correlated with a partial reversal of the EMT phenotype (Watson *et al*., 2003).

Similar to their human counterparts, the papillomaviruses from Rhesus monkeys (RhPV) as well as the cottontail rabbit (CRPV) also cause anogenital malignancy. The E6 proteins from CRPV (LE6/SE6) have PDZ binding activity and have been shown to interact with but not lead to the degradation of hDlg, a PDZ substrate (Du *et al*., 2005). Moreover, a CRPV SE6 mutant which was unable to form papilloma's was also deficient for binding to hDlg, suggesting that an interaction with hDlg was an important biological function of CRPV E6 (Du *et al*., 2005). Interestingly, the RhPV E6 protein does not contain a functional PDZ binding motif; instead it confers its PDZ binding activity via the E7 protein (Tomaic *et al*., 2009) (Figure 1.8).

A conserved PBM is also a feature of a number of other viral proteins including the adenovirus type 9 E4-ORF1 protein and the HTLV-1 Tax protein, which in both cases, is required for binding PDZ substrates and transforming activity (Lee *et al*., 1997) (Figure 1.8), suggesting that the conservation of this domain is a requirement for a number of transforming proteins.

1.7.4.1 E6 and PKA

As well as the structure of the E6 PBM regulating targeting of PDZ substrates, phosphorylation of E6 by protein kinase A (PKA) has been shown to negatively regulate HPV18 E6-PDZ interactions. The PKA pathway has been shown to selectively regulate the function of high risk but not low risk E6 proteins (Kuhne *et al*., 2000). *In vitro* binding experiments show that E6 phosphorylation by PKA reduces binding to hDlg and the E6 dependent degradation of hDlg in cells with high PKA levels is inhibited suggesting that this inhibition is dependent upon the phosphorylation of the PDZ binding site (Kuhne *et al*., 2000). Moreover, a study by Watson and colleagues show that loss of negative regulation was associated with an increase in EMT and actin cytoskeleton organisation in SV40-immotliased keratinocytes (Watson *et al*., 2003).

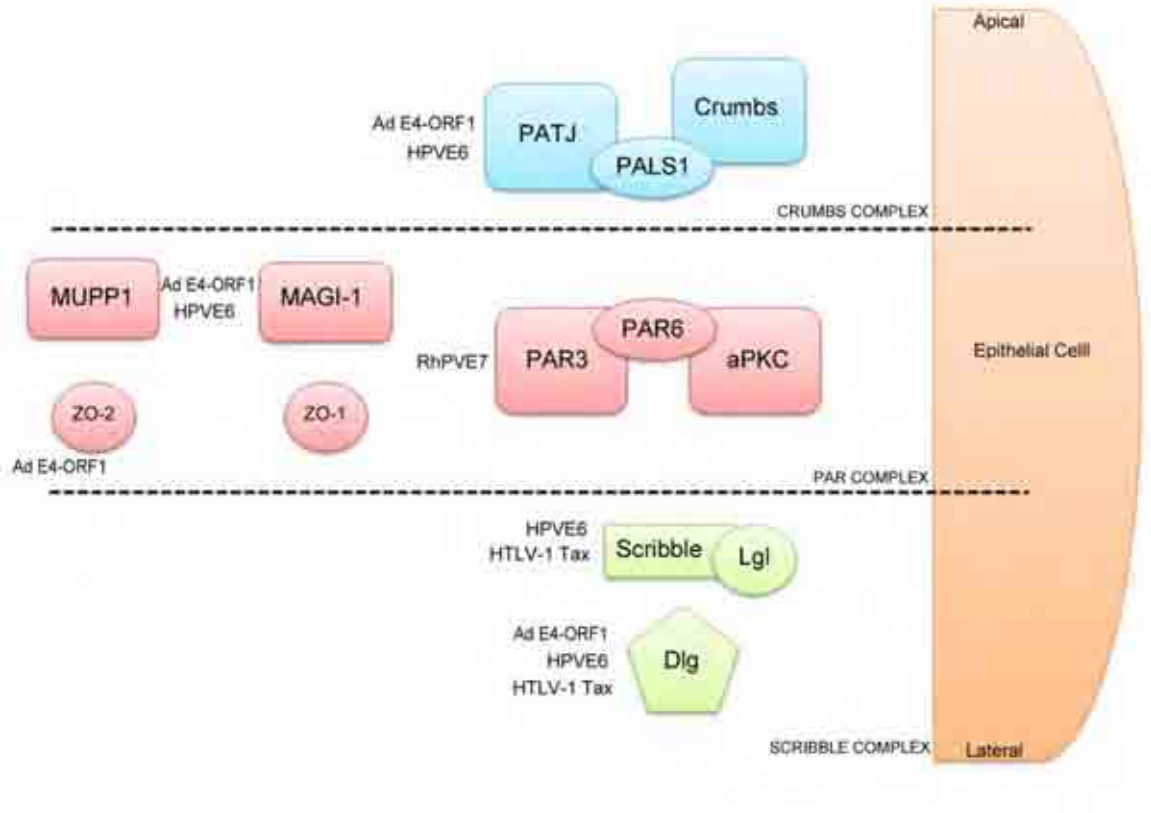

Figure 1.8 PDZ substrates are targets for multiple viral proteins. The polarised epithelial cell can be divided into the apical and lateral regions. The cellular PDZ proteins and their epithelial localisation are shown. The HTLV-1 Tax, HPV E6 and Ad E4-ORF1 specifically target a number of these PDZ substrates. RhPV confers PDZ binding through E7 to the PAR3 protein. Adapted from Javier and Rice, 2011.

1.7.4.2 hDlg

The first PDZ protein to be identified as a target of the high risk E6 proteins was hDlg; the human homologue of the *Drosophila lethal (1) discs large-1* tumour suppressor protein (Dlg) (Kiyono *et al*., 1997; Lee *et al*., 1997). As previously discussed, hDlg contains three PDZ domains and is a member of the MAGUK family of proteins. The MAGUK protein family act as molecular scaffolds to form protein complexes and are detected on the cell membrane and at sites of cell-cell contact (Ide *et al*., 1999). In *Drosophila*, Dlg localises to the cytoplasmic face of septate junctions between epithelial cells and the imaginal discs. Recessive mutations in the *Drosophila* Dlg locus result in loss of cell polarity and differentiation, neoplastic progression and ultimately larval death (Woods & Bryant, 1989). Further studies have revealed that mutations in the SH3 and GUK domains of this protein cause a loss of normal cell proliferation (Woods *et al*., 1996). Based on these data it was suggested that Dlg functions to organise signalling complexes at cell junctions and thereby negatively regulates the growth of epithelial cells. Interestingly mammalian forms of the hDlg protein can rescue the phenotype of *Drosophila* Dlg mutants, implying that the human homologue of this protein is also involved in controlling epithelial cell growth (Thomas *et al*., 1997). A seminal study by Gardiol and colleagues identified hDlg as a target for high risk HPV18 *in vitro* and *in vivo* (Gardiol *et al*., 1999). Moreover, subsequent studies by Kuhne and colleagues showed that high risk E6 proteins are phosphorylated at their PBM by a PKA like activity (Kuhne *et al*., 2000).

First identified from human B lymphocyte cDNA, hDlg encodes a 100kDa protein which shares 60% homology with Drosophila Dlg and 70% similarity with rat SAP90. Consistent with the cellular localisation of Dlg to septate junctions hDlg localises to regions of cell-cell contact in cells where it modulates cell polarity and proliferation (Lue *et al*., 1994). hDlg exists as a number of alternatively spliced isoforms. A number of insertions have been

identified including a 33 amino acid insertion termed I1 which is found in the N-terminal domain upstream of the first PDZ repeat (I1a and I1b), a 12 amino acid I2 insertion and a 34 amino acid I3 insertion (McLaughlin *et al*., 2002; Roberts *et al*., 2007). Both the I2 and I3 isoforms are located between the SH3 and GUK domains in the C-terminal region. In response to differentiation, the I2 isoforms relocalise from the nucleus and the I3 isoforms are selectively upregulated (Roberts *et al*., 2007). While membrane localisation of hDlg is evident in differentiating keratinocytes, it is believed that nuclear forms are important for the cell cycle, mediated by the I2 isoform (Roberts *et al*. 2007).

Another homologue of *Drosophila* Dlg is the hDlg-4 protein, also known as PSD95, which bares a PDZ domain and is targeted for degradation by the high risk HPV18 E6 protein (Hanada *et al*., 2007). Although its function is not yet known, Hanada and colleagues found that overexpression of PSD95 in HPV cervical cancer was able to inhibit tumourigenicity, suggesting a role for this protein in tumour suppression – particularly, during HPV infection (Hanada *et al*., 2007).

It is hypothesised that hDlg-1 tumour suppressor activity is not directly related to its membrane localization, and is instead carried out by binding to two classic tumour suppressors PTEN (a negative regulator of the PI3K pathway) and APC (adenomatous polyposis coli), another tumour suppressor protein which is a negative regulator of the Wnt differentiation signalling pathway (Ishidate *et al*., 2000). When hDlg-1 complexes with APC, it prevents the cell from progressing into the first stage of cell cycle progression, DNA synthesis or S-phase (Ishidate *et al*., 2000). hDlg-1 phosphorylation helps to determine its localization within the cell, and following the observation that this was affected by cell cycle progression (Massimi *et al*., 2003), Narayan and colleagues determined that hDlg-1 is also a substrate for cyclin-dependent kinases 1 and 2 (Narayan *et al*., 2009). This places hDlg- 1 phosphorylation under the same regulation system as the cell cycle itself, suggesting that

hDlg-1 is only capable of complexing with APC in particular phosphorylation states to inhibit cell cycle progression.

The PDZ binding motif of E6 has been shown to contribute to the reduced localisation of hDlg to sites of cell adhesion and disruption of intracellular junction formation. This domain has also been shown to contribute towards the E6-dependent morphological transformation of keratinocytes (Watson *et al*., 2003). The presence of hDlg at sites of cell: cell contact diminishes whilst intracellular cytoplasmic levels increase significantly in high grade but not low grade cervical neoplasias (Watson *et al*., 2002). This indicates that a loss of hDlg from cell: cell periphery could be an initial risk factor for disease progression (Watson *et al*., 2002; Cavatorta *et al*., 2004; Lin *et al*., 2004). Interestingly, E6 mutants which are unable to bind E6AP are still able to target hDlg for degradation, suggesting that hDlg degradation although still proteasome mediated is independent of an E6AP interaction, and suggests a novel ubiquitin ligase in this process (Pim *et al*., 2000).

A role for hDlg in the regulation of cell growth is further supported by studies showing that hDlg is a target for viral transforming proteins, including HPV E6, adenovirus 9ORF1 and HTLV-1 Tax. In each case the viral protein abolishes the normal function of hDlg albeit via different mechanisms (Lee *et al*., 2000; Gardiol *et al*., 1999). It is thought that 9ORF1 and HTLV-1 Tax form complexes with hDlg. These complexes prevent the formation of hDlg: APC complexes (which negatively regulate cell cycle progression) and thereby contribute to the promotion of unregulated cellular proliferation (Lee *et al*., 1997).

It is not only other viral transforming proteins which are able to target hDlg for degradation. Spliced isoforms of the E6 protein are also able to direct degradation of hDlg. Overexpression analysis of E6*I protein from high risk mucosal papillomavirus types showed that E6*I is able to direct the degradation of cellular PDZ proteins including MAGI-1 and hDlg (Pim *et al*., 2009). Interestingly, expression of an E6 mutant of the E6 PBM in the same study,

which is unable to bind to hDlg, but still expresses the E6* spliced product, is also able to target hDlg for degradation (Pim *et al*., 2009). Moreover, mutation of the splice donor site in the same lines was negative for hDlg degradation (Pim *et al*., 2004). These data suggest that a significant proportion of the ability to degrade hDlg is due to E6* rather than full-length E6 and propose that E6* expression can lead to hDlg degradation independently of full length E6 (Pim *et al*., 2009).

The sequence of the E6 PDZ binding domain is not identical between all high-risk HPV types. The E6 proteins of HPV type 18 and 31 contain an E-T-Q-V whilst HPV16 E6 has a leucine as a final amino acid (E-T-Q-L) (Figure 3.1). A study by Thomas and colleagues have shown that HPV18 E6 and HPV16 E6 exhibit different preferences for PDZ substrates and this is dependent on the last amino acid of the PDZ binding motif (Thomas *et al*., 2003). HPV18 is able to bind and degrade hDlg more efficiently than HPV16 whilst binding to another PDZ protein hScrib(Scribble) is weak and degradation, poor. The converse is true for HPV16 E6. A replacement of the valine with leucine switches HPV18 E6 preference for PDZ substrates from hDlg to hScrib (Thomas *et al*., 2003; Zhang *et al*., 2007). This reinforces the results from a study by Watson and colleagues which showed that hDlg is predominantly targeted by HPV18 E6, while HPV16 E6 predominantly targets hScrib (Watson *et al*., 2005). It has been hypothesised that these interactions represent a fundamental difference in the biological activities of the E6 proteins with respect to their PDZ substrates and the differential targeting may relate to differences in the pathogenicity between high risk HPV types.

1.7.4.3 hScrib

hScrib, the mammalian homologue of the *Drosophila* Scribble protein is a member of the LAP family. The protein contains four PDZ domains and a leucine rich domain, required for its basolateral localisation in epithelial cells (Kim, 1997; Bilder & Perrimon, 2000) (Figure 1.7). hScrib was isolated in a screen for proteins targeted for ubiquination by HPV E6

proteins in an E6AP-dependent manner, and over expression of Scribble has been shown to inhibit transformation of rodent epithelial cells by HPV E6 (Nakagawa & Huibregtse, 2000; Nguyen *et al*., 2003). hScrib is considered to have tumour suppressor activity, as significantly reduced expression of this protein is observed in a large number of carcinomas including breast and endometrial cancers (Zhan *et al*., 2008; Ouyang *et al*., 2010). hScrib has also been shown to inhibit cell cycle progression to DNA synthesis (Nagasaka *et al*., 2006). As with hDlg-1, hScrib is known to interact with APC; interruption of this interaction prevents hScrib from localizing to adherens junctions, thus retarding adherens junction formation (Takizama *et al*., 2006).

1.7.4.4 MAGI-1, 2, 3

MAGI-1,-2, and -3 are all PDZ-containing proteins that are targeted by high-risk E6 proteins for degradation (Glaunsinger *et al*., 2000; Thomas *et al*., 2002). Unlike other PDZ proteins, members of the MAGI family have an inverted domain structure with guanylate kinase homology at the amino terminus. MAGI proteins contain at least five PDZ domains (Wu *et al*, 2000a; Wu *et al*., 2000b). MAGI-1 is found in a complex with β-catenin (Dobrosotskaya *et al*., 1997), a protein which is downregulated in a number of human cancers. MAGI-2 and MAGI-3 are involved in the regulation and activation of the PTEN tumour suppressor protein to prevent its degradation (Marte & Downward, 1997). Degradation of MAGI-1 leads to mislocalisation of the PTEN tumour suppressor, which releases inhibition of the PI3K/AKT pathway and loss of control of cell proliferation (Liu *et al*., 2008). The pathway used by high risk E6 protein is distinct from those used for the degradation of p53 and hDlg (Thomas *et al*., 2001). Similar to hDlg, HPV18 demonstrates a higher affinity of MAGI binding compared with HPV16 (Thomas *et al*., 2001). MAGI-1 has been shown to inhibit cell transformation in a HPVE7/ras or Adenovirus E1A/ras oncogene cooperation assay in primary rodent cells (Massimi *et al*., 2004).

1.7.4.5 MUPP1

MUPP1 is another of the PDZ-containing proteins targeted by high-risk E6 proteins for degradation (Lee *et al*., 2000). This protein contains thirteen PDZ domains (Ullmer *et al*., 1998) and is thought to be involved in signal transduction (Adachi *et al*., 2009). MUPP1 shares similar binding partners with another PDZ protein- PATJ such as ZO-1 and Par6, although it has a higher affinity for Claudin-1 and JAM (junctional adhesion molecule) (Adachi *et al*., 2009). Despite the similarities in localization and binding partners, MUPP1 is not deemed essential for tight junction establishment or epithelial cell polarity, as abolition of MUPP1 expression alone does not severely affect cell polarity (Adachi *et al*., 2009).

1.7.4.6 TIP-1

TIP-1 (Tax-interacting protein 1) was first identified in its interaction with the HTLV-1 Tax oncoprotein via the PDZ domain (Reynaud *et al*., 2000). Unlike other PDZ proteins, TIP-1 is found in the cytoplasm and has thus far not been found to be membrane-bound (Hampson *et al*., 2004; Reynaud *et al*., 2000). The exact role for TIP-1 is unclear however it appears to complex with rhotekin and RhoA to activate serum response element (SRE) transcription (Reynaud *et al*., 2000). This interaction subsequently leads to the transcription of elements responsible for cell cycle progression, apoptosis, and differentiation (Thomas *et al*., 2008). Recent studies have suggested that TIP-1 may also act as a PDZ domain inhibitor (Thomas *et al*., 2008). High-risk E6 interacts with TIP-1 via its PDZ domain, but does not induce its degradation; instead, the cellular protein appears to aid E6 in inducing cell mobility, as knockdown of TIP-1 expression inhibits the mobility of cervical carcinoma cells in tissue culture (Hampson *et al*., 2004).

1.7.4.7 TIP-2/GIPC

GIPC (GAIP-interacting protein, C-terminus) is another PDZ-containing binding partner of high-risk HPV18 E6 (Favre-Bonvin *et al*., 2005). It is also known as TIP-2 due to its discovery as a Tax-interacting protein (Rousset *et al*., 1998). GIPC has a variety of roles in cell signalling. GIPC is also known to interact with the transforming growth factor-β (TGF-β) receptor type III (TβRIII) (Blobe *et al*., 2001). TGF-β is a cytostatic cytokine whose function is to inhibit cell proliferation (Blobe *et al*., 2001). By inducing degradation of GIPC, E6 interrupts this signalling pathway leading to the loss of control of cell proliferation (Favre-Bonvin *et al*., 2005).

1.7.4.8 CAL

CAL (cystic fibrosis transmembrane regulator-associated ligand) is another binding partner of high-risk E6 that is not found associated with the cell membrane, but is instead localized to the Golgi apparatus (Jeong *et al*., 2007). Similar to hScrib, CAL shows preferential targeting by HPV16 E6 as opposed to HPV18 E6 (Jeong *et al*., 2007). The role of CAL in the cell is believed to involve vesicle trafficking of cell membrane proteins as they pass through the Golgi, and ensuring that they are targeted to the cell membrane (Jeong *et al*., 2007). This trafficking is probably important for the establishment of cell polarity in cells.

1.7.4.9 NHERF-1

NHERF-1 (N+/H+ exchange regulatory factor 1) is a multidomain scaffolding protein which regualtes the trafficking and signalling of several G protein coupled receptors (GPCRs). The protein has a PDZ binding motif and a 14 amino acid motif which regulates the interaction with the actin-binding protein ezrin, contributing to several signalling events (Mahon *et al*., 2002). Furthermore, the NHERF protein is also able to regulate cell shape and migration and

has been shoen to interact with a number of proteins involved in transformation (Georgescu *et al*., 2008).

1.7.4.10 PTPN3 and PTPN13

The final proteins which contain a PDZ domains belong to a family of protein tyrosine phosphatases, nonreceptor type (PTPNs) as discussed earlier. Members of this family include PTPN3 and PTPN13. PTPN13 has a cell membrane localization sequence along with its PDZ domain, and has been implicated as a tumour suppressor due to its loss of function in many colon cancers (Wang *et al*., 2004). PTPN13 appears to be involved in suppressing proto-oncogenes such as *c- src* by dephosphorylating nuclear factors associated with their induction (Erdmann, 2003). Recent studies have shown that inhibition of PTPN13 by E6 results in anchorage-independent growth, leading to invasive growth in mouse and human epithelial cells *in vivo*. Moreover complementation of PTPN13 in this system restored normal cell growth (Spanos *et al*., 2008).

PTPN3, also known as PTPH1, is another member of this PDZ family which has been implicated in colon cancers (Wang *et al*., 2004), and has been shown to be targeted by E6 for E6AP-mediated degradation (Jing *et al*., 2007). One of its few identified substrates is a cell cycle protein VCP (valosin-containing protein). Overexpression of PTPN3 leads to growth inhibition in cell culture (Zhang *et al*., 1999).

1.8 Hypothesis and Aims

Conservation of an E6 PBM between high risk HPV types but not low risk HPV types makes it a marker of oncogenicity. Many of the targets are relevant functionally to cell polarity and maintenance of intercellular tight junctions, whilst others also show tumour suppressor activity. A number of studies have highlighted the importance of the PBM in transformation of rodent cells as well as E6-induced anchorage independent cell growth and induction of EMT – hallmarks of cancer and metastatic progression. In mouse skin and cervical carcinogenesis an E6 PBM required for efficient tumour formation, whilst in the context of the virus life cycle, the E6 PBM was involved in maintenance of viral copy number as well as growth rate of immortalised cells. The indication that this domain can be regulated by phosphorylation suggests that changes in kinase signalling pathways such as the cAMP dependent kinase during the life cycle can be expected to regulate this E6 function. It is not clear which signalling pathways are impacted by E6 binding to PDZ proteins or which PDZ proteins are most important for the phenotypes which have been observed. The understanding of these pathways will in turn, help our understanding of the HPV pathogenesis.

The aims of this thesis are:

1. Establish a role for the E6 PBM in the HPV18 replication cycle by construction of an HPV18 genome which lacks an E6 PDZ binding motif and introduce into a primary human keratinocyte system which supports virus replication. (*Chapter 3 and Chapter 4)*

2. To establish whether PKA phosphorylation of the HPV18 E6 PDZ binding domain motif has a physiological role in the infection cycle of the virus, using a mutant HPV18 genomes which is unresponsive to PKA recognition. (*Chapter 3* and *Chapter 4*)

3. To determine the effect of changes in the activity of the PBM on potential cellular PDZ substrates. (*Chapter 5*)

CHAPTER 2 MATERIALS AND METHODS

2.1 Molecular biology techniques

2.1.1 Bacterial hosts, growth and storage

The DH5α strain of *Escherichia coli* (*E. coli*) was used as a bacterial host for the growth of the pcDNA 3.1 and pGEMII plasmid vectors for preparation of plasmid DNA. Bacteria were streaked out and stored on agar plates for short periods of time, or for longer periods of time, stored as a glycerol stock.

Agar plates were made by dilution of 1.5% (wt/vol) agar in Luria-Bertani (LB) medium (1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) bacto-yeast extract, 1% (wt/vol) NaCl). Following autoclaving, the agar was boiled then cooled to approximately 50°C before the appropriate antibiotic was added to a final concentration of 50 µg/ml and 25 ml of agar dispensed into each petri dish. Bacteria were streaked out from glycerol stocks by streaking of the bacteria across the surface of the agar with a fine loop, and then incubated at 37°C overnight to allow the growth of bacterial colonies.

Glycerol stocks were made following the growth of bacteria in LB media supplemented with 50 µg/ml antibiotic. Bacteria were grown in LB media overnight at 37°C with shaking before a 400 µl aliquot of bacteria was taken and added to 600 µl of sterile 80% (vol/vol) glycerol solution. Glycerol stocks were stored in 1 ml aliquots at -80°C.

2.1.2 Growth of *E. coli*

A starter culture of host cells was prepared by inoculating 6 ml of L-broth with a single bacterial colony, followed by the overnight incubation of this culture in a shaker at 37°C. The overnight preparation was used to inoculate 200 mls of L-broth, which was grown overnight.

2.1.3 Long-term storage of bacterial cultures

A 500 µl aliquot of an overnight bacterial culture was mixed with 500 µl of sterilized 50% (w/v) glycerol and stored at -80°C. To recover frozen bacteria, a small amount of the frozen glycerol stock was streaked out onto the appropriate agar plate and incubated at 37°C.

2.1.4 Transformation of competent *E.coli* with plasmid DNA.

Chemically competent *E.coli* were first thawed on ice, prior to addition of 30-50 µl of cells in a pre-cooled Falcon tube. One to two micrograms of plasmid DNA were typically added directly into the thawed cells. Following incubation on ice for 30 min the *E.coli* were heat shocked at 42°C for 45 sec, followed by a 2 min incubation on ice. 500 µl LB media was subsequently added, followed by incubation at 37°C with shaking at 200 rpm for 1 h. 250 µl were then plated onto ampicillin selection agar plates and incubated overnight at 37°C.

2.2 Preparation of plasmid DNA

2.2.1 Small preparation of plasmid DNA

A single bacterial colony was grown in 6 ml LB media with selection antibiotic at 37°C for 16 h with shaking at 200 rpm. The following day, 3 ml of the overnight culture, was subsequently pelleted by centrifugation at 16100 xg at 4°C for 5 min, followed by DNA extraction using the QIAprep® Spin Miniprep Kit (Qiagen), following manufacturer's instructions. DNA was eluted into 50 µl of 1X Tris-EDTA (TE) buffer and stored at -20°C.

2.2.2 Large-scale preparation of plasmid DNA

Qiagen Maxiprep

A 6ml starter culture of LB media with antibiotics was inoculated with a single colony and grown at 37°C with shaking at 200 rpm. After 6-8 h 200 ml of selective L-broth was inoculated with the starter culture and grown overnight at 37°C in an orbital shaker. Cells

were pelleted by centrifugation at 13,000 rpm in a Sorvall GS3 rotor for 10 min and re-suspended in 4 ml, 50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0. Between 10 and 15 mg of lysozyme were added and the mixtures incubated at 37°C for 20 min. 8 ml of a freshly prepared solution of 0.2 M NaOH, 1% (w/v) SDS was added and the samples left on ice for 5 min before the addition of 6 ml ice-cold 3 M sodium acetate.

Following 5 min incubation on ice, samples were centrifuged at 13,000 rpm in a Sorvall SS-34 rotor for 15 min and the aqueous phase transferred to an equilibrated Qiagen-tip 500 (Qiagen). The tip was washed twice with 30 ml washing buffer (1 M NaCl, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol) and the plasmid DNA was eluted with 15 ml elution buffer (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% (v/v) isopropanol). DNA was precipitated by addition of 0.7 volumes of isopropanol (Fisher) and the sample was then centrifuged at 9,000 rpm in a Sorvall SS-34 rotor for 40 min at 9°C. The pellet was then washed with 5 ml 70% (v/v) ethanol and centrifuged for a further 15 min at 4°C in a microcentrifuge. Plasmids were air-dried are re-suspended in 0.5-1 ml of TE buffer or sterile distilled water and stored at 4°C short term or -20°C long term. The concentration of DNA was determined by spectrophotometry (Nano-Drop).

2.3 Molecular cloning

2.3.1 Plasmid DNA vectors

The pGEMII-HPV18 plasmid (a gift from Frank Stubenrauch, University of Tubingen and used in the previous study (Wilson *et al*., 2007) contains the total HPV18 genome (accession number: NC 001357) cloned into the *pGEMII* vector at the Eco RI restriction site at residue 2440. The *pCDNA* 3.1 expression plasmid was generated by cloning the E6 protein of HPV18 into the *pCDNA*3.1 expression vector. The p53 untagged construct was a kind gift from Professor Lawrence Banks (Trieste, Italy).

2.3.2 Polymerase chain reaction (PCR)

PCR reactions were typically performed with 1 µg of plasmid DNA using the Expand High Fidelity PCR system (Roche), following manufacturer's instructions. PCR reactions were carried out in a total volume of 100 µl in expand high fidelity buffer supplemented with 15 mM $MgCl_2$, 2 mM dNTPs, 25 pmol forward and reverse primers (generated by AltaBiosciences), 1% (vol/vol) DMSO with 3.5 U of Taq DNA polymerase. Amplification of the DNA was performed on a thermal cycler (2720, Applied Biosciences) using a 2 min 94°C hot start followed by 25 repeats of 94°C for 1 min, 55°C for 30 s and 72°C for 3 min, followed by 8 min at 72°C before cooling to 4°C. The PCR products were often cleaned with High Pure PCR product Purification Kit (Roche) according to the manufacturer's instructions. DNA was eluted in 50 µl dH_20 and stored -20°C.

2.3.3 Agarose gel electrophoresis

Agarose (Sigma) was melted into a 1x solution of Tris/Boric EDTA (TBE) (45 mM Tris HCl, 45 mM orthoboric acid and 1 mM EDTA, pH 8.0) to a final concentration of 0.8-2.0% (w/v) depending on the size of fragment to be separated. Ethidium bromide was added to a final concentration of 0.5 µg/ml prior to pouring into a Mini Sub Gel GT Electrophoresis Tank (BioRad). DNA samples were mixed with loading buffer (30% (v/v) glycerol, 0.3% (w/v) bromophenol blue, 0.1% xylene cyanol FF 10x TBE) prior to loading. For size comparison a 1 kb Plus DNA marker (Gibco) was run alongside the samples. Gels were routinely run at 80 V for 1 hr in TBE running buffer and DNA bands were visualised with a Gene Flash UV light box (Syngene Bio Imaging). For resolution of genomic DNA isolated from HFK prior to southern blot analysis 0.8% (wt/vol) agarose gels were prepared as described and cast in Fisherband horitzaontal gel electrophoresis tanks (Fisher Scientific), with gels run at 50 V overnight.

2.3.4 Purification of DNA fragments

DNA fragments were purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufactures instructions. This involved the required band to be excised from the agarose gel using a clean scalpel and heated in a solubilisation buffer for 10 min at 50°C to dissolve the agarose. The sample was then applied to a QIAquick column and then centrifuged at 13,000rpm for 60 sec in a microcentrifuge to allow the binding of the DNA. The bound DNA was washed by applying 750 µl of wash buffer to the column and then centrifuged at 13,000 rpm for a further 60 sec. DNA was then eluted into an eppendorf by applying between 15-30 µl of TE and centrifugation at 13,000 rpm for 60 sec and stored at -20°C.

2.3.5 Restriction enzyme digestion

Restriction enzyme digestions were typically performed using 2-10 µg of plasmid DNA incubated with restriction enzymes in the appropriate buffer provided with the enzyme in a total volume of 20-50 µl. Restriction enzymes used in this study include *Eco*RI (20 units), *Bgl*II (10 units), *Bam*HI (20 units) (New England Biolabs) and *Dpn*I (10 units) (Roche). Following addition of the restriction enzyme, reactions were vortexed and centrifuged briefly, before incubation at 37°C for 1-2 hr or overnight. To ensure complete digestion, 200 ng of the digested product was analysed on an agarose gel. For digestion with multiple restriction enzymes these were carried out simultaneously by selecting an appropriate buffer in which both restriction enzymes were active.

2.3.6 Purification of DNA fragments

DNA fragments were purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufactures instructions. In brief, the excised band is excised form the agarose gel and heated in a solubilisation buffer at 50°C for 10 min to dissolve the agarose. The sample is applied to a QIAquick column and then centrifuged at 13,000 rpm for 60 sec in a

microcentrifuge to allow binding of the DNA. The captured DNA is then washed by applying 750 µl of wash buffer to the column and centrifugation at 13,000 rpm for a further 60 sec. DNA was eluted into an eppendorf by applying 30 µl TE and centrifugation at 13,000 rpm for a further 60 sec and then stored at -20°C until use.

2.3.7 DNA ligation reactions

Following restriction digests, enzymes were heat-inactivated prior to a ligation reaction by incubation of reactions at 65°C for 10 min, then cooling on ice for 2 min. Ligations were performed by incubation of vector and insert DNA at rations typically of 1:3, 1:5 or 1:10 with between 4-8 units of T4 DNA ligase (New England Biolabs) and ligase buffer (660 mM Tris-HCL pH 7.5, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10mM ATP) and incubated overnight at 16°C. Ligated DNA was then transformed into bacteria and sequenced following mini-preparation of plasmid DNA.

2.3.8 PCR sequencing

One µg of plasmid DNA was mixed with 2.5 pmol primer, 1 µl BigDye® terminator ready reaction mix (Applied Biosystems) and relevant buffer in a total volume of 20 µl. The reaction was carried out with 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min on a thermal cycler. Oligonucleotide primers utilised for the sequencing of plasmid DNA are listed in Table 2.1, and were generated by Altabiosciences, University of Birmingham. The DNA was precipitated with 5 µl 125 mM EDTA and 60 µl 100% ethanol at room temperature for 15 min, and the sample centrifuged at 16100 x g for 45 min at 4°C. The supernatant was removed and the pellet washed in 70 µl 70% ethanol, the sample was then centrifuged at 16100 x g for 15 min at 4°C. The supernatant was removed and the pellet air dried and re-suspended in 10 µl HiDi Formamide (Applied Biosystems). The reaction was heated at 95°C for 5 min before loading into a 96 well plate in a 3100 ABI Prism™ DNA capillary sequencer

(Applied Biosystems). The sequencer capillary array was filled with 3100 Performance Optimised Polymer 6™ Performance Optimized Polymer (Applied Biosystems). Sequencing data was collected using the 3100 data collection software version 3.1 and was analysed using ABI sequencer version 3.6.1 or FinchTV Version 1.4.0 (Geospiza).

Table 2.1 Sequencing primers

Primer	Direction	Primer Sequence
E6 SEQ	F	5' GAC AGT ATA CCG CAT GCT GCA TGC C 3'
E6ΔPDZ	F	5' GAA CGA CTC CAA CGA CGC AGA TAA TGA CAA GTA TAA TAT TAA GTA TG 3'
	R	5' CAT ACT TAA TAT TAT ACT TGT GTT TCT CTG CGT CGT TGG AGT CGT TC 3'

2.3.9 Generation of mutations within the HPV18 E6 *pCDNA* 3.1 expression vector

Site-directed mutagenesis was performed using the Quick-Change Kit (Stratagene) following manufacturer's instructions. The *pCDNA*3.1 HPV18 E6 expression vector was used as template DNA using the primers listed in Table 2.1, with the number of PCR cycles used between 18 and 25. All of the oligodeoxyribonucleotide site-directed primers used in this study were generated by Altabiosciences, University of Birmingham. PCR products were digested with *Dpn* 1 and transformed into XL-1 blue E-coli. Colonies were grown in LB media and mini-preparation performed (according to manufacturer's instructions). Extracted DNA was sequence using the sequencing primer listed in Table 2.1 to confirm correct introduction of mutants.

2.4 Protein biochemistry

2.4.1 Bradford assay

Protein concentration was determined using the Bradford method using a Bio Photometer (Eppendorf). Readings were taken at 0, 4, 8, 16 and 20 µg of 1mg/nl BSA in 1 ml of diluted protein assay reagent (Bio-Rad). A known amount (1-5µl) of the protein samples were then added to the reagent and the concentration determined.

2.4.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were typically resolved on 12.5% polyacrylamide separating gels (350 mM Tris-HCL, 12.5% [wt/vol] polyacrylamide (acrylamide : bis-acrylamide 37.5 : 1), 1% [wt/vol) SDS, polymerised with 0.4% [wt/vol] ammonium persulphate (APS) and 0.08% [vol/vol] TEMED). To ensure a level surface of all polyacrylamide gels, a layer of isopropanol was added to the surface immediately following pouring. Once the separating gels had set, isopropanol was removed and a stacking gel (125 mM Tris pH 6.8, 4.5% [wt/vol] acrylamide, 1% [wt/vol] SDS, polymerised with 0.5% [wt/vol] APS and 0.125% [vol/vol] TEMED) was poured on top of the resolving gel and the gel combs inserted. Once set, the wells were filled with running buffer (25 mM Tris, 192 mM Glycine, 0.1% [wt/vol] SDS, pH 8.3). Protein samples were prepared for SDS-PAGE by addition of Laemelli sample loading buffer supplemented with 5% [vol/vol] β-Mercaptoethanol, and boiled at 100°C for 5 min. Samples were loaded into wells of the gel with gel-loading pipette tips (Biorad). Electrophoresis was performed in running buffer using vertical gel slab units. The Mini Protean® 3 Cell (Bio-Rad) was run at 25 mA for 1-2 h for smaller gels whilst for larger gels, the Sturdier system (Amersham Biosciences) was used and run overnight at 55 V or until the correct separation had been achieved.

2.4.3 Western blot analysis

2.4.3.1 Electrophoretic transfer of proteins

Following separation by SDS-PAGE, proteins were transferred to BioTrace® NT nitrocellulose blotting membrane (Pall Life Sciences) using a Trans-Blot® Cell transfer tank (BioRad), in transfer buffer (25 mM tris, 192 mM glycine, 20% (vol/vol) methanol, pH 8.3), at 350 mA for 3.5 h, or overnight at 15 V. To ensure transfer of proteins was successful, membranes were stained in Ponceau stain (1% (wt/vol) Ponceau Red, 3% (wt/vol) trichloroacetic acid) to visualise proteins, before de-staining by washing in PBS containing 0.1% (vol/vol) Tween-20 (PBS-T). Nitrocellulose membranes were incubated in blocking buffer consisting of 2% (wt/vol) dried skimmed milk in PBS-T at room temperature for 30 min or overnight at 4°C. Primary antibodies were diluted in 5% milk in PBS and incubated with membranes for between 2 h to overnight depending on the antibody. For a full list of the antibodies used in this study, see Table 2.2. To control for equal loading, GAPDH levels were determined using a mouse anti-GAPDH MAb. Following incubation with primary antibodies, membranes were subjected to two 15 min washed with 0.2% Tween (v/v) in PBS. Typical secondary antibodies used in this study were a horseradish peroxidise-conjugated anti-mouse (Sigma-Aldrich) or anti-rabbit immunoglobulins (DAKO) (Table 2.2) visualised by chemiluminesence, and detected using autoradiography (GE Heathcare).

Table 2.2 Antibody dilutions used in Western blotting

Antibody Name	Manufacturer	Species	Dilution
Cyclin A	Santa Cruz	Mouse	1:1000
Cyclin E	Santa Cruz	Mouse	1:1000
Chk1	Santa Cruz	Mouse	1:1000
Rb	Santa Cruz	Mouse	1:1000
pH3 (ser10)	Cell Signalling	Mouse	1:100
CDC20	Santa Cruz	Mouse	1:1000
Cyclin B1	Santa Cruz	Mouse	1:1000
E6	Arbor Vita	Mouse	1:1000
E7	Abcam	Mouse	1:1000
DO-1 (p53)	*Gift from Prof David Lane	Mouse	1:5
E-cadherin	BD Transduction Labs	Mouse	1:500
Tip-2	Favre-Bonvin *et al*., 2005	Goat	1:1000
MUPPP-1	Upstate Biotechnology	Sheep	1:5000
GAPDH	Santa Cruz	Mouse	1:1000
Dlg (2D11)	Santa Cruz	Mouse	1:1000

Dlg (NAG)	Roberts *et al*., 2003	Rabbit	1:200
MAGI-1	Sigma	Rabbit	1:500
hScrib	Sigma	Goat	1:500
PTPN13	*Gift from WijianHendricks	Rabbit	1:5000
Anti-mouse IgG (whole molecule)-Peroxidase	Sigma	Goat	1:3000
Polyclonal Swine Anti-Rabbit Immunoglobulins/HRP	DAKO	Swine	1:3000
Anti-goat IgG (whole molecule)-Peroxidase	Sigma	Rabbit	1:3000

2.4.3.2 Densitometry

Protein levels from western blot films were examined using a Scanning Densitometer GS-800 (BioRad) and images were analysed using the Quality One 4.6.7 software (BioRad).

2.5 Tissue culture techniques

2.5.1 General information

Tissue culture media and solutions were prepared as described below, or purchased from indicated suppliers as detailed.

2.5.2 Tissue culture solutions

Dulbecco A solution (DulbaA-Phosphate buffered saline-PBS)

Dulbecco A solution (0.8 g/L NaCl, 0.02 g/L KCl. 0.115 g/L Na_2HPO_4, 0.02 g/L KH_2PO_4 pH7.3) was made up in deionised water and was sterilised by autoclaving.

EDTA (Ethylene diamintetra-acetic acid)

Dulbecco A was prepared as described above, and EDTA added to a final concentration of 0.02% (w/v). The solution was adjusted to pH 7.4, filter sterilised and stored at 4°C until use.

Saline Solution

A 0.85% NaCl solution was prepared by dissolving one saline tablet in 500 ml of deionised water. Saline solution was subsequently sterilised by autoclaving and stored at 4°C.

2.5.3 Tissue culture media supplements

Antibiotics

A 1/100 volume of a stock solution of penicillin (10,000 units) and streptomycin (10 mg/ml) (Sigma) was added to all media to prevent bacterial growth.

Bovine serum

Newborn calf serum was purchased from Gibco BRL and dispensed into 50ml aliquots under sterile conditions and stored at -20°C.

Foetal calf serum (FCS)

Sterile serum was purchased from Gibco BRL and dispensed into 50 ml aliquots and stored at -20°C until use.

Geneticin G418 sulphate (G418)

Geneticin G18 sulphate powder was purchased from Gibco BRL and dissolved in saline solution to a final concentration of 50 mg/ml. G418 stock solutions were passed through a 0.2µm filter (Pall), aliquoted and stored at -20°C.

Glutamine

A solution of 0.2 M L-glutamine was prepared, filtered through a 0.2 µm filter (Pall) and dispensed into 10 ml aliquots under sterile conditions and stored at -20°C. A single aliquot was added to 500 ml media, giving a final concentration of 4 mM.

Hydrocortisone

A sterile ampoule of 100 mg hydrocortisone (Upjohn Ltd.) was added to 20 ml of a 1:1 ethanol: water solution. This 5 mg/ml stock solution was subsequently filter sterilised and stored at -20°C. 40 µl of the stock solution was added to 500 ml of media as required.

Non-essential amino acids

A 1/100 volume of non-essential amino acids (Gibco BRL) was added to media when required.

2.5.4 Maintenance of cell cultures

All cell cultures were maintained on plastic tissue culture dishes (Iwaki) in the appropriate growth medium. Cells were incubated in a cell culture incubator at 37°C, with a humidified atmosphere containing 5% CO_2 in air.

2.5.5 Epithelial cell sub-culturing

Cells were harvested at around 80% confluency the media was removed from the dishes and the cell monolayer was washed with pre-warmed DulbA. 1 ml of 0.05% Trypsin was added

to each dish and then dishes were incubated at 37°C until the cells had detached (typically 1-5 min). To inactivate the trypsin, the cells were re-suspended in 9 ml appropriate growth medium and counted using a haemocytometer. Cells were either pelleted by centrifugation at 1400 rpm for 5 min in a bench-top centrifuge or plated out at the required density.

Table 2.3 Mammalian cell lines used in this study

Cell line	Cell type
HeLa	Cervical adenocarcinoma derived cell line, contains integrated HPV18 genomes
NIH-3T3 J2	Mouse embryonic fibroblast cell line
SAOS-2	Osteosarcoma derived cell line
HEK 293	Human embryonic kidney cell line that contains adenovirus E1A
HaCat	Transformed keratinocytes from normal skin. HPV negative.

The cell lines , HeLa, HEK 293 and SAOS-2 were all cultured in Dulbecco's modified Eagle medium (DMEM) HEPES modification (Sigma-Aldrich) supplemented with 4 mM glutamine and 10% (v/v) foetal calf serum. NIH 3T3 J2 cells were grown in (DMEM) HEPES modification (Sigma-Aldrich) supplemented with 10% (V/V) bovine serum and 4 mM glutamine. HaCat cells were grown in (DMEM) HEPES modification (Sigma-Aldrich) supplemented with 4 mM glutamine, 0.4 µg/ml hydrocortisone and non essential amino acids (Gibco BRL). Cells were kept at 37°C, 5.0% CO_2 in an incubator and were routinely grown in 75 cm^3 flasks (Iwaki) or 10 cm tissue culture dishes (Iwaki).

2.5.6 Cryopreservation of mammalian cells

For long-term preservation, cells were stored in liquid nitrogen. Cells were harvested by trypsinization when approximately 80% confluent and pelleted by centrifugation at 538 x g for 10 min. The pellets were then re-suspended in 1 ml growth medium containing 10% (v/v) DMSO and transferred to labelled NUNC cryotubes at a concentration of 2 x 10^6 cell/viol (Nalgene-Nunc). Tubes were stored in a Cryo Freezing container (Nalgene®MrFrosty) and stored at -80°C overnight to allow slow cooling. When frozen, cells were transferred to the vapour phase of liquid nitrogen for permanent storage.

2.5.7 Retrieval of frozen cells

To retrieve frozen stocks of cells, cryotubes were placed into a water bath at 37°C and left until completely thawed, typically 1-2 min. The contents were then transferred drop-wise into 10 mls pre-warmed fresh culture medium. Cells were pelleted by centrifugation at 538 x g for 5 min, resuspended in fresh culture medium and seeded onto tissue culture dishes at the required density.

2.5.8 Transfection in 5 cm dishes

Cells were seeded out at 5 x 10^5 cell per dish and grown to 50-60% confluence in 5 cm dishes. Cells were transfected with 20 µg DNA and Lipofectamine™ 2000 (10µl/µg DNA transfected) (Invitrogen) reagent or Lipofectamine™ LTX (10µl/µg DNA)(Invitrogen) with PLUS reagent (1 µl/µg DNA) in 500 µl Opti-mem® (Invitrogen) according to the manufacturers instructions. After 4 hours, the transfection reaction was stopped by the addition of growth media. At 48 h cells transfections were harvested using the appropriate method.

2.5.9 Harvesting of cells and protein extraction

Cells were harvested from dishes using 0.05% trypsin EDTA as previously described (2.5.5). Following neutralisation of the trypsin, the cells were pelleted by centrifugation at 538 x g for 5 min before being washed in PBS and re-pelleted twice. The pellet was then re-suspended in UTB (8 M Urea, 25 mM Tris-HCL pH 8.0, 0.15 M β-Mercaptoethanol) and incubated on ice for 20 min. The lysate was sonicated for 20 s at 2 watts using microson ultrasonic cell disrupter (Misonix) and the insoluble material removed by high speed centrifugation at 16100 x g for 10 min. The proteins were then analysed using a Bradford assay (2.4.1) before resolution by SDS PAGE (2.4.2).

2.6 Immunocytochemical techniques

2.6.1 Preparation of samples for indirect immunofluoresence

Cells were harvested as described in section (2.5.5), counted on a haemocytometer and re-suspended in normal growth medium at the required cell density, normally 3-5 x 10^4 cells per spot. Aliquots were pipetted onto the spots of sterile microdot multispot glass slides (Hendley Essex) or coverslips (VWR international). These were left for several hours to allow the cells to adhere to the glass and slides and then the slides were covered with the appropriate culture medium. Cells were grown until they reached the required level of confluency, typically 60-70%.

2.6.2 Fixation and permeabilization

A number of different methods were used to fix and permeabilize cells and tissues prior to direct immunofluorescence (IF) staining. To fix in acetone and or methanol, samples were washed twice in ice-cold saline and then immediately immersed in cold (-20°C) acetone, methanol or a 1:1 mixture of both, and left for 10 min. Slides were air dried before storage at -20°C. Alternatively, samples were fixed in 4% (w/v) paraformaldehyde, freshly prepared in

PBS, for a desired length of time prior to permeabilization. Paraformaldehyde-fixed slides were washed twice in PBS and either immersed in cold (-20°C) acetone for 10 min, or incubated in 0.1% (v/v) Triton X-100 (Sigma) in PBS for 5 min at room temperature. Following Triton X-100 treatment, slides were washed in agitated PBS to remove excess detergent, and stored in PBS until use.

2.6.3 Indirect immunofluorescence microscopy

During the staining procedure, slides were kept in a humidified box to prevent evaporation of anti-body solutions. Fixed cells or tissue sections were blocked against non-specific antibody binding by the addition of blocking buffer (20% heat inactivated normal goat serum (HINGS), 0.1% (w/v) Bovine Serum Albumin (BSA) and 0.1% (w/v) sodium azide in PBS) and incubated at room temperature for 1 h. The blocking buffer was then replaced with the required dilution of primary antibody, diluted in blocking buffer, and slides incubated at 37°C overnight. A list of antibodies used for immunofluorescence can be seen in table 5. Excess antibodies were removed by washing the slides for 30 min in two changes of agitated PBS. The appropriate dilution of Alexa labelled anti-species specific antibody conjugates, (Molecular Probes) diluted in blocking buffer, were added to cells and sections. Slides were incubated at 37°C for a further 60 min. Finally, slides were washed for a further 30 min in two changes of PBS and nuclei counterstained with DAPI (4’6-Diamidino-2-phenylindole) (Sigma). Cells and sections were mounted in 80% (v/v) glycerol in PBS containing 2% (w/v) DABCO (1, 4 diazobicyclo-[2, 2, and 2] octane). Alternatively, sections were mounted in Pro-Long anti-fade mountant (Molecular Probes). Immunofluorescence was observed using a Nikon Eclipse E600 microscope and images captured using a Leica DC200 camera and software. The dilutions of the antibodies used in immunofluorescence microscopy are listed in Table 2.4.

Table 2.4 Dilutions for antibodies used in immunofluorescence

Antibody Name	Manufacturer	Species	Dilution
E-cadherin	BD Transduction Laboratories	Mouse	1:200
Filaggrin	Biogenesis	Mouse	1:200
hDlg 2D11	Santa Cruz	Mouse	1:30
BRDU	Becton-Dickinson	Mouse	1:3
Connexin-43	*Gift from Dr Sheila Graham	Mouse	1:200
hScrib	Sigma	Goat	1:500
860	Sigma	Mouse	1:20-40
K5	*Gift from Prof Birgette-lane	Rabbit	1:150
AlexaFluor® 488 Goat anti-mouse IgG	Molecular Probes (Invitrogen)		1:500
AlexaFluor® 488 Goat anti-mouse	Molecular Probes (Invitrogen)		1:500
*****AlexaFluor® 594 Rabbit anti-goat IgG2a	Molecular Probes (Invitrogen)		1:500
AlexaFluor® 594 Goat anti-rabbit IgG	Molecular Probes (Invitrogen)		1:500

2.6.4 Microscopy

Epifluorescence microscopy was performed on a Nikon Eclipse E600 microscope and images were captured using Nikon DXM1200F digital camera.

2.6.5 Immunoprecipitation

Cells were washed with ice cold PBS and then lysed in NP-40 buffer or RIPA buffer and harvested using a cell scraper. Following incubation on ice for 10 min aliquots, the lysates were cleared by centrifugation at 16100 x g for 10 min and an aliquot taken as a control sample. The lysate was mixed with the relevant antibody for 1 h and a 4°C and then (500 µl) of a 50% slurry of protein A/G sepharose was added and rotated at 4°C for 1 h. The beads were washed 5 times with lysis buffer and re-suspended in 30 µl Laemmli buffer.

2.7 Generation and maintenance of HFK cell lines containing HPV 18 genomes

Generation of HFK cell lines was undertaken according to the methods described by Wilson and Laimins (Wilson & Laimins, 2005) unless otherwise stated.

2.7.1 Maintenance of HFKs prior to transfection

Normal primary human foreskin keratinocytes (HFK), derived from neonate foreskins were kindly provided by Dr Joseph Spitzer and arranged under the REC notification RG_06-095 (Roberts). All the primary human foreskin keratinocytes used in this study were isolated by the foreskin tissue by Dr Sally Roberts following the protocol described by Meyers and Laimins (Meyers & Laimins, 1994) or purchased commercially (Clonetics™ Lonza,Walkersville USA). HFKs were cultivated in serum-free keratinocyte growth medium

(SFM, Invitrogen) replaced every 2 days and cells were grown to 80% confluency. All cells were maintained in an incubator with 5% CO_2 at 37°C. Cells were passaged by removal of media followed by washing twice in PBS before addition of 1 ml of 0.05% trypsin/EDTA and incubation at 37°C. After 5-10 min, the trypsin was inactivated by addition of 1 ml trypsin neutralising solution (TNS, Invitrogen 0.25 mg/ml soyabean trypsin inhibitor in Dulbecco's PBS without calcium or magnesium, pH 7.2) before transfer of cells to a 15 ml conical tube. Cells were pelleted by centrifugation at 538 x g for 5 min at room temperature. The supernatant was subsequently removed and the pellet re-suspended in 10 ml SFM and transferred to a 10 cm tissue culture dish or, prior to transfection into a 5 cm dish at the required density.

2.7.2 Freezing of HFKs

Cells were frozen as described in section (2.5.6), with resuspension of HFKs at a density of between 1 and 2 $x10^6$ cells per vial. Freezing media for these cells was made up of SFM supplemented with 10% (vol/vol) FBS and 10% (vol/vol) DMSO or following transfection with HPV18 genomes, in E-medium supplemented with 10% (vol/vol) FBS and 20% (vol/vol) glycerol.

2.7.3 Preparation of E-medium

Following transfection of HFKs with HPV18 genomes, cells were grown in E-medium. To make 2 litres of E-medium the following components were mixed: 1200 ml DMEM HEPES modification; 640 ml Ham's F-12, 20ml of 100 X cocktail (described below); 40 ml of 100 X Penicillin Streptomycin (PAA Laboratories); 100 ml FBS (5% (vol/vol)); 2ml 1000 X Cholera toxin (ICN Biomedical) and 2 ml 1000 X Hydrocortisone (Sigma).

100 X Cocktail

To make 200 ml of 100 X cocktail the following components were mixed: 20 ml 0.18 M Adenine (Sigma, 0.486 g in 15 ml H_20 with addition of 10 m HCL until dissolved, then addition of 5ml H_20); 20 ml 5 mg/ml Insulin (Sigma, 0.1g added to 20 ml 0.1 M HCL); 20 ml 5 mg/ml transferring (Sigma, 0.1g added to 20 ml PBS); 20 ml 2 x 10^{-8} M 3,3',5-Triiodo-L-thyronine (T_3) in PBS (Sigma, 13.6 g added to 100 ml 0.02 M NaOH to make 2 x 10^{-4} M T_3 further diluted in PBS to make 2 x 10^{-8} M). The 100 X cocktail mix was filter sterilised and frozen prior to use.

Following mixing, E-medium was then filter sterilised and kept at 4°C. Prior to use, the E-medium was supplemented with 2 mM L-glutamine and 5 ng/ml epidermal growth factor (EGF, BD Biosciences).

2.7.4 Maintenance and irradiation of J2-3T3 mouse fibroblasts

Following transfection of HFK with HPV 18 genomes, cells were cultivated with a feeder layer of irradiated NIH 3T3 J2 mouse fibroblasts. J2 3T3 cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) HEPES modification (Sigma-Aldrich) supplemented with 10% [vol/vol] new born bovine serum, in 10 cm tissue culture dishes (Iwaki) and were grown to a confluency of no greater than 80% before passaging. J2-3T3 cells were grown to no more than passage 25 and then replaced with earlier J2-3T3 cells. J2-3T3 cells were passaged as described previously (2.5.5) and were frozen in DMEM supplemented with 20% [vol/vol] bovine serum, and 10% [vol/vol] DMSO. When J2-3T3 cells were required as feeder cells, they were harvested and re-suspended in E-medium at 2 x 10^6 cells/ml in a 50 ml tube (Corning). Cells were irradiated using 50 Gray of a Caesium-137 source. Irradiated J2-3T3s were plated out at 2 x 10^6 cells per 10cm dish in E-medium and allowed to settle for at least 2 h before the addition of HFKs. J2-3T3 that had been irradiated and were not required straight away were stored in E-medium at 4°C for up to 3 days.

2.8 Generation of HFK HPV18 genome containing cell lines

2.8.1 Construction of mutant genomes

To generate mutations within the HPV18 genome a number of different primer pairs were used in site-directed mutagenesis (See table 2.5) using the QuikChange Kit (Stratagene) following manufacturer's instructions. The pGEMII-HPV18 plasmid was used as the template DNA and the number of PCR cycles used was between 18 and 25.

Table 2.5 Mutants generated in pGEMII-HPV18 genome expression vector and their sequencing primers

Primer	Direction	Primer Sequence
E6ΔPDZ	F	5' CGA CTC CAA CGA CGC AGA TAA TGA CAA GTA TAA TAT TAA GTA TG 3'
	R	5' CAT ACT TAA TAT TAT ACT TGT CAT TAT CTG CGT CGT TGG AGT CG 3'
E6ΔPKA	F	5' CTC CAA CGA CTC AGA GAA ACA CAA GTA TAA 3'
	R	5' TTA TAC TTG TGT TTC TCT GAG TCG TTG GAG 3'

The complete HPV18 wild type and mutant genomes were sequenced using the primers used in table 2.6, which cover the entire HPV18 genome, to ensure that only the mutations generated by site-directed mutagenesis were changed within the genome.

Table 2.6 Sequencing primers for the total HPV 18 genome

Primer	Direction	Primer Sequence
E6	Forward (5')	5' CC GAA AAC GGT CGG GAC CG 3'
E7	Forward (5')	5' CAC AAC ATA CGT GGG CAC TAT AG 3'
E1 (1)	Forward (5')	5' CCA GAA GGT ACA GAC GGG GAG 3'
E1 (2)	Forward (5')	5' GA CGG GGG CAC AGA GGG C 3'
E1 (3)	Forward (5')	5' GCC CTG TTG CGT TAC AAA TG 3'
E1 (4)	Forward (5')	5' G CAA ACA TTA TAG GCG AGC CC 3'
E1 (3)	Reverse (3')	5' G CAG ACA CCG AAG GAA ACC C 3'
2F	Forward (5')	5' CCA GCA AAG GAT AAT AGA TGG CC 3'
3F	Forward (5')	5' GGC CCT ACA AGG CCT TGC AC 3'
E2F	Forward (5')	5' GGA GAT TGT ATT ATG TAA AGC 3'
4F	Forward (5')	5' GGA ATA CTG ACT GTA ACA TAC C 3'
5F	Forward (5')	5' CCC CTG CCA CAG CAT TCA CAG 3'
L2 (1)	Forward (5')	5' CAC GTC CCC CAG TGG TTA TTG 3'
L2 (2)	Forward (5')	5' CCT GAG TTT CTT ACA CGT CCA TCC 3'
L1 (1)	Forward (5')	5' GCC TGT ATA CAC GGG TCC TG 3'
L1 (2)	Forward (5')	5' CA CAA CGT TTA GTG TGG GCC 3'
L1 (3)	Forward (5')	5' GCA GAT CCT TAT GGG GAT TCC ATG 3'
L1 (4)	Forward (5')	5' G CAG TAT AGC AGA CAT GTT GAG G 3'
End (1)	Forward (5')	5' GCG TGT GCG TGT ACG TGC CAG 3'
End (2)	Forward (5')	5' CAA TTG GCG CGC CTC TTT GG 3'

2.8.2 Preparation of HPV18 genomes for transfection into HFK

To extract the HPV18 genome from the bacterial vector, 10 µg of pGEMII-HPV18 wild type or mutant genomes were digested with *Eco*RI in a total volume of 50 µl. Complete digestion of the bacterial product was verified by running 200 ng of the product on an agarose gel. Following verification, the reaction was heat inactivated at 65°C for 20 min. HPV18 genomes were then re-circularised in a 900 µl total volume ligation reaction mix (with T4 DNA ligase containing 400 U/ml, New England Biolabs,) to encourage self-ligation of the

HPV genomes, and incubated overnight at 16°C. The DNA was then precipitated by the addition of 2 volumes of isopropyl alcohol and 1/5 volumes of 5 M NaCl followed by vortexing before incubation at -20°C overnight. Following centrifugation at 16,100 g for 30 min at 4°C, the supernatant was removed and the pellet washed with 70% ethanol (pre cooled to -20°C), prior to centrifugation at 16,100 g for a further 15 min. The supernatant was subsequently removed and the DNA pellet re-suspended in 12 µl of 1 X TE buffer. The DNA concentrations were then estimated by running a 200ng aliquot on an agarose gel.

2.8.3 Transfection of HFKs with HPV18 genomes

HPV18 genomes were co-transfected into HFKs with *pcDNA*3.1 (Invitrogen), a plasmid which carries the neomycin resistance gene. Control transfections were also carried out in parallel, including *pcDNA*3.1 (Neo) alone with a green fluorescent protein (GFP, Clontech) reporter expression vector and no plasmid vector DNA. For each transfection, 94 µl of SFM was dispensed into a polypropylene falcon tube, followed by the addition of 6 µl FuGene 6 Transfection Reagent (Roche). In a separate falcon tube, 100 µl of SFM, 1 µg of HPV genomic plasmid DNA or control plasmid together with 1 µg of Neomycin resistance plasmid were mixed, prior to the addition of the SFM/FuGene 6 mixture. This was incubated at room temperature for 30 min. HFKs at 50-60% confluency were selected, and the media replaced with SFM media (Invitrogen-Gibco), prior to the addition of the transfection mixture. The dish was rocked gently to mix.

The transfection efficiency of the GFP transfection was established the following day using a Zeiss Axiovert 100 microscope, with GFP-expressing cells typically representing 5% of the total population. Tranfected keratinocytes were then trypsinised and re-plated onto 100 mm tissue culture dishes with 2 x 10^6 irradiated J2-3T3 fibroblasts in the presence of E-medium. Over the next 8 days the HFKs underwent a process of selection in which, on alternate days, the cells were treated with either G418 or fresh irradiated J2-3T3 fibroblasts. The selection

period began with the replacement of E-medium with E-medium supplemented with 100 µg/ml G418 (PAA Laboratories). On the days following selection, 1 x 10^6 irradiated J2-3T3 cells were added. The concentration of G418 was increased to 200 µg/ml for the latter two of the four selection days and the following the last day of G418 treatment the media was replaced with E-medium without selection and 2 x 10^6 irradiated J2-3T3 cells. In the event that cells reached 80% confluency during the selection process, the dish was split equally onto two dishes of irradiated J2-3T3 fibroblasts in the presence of E-medium and the selection process was resumed the following day.

Once colonies reached approximately 2 cm in diameter, routinely observed after 1- weeks post selection, the plates were pooled and transferred onto a single plate with irradiated J2-3T3 cells. Upon reaching 80% confluency, the HFKs were passaged into five, 100 mm tissue culture dishes and once 70-80% confluency was reached, cells from four of the five dishes were taken for stocks and frozen in liquid nitrogen.

2.8.4 Maintenance of HFKs following transfection of HPV18 genomes

HFKs were routinely grown on a feeder layer of 2 x 10^6 irradiated J2-3T3 fibroblasts, in E-medium supplemented with EGF and L-Glutamine prior to use. The media was replaced every two days and the cells grown to a confluency of no greater than 80% prior to passaging. Irradiated J2-3T3 cells were plated out in E-medium at least 2 h, or on the previous day, prior to addition of the HFK. HFKs were passaged by first removing the J2-3T3 feeder layer, using 0.5 mM EDTA in PBS. Once the feeder cells were detached, a transfer pipette was used to aspirate the EDTA and the dish was then washed twice in warmed PBS. HFKs were then removed with 2 ml trypsin/EDTA (0.25% [wt/vol] trypsin/ 1 mm EDTA) and incubation at 37°C for 5-10 min with regular checks. The cells were harvested in 10 ml media and transfer to a 15 ml conical tube. A further 3 ml of media was then used to wash the dish and the HFKs spun by centrifugation at 538 g for 5 min at room temperature. The supernatant was

removed and the cells washed in 10 ml PBS, prior to a further 5 min centrifugation and re-suspension in E-medium. HFKs were routinely seeded out at 2 x 10^5 cells/dish on irradiated J2-3T3 cells. The plates were rocked several times to ensure thorough mixing and the cells incubated at 37°C. The media was replaced every 2 days, with cells taking typically 7 days to reach a confluency of 80%.

2.8.5 Analysis of HFK cell lines harbouring HPV18 genomes

2.8.5.1 HFK cellular proliferation assays

HFKs were plated out at 2 x 10^4 cells/well in a 6 well plate (Iwaki) containing 2 x 10^5 irradiated J2-3T3 cells/well. Cells were harvested at various times, with each time point performed in duplicate. Viable HFKs were counted following removal of the feeder fibroblasts.

2.8.6 Differentiation of HFKs in semi-solid media

2.8.6.1 Preparation of semi-solid medium

Semi-solid medium (1.5% [wt/vol] methylcellulose (Sigma) in E-medium supplemented with 5% [vol/vol] FBS, was prepared by dispensing 6 g of methylcellulose (Sigma) into a Pyrex bottle with a stir bar and autoclaving. 200 ml of E-medium containing no EGF, was subsequently added, and the solution gently swirled before placing in a water bath at 60°C. At regular intervals of approximately 5 min, the solution was gently swirled and after 30 min the bottle was cooled on ice for 2 min. A further 180 ml of E-medium (-EGF) was added to the bottle, before being left to stir overnight at 4°C. The following day, 20 ml of FBS was added and a further 2 h of stirring at 4°C was performed prior to storage of the semi-solid media at 4°C for up to 2 weeks.

2.8.6.2 Suspension of HFK in semi-solid medium

HFKs at a confluency of approximately 80% were harvested following removal of J2-3T3 fibroblasts as described previously (2.8.4). Cells were pooled and re-suspended in E-medium (-EGF) to form a single cell suspension. The cell suspension was then added to a Petri dish containing 25 ml semi-solid media, pre warmed to 37°C, by dispensing the cells, in a volume of 1 ml, to each dish in a drop-wise manner. To ensure an even distribution of the cells, the semi-solid media was mixed extensively before incubation at 37°C.

2.8.6.3 Harvesting of HFKs from semi-solid medium

HFKs were harvested at 24 h or 48 h following suspension in methylcellulose. Using a cell scraper, the semi-solid medium was transferred equally into 4 x 50 ml tubes and the dish washed three times in ice-cold PBS to remove the remaining cells. The 50 ml tubes were filled with PBS and mixed, prior to centrifugation at 537 g for 10 min at 4°C. Following centrifugation, the supernatant was removed to leave 10 ml remaining in each tube and the cells re-suspended in this volume and pooled into 2 tubes. PBS was added to 50 ml and centrifugation step was repeated. Finally the cells were pooled into 1 tube and after centrifugation, were re-suspended in 10 ml PBS, transferred to a 15 ml conical tube, pelleted, then divided into aliquots for DNA and protein analysis.

2.8.7 Organotypic raft cultures

Organotypic raft cultures were prepared as described in (Wilson and Laimins, 2005). Typically, 1.5- 2 x 10^6 HFKs were grown on a collagen plug in a 2 cm tissue containing 2 x 10^6 J2-3T3 fibroblasts. The plugs were allowed to expand until the collage turned yellow (typically after 24-48 h) after which, the collagen plug was carefully lifted and placed on a wire mesh platform in E-medium (-EGF) to create an air/liquid interface (Figure 2.1). The rafts were allowed to grow for 13 days with the media changed with fresh E-medium every 2

days. 5-Bromo-2'-deoxyuridine (BrdU) was added 12-15 h before fixing. The rafts were fixed by flooding the rafts with 4% (w/v) paraformaldehyde in DMEM. Rafts were paraffin-embedded and sections (4 microns) prepared for staining by Propath Ltd.

2.8.7.1 Protein extraction from Raft Cultures

Protein was extracted from raft cultures by peeling of HFKs from the collagen layer followed by homogenisation in 1 ml raft lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1% [vol/vol] NP40 supplemented with protease inhibitors), performed on ice in a glass Dounce homogeniser. Following a 30 min incubation, lysate was transferred with a transfer pipette into a pre-cooled eppendorf tube and underwent centrifugation at 16,100 g for 15 min at 4˚C. The supernatant was subsequently removed and the pellet was solubilised in 250 µl of Laemelli buffer supplemented with 5% [vol/vol] β-Mercaptoethanol, before centrifugation at 16,100 g for 10 min at 4˚C. The supernatant was then heated at 100˚C for 5 min prior to SDS-PAGE and Western blot analysis (as described in sections 2.4.2 and 2.4.3 respectively).

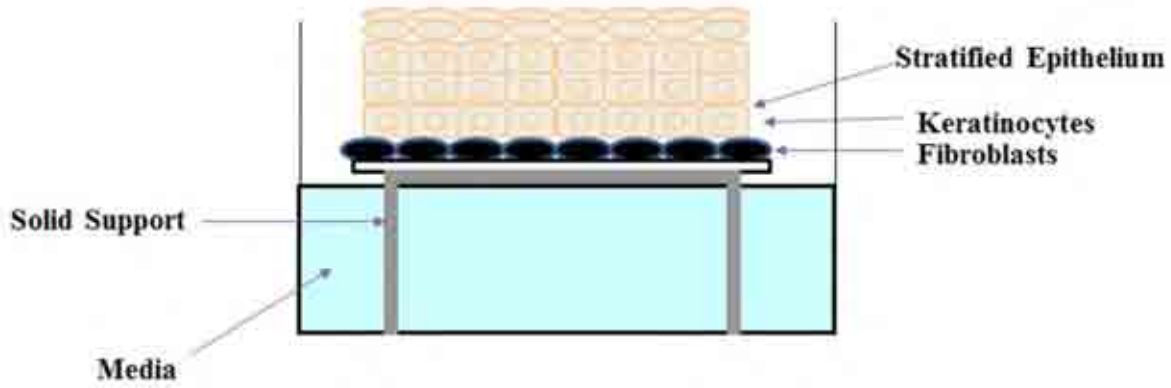

Figure 2.1 Organoytpic Raft Culture Model. Representation of the organotypic raft culture system. HFKs containing HPV genomes were seeded onto collagen plugs containing fibroblasts. When HFKs reached confluence, the collagen plugs were lifted onto stainless steel support grids. HFKs are stimulated to differentiate by the air liquid interface formed and lead to the production of a stratified epithelium as shown above.

2.9 Southern blot analysis

2.9.1 Isolation of genomic DNA

HFKs were harvested following removal of J2 3T3 fibroblasts (2.8.4) or harvested from methylcellulose (2.8.6.3) and pelleted cells were washed once in PBS and resuspended in 3 ml lysis buffer (400 mM NaCl. 10 mM Tris-HCl, 10 mM EDTA, pH 7.4). RNase A was added to a final concentration of 50 µg/ml, cell lysates vortexed, and then incubated for 15 min at room temperature. Proteinase K (Roche) was subsequently added to a final concentration of 50 µg/ml and SDS to 0.2% [wt/vol], prior to vortexing and incubation at 37°C overnight. DNA was sheared by passing the lysate through an 18-gauge needle, up to 10 times. Phenol-chloroform extraction was performed by addition of 6 ml phenol/chloroform/isoamylethanol (25:24:1) (Sigma-Aldrich) prior to centrifugation at room temperature for 5 min at 4838 g. The upper aqueous phase was retained and the extraction repeated a further two times, before extraction of the aqueous phase with chloroform/isoamylethanol (24:1) (Sigma-Aldrich). DNA was then precipitated by the addition of two volumes of 100% ethanol and 1/10th volume of 3 M sodium acetate, and left either at -80°C for 1 h or at -20°C overnight. Following centrifugation at 4°C for 30 min at 4838 g, the supernatant was removed and the DNA pellet washed twice, first in 100% ethanol, then in 70% ethanol before re-suspension in 50-100 µl 1 x TE buffer and incubation at 37°C for 30 min. The DNA yield was determined using a spectrophotometer (Nanodrop™) and samples were stored at 4°C until required.

2.9.2 Preparation of genomic DNA for Southern blot analysis

For analysis of viral episomes, genomic DNA extracted from HFKs was subjected to restriction enzyme digest with *Bgl*II, which has no restriction sites within the HPV18 genome. For analysis of linear genomes, DNA was digested with *Eco*RI which has one site within the

HPV18 genome. *Dpn*I digests of all DNA samples was carried out to remove any residual DNA input. The 5 µg of digested genomic DNA was analysed by electrophoresis in an 0.8% [wt/vol] agarose gel. To enable quantification of genome copy umbers, copy number standards were generated by digestion of pGEMII-HPV18 with *Eco*RI to release the HPV18 genome from the vector and the equivalent 5 and 50 genome copies/cell were run as standards alongside the DNA on the agarose gel.

2.9.3 Capillary transfer of DNA from agarose gel to nylon membrane

Prior to blotting, the agarose gel was washed twice in 250 mM HCL for 20 min at room temperature with shaking, and twice in 0.4M NaOH for 30 min at room temperature with shaking. To set up the transfer of DNA from the agarose gel to the nylon membrane, a tray was filled with 1 L of 400 mM NaOH and a glass plate rested in top of the tray. A 24 x 33 sheet of Whatman™ 3MM paper was soaked in 0.4M NaOH and laid across the glass plate with both ends submerged in the NaOH, thus forming a wick. All the bubbles were removed, followed by three more layers of 24 x 33 Whatman™ paper on top, in the same manner as before. The agarose gel was then placed on top of the paper wick, with the loading wells facing downwards. Bubbles were subsequently removed with a pipette prior to placing a 20 x 22.5 cm sheet of Gene Screen Plus nylon membrane (Perkin Elmer) pre-soaked in NaOH on top, followed by four layers of 21 x 23.5 Whatman™ paper, placed one at a time, with care taken to remove all bubbles. Two stacks of absorbent paper towels, approximately 10 cm in height were placed on top, covering the Whatman™ paper, ensuring that there were no gaps between the stacks. A second, smaller glass plate was placed in top of the towels, and a weight centred on top. SaranWrap was placed between the top and bottom filter paper layers to ensure that they were not in contact, and the gel was left to transfer overnight. Following transfer, the position of the loading wells was marked with a pencil, transfer stack was disassembled, and the DNA auto-cross linked to the membrane using a UV crosslinker

(Stratalinker, Stratagene) on auto-crosslink mode. The membrane was then frozen at - 20°C in SaranWrap, following soaking in 2 x SSC.

2.9.4 Preparation of radiolabelled DNA probe

In preparation for generation of a DNA probe, the pGEMII-HPV18 vector was linearised by *Eco*RI digestion (2.3.5). 50 ng of digested vector was subsequently diluted into 45 µl of 1 x TE buffer and the DNA denatured by heating at 95°C for 5 min followed by incubation on ice for 2 min. The denatured DNA was then used as a template for radiolabelled probe generation using the Ready To Go DNA labelling beads- dCTP kit (Amersham), following manufacturer's instructions. Following re-suspension of the labelling beads with the denatured linear DNA, 50 µCi of [^{32}P] dCTP (Perkin Elmer) was added and the mixture left to incubate at 37°C for 30 min. To purify the labelled probe DNA, the Illustra Probe Quant G-50 microcolumn (Amersham), a radiolabelled probe purification kit, was used following manufacturer's instructions.

2.9.5 Hybridisation of radiolabelled probe to immobilised DNA

To prepare the hybridisation buffer: a 2 x hybridisation solution (5 x SSC (1.5 M sodium citrate, 750 mM NaCl, pH 7.0), 10 x Denharts (0.2% [wt/vol] Ficoll 400, 0.2%[wt/vol] Polyvinylpyrolilone, 0.2% [wt/vol] BSA fraction V (Sigma-Aldrich)) and 20% [wt/vol] Dextran Sulphate, was diluted prior to use 1:1 with formamide (Sigma-Aldrich) and addition of SDS to a final concentration of 0.1% [wt/vol]. 200 µl of (10 mg/ml) salmon sperm DNA (Invitrogen) as denatured by heating at 95°C for 5 min, followed by cooling on ice for 2 min and was subsequently diluted into 10 ml hybridisation buffer. The nylon membrane on which the DNA was immobilised was then rolled and carefully placed into a glass hybridisation canister. The hybridisation buffer containing salmon sperm DNA was then added and the canister placed into a hybridisation oven with rotation at 42°C for 1 h. Following the pre-

hybridisation step, hybridisation buffer containing the radiolabelled DNA probe was prepared by addition of 200 µl of (10 mg/ml) salmon sperm DNA to the probe, followed by boiling for 5 min, cooling and addition to the 10 ml hybridisation buffer as described above. The pre-hybridisation buffer was removed and the hybridisation buffer containing the probe was added and left to rotate overnight at 42°C.

2.9.6 Stringency washes

Following the removal of the membrane from the hybridisation cassette, the membrane was rinsed in buffer 1 (600 mM sodium acetate, 300 mM NaCl pH 7.0, 0.1% (w/v) SDS, using a sponge to wipe the surface of the membrane to clear any excess unbound probe. Two further washes in the same buffer followed, with gentle agitation. The membranes were then washed for two 15 min washes with buffer 2 (150 mM sodium citrate, 75 mM NaCl pH7.0, 0.1 % (w/v) SDS and then 2 x 15 min washes with buffer 3 (30 mM sodium citrate, 15 mM NaCl pH 7.0, 0.1% (w/v) SDS). Following this, the membrane was then washed once for 30 min at 65°C with buffer 4 (30 mM sodium citrate, 15 mM NaCl pH 7.0, 1% (w/v) SDS). The membrane was then wrapped in SaranWrap and exposed to autoradiography film.

2.10 Flow cytometric analysis

For DNA and cell cycle analysis, cell pellets were resuspended in 70% ethanol and fixed at -20°C for 1 h. Cells were then pelleted by centrifugation at 16 100 x g for 10 min and washed twice with phosphate buffered saline (PBS). After the second PBS wash, 1 ml of PBS containing 10 µg/ml of RNase (Sigma Chemicals) was added and the samples were incubated at 37°C for 1 h. The cells were pelleted by centrifugation and resuspended in 500 µl of PBS containing 20 µg/ml of propidium iodide (PI). The relative cellular DNA content of stained cells was measured using an EPICS XL flow cytometer (Coulter Electronics). PI fluorescence was collected by using a 488-nm long-pass filter, followed by a 635-nm-band-

pass filter. Single cells were selected for analysis by using the distribution of PI fluorescence signal integral against PI fluorescence signal peak to discriminate doublets and debris. The relative size of PI fluorescence signal integral (DNA content) in single cells was plotted as a frequency histogram. Equal numbers of cells (20,000) were analysed by fluorescence-activated cell sorting. The proportions of cells in phases G_0/G_1, S and G_2/M were sorted using the Multicycle dedicated cell cycle analysis software (Phoenix Flow Systems).

2.11 G_2/M assay

For G_2/M checkpoint analysis, cell pellets were resuspended in 70% ethanol and fixed at -20°C for 1 h. Cells were then pelleted by centrifugation at 16 100 x g for 10 min and washed twice with 20 ml phosphate buffered saline (PBS). After the second PBS wash, the cells were resuspended in 10 ml ice cold 0.25% Triton x 100 in PBS and left to permeabilise on ice for 15 min. Following permeabilisation, cells were pelleted by centrifugation at 16 100 x g for 5 min and the supernatant removed. Cells were resuspended in 100 µl of 1% BSA in PBS followed by centrifugation of the cells at 16 100 x g for 5 min and removal of the supernatant. Cell pellets were resuspended in 100 µl of 1% BSA containing anti-phospho histone H3 (ser 10) antibody (Cell Signalling) (used as a marker for cells in mitosis) and transferred to a 1.5 ml eppendorf and left to incubate 1-3 hours. Following a primary incubation, cells were washed twice in 1ml 1% BSA/PBS at 16 100 x g for 5 min. After the removal of the supernatant, cells were resuspended in 100 µl 1% BSA/PBS and 1:50 dilution of FITC-anti rabbit antibody and left to incubate for 30 min in the dart at room temperature. Following the secondary incubation, cells were washed once with 1%BSA/PBS and a further wash with 1 x PBS. Cells were stained with PI (25 µg/ml) and 0.1 mg/ml RNAse at room temperature for 30 min prior to analysis on the EPICS XL flow cytometer (Coulter Electronics).

2.12 Effect of PKA inhibitors/activators on keratinocyte cell growth

HFKs containing either wild type or mutant HPV18 genomes were seeded into a 6-well plate containing an irradiated J2-3T3 fibroblast feeder layer at a concentration of $5x10^4$ cells/well. Each cell line was set up in duplicate at each time point for scientific accuracy. Cells were allowed to stratify in these wells for 48 h before the addition of specific activating/inhibiting PKA analogues. Forskolin (FK) and 3-isobutyl-1-methylxanthine (IBMX) were used in combination at concentrations of 50 µM and 1 mM respectively. Dibutryl cAMP (Bt_2cAMP, Calbiochem) was dissolved in Hanks balanced salt solution and diluted in culture medium to a final concentration of 1 mM. The general PKA inhibitor, H89 (Calbiochem) was dissolved in DMSO and added to the culture medium at a final concentration of 10 µM. Cells were harvested at various time points up to 96 h, with each activator/inhibitor performed in duplicate. Viable HFKs were counted following removal of feeder fibroblasts.

2.13 Cell invasion assays

For cell invasion assays, 8 µm polycarbonate transwell membrane inserts were pre prepared by coating the underside of the insert with 100 µl of 2-20 µg/ml collagen and left to set overnight at 4°C. The next day, HFKs were harvested as detailed in section (2.8.4) and 400 µl of HFKs containing wild type or mutant genomes (0.5×10^6/ml) cells in serum free media were inserted into the top of each transwell insert in quadruplicate for each condition (Two total cell number samples and two migration samples). The outside of the transwell was filled with 400 µl serum free media and transwells were incubated at 37°C. After 30 min, "total" control samples were removed and fixed. After 5-8 hours incubation at 37°C, the "migrated" samples were fixed. During the fixing procedure, both sides of the transwell membrane were washed carefully with PBS. For migrated samples only, the cells on the upper surface of the membrane were scraped with a cotton swab to remove any non migrated cells from the inside

surface of the membrane. Both control and migrated transwells were fixed in 4% paraformaldehyde for 10 min and then washed again with PBS. To permeabilise the cells, transwells were subjected to a 0.1% triton x-100 wash for 90 s followed by a DAPI wash for 5 min to stain nuclei. Membranes were cut carefully from the transwells and mounted in 80% (v/v) glycerol in PBS containing 2% (w/v) DABCO (1, 4 diazobicyclo-[2, 2, and 2] octane). 4-5 fields for each transwell were observed using a Nikon Eclipse E600 microscope and images captured using a Leica DC200 camera and software. The percentage of invasion was calculated by normalising the migrated counts to the counts from the control transwells.

2.14 Assessment of HPV E2 gene integrity assay

The integrity of the E2 gene was assessed using sets of specific primers which were designed to amplify the full length of the HPV18 E2 protein. Primer pairs were designed by Dr Constandinou-Williams for another HPV study (Collins *et al*., 2009). Primers were designed to the full length E7 protein as a control in this experiment (Table 2.7). 100 ng of plasmid DNA was mixed with 5 µL of 2.5 pmol primer mix (containing forward and reverse primers), 12.5 µl of 2X GoTaq® reaction mix (Promega) and water to a total volume of 25 µl. The reaction was amplified using a thermal cycler with 1 cycle of 95°C for 5 min followed by 40 cycles of 95°C for 45 sec, 1 min at the annealing temp (55°C for E7 and 53.8°C for E2), 72°C for 2 min followed by 72°C for 10 min. For resolution of DNA fragments, PCR samples were run on a 2% (wt/vol) agarose gel as described at 25mA for 1 hour.

Table 2.7 Primer sequences used in E2 integration assay

Primer	Direction	Primer Sequence
HPV18 E7	F	5’ GTT GAC CTT CTA TGT CAC GA 3’
	R	5’ CAA TTC TGG CTT CAC ACT TA 3’
HPV18 E2	F	5’ TTA GAT GAT GCA ACG ACC AC 3’
	R	5’ CGG TGG GAT ACC ATA CTT TT 3’

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CHAPTER 3 THE FUNCTION OF THE E6 PDZ BINDING MOTIF IN THE EARLY STAGES OF THE HPV18 LIFE CYCLE

3.1 Introduction

3.1.1 The development of expression systems for studying the papillomavirus life cycle

Whilst ectopic over-expression studies have enabled assignment of numerous functions to papillomavirus proteins, they may be inadequate for identification of the complete and genuine functional repertoire of these proteins. The fact that the papillomavirus life cycle strictly requires keratinocyte differentiation has posed a substantial barrier to the study of papillomaviruses in the laboratory. To understand the significance of viral proteins in the context of the HPV life cycle it is therefore desirable to utilise an expression system which permits completion of the viral replication cycle, in which viral proteins are expressed in a timely manner and at physiological levels which most closely resemble events of a true infection.

A number of cell lines have been established which allow HPV genomes to be stably maintained, including the HPV16 W12 cell line (Stanley *et al*., 1989) and the HPV31 CIN612-9E cell line (Bedell *et al*., 1991). Differentiation of these cell lines permitted the study of the viral life cycle within a tissue culture setting, however genetic analysis could not be performed and the functional contribution of the viral proteins could not be elucidated. Other systems were required, in which viral genes could be manipulated, and their effects of the HPV life cycle examined. One of the most reliable systems for studying the replication

cycle of the virus and one that allows the genetic analysis of virus gene function is based on normal primary human foreskin keratinocytes (HFKs) derived from neonatal foreskin as a host cell. These cells can be co-transfected with HPV genomes derived from high risk types together with a drug resistance marker (Frattini *et al*., 1996). Following drug selection, the population of cells harbouring viral genomes is expanded, generating a cell line which stably maintains HPV genomes as extrachromosomal elements. These cell cultures can subsequently be grown as organotypic raft cultures or induced to differentiate following suspension in a semi-solid media composed of methylcellulose, or in the presence of high concentrations of calcium ions. The cells grown in a 'raft' culture are held at the liquid medium-air interface until they stratify, differentiate, and reach the thickness of normal epithelia, forming sheets of tissue virtually indistinguishable from real skin. If the viral DNA is introduced into the keratinocytes before they are placed into the raft culture environment, it carries out its full reproductive program including controlled RNA transcription, DNA replication, and packaging into the capsid proteins, as it would in the human body. Such progeny viruses are then capable of re-infecting fresh skin cells and repeating the entire infection cycle (Myers *et al*., 1997). These systems have been crucial for the understanding of a number of the HPV viral proteins and the regulation of viral gene expression and replication.

3.1.2 Conditional regulation of E6 PBM function

Functions of HPV E6 proteins in human carcinogenesis have been extensively studied; however their roles in the viral life cycle are still poorly understood. In this regard, transfection studies of human keratinocytes with E6 viral proteins from HPV31 and HPV16 have demonstrated a distinct E6 requirement for the long term maintenance of viral episomes (Thomas *et al*., 1999; Park & Androphy, 2002). Similar experiments with HPV31 genomic DNA containing mutations in the E6 PDZ binding domain (E6 PBM) revealed that abrogation

of this domain function was associated with decreased cellular proliferation and a reduction in viral genome copy number (Lee & Laimins, 2004), thus demonstrating that E6 binding to PDZ proteins is necessary for optimal support of viral DNA maintenance. Whether these functions of the E6 PBM in the life cycle are shared amongst other high risk HPV types such as HPV18 remains to be determined and forms the basis of this initial study.

As well as the structure of the E6 PBM facilitating binding to PDZ substrates, phosphorylation of HPV18 E6 by the kinase, protein kinase A (PKA) negatively regulates E6-PDZ interactions. The C terminal motif of HPV18 E6 contains an RXXT consensus sequence for the cyclic AMP-dependent PKA which overlaps with the E6 PBM. *In vitro* binding experiments have shown that HPV18 E6 phosphorylation by PKA reduces its binding to hDlg (a substrate of the PDZ binding motif), and the E6-dependent degradation of this PDZ protein in cells with high PKA levels is inhibited and this is dependent on phosphorylation of the PDZ binding site in E6 (Kuhne *et al*. 2000). Further studies have shown that the Thr156 of the PBM of HPV18 E6 is critical for the binding to PDZ substrates including hDlg, with both neutral and acidic charge mutations abolishing binding (Gardiol *et al*., 1999). These data, support *in vivo* data, whereby induction of PKA leads to the stabilisation of the hDlg protein in HPV positive cervical carcinoma cells, whereas in an HPV negative cell line (HaCaT), hDlg levels are not altered (Kuhne *et al*., 2000). Furthermore, an E6 mutant that is capable of binding hDlg but which is no longer phosphorylated by PKA further demonstrated the involvement of the conserved PKA consensus site in the regulation of the PDZ binding domain of HPV18 E6 (Kuhne *et al*., 2000).

PDZ proteins are localised at the membrane cytoskeleton at sites of cell-cell contact where they have been shown to regulate cell growth, polarity and adhesion in response to cell-cell contact (Fanning & Anderson, 1999). Moreover, studies from *Drosophila* have shown that mutation of the Dlg or Scrib proteins results in hyperproliferation and a loss of cell polarity

(Bilder *et al*., 2000; Woods *et al*., 1996; Goode & Perrimon 1997). Our understanding of E6 PBM-PDZ interactions has largely came from overexpression studies (Lee *et al*., 2000; Nakagawa & Huibregtse, 2000; Pim *et al*., 2000; Thomas *et al*., 2002), therefore the true physiological significance of a regulated, conditional function of the E6 PBM, in the context of an HPV18 infection remains unclear. As such, the contribution of the E6 PBM to the early and late viral life cycle functions of HPV18 was investigated and the significance of this domain during early viral life cycle stages forms the initial basis of this study.

3.2 Results

3.2.1 A molecular signature for malignant potential

As mentioned previously, of those HPV types within the alpha genus implicated as aetiological agents for the development of cervical cancer, all of them express an E6 protein with a four amino acid PBM at the extreme C-terminus. None of the E6 proteins from low risk HPV types have this motif, nor is it present in E6 proteins of beta types linked to the development of skin cancers. A multiple alignment of these E6 protein sequences reveals a strictly conserved PKA consensus recognition motif (PKA-RM) overlapping the PBM with a threonine, or occasionally a serine at position -3, being the putative phospho-acceptor site in protein of HPV types most frequently found in cancer (Group 1, Figure 3.1). In viruses classified as probably, or possibly carcinogenic, a PKA-RM is often present within the extreme C-terminus of E6, however overlap with the PBM is infrequent (Group 2B, Figure 3.1) (Bouvard *et al*., 2009). Significantly, a C-terminal PKA-RM is absent in E6 proteins classified as non-carcinogens (Group 3, Figure 3.1). Overall, this suggests that PKA regulated E6 degradation of PDZ domain containing substrates is only a function of high risk HPV types that have a strong association to carcinogenesis development.

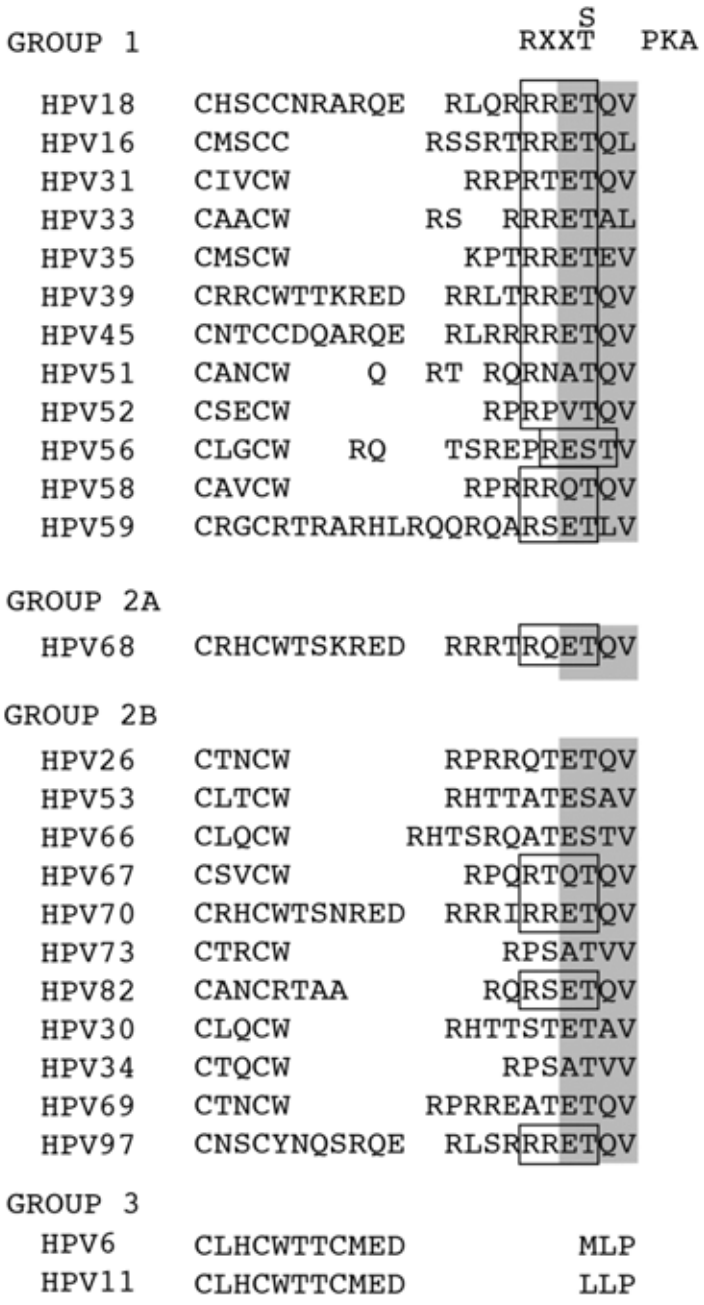


Figure 3.1 Sequence alignment of high risk HPV types. An alignment of HPV sequences from high risk and low risk types revealed that all of the HPV types within the alpha genus recognised as high risk types (Group 1 and 2A and 2B) contain a PBM at the extreme C-terminus. The grey area highlights how conserved this domain is between the high risk typesOf the types frequently found in cancer (Group 1) a PKA consensus motif (RXXT) overlapping the E6 PBM is a conserved feature.

3.2.2 Construction of HPV18 genomes containing mutations within the E6 open reading frame that alter PDZ binding activity

To study the function of the PBM of E6 in the HPV18 life cycle, two mutant viral genomes were constructed by site directed mutagenesis (Chapter 2: Materials and Methods, Section 2.8.1). The plasmid vector pGEMII contains the complete HPV18 genome cloned into the *Eco*RI restriction site at nucleotide position 2440 and was used as a template for site-directed mutagenesis. The mutation referred to as E6ΔPDZ contains three nucleotide substitutions (G^{567}->T, A^{570}->T, C^{571}->G) within the E6 open reading frame (ORF) (pGEMII18-E6ΔPDZ) and substitutes translation termination codons at amino acid positions 155 and 156 of the E6 protein (Figure 3.2). The E6 protein expressed from this genome therefore lacks the extreme four amino acids of the PBM. The second mutant genome generated, E6ΔPKA contains one nucleotide substitution (G^{565}->T, pGEMII18-E6ΔPKA) within the E6 ORF and substitutes amino acid arginine 153 for leucine (Figure 3.2). This mutation disrupts the integrity of the PKA recognition signal (^{153}RRET^{156} -> ^{153}LRET^{156}) that overlaps with the PBM and abrogates recognition of E6 by PKA and thus phosphorylation of the threonine acceptor (Threonine 156) within the PBM (Figure 3.2) (Watson *et al*., 2003).

Bidirectional DNA sequencing of the complete HPV18 genomes confirmed insertion of the nucleotide changes and the absence of mutations outside these nucleotide positions (Chapter 2: Materials and Methods, Section 2.8.1).

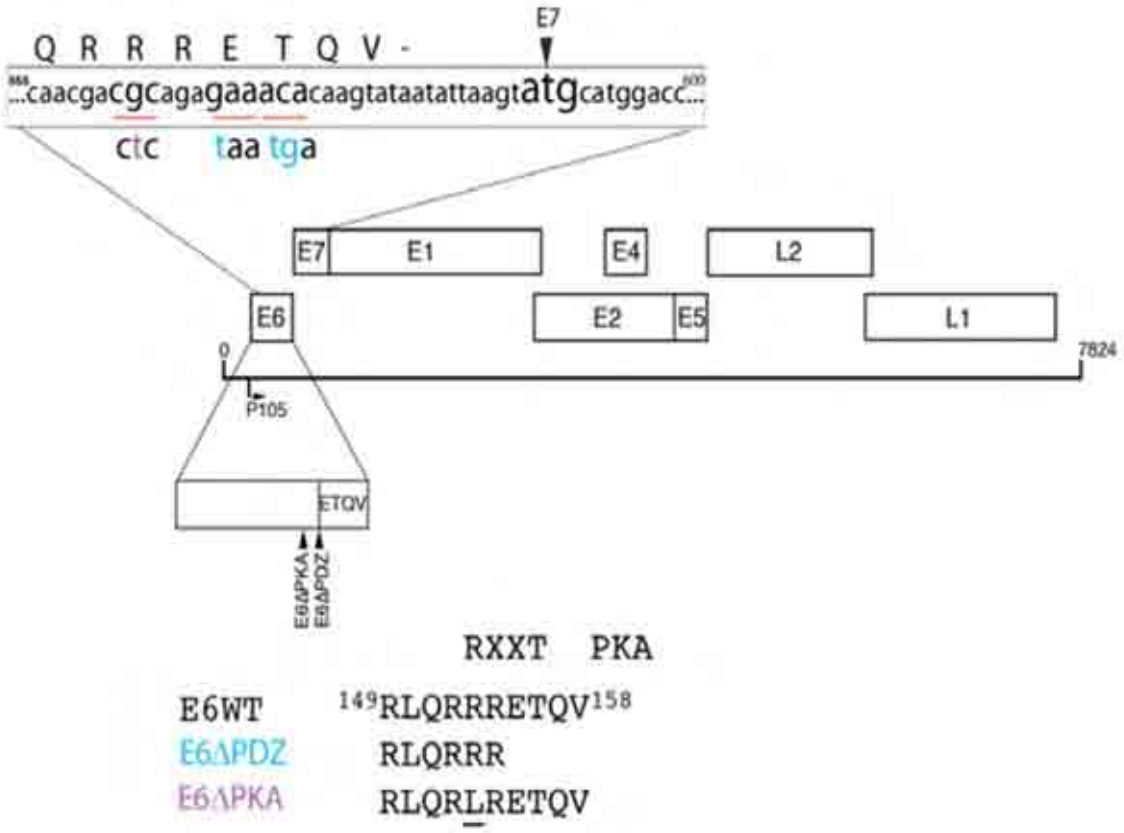


Figure 3.2 Construction of mutants within the E6 ORF. The mutation referred to as E6ΔPDZ (shown in blue) contains three nucleotide substitutions (G^{567}->T, A^{570}->T, C^{571}->G) within the E6 open reading frame (ORF) and substitutes translation termination codons at amino acid positions 155 and 156 of the E6 protein. The second mutant genome generated, E6ΔPKA (shown in purple) contains one nucleotide substitution (G^{565}->T) within the E6 ORF and substitutes amino acid arginine 153 for leucine. Following the insertion of the E6ΔPDZ and E6ΔPKA genomes, total genome sequencing was carried out to check the integrity of the genome.

3.2.2.1 The mutations within the E6 coding sequence do not alter p53 degradation assay

Before characterising the effects of expression of the different E6 protein upon the HPV18 life cycle, it was first necessary to confirm that they were biologically active with regards to functions not mediated by the extreme C-terminal sequences. The most well characterised function of E6 is inactivation of p53 through E6AP-mediated proteasomal degradation. Therefore to assess ability of the E6ΔPDZ and E6ΔPKA mutant proteins to target p53 for degradation, SAOS-2 cells were transfected at 50-60% confluency with equal amounts of *pcDNA* 3.1 based plasmids containing the E6 cDNAs, or an empty vector as a control, together with an equivalent amount of a construct encoding untagged p53 (gift from Lawrence Banks). Cells were harvested 24 hours after transfection and p53 levels determined by western blotting. As shown in figure 3.3, p53 levels in control cells were detected strongly after 24 hours, whereas the levels of p53 were diminished in cell transfected with the wild type or mutant constructs (Figure 3.3), indicating that the E6 proteins are active for p53 degradation.

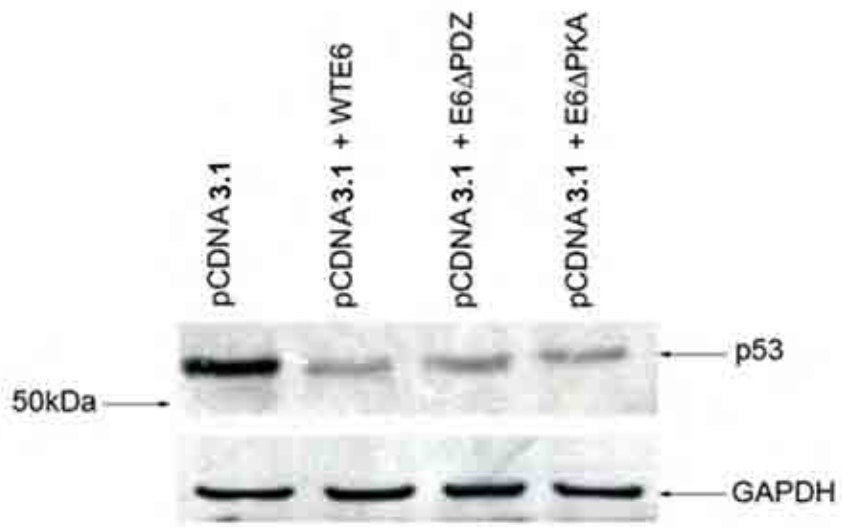


Figure 3.3 Changes made within the PBM do not affect the ability of E6 to degrade p53. To assess the ability of mutants of E6 to carry out known functions of E6 protein, SAOS-2 cells were transfected with 10 µg of pCDNA vector containing E6 wild type, E6ΔPDZ or E6ΔPKA cDNA's together with 10 µg untagged p53. Cells were harvested after 24 h and p53 levels were analysed by western blot analysis with an anti-p53 antibody. GAPDH levels were analysed to control for protein loading.

3.2.3 Stable transfection of primary HFKs with HPV18 genomes

For stable transfection into HFKs, the HPV18 genomes were excised from the pGEMII plasmid by *Eco*RI restriction digestion and the viral genome recircularized in the presence of T4 DNA ligase (Chapter 2: Materials and Methods, Section 2.8.2). Low passage HFKs (between passage (P) 0 and 2 depending on donor used) were co-transfected with each of the mutant viral genomes and a plasmid that expresses the drug resistance marker, neomycin (Chapter2: Materials and Methods, Section 2.8.3). Following a short period of exposure to the drug G418-whereby only cells transfected with the neomycin resistance gene are provided with resistance, drug resistant colonies emerged after 10 to 14 days. These colonies were then pooled and expanded on a feeder layer of γ-irradiated J2-3T3 cells in the absence of G418. Sequencing of extracted DNA from these cell lines with a primer covering the E6 PBM confirmed that the cell lines contained the correct HPV18 genomes.

To control for HFK donor effects, the E6ΔPKA genomes were transfected into HFK isolated from three different donors, whilst E6ΔPDZ was transfected into HFK from four different backgrounds. For each donor used, stable transfections with the wild type genome were also carried out in parallel. Drug-resistant colonies were obtained for each transfection. During the establishment of these lines it was noted that the appearance of drug resistant colonies was delayed in cultures carrying E6ΔPDZ genomes, compared with HPV18 wild type or E6ΔPKA genome containing HFK cultures. The number of colonies also varied between the different mutant lines, with E6ΔPDZ genome containing lines presenting only 2 or 3 large colonies following drug selection, compared to anything between 20 and 30 colonies established with wild type and E6ΔPKA containing donors.

3.2.4 The E6 PBM is important for efficient establishment of viral genomes

To confirm that the transfected HFK cell lines had established episomal copies of HPV18 genomes, DNA was extracted and analysed by Southern blot analysis using a HPV18 genomic probe (Figure 3.4, A/B). Total DNA was isolated from each of the cell lines that had been expanded in monolayer cell culture to passages P3-P5. To maintain these cells in an undifferentiated state, cells were not allowed to reach a 100% confluence and were routinely harvested for DNA extraction when between 70-80% confluent. Equal amounts of DNA was digested with the restriction enzyme *Eco*RI which cuts the HPV18 genome only once and therefore will linearize viral genomes that are replicating as extrachromosomal plasmids. Digestions were also carried out in the presence of *Bgl*II; the HPV18 genome does not contain any restriction sites for *Bgl*II and therefore episomal genomes remain intact and migrate as both supercoiled (SC) and open circular (OC) plasmid DNA in both wild type and E6 mutant cell lines. The restriction enzyme *Dpn*I was added to each digestion, to digest any residual background input plasmid DNA. The HPV18 genomic DNA was labelled by preparing a radiolabelled DNA probe (Chapter 2: Material and Methods, Section 2.9.4) and used to probe the Southern blots. Ethidium bromide stained gels were used to provide an indication of DNA loading in all Southern blots.

As shown in figure 3.4, the wild type genomes in HFKs established successfully with a similar copy number present across all donor lines at between 40- 50 copies per cell. In contrast, whilst E6ΔPDZ genomes established as episomes, the level of replication was reduced compared to wild type genomes, with HFKs carrying E6ΔPDZ genomes establishing at a copy number of between 8 - 19 copies per cell. Similar results were obtained in three independent transfection experiments, using different HFK isolates (Figure 3.4A). The E6ΔPKA genomes established at a similar copy number to wild type genomes (40 - 50 copies

per cell, Figure 3.4B.). This data suggests that the function of the E6 PBM is important for the efficient establishment replication of HPV18 genomes and concurs with a previously reported study investigating the function of the E6 PBM in HPV31 replication in primary keratinocytes (Lee & Laimins, 2004). Moreover, disruption of the PKA-RM had no affect upon establishment replication of episomes suggesting that loss of negative regulation of the E6 PBM function is not important in the establishment of viral episomes in HFKs.

3.2.5 Alteration of E6 PBM function is associated with changes in growth and morphology of cells containing HPV18 genomes

It was noted that during the routine handling of the cell lines there were obvious differences in both the growth characteristics of the cells and in their cellular morphology. Therefore, to assess the contribution of the E6 PBM towards the growth of HPV18 transfected HFKs, cell growth assays were performed with cells containing wild type, E6ΔPDZ and E6ΔPKA genomes. Cells containing wild type or mutant genomes were seeded at low density onto γ-irradiated J2-3T3 fibroblasts in 6-well plates. At various times (routinely 2, 4 and 6 days post seeding), keratinocytes were harvested after removal of feeder cells, counter stained with trypan blue to detect viable cells (Chapter 2: Materials and Methods, Section 2.8.5.1).

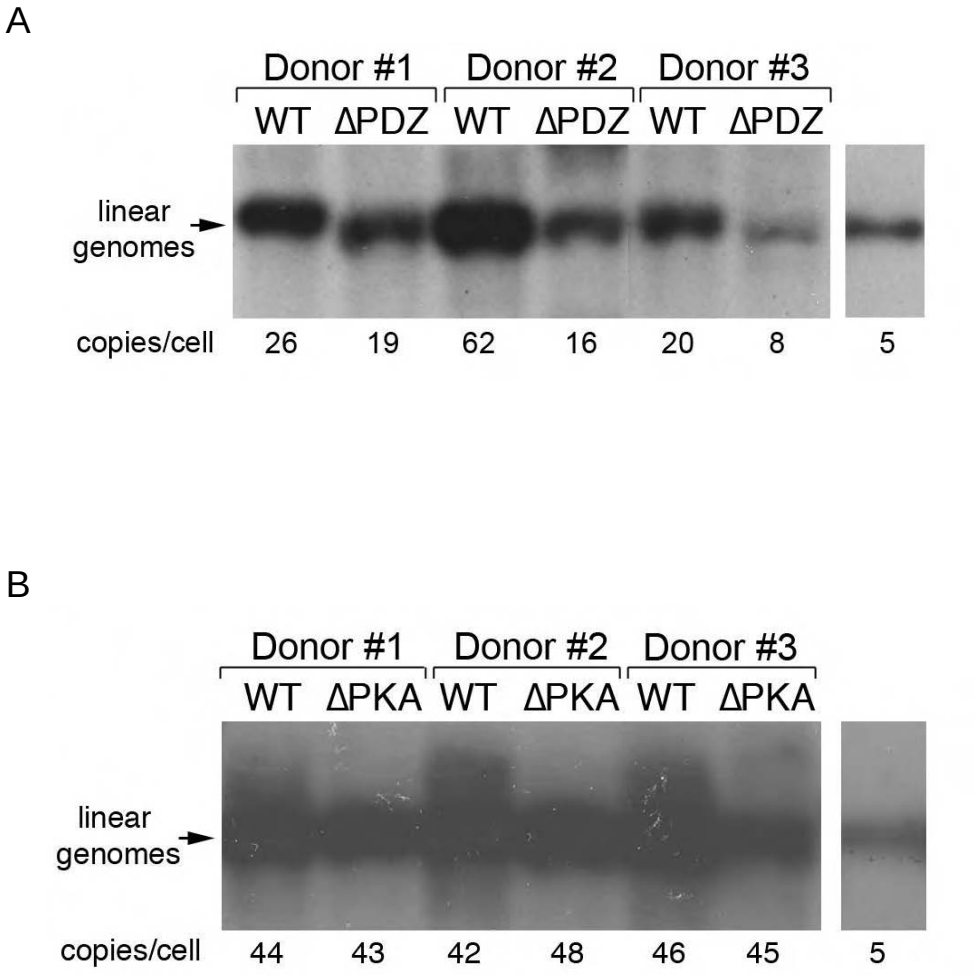
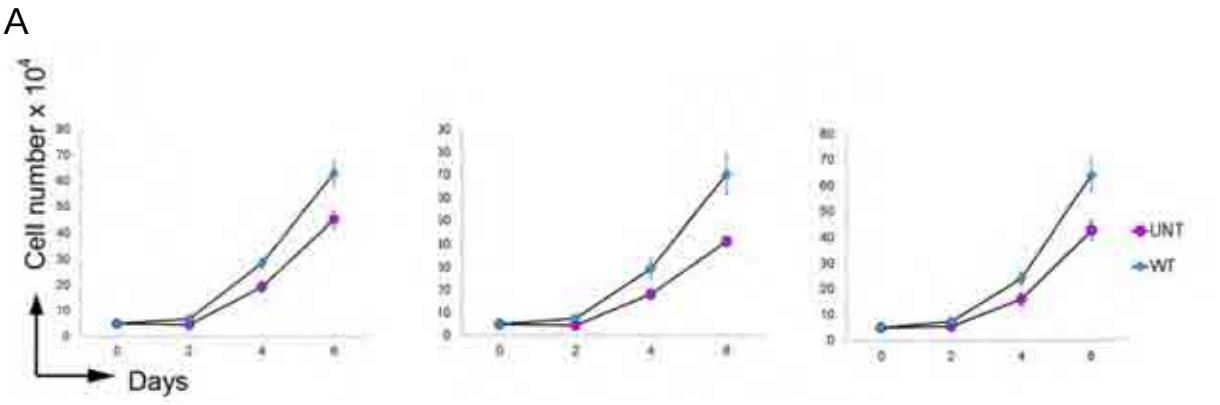
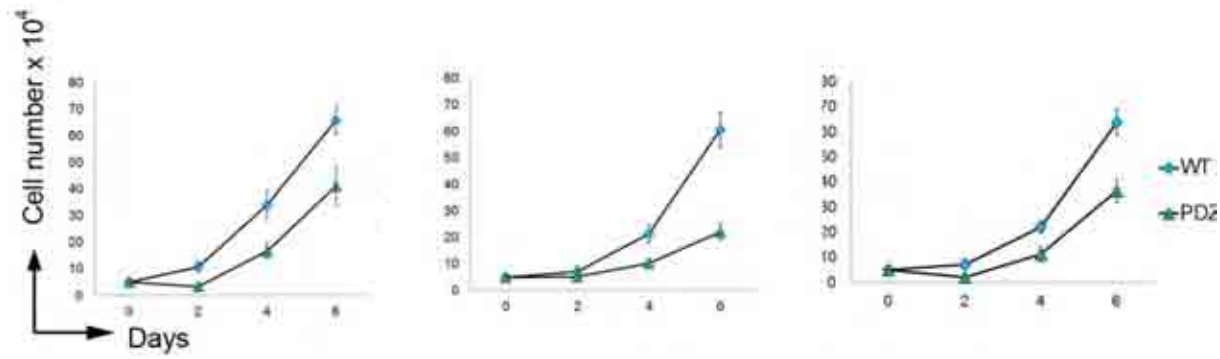
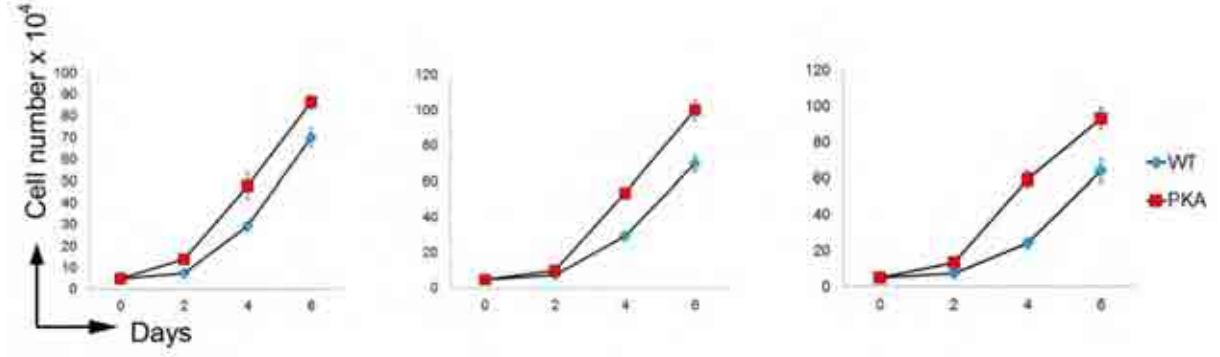


Figure 3.4 Abrogation of the PBM results in a reduction of viral genomes established in HFKs. Total DNA was isolated from 3 donor lines transfected with wild type, E6ΔPDZ or E6ΔPKA genomes and digested with *EcoR1***,** which linearizes the HPV18 genome. The HPV18 genome was also digested with *Dpn*1 to remove residual input DNA and southern blot analysis was performed with HPV18 genomic DNA as a probe. Copy number was calculated from phosphoimaging analysis of autoradiographs compared with a copy number control. Copy numbers are indicated. (**A**) Southern blot analysis of HFKs containing wild type genomes and HFKs containing E6ΔPDZ genomes. (**B**) Southern blot analysis of HFKs containing wild type genomes and HFKs containing E6ΔPKA genomes.

The growth rate of HFK cells was significantly enhanced as expected, following transfection of HPV18 genomes, with un-transfected HFKs growing at a significantly lower rate in comparison with HFKs harbouring wild type HPV18 genomes (Figure 3.5A). HFKs carrying the E6ΔPDZ genomes showed a significant reduction in their growth, when compared to wild type keratinocytes (Figure 3.5B). This result concurs with findings from studies of HPV31, whereby deletion of the PDZ binding motif results in a significant retardation in cellular growth of HPV31 transfected HFKs (Lee & Laimins, 2004). In contrast, cells that contained the E6ΔPKA genomes grew at a faster rate than cells transfected with wild type HPV18 genomes (Figure 3.5C). Taken together, these data suggest that the E6 PBM function facilitates efficient growth of the HPV genome-containing keratinocytes and that the negative regulation of E6 mediated targeting of cellular PDZ proteins by phosphorylation of the E6 PBM has a role to play in controlling the growth of HPV18 genome containing keratinocytes. The growth characteristics of the different cell lines were consistent between all HFK donors. A combined analysis of HFK growth rates from all three donor backgrounds verified the significant change in growth rates between HFKs containing wild type genomes and those containing E6ΔPKA genomes at 6 days post seeding ($<p=0.001$). Furthermore, the difference in growth rates between HFKs containing wild type genomes and those containing E6ΔPDZ genomes was also significant at the same time point ($p=0.01$) (Figure 3.5D).


A
Cell number x 10^4
Days
UNT
WT


B
Cell number x 10^4
Days
WT


C
Cell number x 10^4
Days
WT
PKA

D

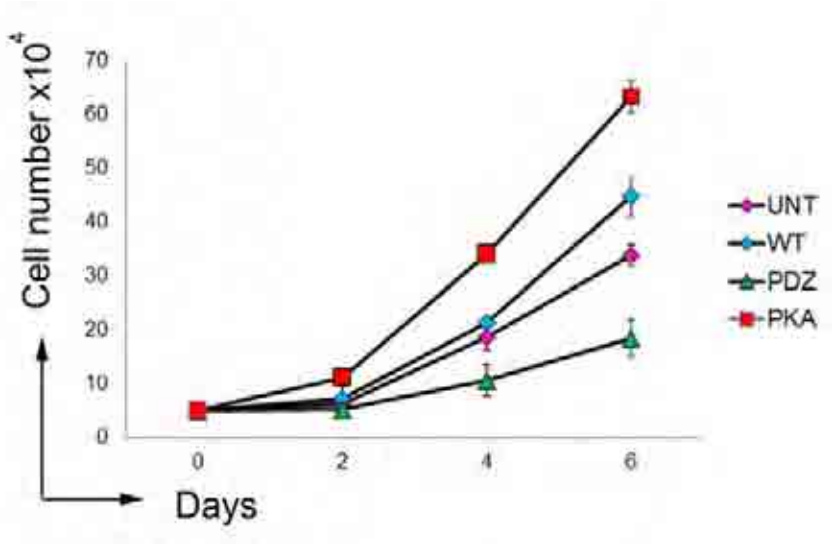


Figure 3.5 Abrogation of the E6 PBM is associated with decreased cell growth, whilst constitutive activation of the PBM is associated with an increase in cell growth. (**A/B/C**) Cellular proliferation profiles of normal HFKs as well as HFKs harbouring wild type, E6ΔPDZ or E6ΔPKA genomes. Cell growth profiles were performed in duplicate in 3 donor lines. Errors bars represent the standard deviation of the experiments shown. Growth profiles are representative of 3 sets of independent experiments. (**D**) Combined analyses of all data. HFKs containing wild type genomes grow significantly faster than HFKs which contain E6ΔPDZ genomes at 6 days ($p=0.01$) and significantly slower than HFKs which contain E6ΔPKA genomes at same time point ($p=0.001$).

A difference in morphology of the cell cultures was also observed. Phase contrast images of the cell colonies were taken when cultures had reached a confluence of between 40 to 50% for further analysis. In monolayer cultures of cells at early passage (P6-P7), cells containing the E6ΔPDZ genomes showed a reduced ability to form colonies consisting of tightly adhered cells with a uniformed cobblestone appearance, compared to the untransfected cells or cells transfected with the wild type or E6ΔPKA genomes (Figure 3.6). Cell cultures of HFKs carrying the wild type genome are characterised by the presence of cells with multiple nuclei and a high frequency of large cells, an observation reported by others (Duensing *et al*., 2000; Duensing *et al*., 2001). Whilst the occurrence of cells with similar appearance is a feature of HFKs carrying the wild type genome and mutant E6ΔPKA genome containing cell lines, there is a significant increase in the number and degree of cellular multinucleation in the presence of E6ΔPDZ genomes (Figure 3.6). This phenotype was consistent across multiple donor cell lines, containing the same mutant genome.

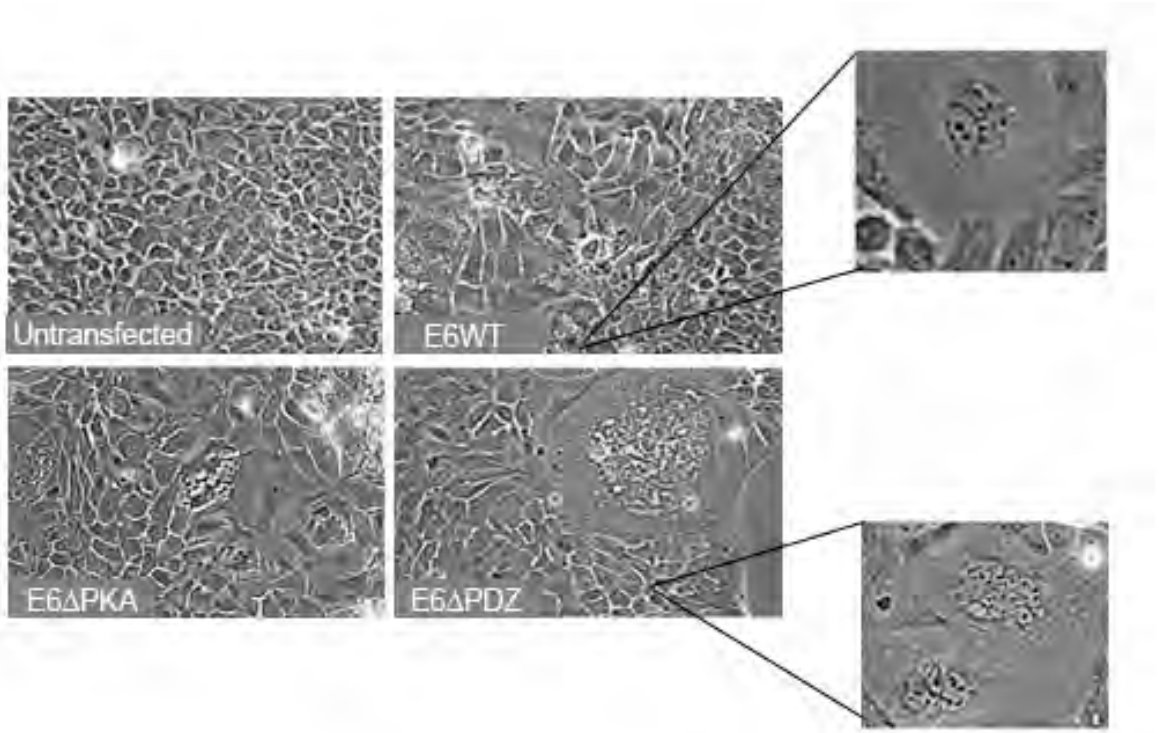


Figure 3.6 Growth of HFKs containing E6ΔPDZ genomes in monolayer cultures is characterised by the presence of large multi-nucleate cells. HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes as well as normal HFKs were grown in monolayer culture. When HFKs reached 50% confluency, phase contrast images were taken using a Nikon Eclipse E600 microscope. Images are representative of phenotypes observed in 3 separate donor backgrounds.

3.2.6 Disruption of the E6 PDZ binding motif induces extensive nuclear abnormalities and changes in cell

Nuclear atypia is an important marker for the diagnosis of cervical neoplasia as well as other malignancies. Nuclear abnormalities can show different degrees of severity beginning with nuclear enlargement and irregular size and shape, but more advance stages frequently include the formation of multiple irregular nuclei. To address whether changes to the PDZ binding activity affect the degree of nuclear atypia seen in monolayer cultures, HFKs containing HPV18 wild type, E6ΔPKA or E6ΔPDZ were grown on coverslips to 80% confluency, fixed in 4% paraformaldehyde (PFA) and stained with DAPI to detect cell nuclei. Cells were then visualised under the microscope for the presence of atypia and any atypic nuclei were marked against a panel of defined nuclear abnormalities including large misshapen, bi-nucleate, multinucleate, aberrant chromosome and aberrant metaphase (Figure 3.7A). Approximately 2000 cells were assessed for nuclear abnormalities from normal HFKs and HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes. The results shown are the individual counts from 1 experimental count across 3 donor backgrounds and the average data from 2 separate counts across 3 donor backgrounds (Figure 3.7, B and C).

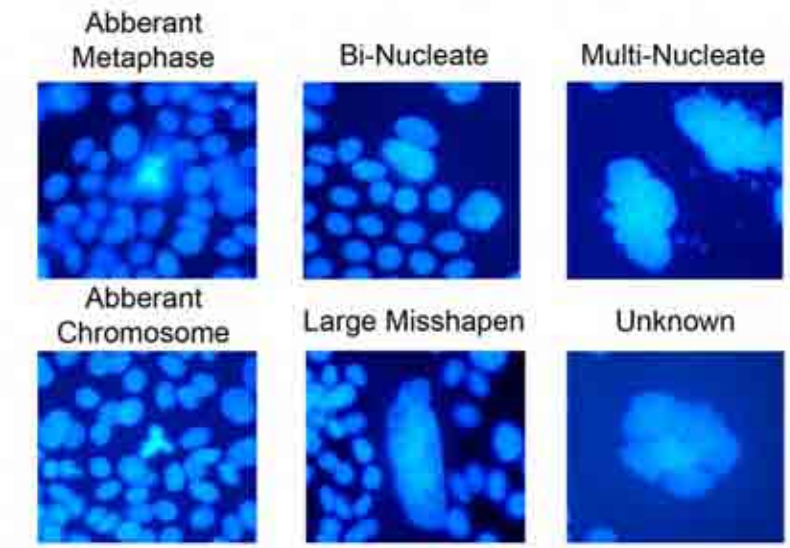
Disturbances of polarity during mitosis including cellular multi-nucleation were observed in cells containing the wild type, E6ΔPDZ and E6ΔPKA genome containing lines. There was an overall increase of 4.5% ($p=0.01$) in the number of atypic mitoses in HPV18 wild type cells compared to normal HFKs. This result is consistent with previous findings from Duensing and others (Duensing *et al*., 2000; Duensing *et al*., 2001). Abrogation of the E6 PBM resulted in a further increase of atypic nuclei (1.8 %) formation compared to wild type cells ($p=0.03$) and nomal HFK cell cultures ($p=0.01$), due mainly to the presence of a marked increase of bi and multinucleate cells in these cultures (Figure 3.7C). Monolayer cultures containing the constitutively active mutant (E6ΔPKA) have less aberrant nuclei than cells containing the

wild type genomes and the atypia present in these cultures is mainly bi nucleate cells (Figure 3.7B). These data together suggests that the enhancement of nuclear atypia in the E6ΔPDZ cells, particularly multinucleation might be the basis for the loss of viral episomes seen in HFKs carrying these mutant E6ΔPDZ genomes.

3.2.7 The E6 PBM is involved in the maintenance of viral copy number

Southern blot analysis of total DNA extracted from the cell lines has already shown that loss of the E6 PBM was associated with reduced establishment replication of the viral genome (replication). To determine whether the PBM of HPV18 E6 had any effect in the stable maintenance of HPV18 episomes, DNA was extracted from HFKs upon increasing passage (up to P15) and Southern blot analysis was performed on the total DNA extracted from these monolayer cell cultures. In HPV18 wild type cells, episomes were maintained at a comparable copy number (40 to 60 copies per cell) (Figure 3.8A). In cells carrying genomes which are constitutively active for binding PDZ substrates (E6ΔPKA), episomes are maintained in a fashion similar to the HPV18 wild type genome containing cells (approximately 40 copies per cell) (Figure 3.8C). However, analysis of episomal maintenance of the E6ΔPDZ genomes showed a reduction in the copy number of viral episomes upon increasing passage (from 21 copies per cell to 5 copies per cell), with significant loss occurring between passages P10 and P15 (Figure 3.8B). An analysis of linear forms of the genome in wild type HFKs and those harbouring E6ΔPDZ genomes confirms the previous findings from figure 3.7A/B (Figure 3.8D). This suggests that the loss of an intact PBM has a deleterious effect on the maintenance replication of viral genomes in primary keratinocytes.

A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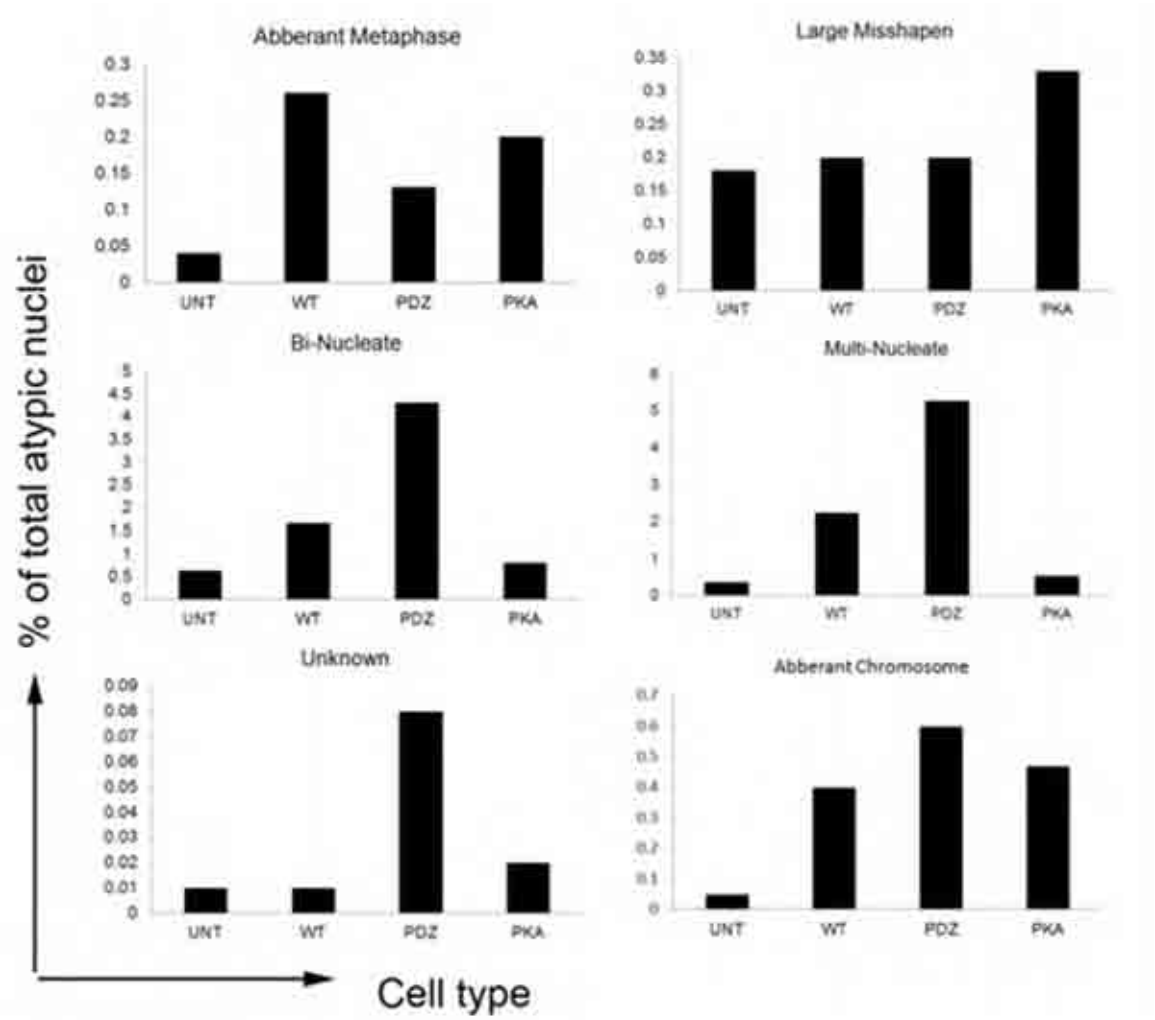
Bi-Nucleate
Multi-Nucleate
Abberant Chromosome
Large Misshapen
Unknown

B


Abberant Metaphase
Large Misshapen
Bi-Nucleate
Multi-Nucleate
Unknown
Abberant Chromosome
UNT
WT
PDZ
PKA
% of total atypic nuclei
Cell type

C

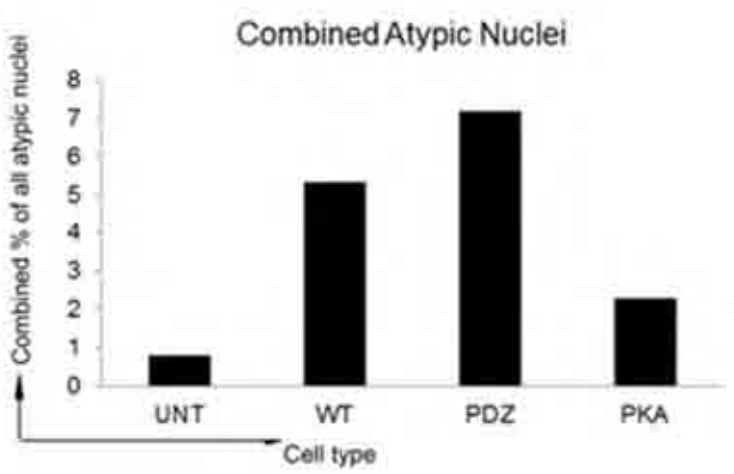
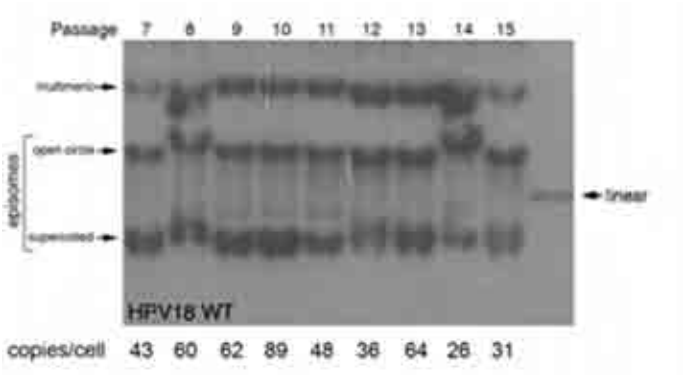


Figure 3.7 Abrogation of the E6 PBM induces extensive nuclear abnormalities. Monolayer cultures of normal HFKs and HFKs containing wild type, E6ΔPDZ and E6ΔPKA genomes were assessed for the presence of atypic mitoses. HFKs containing wild type or mutant genomes were grown over coverslips to 80% confluency, fixed in 4% paraformaldehyde and stained with DAPI (blue) to detect the presence of nuclei. 2000 nuclei were counted from each cell line and marked against a panel of defined nuclear abnormalities. (**A**) Representative images of the panel of defined nuclear abnormalities used to score the nuclei. Any nuclei which could not be defined were marked as 'unknown'. (**B**) Bar graphs represent the data collected from 1 experimental count across 3 donor backgrounds. There is a marked increase in the presence of bi-nucleate and multi nucleate cells in HFKs containing E6ΔPDZ genomes. (**C**) Bar graph represents the data from 2 separate counts across 3 donor backgrounds. Overall there is a significant increase in the percentage of total atypic nuclei in HFKs containing wild type genomes compared to normal HFKs ($p=0.01$). This is further increased in HFKs containing E6ΔPDZ genomes compared with wild type genomes ($p=0.03$).

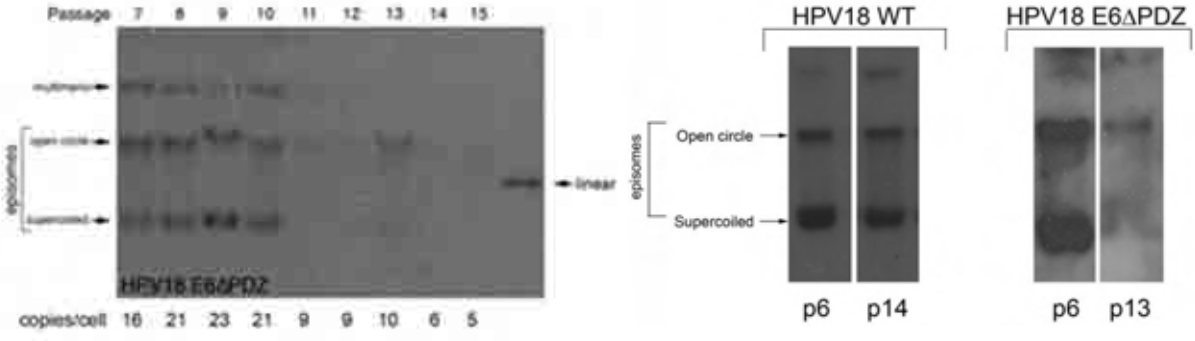
Within the four different donor backgrounds in which this E6ΔPDZ mutant HPV18 genome was established, three showed a consistent reduction in maintenance of the mutant episomes, with much reduced levels by passage P15; however in one keratinocyte host, a reduction of mutant episomes occurred only upon prolonged passaging of cells.

Interestingly, the morphological phenotype of E6ΔPDZ genome-containing cells observed at early passages (P6) was lost at later passages (P15) and the cells revert to a growth pattern of the 'normal' cobblestone phenotype of cultures containing the wild type and E6ΔPKA genome containing HFKs. Analysis of the growth patterns of E6ΔPDZ cells at later passages showed that they had an increased growth rate compared to earlier passages of cells in which maintenance replication of the viral genomes was supported (Figure 3.8E). However, the growth rate of E6ΔPDZ cells at the later passage still did not exceed that of wild type or E6ΔPKA genome containing cells (Figure 3.8F). No changes were observed in the growth rate or phenotype of cultures containing E6ΔPKA and wild type genomes at later passages (Figure 3.7F).

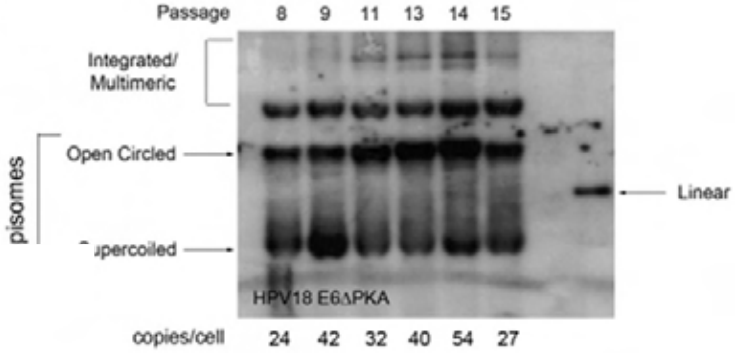
A


Passage 7 8 9 10 11 12 13 14 15
multimeric
open circle
episomes
supercoiled
linear
HPV18 WT
copies/cell 43 60 62 89 48 36 64 26 31

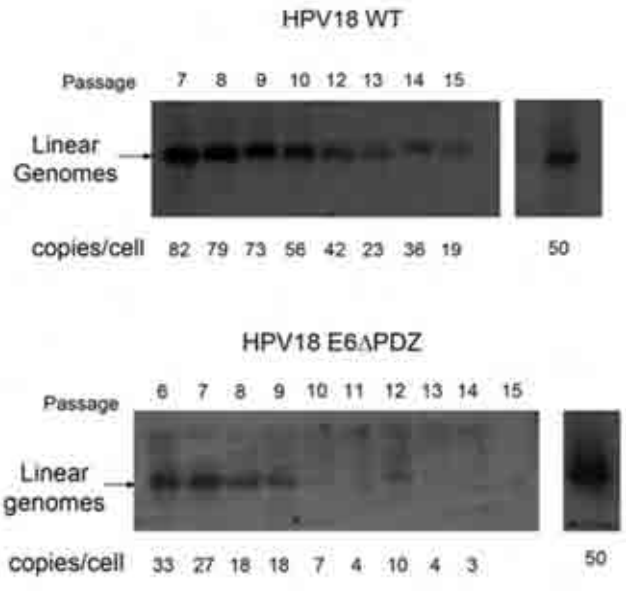
B


Passage 7 8 9 10 11 12 13 14 15
episomes
linear
HPV18 E6ΔPDZ
copies/cell 16 21 23 21 9 9 10 6 5
HPV18 WT
Open circle
Supercoiled
episomes
p6 p14
HPV18 E6ΔPDZ
p6 p13

C


Passage 8 9 11 13 14 15
Integrated/
Multimeric
Open Circled
pisomes
Linear
HPV18 E6ΔPKA
copies/cell 24 42 32 40 54 27

D


HPV18 WT
Passage 7 8 9 10 12 13 14 15
Linear
Genomes
copies/cell 82 79 73 56 42 23 36 19
50
HPV18 E6ΔPDZ
Passage 6 7 8 9 10 11 12 13 14 15
Linear
genomes
copies/cell 33 27 18 18 7 4 10 4 3
50

E

F

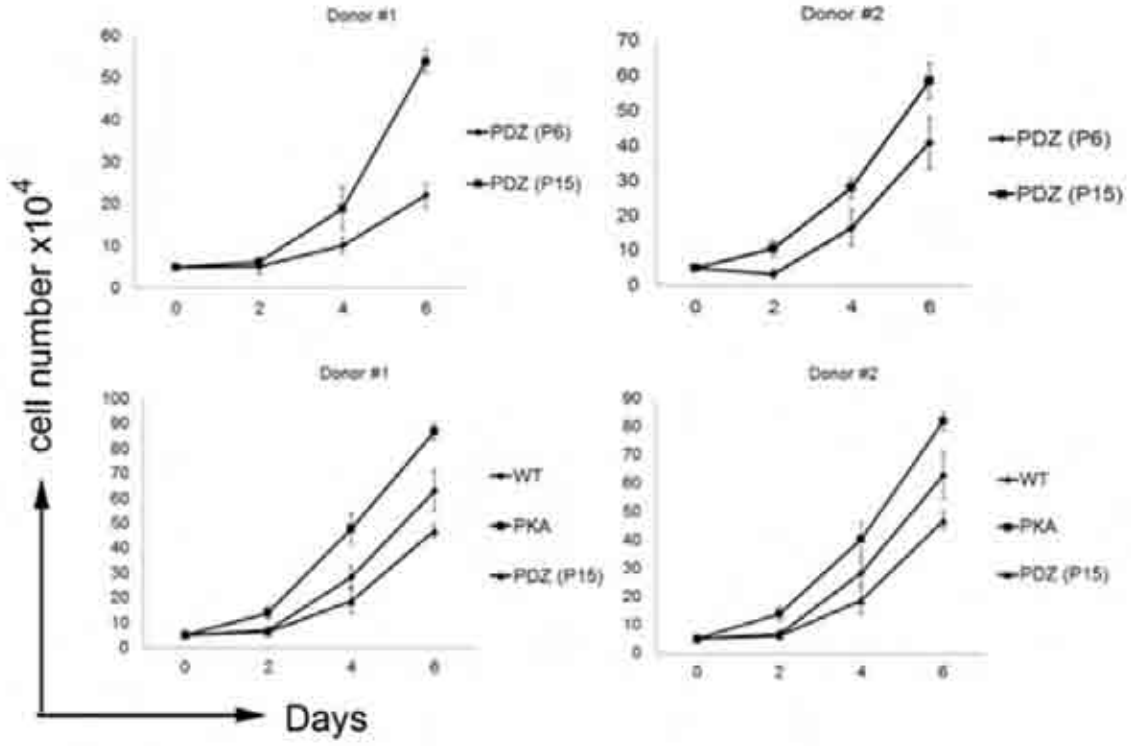
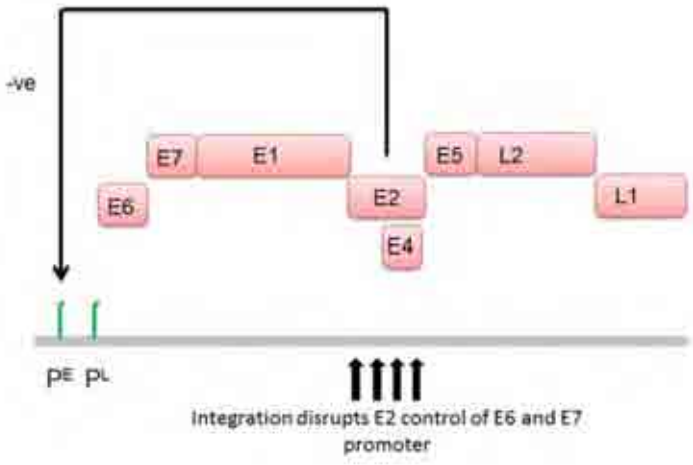


Figure 3.8 HFKs containing E6ΔPDZ genomes are associated with a reduction in viral copy number upon increasing passage. This reduction correlates with an increase in growth rates of HFKs containing these E6ΔPDZ genomes. Total DNA from HFKs harbouring wild type, E6ΔPDZ or E6ΔPKA genomes were extracted at various passages, digested with *BlgII* (a non-cutter of the HPV18 genome) or *Eco*RI (which linearizes the DNA) and examined by southern blot analysis with an HPV18 genomic probe. Genomes migrate as linear, open-circular or supercoiled DNA. Copy number was calculated form phosphoimaging analysis of autoradiographs compared with a copy number control. Southern blots are representative of data derived from 3 different donor backgrounds. (**A**) HFKs containing wild type genomes are stably maintained as episomes upon repeated cell passaging. (**B/D**) Abrogation of the PBM results in a reduction in viral copy number upon repeated cell passaging. (**C)** Constitutive activation of the E6 PBM (E6ΔPKA) does not affect the stable maintenance of upon repeated cell passaging. (**E**) Cellular proliferation of HFKs containing E6ΔPDZ genomes at later passages (P15) is increased compared with HFK containing E6ΔPDZ genomes at early passages (P6). (**F**) Cellular proliferation of HFKs containing E6ΔPDZ genomes at late passages (P15) grows slower than equivalent passage HFKs containing wild type or E6ΔPKA genomes.

3.2.8 The inability of HPV18 E6ΔPDZ to maintain viral genomes is due to viral integration of episomal DNA.

To determine the fate of the HPV18 genome in cells that are unable to maintain episomes, the HPV18 wild type and E6ΔPDZ genome containing HFKs were analysed in a PCR integration assay. This assay uses the principles described for HPV integration into the host chromosomal DNA (Figure 3.8A), which commonly occurs within the *E2* gene (Collins *et al*., 2009). Primers are designed to the E2 ORF, so if there is a disruption of *E2* sequence due to integration of the viral DNA, or loss of HPV episomes, an *E2* band will not be amplified. PCR was also carried out with primers recognising the *E7* gene. The *E7* gene is not lost upon viral DNA integration, so amplification of an *E7* PCR band will highlight the continued presence of the HPV DNA. This method can be used to investigate episome loss (Collins *et al*., 2009). In HPV18 wild type cells, there is no loss of *E2* or *E7* upon increasing passage (P8-P15) indicating that the majority of HPV DNA is in an episomal state (although it is important to note that integration could have occurred in a subset of cells which isn't visible against a background of high episomes) (Figure 3.8B). In early passages of HFK cells harbouring E6ΔPDZ genomes (P8), *E2* and *E7* bands were detected by PCR (Figure 3.8B). By P9 however, the intensity of the *E2* PCR band was beginning to diminish and by P15, the presence of the *E2* was lost completely (Figure 3.8B). The continued presence of an *E7* PCR band within these later passages suggests that E6ΔPDZ DNA is still present within these HFKs albeit in an integrated form and that the genomes are not simply being 'lost' from these cells suggesting that the inability of HFKs containing E6ΔPDZ genomes to maintain viral episomes is due, in some part to viral integration of the DNA in HFKs harbouring E6ΔPDZ genomes. In agreement with these results, a study of HPV31 and HPV16 epsiomes containing E6 mutants which do not bind PDZ substrates showed that these genomes are often found integrated at later passages (Lee & Laimins, 2004; Nicolaides *et al*., 2011).

A



B

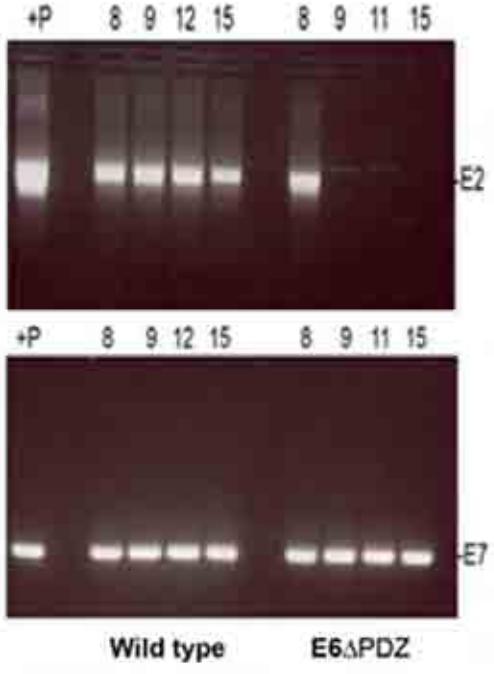


Figure 3.8 Abrogation of the E6 PBM is associated with integration of episomal DNA. HFKs containing wild type and E6ΔPDZ genomes were assessed in a PCR integration assay. (**A**) Graphical representation of the principles underpinning the assay. Integration of the virus results in a disruption of *E2* sequence and so amplification of an *E2* band by PCR would not occur. (**B**) HFKs containing wild type genomes do not integrate their viral genomes as the presence of an in-tact *E2* band by PCR is amplified upon increasing passage of these cells. In HFKs containing E6ΔPDZ genomes, the presence of an *E2* by PCR is lost upon passaging of these cells, suggesting that disruption of the *E2* sequence due to integration has occurred. The presence of an in tact *E7* band by PCR is a control for the sustained presence of viral genomes. This data is representative of 2 independent experiments carried out in 2 donor backgrounds.

3.2.9 Deletion of the PBM or loss of PKA negative regulation does not affect the steady-state stability of the E6 or E7 protein in undifferentiated monolayer cultures

Human papillomaviruses encode the E6 and E7 proteins from a single bicistronic pre mRNA using a common promoter lying immediately upstream of the E6 ORF within the LCR, which is P_{97} in HPV16, P_{99} in HPV31 and P_{105} in HPV18, and a common polyadenylation site (Baker & Calef, 1995; Wang *et al*., 2011). It is feasible that the mutations inserted into the coding sequence of E6 have altered the stability of the E6 protein through the loss of the four amino acids from the C-terminus of the proteins or the arginine to leucine change in the PKA-RM. Also, the mutations in the E6 ORF are close to the ATG initiation methionine of E7 (Figure 3.2) and since E7 is expressed from the bicistronic messenger RNA these changes may affect translation of the E7 protein (Tang *et al*., 2006; Wang *et al*., 2011).

Therefore to examine whether the changes made within the E6 ORF result in changes to the steady state levels of the E6 and E7 proteins, early passage HFKs containing wild type, or mutant (E6ΔPDZ, E6ΔPKA) genomes were grown to 80% confluency, lysed in a buffer containing 8M urea and the solubilized proteins were resolved by SDS-PAGE and subjected to western blot analysis with anti-E6 or anti-E7 monoclonal antibodies as described (Materials and Methods Chapter 2: Section 2.4.3). It is clear from the western blot shown in figure 3.9 that there were no differences in the steady state expression levels of E6, or the E7 protein between HFKs containing wild type or mutant HPV18 genomes (Figure 3.9). These results together suggest that the mutations that have been made to E6 within the context of the HPV18 genome do not affect the steady-state expression levels of the two oncoproteins and it is therefore more likely that the changes observed in the behaviour of the established cell lines in primary keratinocytes relates to the changes in E6 PBM function.

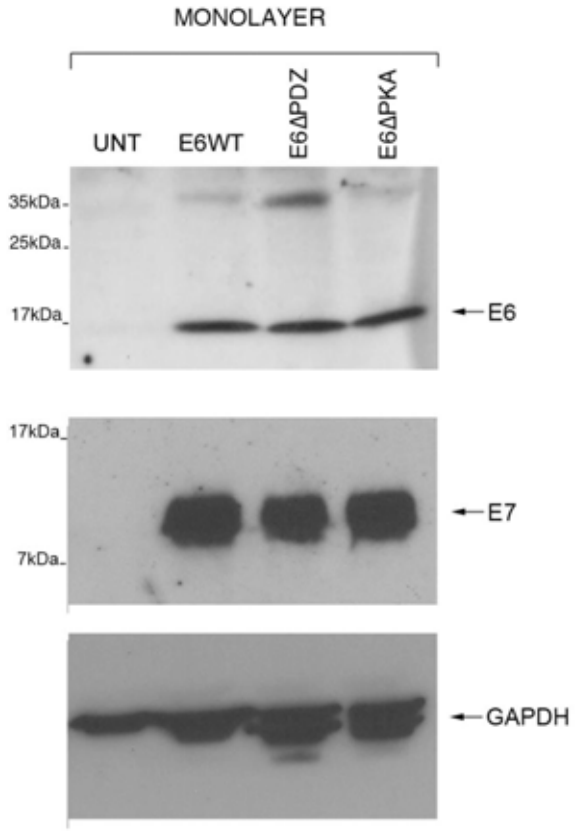


Figure 3.9 Changes to the E6 PBM do not affect the steady state expression of E6 or E7 in monolayer cultures. Monolayer cultures of HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were grown to 80% confluency, harvested and lysed in 8 M urea buffer. Lysates were prepared for western blot analysis with anti-E6 and anti-E7 antibodies. No change was observed in the steady state levels of E6 or E7 expressed from HFKs containing wild type or mutant E6 genomes. GAPDH demonstrates equal loading of protein samples. Western blot is representative of 3 independently performed experiments.

3.3 Discussion

The aim of the initial part of this study was to establish cell lines carrying HPV genomes with mutations that alter the activity of the E6 PBM and characterise the biological function of this domain in the context of cells carrying the complete genome in monolayer cell cultures that represent the early phase of the virus life cycle. By inhibiting and enhancing the interaction of the E6 PBM though genetic changes within the HPV18 genome distinct changes in both the cell morphology and cell behaviour of human keratinocytes was observed. Furthermore, changes in viral DNA replication such as establishment and maintenance replication were also observed. Moreover, these changes appear to be independent of the ability of E6 to target p53 for degradation. Abrogation of the E6 PBM was associated with an increase in nuclear atypia and a reduction in the growth rate of cells and this correlated with a reduction in establishment replication of the mutant genome and in the persistence of episomal DNA. The changes observed were consistent across all donor lines however episome loss occurred much later in one donor line.

Abolition of the E6 PBM in the context of the complete HPV genomes, (E6ΔPDZ), results in a reduction of copy number in the establishment of genomes. This reduction, concurs with the results from parallel studies in HPV31 (Lee & Laimins, 2004) and HPV16 (Nicolaides *et al*., 2011) in which abrogation of the E6 PBM was associated with a reduced copy number upon transfection with viral genomes. Additionally, the level of episomal forms of E6ΔPDZ genomes were reduced and completely lost in some donors after several additional passages; a feature which was not observed in HFKs containing wild type or E6ΔPKA genomes. The inability of E6ΔPDZ genomes to persist in keratinocytes as epsiomes was accompanied by the appearance of cells with integrated copies of the viral genomes suggesting that the function of the E6 PBM is required to maintain episomes. In agreement with these results, similar studies in HPV31 and HPV16 containing E6ΔPDZ mutants showed that these genomes do not

maintain at wild type levels and are found integrated at later passages (Lee & Laimins, 2004; Nicolaides *et al*., 2011). Loss of viral episomes at later passages was not observed in E6ΔPKA cells, indicating that constitutive activity of this domain does not have a detrimental affect on the establishment or maintenance of viral genomes. In this light it is tempting to speculate that if the ability of the E6 PBM to target substrates in inhibited e.g. by PKA phosphorylation, this event might promote integration of the viral genomes and since those genomes that contain a PKA-RM are those that are strongly associated with cancer (e.g. Group 1 Figure 3.1) then changes in PKA signalling could be a risk factor for viral integration which is a risk factor for cervical carcinogenesis.

The results from Nicolaides and colleagues correlate the loss of episomes seen in the E6ΔPDZ line with loss of HPV E6 protein stability, suggesting that PDZ targeting is necessary for the stabilisation of the E6 protein (Nicolaides *et al*., 2011). In this present study, the steady state levels of E6 and E7 proteins were analysed by western blot analysis and no significant changes were observed in the steady state levels of E6 or E7 protein between wild type lines and those harbouring either E6ΔPKA or E6ΔPDZ mutant genomes suggesting that the changes made within the E6 ORF in the context of the complete HPV18 genomes do not compromise the stability of the E6 or E7 proteins in monolayer cultures. These analyses do not consider whether changes to the PBM alter the half life of the E6 or E7 proteins, which is something to be considered and merits further analysis.

It has been proposed that the actions of the E6 and E7 proteins work in synchrony; in a way that loss of one function of one of the proteins increases the action of other functions. HPV E7 initiates DNA synthesis in cells resulting in increased cell growth. By inducing cell survival and delayed apoptosis of cells with DNA damage, via the degradation of p53, E6 allows E7 to sustain its pathological effect. It is unlikely that the changes observed are a response to a defect in E6 mediated p53 degradation as in accordance with (Gardiol *et al*.,

1999), changes to the PBM function by means of the E6ΔPDZ and E6ΔPKA mutations, have no effect on the ability of the E6 protein to target p53 for degradation. One can therefore speculate that the binding of E6 to PDZ proteins may act to create a balance with another activity mediated by E6 and/or E7 and so loss of this interaction (E6ΔPDZ) between E6 and PDZ proteins may result in a reduction of episomes, observed in this study. A recent study by Accardi and colleagues showed that E6 and E7 co-operate in targeting the PDZ protein, Na^+/H^+ exchange regulatory factor (NHERF-1) for degradation (Accardi *et al*., 2011) which shows some precedent for this hypothesis.

In this study, HFKs containing E6ΔPDZ genomes showed a significant reduction in their growth, even when compared to normal HFKs. At later passages, HFKs containing E6ΔPDZ genomes grew significantly faster than cells containing the same genome at earlier passages, but still slower than the wild type genome containing cells. A similar finding was observed by Lee and colleagues in an HPV31 background (Lee & Laimins, 2004). HFKs harbouring E6ΔPDZ genomes appear to integrate into host DNA at later passages. Integration of the viral DNA often provides host cells with a growth advantage due to the loss of E2-mediated inhibition of transcription of E6 and E7, which may explain the changes in growth observed at later passages in these cell lines**.** However, the growth of these cells was still slower than wild type cells suggesting that the function of the PDZ domain does contribute to the enhanced growth potential of genome containing cells.

Compared with wild type and E6ΔPKA genome containing cells, HFKs containing E6ΔPDZ genomes had a reduced ability to form colonies consisting of tightly adhered cells with a uniformed cobblestone appearance in monolayer cultures. In addition this E6ΔPDZ mutation had a tendency to display more nuclear aberrations (in particular bi-nucleate and multi-nucleate cells) when compared with monolayer cultures containing wild type or E6ΔPKA genomes. Centrosome abnormalities in response to HPV E6 expression have been reported

previously and primarily lead to the development of multiple nuclei as well as micronuclei and large multi-lobed nuclei which may have formed as a consequence of a persistent block of cytokinesis (Duensing *et al*., 2001). This may be explained in part by the ability of HPV E6 proteins to relax G_2/M checkpoint control, thereby allowing an increased proportion of cells to enter mitosis.

Targets of the PBM function in a common genetic pathway to regulate apicobasal cell polarity and also play important roles in the control of cell proliferation, survival, differentiation and cell division. The presence of one of these targets in particular, hDlg, at the midbody suggests that hDlg may play a functional role in the process of cytokinesis (Massimi *et al*., 2003; Unno *et al*., 2008). The targeting of this protein by the E6 PBM may be tightly regulated in a way that loss of binding by means of the E6ΔPDZ domain may have a dominant negative effect on cell division and lead to the differences observed in atypic nuclei between wild type and mutant cell lines. These data together suggest that targets of the PBM may behave not only as tumour suppressors but also as oncogenes and that the ultimate outcome is tightly regulated. A recent study by Frese and colleagues revealed that E4-ORF1 acts with hDlg to specifically promote the activation of phosphphatidykinositol 3-kinase (PI3K) in cells, revealing an oncogenic role for this putative tumour suppressor in specific cellular contexts (Frese *et al*., 2003). The changes in hDlg may also explain the differences observed in cell growth as previous studies have shown that overexpression of NE-Dlg (a mammalian homologue of the *Drosophila* Dlg protein) induced growth suppression in cells (Hanada *et al*., 2000).

Abrogating the negative regulation of the PDZ domain by means of the PKA mutation results in a significantly faster growth than normal HFKs and HFKs containing wild type genomes and these cells maintained their episomes upon serial passage. This data suggests that the constitutive targeting of PDZ domain containing proteins leads to an enhancement in cell

growth that cannot be explained by increased levels of E6 and/or E7 oncoprotein, or integration of the viral episomes. Several of the PDZ domain targets of E6 function in some settings as tumour suppressors and negatively regulate cell growth. These include hDlg and hScrib (Ishidate *et al*., 2000; Nagasaka *et al*., 2006). In this respect the constitutive ability of the E6 PBM to bind to PDZ substrates and target them for degradation may explain the changes in growth observed.

CHAPTER 4 THE FUNCTION OF THE E6 PDZ BINDING MOTIF IN THE PRODUCTIVE STAGES OF THE VIRUS LIFE CYCLE AND THE ROLE OF NEGATIVE REGULATION OF THIS MOTIF IN CELL BEHAVIOUR

4.1 Introduction

Previous work from this study has shown that PDZ targeting has a role in the early stages of the virus life cycle, including cell growth control and maintenance of viral episomes and this is sensitive to changes in PKA signalling, suggesting that regulation of this domain by phosphorylation is important in the early stages of the virus life cycle. Understanding the mechanisms which regulate late viral life cycle stages such as differentiation is crucial to the understanding of HPV biology. Previous studies from Lee and colleagues have shown that the E6 PBM is required for HPV31 positive cells to stratify and differentiate (Lee & Laimins, 2004). Furthermore, the function of the E6 PBM has been implicated in cell invasion and anchorage independent growth (Spanos *et al*., 2008) as well as epithelial hyperplasia (Nguyen *et al*., 2003).

As previously discussed, an important feature of E6-PDZ domain interactions is that they are highly defined. E6-PDZ interactions are mediated by a four amino acid motif which can be disabled by point mutations (Gardiol *et al*., 1999; Glausinger *et al*., 2000) or phosphorylation of the threonine within the motif by PKA (Kuhne *et al*., 2000). A number of other HPV proteins have been shown to be phosphorylated *in vitro* and *in vivo,* including E1, E4 and E7 proteins (Ma *et al*., 1999; Doorbar *et al*., 2009; Breitbund *et al*., 1987; Knight *et al*., 2011; Genovese *et al*., 2008). Phosphorylation allows the cell to respond to external stimuli or

intra-cellular control mechanisms. Phosphorylation can lead to a conformational change in the structure of the protein or it may expose or enhance a protein interaction domain, thus allowing protein-protein interactions. The biological activity of a protein can also increase or decrease as a result of phosphorylation by converting a protein into an active or inactive conformation. Furthermore, the activity of protein kinases can themselves be regulated in this manner.

PKA is regulated by fluctuating levels of cyclic AMP within the cell; hence it is a cyclic AMP dependent protein kinase. The outcome of an increase in cAMP can result in an activation or inhibition of cell proliferation and cell cycle progression (Fernandez *et al*., 1995). The catalytic subunit of PKA phosphorylates proteins as the serine and threonine residues and is able to phosphorylate proteins in both the cytoplasm and the nucleus. PKA has previously been shown to be involved in transformation and tumour progression (Cardone *et al*., 2005; Farrow *et al*., 2003; Mantovani *et al*., 2008) as well as proliferation (Robinson *et al*., 2008; Mantovani) and apoptosis (Kim *et al*., 2008; Dohi *et al*., 2007; Paradiso *et al*., 2004) in a number of other cancers including breast and pancreatic cancer.

HPV E6 is a phospho-protein *in vivo* (Kuhne *et al*., 2000) and activation of the PKA pathway leads to an increase in the level of E6 phosphorylation (Kuhne *et al*., 2000). The PKA pathway has been shown to selectively regulate the ability to bind to targets of the PBM of high risk E6 proteins (Kuhne *et al*., 2000). The molecular basis for the differential regulation of the E6 mediated degradation of PDZ substrates such as hDlg is provided by mutation studies which show that there is functional interplay between E6/hDlg binding and PKA phosphorylation due to the overlap of the PKA phosphorylation site with the E6 PBM. What role this conditional regulation plays within the context of the late stages of the virus life cycle remains unclear. Studies from Watson and colleagues in HPV18-E6 expressing SV40 immortalised keratinocytes suggest that the conditional regulation of E6 by PKA contributes

to EMT and the degradation of PDZ proteins including hDlg (Watson *et al*., 2003) and as such work to elucidate the role of PKA regulation of E6 within the context of the HPV18 viral life cycle forms the basis of the second part of this study.

4.2 Results

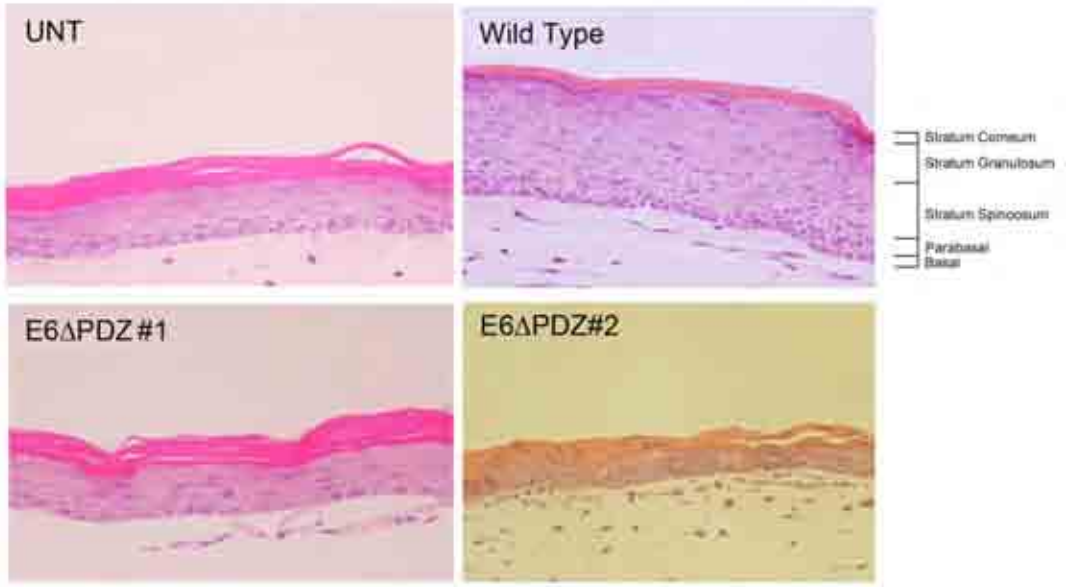
4.2.1 Abrogation of the E6 PBM results in differentiation-dependent morphological changes in organotypic raft cultures

To investigate the effect of changes to the E6 PBM on epithelial stratification and the productive virus life cycle, organotypic raft cultures were grown using primary cells transfected with each of the mutant genomes (E6ΔPKA and E6ΔPDZ) or the wild type genome. Each HPV18 genome containing keratinocyte cell line was grown in organotypic raft culture by seeding the cells onto a collagen matrix embedded with J2-3T3 fibroblasts. The collagen-keratinocyte plug was transferred onto a gridded metal platform when cells reached confluency (typically 1-2 days). Cell stratification was allowed to proceed for 13 days and then fixed with formaldehyde (4%) and embedded in paraffin (Materials and Methods section 2.8.7). For the purposes of morphological examination, representative sections from each donor HFK were stained with haematoxylin and eosin (H and E).

As can be seen from Figure 4.1A, raft cultures of untransfected HFKs were typically 6-7 cell layers thick and exhibited a typical pattern of epidermal differentiation, with each cell layer identifiable; cuboidal basal cells, larger cells of the stratum spinosum, clear evidence of cells of the stratum granulosum with a granular appearance and finally the enucleated cells of the stratum corneum (Figure 4.1A). A similar morphological differentiation pattern is apparent in the rafts formed from the HPV18 wild type cells with the exception that there is significant thickening of the spinous and parabasal cell layers and occurrence of areas where nuclei had been retained throughout all layers of the raft culture (Figure 4.1A). Cross sections of raft

cultures of E6ΔPDZ and E6ΔPKA transfected HFKs revealed striking changes. The overall thickness of raft cultures from E6ΔPDZ genomes was consistently reduced in multiple experiments compared with those formed from primary keratinocytes containing the wild type or E6ΔPKA genomes (Figure 4.1A/B). In addition, the morphology of the raft more closely resembled that of the stratified structures generated from normal keratinocytes than those of cells carrying wild type genomes (Figure 4.1A). Conversely, the morphology of raft cultures of cells containing E6ΔPKA genomes were similar to those formed from cells carrying the wild type genomes, but were generally of greater thickness, a property particularly relevant to the basal and parabasal cell layers (Figure 4.1B). Overall, the morphology of the stratified structures formed from the different HPV18 genomes indicates the function of the E6 PBM is necessary for the hyperproliferative growth of the genome containing cells and concurs with findings from Lambert and co-workers who showed that this domain conferred hyperplasia on keratinocytes in transgenic mice (Nguyen *et al*., 2003). Moreover, the greater thickness of the organotypic rafts formed from E6ΔPKA genome containing cells suggests that a loss of negative regulation of this E6 domain exaggerated the hyperproliferative phenotype. It was also noted that in the E6ΔPKA derived rafts there was evidence of an increase in frequency of areas across the basal cell layer which appeared to be invading into the collagen below (Figure 4.1B). This will be investigated further in section 4.5 of this chapter.

A



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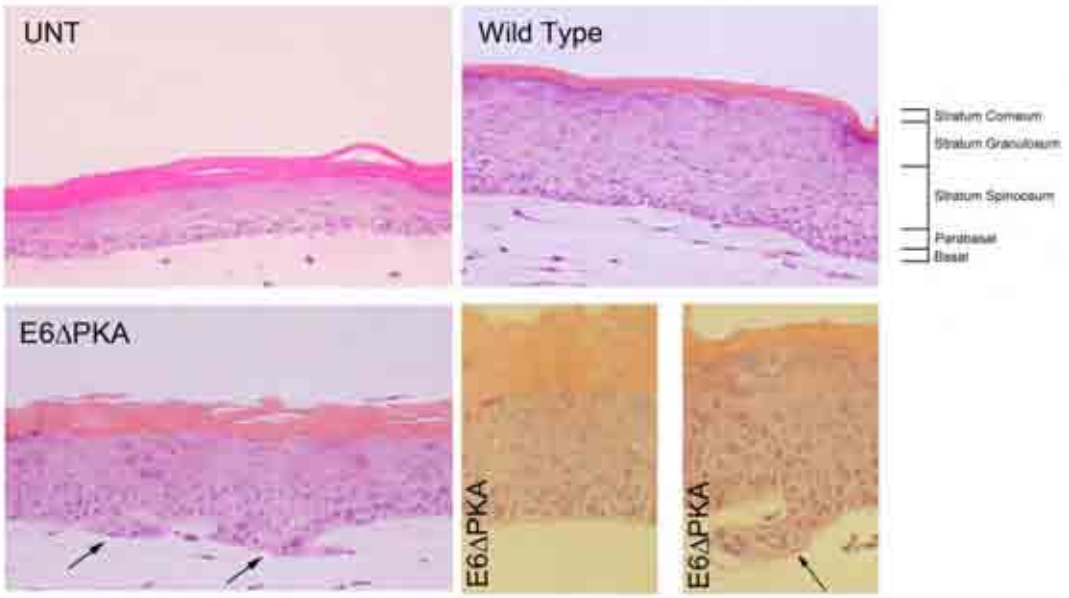


Figure 4.1 Morphology of HPV18 genome containing cells upon stratification. A and B represent Haematoxylin and eosin sections of organotypic raft cultures from untransfected (UNT) HFKs as well as HFKs containing E6ΔPDZ, E6ΔPKA genomes and wild type genomes. The different epithelial layers have been highlighted. Raft cultures were grown for 13 days, harvested and paraformaldehyde fixed. A and B are a representation of the phenotype observed from at least 3 separate donors. Note the areas of invasion seen by HFKs containing E6ΔPKA genomes (**B**) as highlighted by arrows.

4.2.2 Increased suprabasal cellular DNA synthesis upon constitutive activation of the E6 PDZ binding motif

To examine whether changes in the activity of the E6 PDZ binding function affect the vegetative cycle of the virus, cells containing wild type, E6ΔPDZ or E6ΔPKA genomes were stratified in organotypic raft culture as previously described (Materials and Methods section 2.8.7). As previously shown, changes in the activity of the HPV18 E6 PBM are linked to marked changes in the growth of undifferentiated cells (section 3.2.5). Since the virus stimulates infected cells to re-enter S-phase and replicate the host DNA once they have left the basal cell layer, the role of the E6 PDZ binding domain in this viral activity was examined in the first instance. To identify nuclei undergoing cellular DNA synthesis, the organotypic raft cultures were incubated with the thymidine analogue bromodeoxyuridine (BrdU) – a marker for cells in S-phase - 14 hours prior to raft harvesting and paraffin-embedded sections were subsequently stained with an anti-BrdU monoclonal antibody (Beckton-Dickinson).

In organotypic raft cultures generated from normal HFKs, DNA synthesis was largely restricted to the cells of the basal layer. In the presence of HPV18 wild type genomes, BrdU positive nuclei were detected in both the basal compartments and the more differentiated suprabasal epithelial compartments (Figure 4.2A). In rafts derived from the E6ΔPKA genome-containing cells which have a constitutively active PDZ binding domain, there is a significant increase in the number of BrdU positive nuclei in both the basal and suprabasal epithelial compartments compared to the wild type genomes ($p=0.04$) (Figure 4.2 A and B). The E6ΔPDZ genomes, which are deficient for binding to PDZ substrates, were able to induce suprabasal cellular DNA synthesis albeit at a slightly reduced level than the wild type genome, however this difference was not significant ($p=0.80$). Moreover, the number of BrdU positive nuclei in organotypic rafts derived from E6ΔPDZ mutants was significantly lower than the constitutively active PKA mutant (E6ΔPKA) ($p=0.008$) (Figure 4.2A/B).

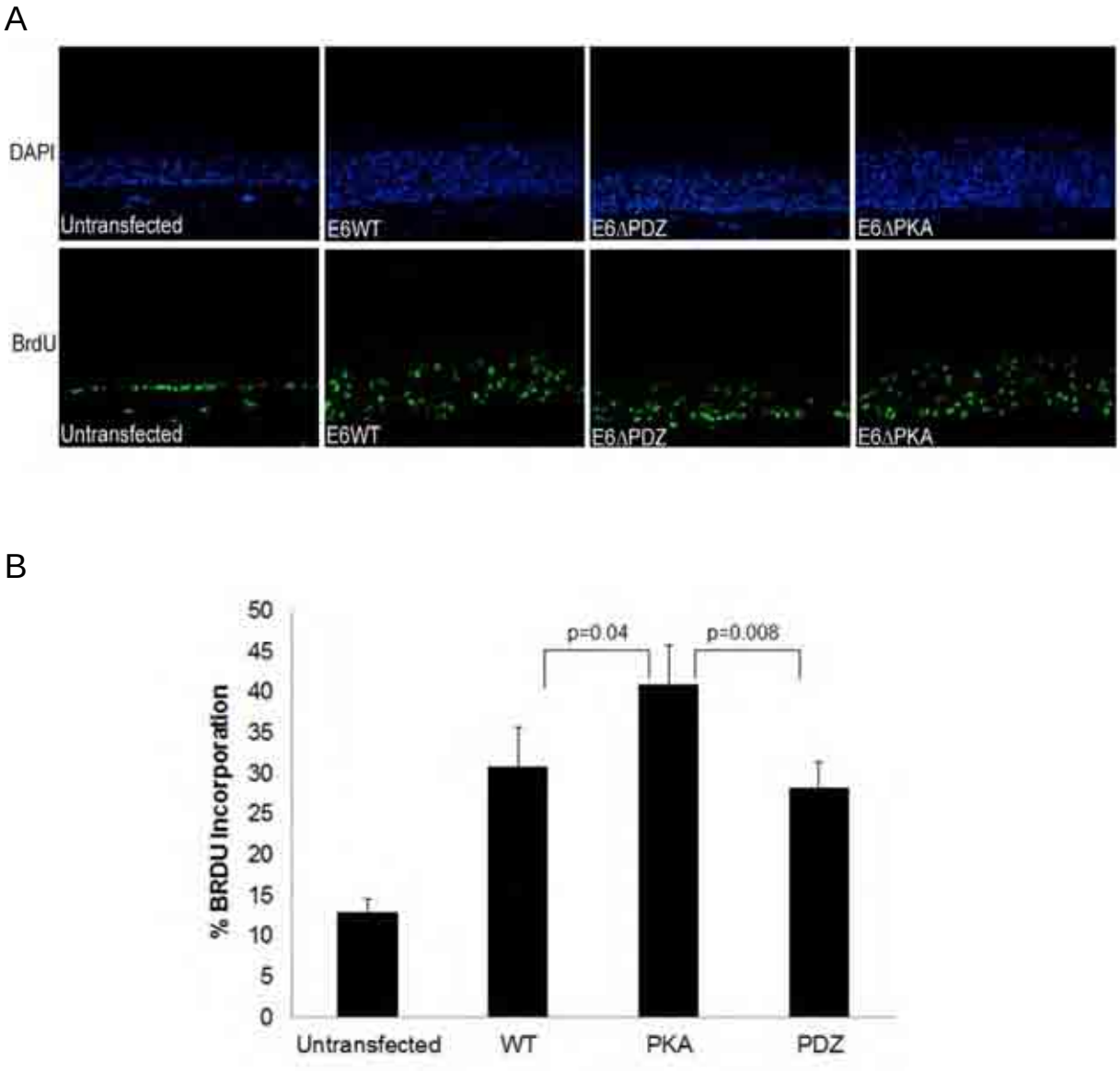


Figure 4.2 Constitutive activity of the PBM results in increased suprabasal DNA synthesis. (A) Paraffin embedded sections of normal HFKs and HFKs containing wild type, E6ΔPDZ and E6ΔPKA genomes were stained with an anti-BrdU antibody (Green) and counterstained with DAPI (Blue) for detection of nuclei by immunofluorescence microscopy. Ten fields of view were taken along each raft section and the total number of nuclei were counted as well as the number of nuclei stained with BrdU to determine a percentage of BrdU incorporation**. (B)** Bar graph of the BrdU incorporation data from 1 donor line. P values were calculated from a one sided T-test performed on normal HFKs and HKS containing wild type, E6ΔPKA and E6ΔPDZ genomes. There is a significant difference in the percentage of BrdU incorporation between wild type cells and E6ΔPKA genome containing cells (p=0.04) and between E6ΔPKA and E6ΔPDZ cells (p=0.008). This data is representative of 3 independently performed experiments in 3 donor lines with similar observations.

These data indicate that although the function of the E6 PBM is not required for induction of suprabasal DNA synthesis, the activity of this domain actively contributes quantitatively to this virally-induced effect.

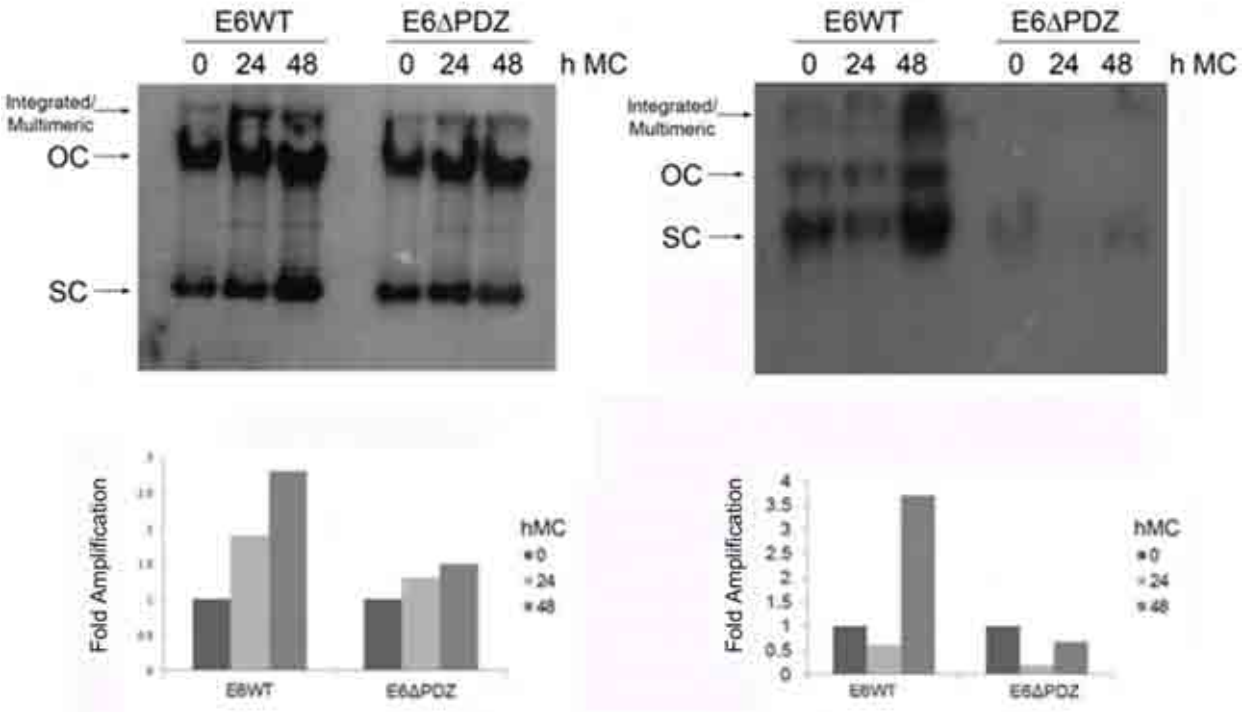
4.2.3 The E6 PBM is required for the differentiation dependent amplification of viral genomes

Suspension of cells containing HPV genomes in a semisolid medium (1.5% methylcellulose) is a well established technique to successfully study the differentiation-dependent HPV late functions, including amplification of the viral genomes. Cells containing wild type, E6ΔPDZ or E6ΔPKA HPV18 genomes were resuspended in 1.5 % methylcellulose for 24 h and 48 h and total DNA extracted from the cells. Equal amounts of the DNA samples were digested with *Dpn*I to digest any residual cellular input DNA and *Bgl*II, a non-cutter of the HPV18 genome, and analysed by Southern blotting with an HPV18 genomic probe. As can be seen in Figure 4.3A, suspension of cells containing the wild type HPV18 genomes induced efficient amplification of the viral genomes at 24 h and 48 h. However, loss of the E6 PBM sequence was associated with poor viral DNA amplification and this was observed in the two separate donors examined (Figure 4.3A). Whilst the wild type genomes amplified by at least 3-3.5 fold by 48 h, the mutant genomes only amplified 0.5 fold. This data indicates that the E6 PBM of HPV18 is necessary for the efficient differentiation-dependent amplification of viral genomes. In addition, organotypic raft cultures of cells containing E6ΔPDZ genomes were compromised for E4 and L1 production, as highlighted by the reduced frequency of E4 or L1 positive cells in raft cultures containing E6ΔPDZ genomes compared with wild type or E6ΔPKA rafts, supporting the idea that the PDZ binding motif is also important for the actiation of late HPV18 life cycle events (data not shown). HPV18 genomes containing the mutation in the PKA-RM were amplified following the induction of differentiation in the two different donor backgrounds examined (Figure 4.3B). The level of amplification was on

average 1.5 and 2 fold at 24 and 48 h respectively (Figure 4.3B) indicating that a loss of cAMP-dependent kinase regulation of the E6 PBM does not affect viral genome amplification and suggests that the role of the E6 PBM in genome amplification is independent of PKA phosphorylation.

In the absence of a functional E6 PBM the level of extrachromosomal episomes decreases upon extended passaging of the cells (Figure. 3.7B; Chapter 3). At these higher passages, the epsiomes are barely detectable following Southern blotting, even upon prolonged exposure of the autoradiographs. Whether these episomes are able to amplify upon differentiation was also examined by suspension of the cells in 1.5% methylcellulose. Minimal differences were observed between fold amplification changes in wild type and E6ΔPKA genomes at higher passages (p=12) with fold changes of 3.2 and 2.6 at 48 h respectively (Figure 4.3C). An equivalent passage of cells transfected with E6ΔPDZ genomes do not amplify the viral DNA even in those donors where low levels of mutant episomes are still detectable, with a fold change of 0.2 at 48 h (Figure 4.3C). Taken together, these results suggest that the E6 PBM plays an important role in the differentiation dependent amplification of HPV18 viral genomes.

A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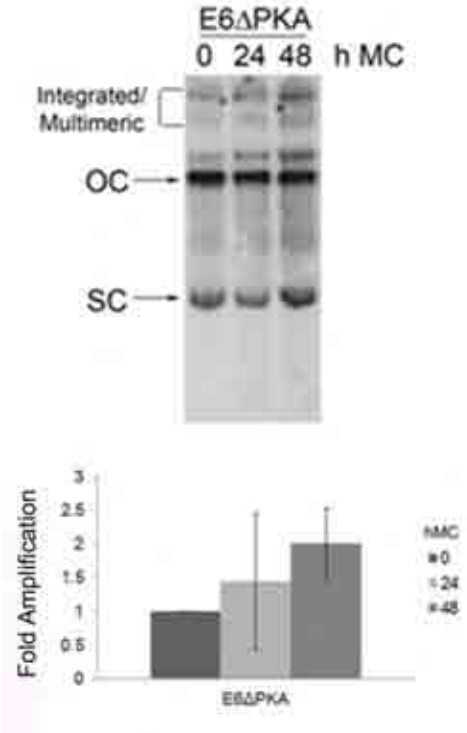
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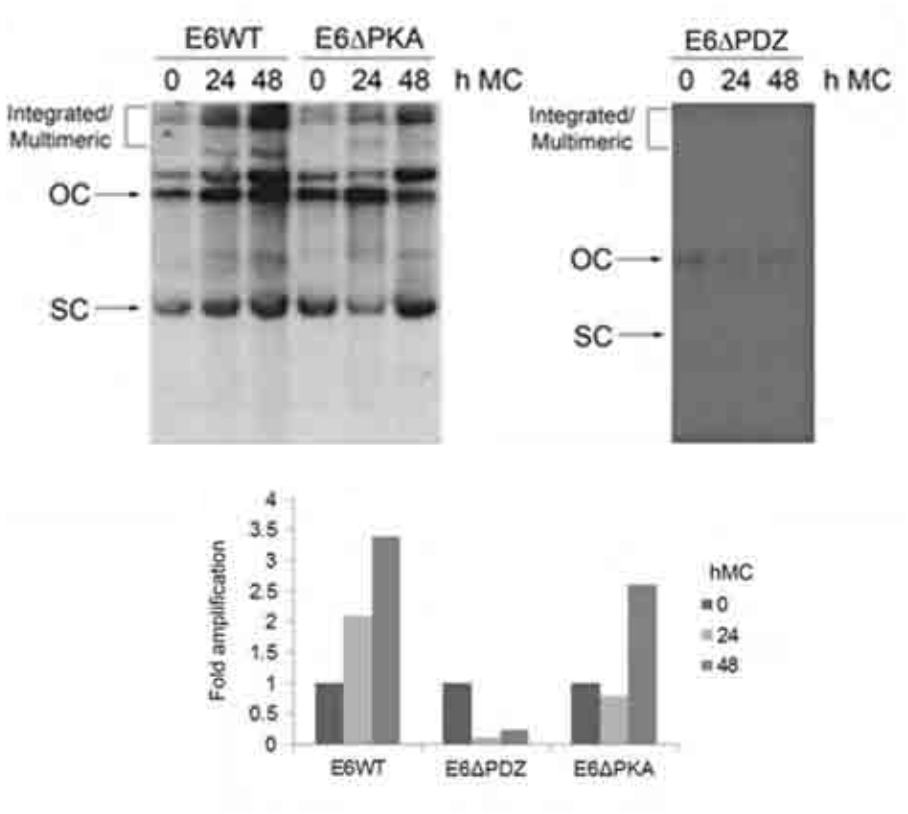


Figure 4.3 Differentiation dependent viral genome amplification of HPV18 genome requires an intact PDZ binding motif. (A) Total DNA was extracted at various times following cellular suspension in semi solid methylcellulose (MC) media of HFKs containing wild type genomes or E6ΔPDZ genomes. Equal amounts of DNA from each cell line were digested with *Dpn*1 and *Blg*II and subjected to Southern blot analysis with an HPV18-specific probe. Bar graphs show the amplification of viral genomes upon differentiation. Data was derived from phosphoimaging data and normalised to monolayer cells at T=0 for each cell line. **(B)** Southern blot analysis of differentiation dependent amplification of E6ΔPKA genomes with bar graph showing phosphoimaging data from 3 separate experiments in 1 donor line. **(C)** Analysis of wild type, E6ΔPKA and E6ΔPDZ genomes at high passage (p=12). Fold amplification changes of HFKs containing wild type and mutant E6ΔPKA genomes were similar; however abrogation of the PDZ binding motif reduces the ability to amplify viral genomes in 1.5% MC. Bar graph showing the phosphoimaging data from experiment C.

4.2.4 Deletion of the E6 PBM or loss of PKA negative regulation does not affect the steady-state stability of the E6 or E7 protein in organotypic raft cultures.

To verify that the phenotypes observed upon stratification of HFKs containing wild type or mutant genomes in organotypic raft cultures was not attributed to changes in the levels of E6 or E7 proteins, organotypic raft cultures of normal HFKs and those containing wild type E6ΔPDZ or E6ΔPKA genomes were grown as previously described (Materials and Methods Chapter 2: Section 2.8.7). Raft cultures were harvested in raft lysis buffer and solubilised in lamelli buffer, then equal amounts of the lysate were analysed by western blot analysis with an anti-E6 and anti-E7 antibody. GAPDH antibody was also used to ensure equal protein loading. No differences were observed in the protein expression levels of E6 or E7 in raft cultures from wild type genomes and those containing E6ΔPDZ or E6ΔPKA mutant genomes (Figure 4.4). These results suggest that the differences in phenotypes observed upon stratification of wild type and mutant genome containing HFKs in organotypic raft cultures is not attributed to changes in the expression levels or stability of the E6 or E7 protein and is more likely that the changes observed in organotypic raft cultures between wild type and mutant containing cells relates to the changes in the E6 PBM function.

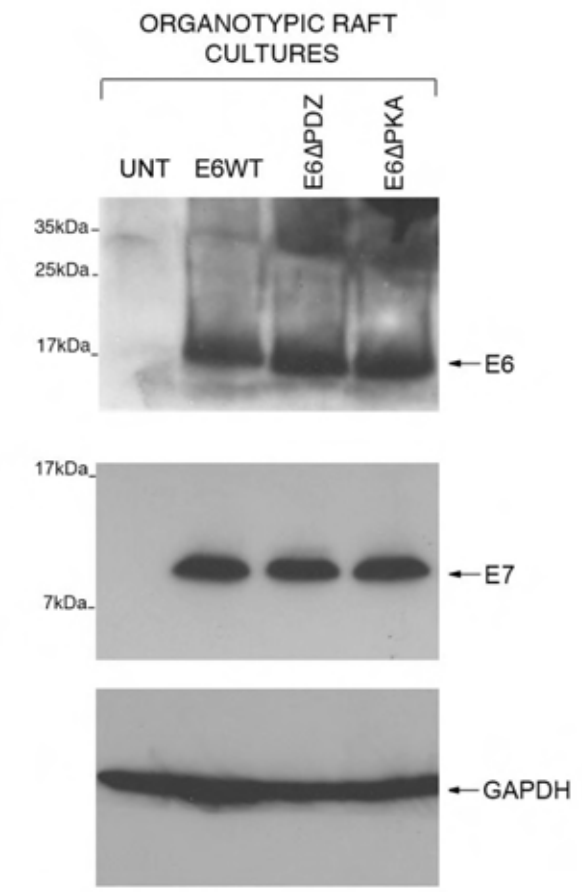
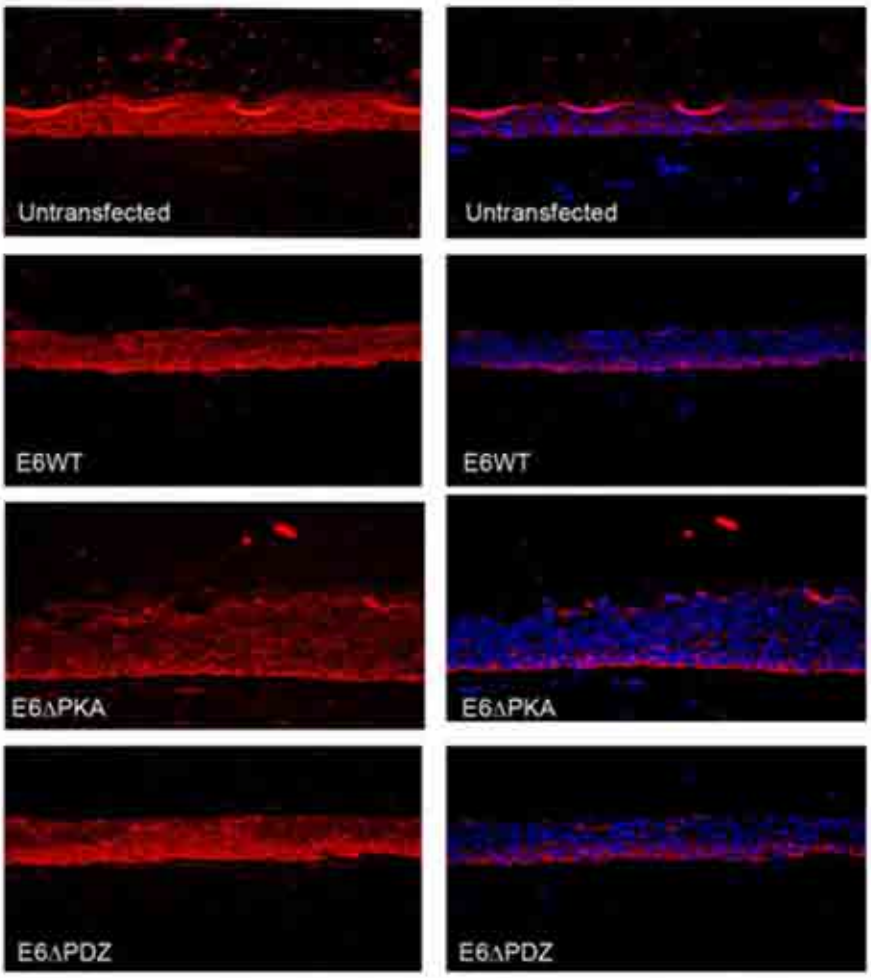


Figure 4.4 Changes to the E6 PBM do not affect the steady state expression of E6 or E7 in organotypic raft cultures. Following differentiation of HFKs harbouring wild type, E6ΔPDZ or E6ΔPKA genomes in organotypic raft cultures cellular lysates were prepared for western blot analysis with anti-E6 and anti-E7 antibodies. No change was observed in the steady state levels of E6 or E7 expressed from HFKs containing wild type or mutant E6 genomes in organotypic raft cultures. GAPDH demonstrates equal loading of protein samples. Western blot is representative of 3 independently performed experiments.

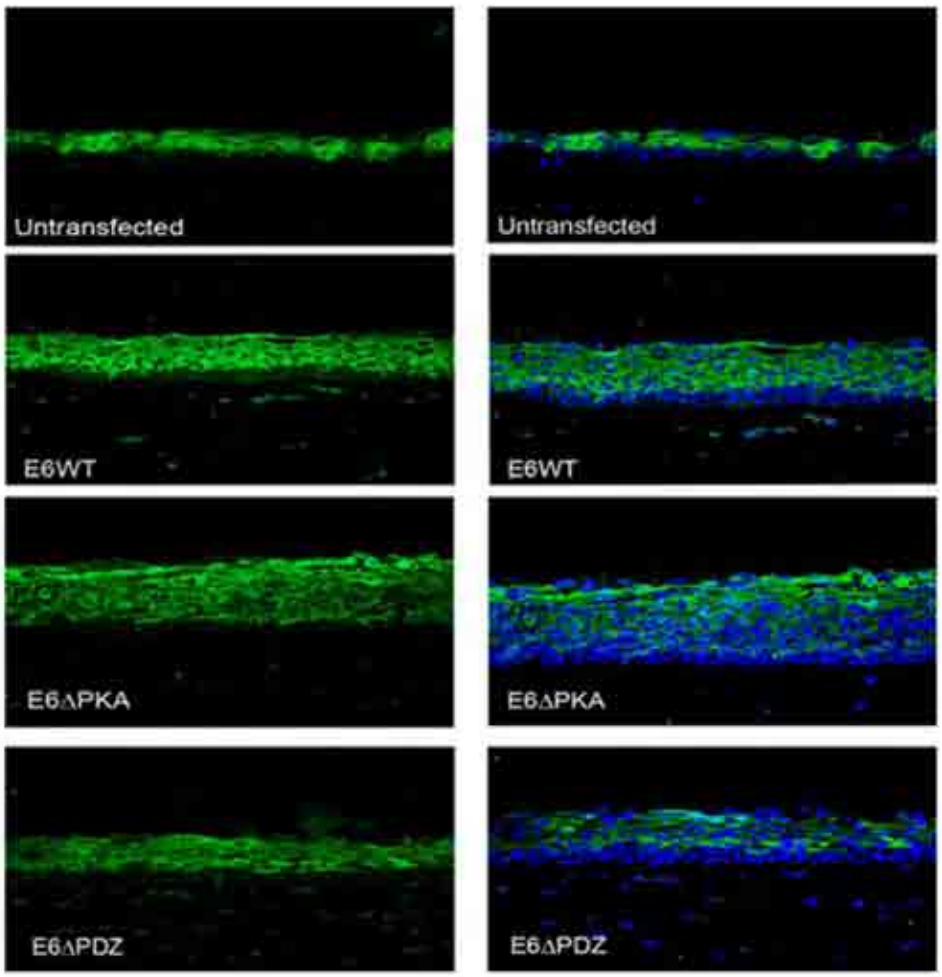
4.2.5 The E6 PBM function does not play a role in the expression of markers of differentiation in organotypic raft cultures.

Amplification of HPV DNA to thousands of copies per cell occurs in the suprabasal cells of the differentiating epithelium and is essential for the production of new virions. Since abrogation of the E6 PBM resulted in a reduced amplification of viral genomes in HFK containing E6ΔPDZ genomes, the ability of HFKs containing wild type genomes as well as those containing E6ΔPDZ and E6ΔPKA genomes to expresses a number of markers of differentiation was analysed by immunofluorescence staining of paraffin embedded sections from organotypic rafts (Materials and Methods section 2.6.3). Organotypic rafts of normal HFKs as well as HFKs containing wild type, E6ΔPDZ and ΔE6PKA genomes were stained for the expression of K5, K1 and filaggrin. K5 is an early differentiation marker and localised to the basal cell layers. The keratin marker K1 represents cells in the suprabasal layers of cells which have left the basal cell layer and have undergone the commitment to terminally differentiate. Filaggrin expression is contained to the granular layer where it is thought to be necessary for the cross linking of keratins that occur in cellular cornification. Normal HFKs, as well as those containing wild type genomes showed a similar pattern of staining for the differentiation markers analysed, with each marker being expressed in the appropriate epidermal compartment (Figure 4.5A-C). Moreover, expression of K5, K1 or filaggrin was not compromised in organotypic raft cultures of HFKs containing either E6ΔPDZ or E6ΔPKA genomes. In addition, organotypic rafts containing E6ΔPKA or E6ΔPDZ genomes showed a staining pattern similar to that of wild type and normal HFKs. Taken together these data suggest that changes to the E6 PBM do not effect the expression of markers of differentiation in organotypic raft cultures.

A K5 distribution



B K1 distribution



C Filaggrin distribution

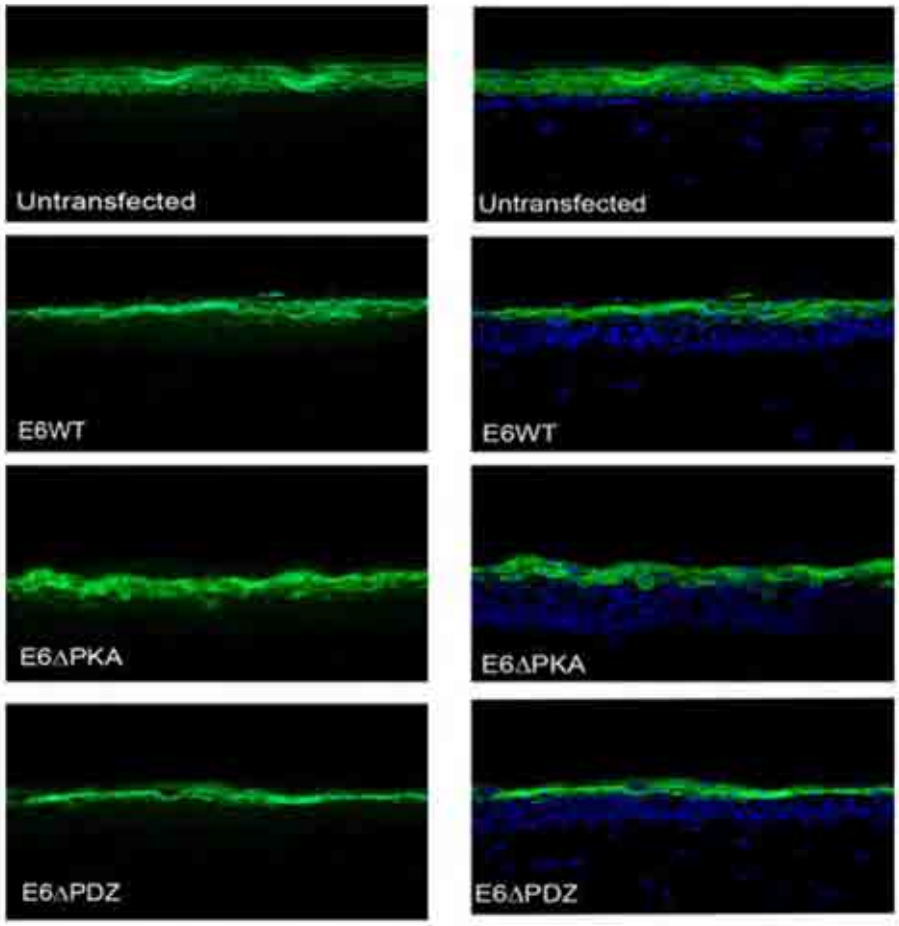


Figure 4.5 Changes to the PDZ binding motif do not effect the expression of epithelial differentiation markers. Organotypic raft cultures of normal HFKs and HFKs containing wild type, E6ΔPKA or E6ΔPDZ genomes were grown to 13 days, fixed in 4% paraformaldehyde and paraffin embedded sections were prepared for indirect immunofluorescence with anti-K5, anti-K1 and anti-Filaggrin antibodies. **(A)** Prepared sections were stained with the anti-K5 antibody (Red) and counterstained with DAPI (Blue) for detection of nuclei. A similar pattern of K5 staining was observed and localised to the basal cell layers. No changes were observed in the expression or localisation of K5 in raft cultures from HFKs containing wild type genomes and those containing E6ΔPKA and E6ΔPDZ genomes. **(B)** Immunostaining of raft sections with anti-K1 antibody (Green). Expression of K1 is strong and localised to the suprabasal cell layers. No changes were observed in the K1 staining pattern in normal HFKs and those which contain wild type, E6ΔPDZ or E6ΔPKA genomes. **(C)** Raft sections were also stained with anti-Filaggrin (Green) – a late differentiation marker and component of the cornified cell envelope. Similar to the finding of K5 and K1, no changes were observed in the expression or localisation of Filaggrin in raft cultures from normal HFKs and those containing wild type genomes. Changes to the PBM in E6ΔPKA and E6ΔPDZ mutant genomes do not effect the expression or localisation of this protein and show a similar pattern of expression to wild type and normal HFKs.

4.2.6 Loss of negative regulation of the E6 PBM results in a more invasive phenotype

It was noted from the H and E staining of HFKs containing E6ΔPKA genomes that these cells were characterised by the presence of a number of 'pockets' of cells which appeared to be invading down into the collagen below. Cell invasion requires a cell to migrate through an extracellular matrix (ECM) or basement membrane extract (BME) barrier by first enzymatically degrading the barrier in order to become established in a new location. This invasion can be detected and calculated by means of a Transwell® invasion assay.

In principal, the upper surface of the insert membrane (8 µm pore size) is coated with a uniform layer of rat-tail derived collagen and a defined number of cells (0.5×10^6) are added into SFM. This layer serves as a barrier to discriminate invasive cells from non-invasive cells. Invasive cells are able to degrade the matrix proteins in the layer and ultimately pass through the pores of the polycarbonate membrane to the underside. After 5-8 hours, the cells are removed from the top of the membrane and the invaded cells are stained with DAPI and quantified and the extent to which they have invaded is calculated (Materials and Methods section 2.14). The percentage of invasion can be calculated by counting the no of DAPI stained nuclei present at 0 h from 4-5 fields of view, compared with the number of nuclei which have been able to invade to the underside of the membrane (Figure 4.6A).

Using this method, the invasive capacity of HFKs transfected with wild type and E6ΔPKA genomes was examined. Normal HFKs would be expected to have a very limited capacity to invade and therefore served as a negative control. Overall, cells transfected with wild type HPV18 genomes had an increased capacity to invade when compared to control normal HFKs ($p=0.05$). Furthermore, cells containing the E6ΔPKA genomes, which have a conditionally active E6 PBM had an increased potential to invade compared with wild type containing HFKS ($p=0.002$) and normal HFKs ($p=0.0002$) (Figure 4.6B).

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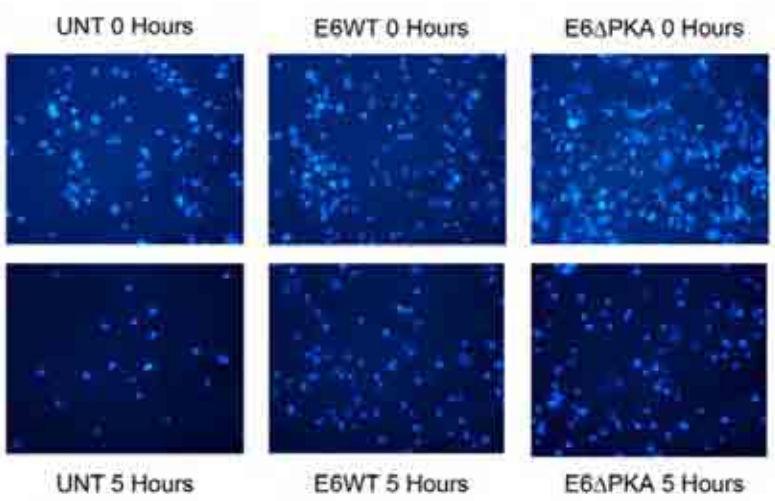

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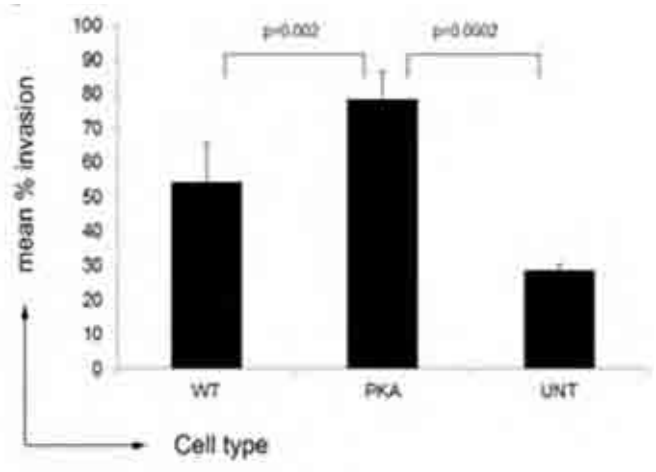

Figure 4.6 The loss of the negative regulation of the E6 PBM results in a more invasive phenotype. (A) Normal HFKs as well as HFKs containing wild type or E6ΔPKA genomes were harvested and seeded in SFM at a concentration of $5x10^5$ into the upper layer of a Transwell® insert separated by a 0.8 µM polycarbonate membrane. Membranes were fixed at 0 h and then after 5 h, counterstained with DAPI to detect nuclei and the percentage of invasion was calculated by counting the no of DAPI stained nuclei present at 0 h from 4-5 fields of view, compared with the number of nuclei which have been able to invade to the underside of the membrane. Representative images collected during the Transwell experiment are shown. **(B)** Combined Transwell® data from 6 independent experiments across 3 different donor lines. Overall, HFKs containing the constitutively active mutant (E6ΔPKA) has an increased potential to invade compared with HFKs containing wild type genomes (p=0.002) and normal HFKs (p=0.0002).

This data is consistent in six separate experiments with three experimental repeats coming from one donor line. Taken together the data from the Transwell assays and from the morphological appearance of the organotypic raft cultures suggest that the constiuitive expression of an E6 PBM confers a more invasive phenotype upon the host keratinocytes.

4.2.7 Changes in PKA signalling are associated with changes in HFKs containing wild type genomes

Previous growth analysis data from this study suggests that an increase in growth of cells containing a genome with an E6 mutation that conferred constitutive activity upon the E6 PBM (E6ΔPKA) could be attributed to changes in PKA signalling. To test the hypothesis that conditional regulation of the E6 PBM has a role in regulating cell growth, the HFKs in this study were treated with a number of activators and inhibitors of the cAMP-dependent kinase; if a correct hypothesis exists then when cells containing a genome with a responsive E6 PBM (i.e. HPV18 wild type genomes) are treated with an inhibitor of PKA they would behave similarly to the E6ΔPKA mutant cells which are not regulated by PKA and grow faster that the vehicle only treated cells. In contrast, treatment with an activator of PKA would enhance the negative regulation of the E6 PBM and provide a growth profile similar to that of the E6ΔPDZ mutant which is defective in binding to PDZ proteins.

PKA is an enzyme whose activity is dependent on the intracellular levels of cAMP. Each PKA is a holoenzyme that consists of two regulatory subunits and two catalytic subunits. Under low levels of cAMP, the holoenzyme remains intact and is catalytically inactive. When the concentration of cAMP rises i.e. by the addition of an activator of PKA such as forskolin (FK), cAMP binds the two binding sites on the regulatory subunits, which leads to the release of the catalytic subunits. The release of the catalytic subunits allows these units to phosphorylate proteins in the cytosol and nucleus. Down-regulation of PKA (by compounds such as H89) occurs by a feedback mechanism. One of the substrates that are activated by the

kinase is a phosphodiesterase which quickly converts cAMP to AMP, thus reducing the amount of cAMP that can activate protein kinase A. 3-isobutyl-1-methylxanthine (IBMX) is a potent cyclic nucleotide phosphodiesterase inhibitor which increases the amount of cyclic AMP in a cell. H89 is a competitive inhibitor and competes with ATP binding to the catalytic subunits and prevents substrate phosphorylation.

Cells containing either wild type or the mutant E6ΔPKA HPV18 genomes were seeded into a 6-well plate containing a γ-irradiated J2-3T3 fibroblast feeder layer at a concentration of $5x10^4$ cells/well. Each cell line was set up in duplicate at each time point. Cells were allowed to grow for 48 h before the addition of the PKA activator or inhibitor. FK and IBMX (Figure 4.7A) were dissolved in DMSO and used in combination at concentrations of 50 μM and 1 mM respectively. Dibutyryl cAMP (Bt_2cAMP, Calbiochem) (Figure 4.7A) was dissolved in Hank's balanced salt solution and diluted in culture medium to a final concentration of 1 mM. The general PKA competitive inhibitor, H89 (Calbiochem) (Figure 4.7B) was dissolved in DMSO and added to the culture medium at a final concentration of 10 μM. All activators and inhibitors were used at concentrations that were non toxic to the HFKs (data not shown). Cells were harvested at various times after the addition of activator/inhibitor cells, by first removal of the J2-3T3 feeder layer using EDTA and the keratinocytes removed with trypsin-EDTA solution (Materials and Methods section 2.8.4). Cell numbers were derived from 5 counts at each time point and two wells were harvested for each time point. Cells containing the E6ΔPKA genomes were used as a negative control in these experiments since they have an intact E6 PBM, but have a mutant PKA consensus motif and are therefore unresponsive to changes in PKA signalling.

Addition of the inhibitor of PKA, H89 to cultures of cells containing the E6ΔPKA genome had no effect upon the growth profile of these cells compared to cells treated with the DMSO alone. However, as predicted, H89-treated wild type cells grew significantly faster than

DMSO-treated cells (Figure 4.7C). The results are representative of 3 experiments carried out in 2 donor lines. The most significant difference in growth is seen at 24 h (p=0.004) and (p=0.0003) respectively (Figure 4.7C). To avoid bias, the inhibitor studies were repeated in a blind study carried out by Dr Sally Roberts. The results from this study correlate with the results from this H89 study. In these experiments, the greatest difference in growth was also observed at 24 h (p=0.0002) and there was no significant difference is observed in the E6ΔPKA line with or without the activator (p=0.8) (data not shown).

FK activates PKA by stimulating cAMP levels, causing the phosphorylation of substrates including the phosphodiesterases (PDEs), which degrade cAMP and thereby reduce PKA activity. IBMX selectively inhibits phosphodiesterases. There is no change in the growth profiles of cells containing E6ΔPKA genomes, with or without the addition of the general activators FK/IBMX (Figure 4.7D) (p=0.5 at 24 h post-addition of activator). However, as predicted, wild type cells treated with FK/IBMX grew significantly slower than untreated wild type cells at 12, 24 and 48 h post treatment (Figure 4.7D). Similar to the activator studies, the greatest effect of the inhibitor could be seen at 24 h post treatment (Figure 4.7D). This result was consistent in 3 experiments across the 2 donor backgrounds tested.

Since FK is known to potentially have off target effects, a specific activator of PKA, dibutyryl-cAMP was used in this assay to confirm the previous findings observed. The data from the dibutyryl-cAMP experiments confirms the results from the FK/IBMX experiments. Dibutyryl-cAMP treated wild type HPV18 genome containing cells grew slower than wild type cells which had been untreated (Figure 4.7E). The data for cells derived from two separate donors is shown. The difference in growth between treated and untreated wild type cells can be seen at 12, 24 and 48 h with the greatest effect seen at 24 h in all donor lines (p=0.0005, p=0.02 respectively). As expected there is no difference in growth between E6ΔPKA cells treated or untreated with dibutyryl-cAMP (p=0.7, p=0.6 respectively) (Figure

4.7E). Untransfected HFKs were also counted with and without the addition of dibutyryl-cAMP to confirm the changes observed were due to the presence of HPV virus, in particular, E6 and no significant difference was observed in the growth profiles ($p=0.5$) observed (data not shown). Taken together the data from the activator and inhibitor studies is in agreement with the hypothesis supporting the notion that the activity of the E6 PBM is conditionally regulated by PKA in primary human keratinocytes. Changes in PKA activity by addition of specific activators and inhibitors of PKA were associated with a change in the growth of the HPV18 genome-containing cells; an increase in cell growth correlating with loss of negative regulation and reduced cell growth correlating with loss of E6 PBM function.

4.2.8 The effect of E6 PBM activity on cell cycle

4.2.8.1 Changes to E6 PBM activity does not affect the expression of cyclins

The basis for the effect of the E6 PBM on cell growth may reflect changes in cell cycle progression, therefore to address this question the expression of a number of cyclins involved in cell cycle progression were analysed. The cell cycle is governed by a family of cyclins and CDKs which mediate activating and inactivating phosphorylation events. Different cyclin-CDK combinations determine the downstream proteins targeted. CDKs are constitutively expressed in cells whereas cyclins are synthesised at specific stages of the cell cycle, in response to various molecular signals. A change in the expression of these cyclins in HFKs transfected with wild type or mutant genomes may explain the changes observed in growth rates and extensive nuclear abnormalities observed and so attention has been focused on altered expression of cyclins as they have been shown to be important in the events leading to cell proliferation and differentiation within the cell cycle (Fehrmann *et al*., 2003).

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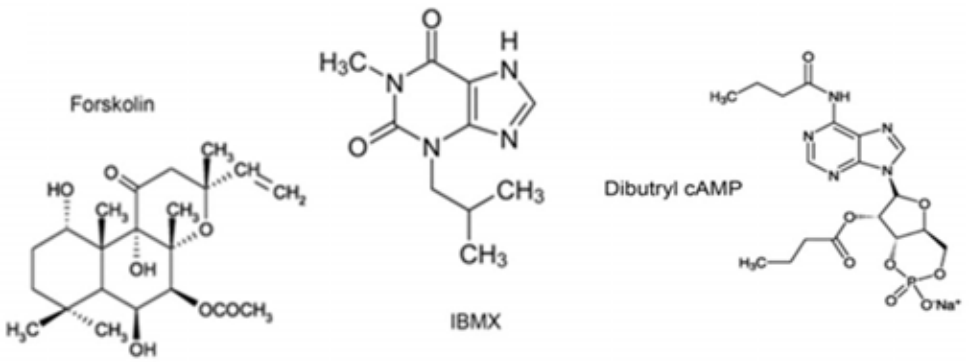
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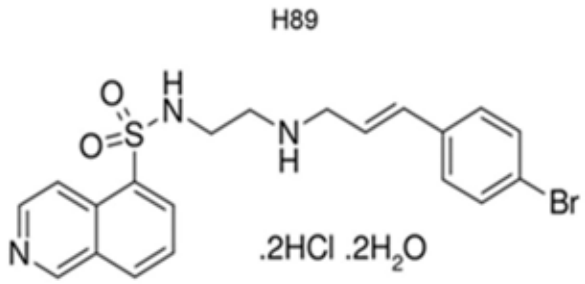
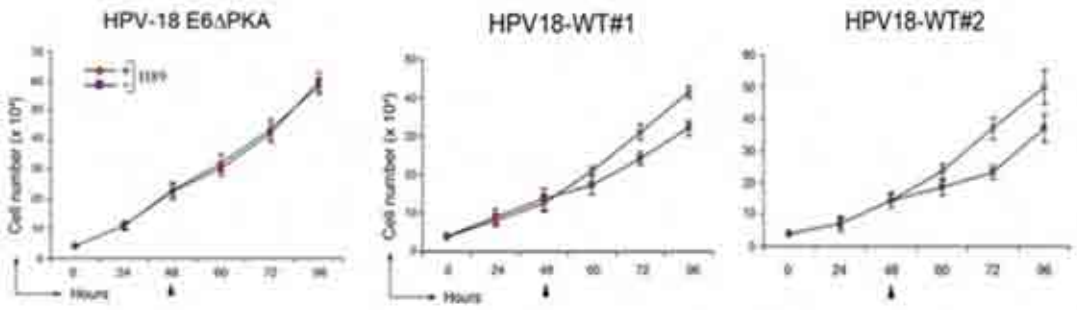
IBMX
Dibutryl cAMP

B


H89
.2HCl .2H2O

C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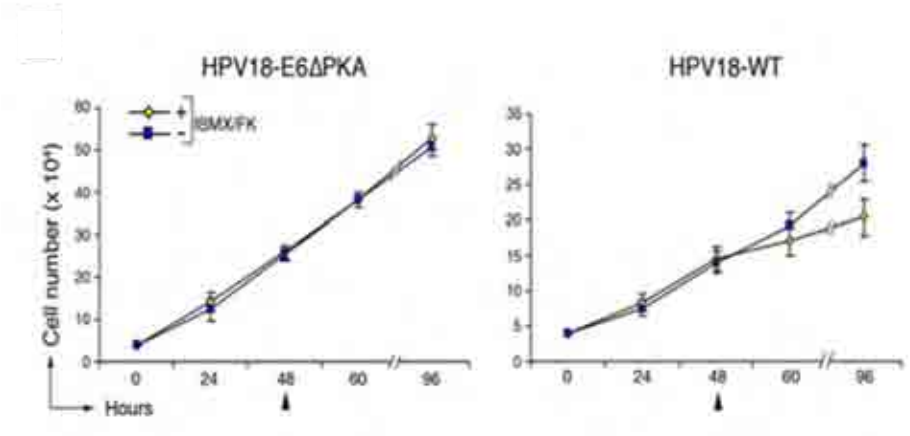
HPV18-WT#1
HPV18-WT#2
Cell number (x 10⁴)
Hours

D


HPV18-E6ΔPKA
HPV18-WT
IBMX/FK
Cell number (x 10⁴)
Hours

E

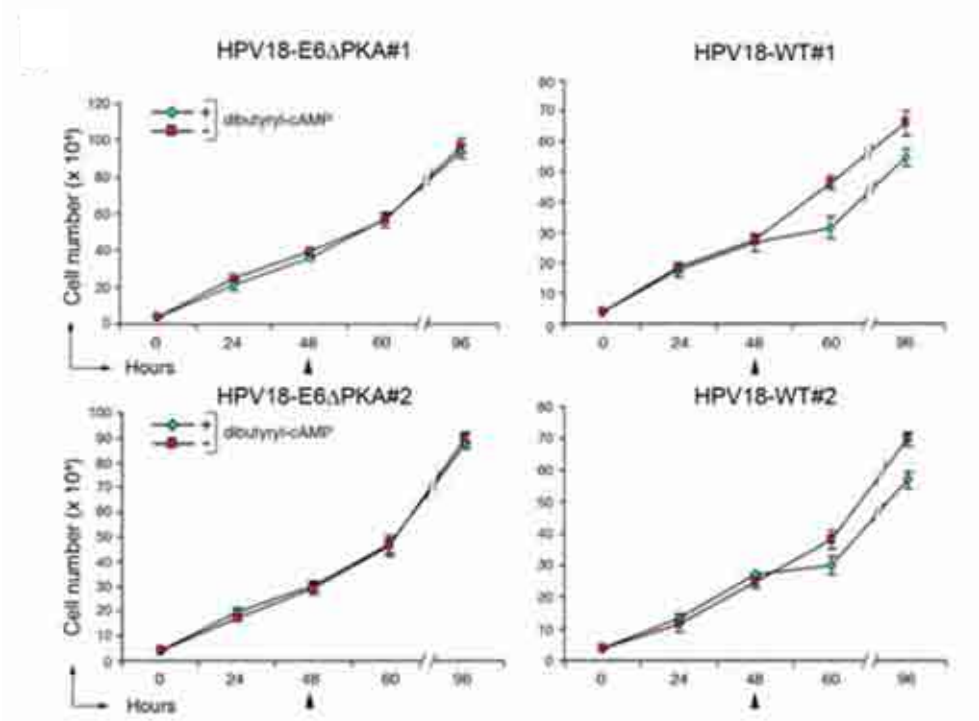


Figure 4.7 Treatment of wild type cells with activators or inhibitors of PKA correlate with changes in keratinocyte cell growth. **(A)** Chemical structures of the activators IBMX/FK and Dibyutryl cAMP used in this study. **(B)** Chemical structure of the general inhibitor H89, used in this study. **(C)** HFKs containing wild type or E6ΔPKA genomes were seeded out on γ-irradiated J2 fibroblasts at a concentration of $5x10^4$ cells and treated with a variety of activators and inhibitors of PKA. Cells were treated at 48 h post seeding and cell counts taken at 12 h, 24 h and 48 h post drug treatment. Cell counts were carried out in duplicate and each time point was counted 5 times. Errors bars represent the standard deviation of the experiment. P values were calculated from a one sided student T-test. Treatment of wild type cells with the general inhibitor H89 (10 μM) results in a significant increase in growth compared to wild type untreated controls, with the greatest effect seen at 24 h post treatment (p=0.004 and p=0.0003 respectively). HFK s containing E6ΔPKA genomes are not affected by the treatment with the general H89 inhibitor (p=0.8). These data are representative of 3 independent experiments in 2 donor backgrounds. **(D)** FK/IBMX activation of PKA reduces the growth of HPV18 wild type genomes. HPV18 wild type and E6ΔPKA genomes were harvested and seeded as previously discussed and incubated with or without FK (50 μM) and IBMX (1 mM) after 48 h. Cell counts were taken at 12 h, 24 h and 48 h post treatment with activator. Treatment of wild type cells with FK/IBMX significantly reduces the growth compared with untreated control wild type containing HFKs. The greatest effect is seen at 24 h post treatment. No significant changes were observed in E6ΔPKA genome containing cells with or without treatment with FK/IBMX. Data is representative of 3 separate experiments carried out in 2 donor lines. **(E)** Analysis of wild type treated with the specific activator Dibutryl cAMP revealed similar findings to the FK/IBMX study. HFKs containing wild type genomes treated with Dibutryl cAMP (1 mM) grew significantly slower than untreated wild type controls, with the greatest change in growth seen at 24 h post treatment (p=0.0005 and p=0.002) respectively. No significant changes were observed in E6ΔPKA cells with or without the treatment of Dibutryl cAMP (p=0.7 and 0.6 respectively). Data is representative of 3 independent experiments carried out on 2 donor lines.

From monolayer cell cultures, early passage cells (P6) containing wild type, E6ΔPKA or E6ΔPDZ genomes were harvested in lysis buffer containing 8M urea and equal amounts of the protein lysates analysed by western blotting to determine the protein expression levels of cyclins E, A and B1- cyclins which regulate G1 to S and G2-M cell cycle progression. The results from western blot analysis showed that cells containing wild type, E6ΔPDZ or E6ΔPKA genomes contained detectable levels of all three cyclins (Figure 4.8) and all three were expressed at higher levels than in the untransfected cells. This increase in cyclin expression by HFKs containing HPV genomes have been previously reported (Fehrmann *et al*., 2003). No marked changes were observed in protein expression levels of the cyclins B1 and A in either mutant line compared with the wild type genome containing cells (Figure 4.8). Western blot analysis of cyclin E showed a slight reduction in expression of cyclin E in HFKs carrying E6ΔPDZ genomes when quantitated against GAPDH protein loading, control however this change was not significant when compared with the wild type control ($p=0.67$). These experiments were repeated three times with similar results. These studies confirm the induction of cyclin A, cyclin B1 and cyclin E in HFKs containing wildtype, E6ΔPDZ or E6ΔPKA genomes but demonstrate that the E6 PBM does not influence this process.

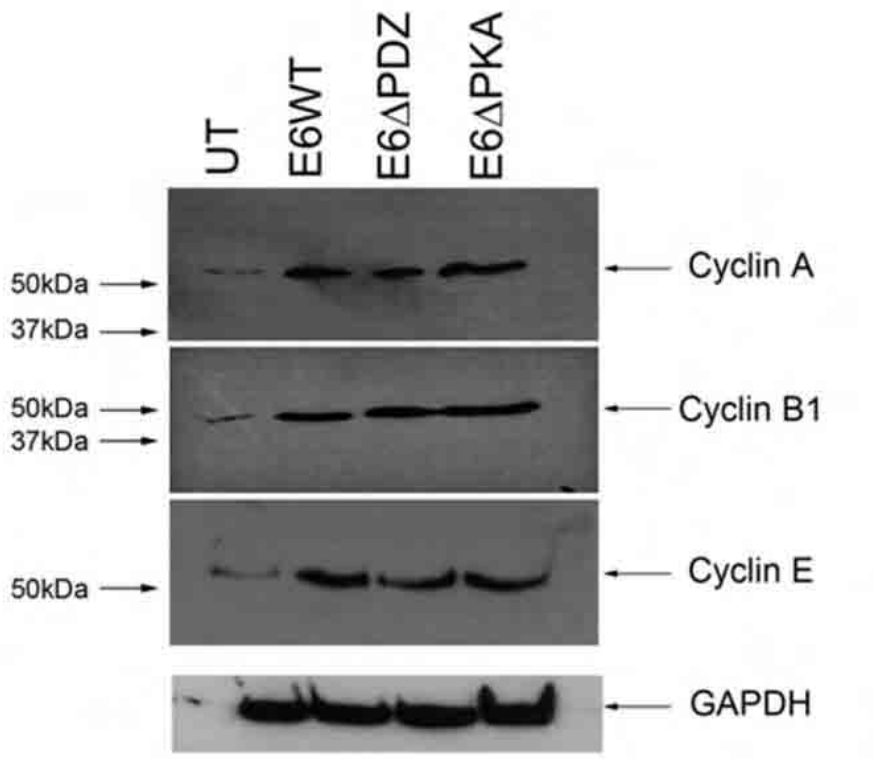


Figure 4.8 Changes to the E6 PBM do not affect the expression levels of cell cycle markers. Lysates from normal HFKs as well as those containing wild type, E6ΔPKA and E6ΔPDZ genomes were grown to 80% confluency, harvested and subjected to western blot analysis with the cell cycle markers Cyclin E , Cyclin A and Cyclin B1. No significant changes were observed in the Cyclin A, Cyclin E and Cyclin B1 protein expression levels between cell carrying wild type, E6ΔPDZ or E6ΔPKA genomes suggesting that abrogation of the E6 PBM or constitutive expression of this domain does not effect the levels of cell cycle markers. GAPDH is shown as a marker for loading control. The western blots are representative of 3 separate experiments from 1 donor line.

4.2.8.2 Changes to the E6 PBM do not affect the ability of cells to progress through the cell cycle

Since no significant differences were observed in the cyclin expression between HFKs containing wild type or mutant genomes, the cell cycle profiles of normal HFKs or HFKs containing wild type or E6ΔPDZ genomes were determined at various passages post transfection by flow cytometry analysis.

For DNA cell cycle analysis, cell pellets were resuspended in 70% ethanol and fixed as described (Materials and Methods section 2.11) and cell pellets resuspended in PBS containing propidium iodide (PI). The relative cellular DNA content of stained cells was measured by flow cytometry and the proportions of cells in phases G_0/G_1, S and G_2/M were calculated using the multicycle dedicated cell cycle analysis software (Phoenix flow systems, San Diego, Calif).

Cells carrying E6ΔPDZ genomes or wild type genomes produced cell cycle profiles that were similar to untransfected cells at both low and high passages (P5, P15 respectively) (Figure 4.9A) and this was consistent across multiple donors (data not shown). A combined analysis of FACS profiles from a number of low and high passages cells further confirmed there was no significant changes in the proportions of cells in G_0/G_1, S and G_2/M phase between HFKs containing wild type genomes or E6ΔPDZ genomes ($p=0.2$, $p=0.7$ and $p=0.3$ respectively) (Figure 4.9B). Moreover, no significant changes were observed in the G_2 + M:G_1 ratios at early and late passages between wild type expressing and E6ΔPDZ genome containing cells (Figure 4.9C). These results taken together suggest that change observed in the growth profiles of cells containing E6ΔPDZ genomes may not be attributed to changes in the ability of cells to progress through cell cycle as similar cell populations were observed in HFKs containing wild type and E6ΔPDZ genomes at both low and high passages.

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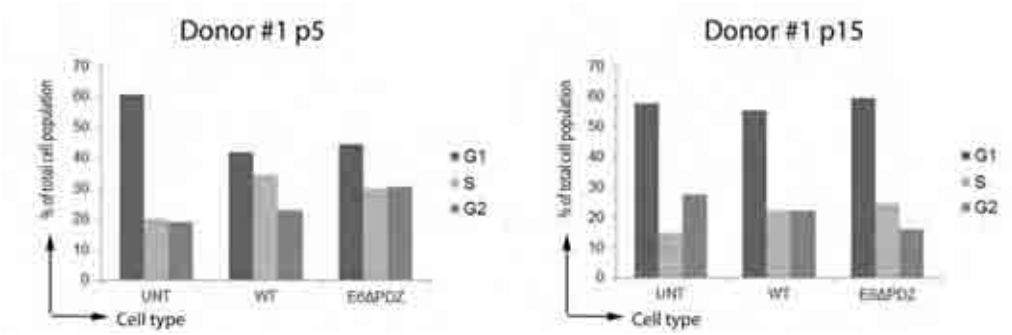

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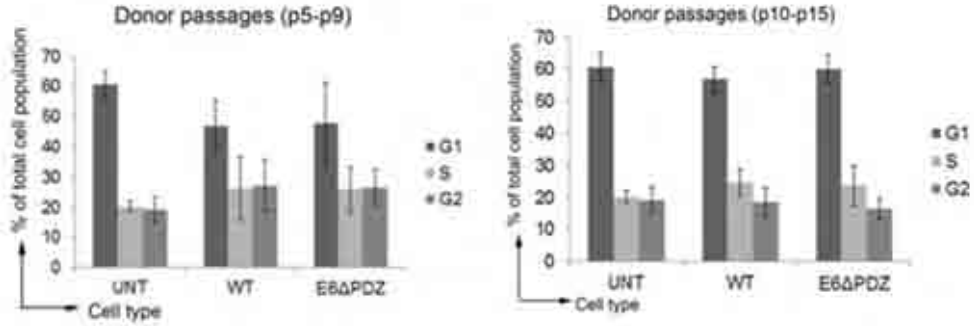

C

Cell Line	G_1		S		G_2/M		G_2/M:G_1
E6ΔPDZ passage 5-9	47.7	+/- 4.4	25.9	+/- 6.0	26.5	+/- 3.3	0.55
E6ΔPDZ passage 10-15	59.9	+/- 13.7	23.5	+/- 7.4	16.5	+/- 6.1	0.27
E6WT passage 5-9	46.8	+/- 8.4	26.1	+/- 10.5	27.1	+/- 8.5	0.57
E6WT passage 10-15	56.8	+/- 3.9	24.6	+/- 4.0	18.5	+/- 4.4	0.32
Untransfected	60.7	+/- 4.4	20.1	+/- 1.8	19.1	+/- 4.0	0.31

Figure 4.9 Abrogation of the E6 PBM does not affect the ability of cells to cycle. HFKs containing wild type or E6ΔPDZ genomes were grown to 80% confluency, harvested, fixed and incubated with propidium iodide (PI). The relative cellular DNA content of stained cells was measured by flow cytometry and the proportions of cells in G_0/G_1, S and G_2/M were calculated. **(A)** Cell cycle profiles of HFKs containing wild type or E6ΔPDZ genomes at low (P5) and high (P15) passages are shown. (**B**) A combined analysis of FACS profiles from multiple low passage (P5-9) and multiple high passage (P10-15) donors revealed no significant differences in the proportion of cells in G_1, S or G_2 between normal HFKs and those containing wild type or E6ΔDZ genomes. Errors bars represent the standard deviation of the experiment shown. (**C**) The relative G_2/M:G_1 populations were calculated. No significant changes were observed in the G2/M:G_1 ratios of HFKs containing wild type genomes or E6ΔPDZ genomes at multiple low passages or multiple high passages.

4.2.8.3 Abrogation of the E6 PBM is associated with an increased population of cells in mitosis

Single parameter DNA histograms cannot discriminate G_2 from M-phase cells so to address the question whether the cells may be arresting in mitosis (M), the G2/M checkpoint assay using the marker for M phase cells, phospho-histone 3 (pH3) was used to determine the population of cells that are in M phase. Briefly, cells are fixed and incubated with an anti-phospho histone H3 (Ser10) antibody (Cell Signalling) and positive cells identified using a FITC conjugated secondary antibody and the percentage of positive cells determined using flow cytometry (Materials and Methods section 2.12).

Overall, cells carrying E6ΔPKA or wild type HPV18 genomes showed, on average, similar populations of cells in M phase (1.7% and 5% respectively) compared with untransfected keratinocytes (0.18%) (Figure 4.10A). The difference in M phase populations between wild type and E6ΔPKA cells is not significant ($p=0.2$). Interestingly, cells containing the mutant E6ΔPDZ genomes had a significantly greater population of cells in M phase when compared with wild type ($p=0.008$) or E6ΔPKA cells ($p=0.005$). Twenty percent of cells carrying E6ΔPDZ genomes were found to be in M phase compared to 5% of the cells containing the wild type HPV genomes (Figure 4.10A). The data presented, represents the combined results of 3 separate experiments from one donor.

An assessment of pH3 protein levels by western blot analysis confirmed the results seen by the G2/M assay. Western blot analysis of cell lysates revealed a distinct pH3 band in lysates prepared from cells carrying the E6ΔPDZ genomes (Figure 4.10B), which may explain the higher population of cells we see in this mutant cell line in the G2/M assay. No pH3 band was detected in lysates from untransfected, wild type or E6ΔPKA genome containing HFKs (Figure 4.10B), concurrent with the lower populations of M cells in the G2/M assay.

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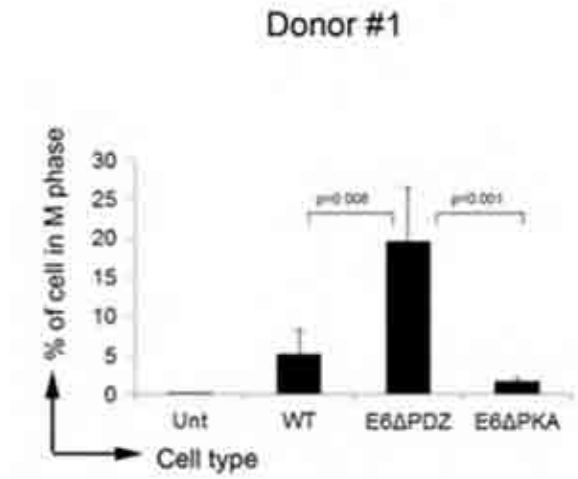

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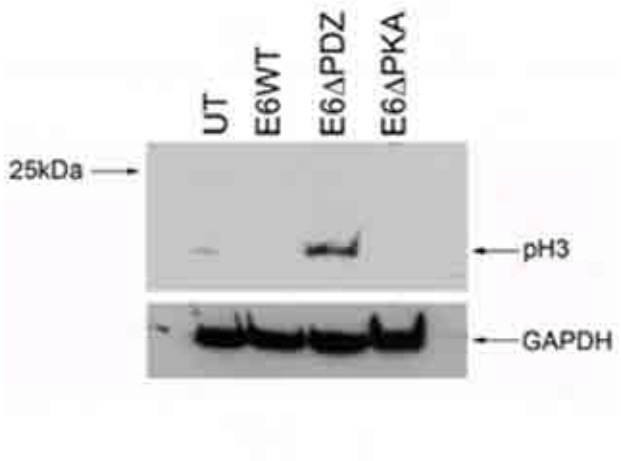


Figure 4.10 Abrogation of the E6 PBM results in an increase of cells in M phase. (**A**) Normal HFKs as well as HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were grown to 80% confluency, cells were harvested, fixed and incubated with an anti-pH3 (ser 10) antibody followed by a FITC conjugated secondary antibody. The percentage of cells in M phase was determined by flow cytometric analysis. Bar graph is representative of 3 independent experiments carried out in 1 donor line. Overall HFKs carrying the E6ΔPDZ genomes have a greater % of cells in M phase compared with matched HFKs containing wild type genomes (p=0.008) and E6ΔPKA genomes (p=0.001). P values were calculated from a one sided student T-test. **(B)** Western blot analysis of lysates from the same donor line. Cells were harvested and probed for immunoblotting by an anti-pH3 antibody Western blot analysis shows a distinct pH3 in lysates from HFKs carrying E6ΔPDZ genomes which is absent in matched donors of normal HFKs as well as those containing wild type or E6ΔPKA genomes.

Taken together these data suggest that abrogation of the E6 PBM may be important during the final stages of cell cycle since an increased proportion of cells in M phase were observed by FACS analysis and confirmed by western blot analysis in HFKS containing E6ΔPDZ genomes.

4.3 Discussion

To understand if the function of the E6 PBM was necessary for late viral functions, the vegetative life cycle of the virus was induced within HFKs containing wild type and mutant (E6ΔPDZ or E6ΔPKA) genomes either by suspension of the cells in methylcellulose or by stratification in organotypic raft culture. In histological cross sections of normal HFKs as well as HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes, abrogation of the E6 PBM by means of the E6ΔPDZ mutation was consistently found to present a reduced thickness of the basal cell layer with morphology more similar to that of normal HFKs than those containing HPV18 wild type genomes. This finding is consistent with comparable analyses of E6ΔPDZ genomes in HPV31 genome containing cells and in mice, both of which showed similar histology (Lee & Laimins, 2004; Nguyen *et al*., 2003). Conversely, histological cross sections of HFKs containing the E6ΔPKA genomes showed an enhanced hyperplasia upon constitutive activation of the E6 PBM. Changes in basal layer thickness between E6ΔPDZ and E6ΔPKA genome containing cells could possibly be explained by the changes we see in proliferation rates between the two mutants since HFKs containing E6ΔPKA genomes have been shown to grow significantly faster than those containing wild type or E6ΔPDZ genomes (Chapter 3 Section 3.2.5) and this correlates with the increased hyperplasia in cells harbouring E6ΔPKA genomes compared with those containing wild type or E6ΔPDZ genomes.

The studies described above suggest that the PBM of E6 is involved in modulating proliferation as well as impacting on the early viral functions such as the establishment of episomes and maintenance of viral copy number. Since the late phases of the viral life cycle are linked to differentiation of keratinocytes, the E6 PBM function was assessed following differentiation in methylcellulose. HFKs containing wild type or E6ΔPKA genomes were able to amplify their genomes to a similar level, and this was consistent across a number of donors investigated. Interestingly, HFKs carrying a mutant of E6 which is unable to bind to PDZ substrates (E6ΔPDZ) failed to amplify their genomes in methylcellulose suggesting that the E6 PBM is required for differentiation dependent amplification. The data from these experiments are in variance to the studies from Lee and Laimins, who show that in an HPV31 background, the PBM of E6 is not required for the differentiation dependent amplification of viral genomes (Lee & Laimins, 2004). Interestingly, the study by Lee and colleagues do show a moderate decrease in genome amplification in HFK containing E6ΔPDZ genomes compared to wild type genomes (2.7 fold compared with 4.0 fold respectively) suggesting that the E6 PBM may be required for efficient amplification of viral genomes (Lee & Laimins, 2004). Moreover, the differences observed in amplification of E6ΔPDZ genomes between this study and that of Lee and colleagues could be attributed to an HPV subtype specific difference.

The loss of viral genome amplification in cells harbouting E6ΔPDZ genomes could not attributed to changes in the stability or expression of E6 or E7 proteins, since raft lysates of HFKs harbouring wild type or mutant genomes showed a similar expression pattern of both E6 and E7 proteins. Loss of efficient viral genome amplification in cells harbouring E6ΔPDZ genomes could also not be explained by the inability of the suprabasal cells to re-enter S phase since the level of BrdU incorporation (a marker of S phase activity) in suprabasal E6ΔPDZ cells was comparable to cells carrying wild type genomes. This may not be that

surprising since the expression of the E7 protein of both high risk and low risk HPV types has been shown to be capable of inducing S phase reactivation in suprabasal cells of differentiated keratinocytes (Cheng *et al*., 2005). The difference in BrdU incorporation between HFKs carrying E6ΔPKA genomes and those carrying wither wild type or E6ΔPDZ genomes was significant suggesting that phosphorylation of the E6 PDZ binding motif may be an additional means of regulating S-phase re-entry and replication of HPV18 genomes during the productive stages of the HPV18 life cycle.

As previously mentioned, loss of the conditional regulation of the E6 PBM within HPV18 genome by means of the E6ΔPKA mutation has been shown to be associated with a significant increase in cell growth (Chapter 3 Section 3.2.5). This suggests that changes in PKA signalling in HFKs carrying wild type genomes may lead to changes in keratinocyte cell growth. Indeed, the treatment of cells harbouring wild type genomes with activators of PKA including FK/IBMX and dibutryl-cAMP resulted in a reduction in keratinocyte cell growth, whilst the treatment of wild type cells with an inhibitor of PKA, H89, resulted in a significant increase in keratinocyte cell growth. Taken together these data suggest that changes in PKA signalling have a significant effect on keratinocyte cell growth. It is important to note that H89 is known to have off target effect in other cell types and can inhibit other kinases (Lochner & Moolman ,2006) so future experiments would be to confirm findings of the inhibitor data using a specific inhibitor of PKA such as (R_p)-8-Cl-cAMPS.

Modulation of E6 PDZ targeting was also linked to cell invasion since HFKs containing the E6ΔPKA genomes showed an increased invasive potential compared with wild type genomes that could still be regulated by PKA. E6 PBM substrates have previously been shown to be implicated in cell invasion. A study by Goode and Perrimon revealed that *Drosophila* Dlg was required to block cell invasion as loss of Dlg was associated with an increase potential of follicle cells to change shape and invade (Goode & Perrimon, 1997). Moreover, a study by

Dow and colleagues also implicate the PDZ substrate hScrib in invasion, as loss of hScrib in human epithelial cells expressing oncogenic *ras* was associated with the promotion of cells through the extracellular matrix in an organotypic raft culture system (Dow *et al*., 2008). In this light it is tempting to speculate that constitutive degradation of PDZ proteins in cells harbouring the E6ΔPKA mutation may explain the enriched phenotype observed.

Data from this study has shown that abrogation of the E6 PBM results in an accumulation of mitotic defects in cells lacking an intact PBM including an increase in populations of bi-nucleate and multi-nucleate cells. Furthermore, HPV18 mutants which lack the PBM have been shown to grow significantly slower than matched wild type controls. In an attempt to elucidate a mechanism for the phenotypes observed, FACS analysis on normal HFKs as well as HFKs carrying wild type or E6ΔPDZ genomes was carried out. An analysis of multiple samples from both low and high passages showed no significant difference in the populations of G_1, S or G_2 cells derived from wild type or E6ΔPDZ genomes suggesting that the PBM of E6 does not play a significant role in the progression of cells through cell cycle. An analysis of cyclin levels in cells containing wild type or E6ΔPDZ genomes revealed no significant changes which further supports the argument that the E6 PBM does not affect the ability of cells to process through cell cycle.

Interestingly, on further scrutiny, a detailed analysis of cells specifically in mitosis revealed that abrogation of the E6 PBM resulted in a significant increase of cells in M phase compared with wild type cells. This was verified by western blot analysis of lysates from wild type and E6ΔPDZ cells for the M phase marker pH3. Constitutive activity of the E6 domain was not associated with an increase in M phase populations, since no significant differences were observed from HFKs carrying wild type genomes and those carrying the constitutively active mutant, E6ΔPKA. The increase in population of cells in mitosis is consistent with the aberrant nuclear morphology and replication associated with cells lacking an in tact PBM,

suggesting that the function of the E6 PBM may be important during the final stages of mitosis/cytokinesis, during the separation of daughter cells. Work from Unno and colleagues have shown that hDlg- a substrate of the PBM plays an important role in cytokinesis, via its GUK domain (Unno *et al*., 2008). Moreover, overexpression of the hDlg protein in U2OS and Hela cells impaired cytokinesis (Unno *et al*., 2008). In this light, it is tempting to speculate that abrogation of the PBM, leads to an increase in target substrates, such as hDlg and may explain the aberrations in mitosis and the phenotypes observed.

CHAPTER 5 AN ANALYSIS OF THE EXPRESSION OF PDZ DOMAIN CONTAINING SUBSTRATES OF E6 IN HPV18 GENOME CONTAINING KERATINOCYTES

5.1 Introduction

Nearly all cells in human tissue require polarisation to carry out their normal function. Polarisation of epithelial cells helps the cell to identify what ions and molecules should be taken up and what should be excreted and on which surface this should occur (Thomas *et al*., 2008). Three polarity modules are important in the establishment and maintenance of apicobasal cell polarity in epithelial cells, known as the Scribble, Par and Crumbs polarity modules. These three complexes have been described as a polarity 'super complex,' whereby each complex is required to localize all of its proteins at the appropriate membrane positions in order to achieve and maintain epithelial cell polarity (Macara, 2004). Cell polarity is thought to be involved in tissue arrangement and in cell migration (Gonzalez-Mariscal *et al*., 2003). In epithelial tissues, the apical and basolateral membrane domains are separated by a physical barrier called the apical junctional complex (AJC). This is the most significant epithelial cell-cell adhesion structure and comprises tight junctions (TJ) and adherens junctions (AJ) (Hartsock & Nelson, 2008). TJ provide a seal between the membranes of neighbouring cells whilst AJ use the actin cytoskeleton to keep the cellular membranes of neighbouring cells together (Hartsock & Nelson 2008; Perez-Moreno & Fuchs, 2006).

The Scribble polarity complex is comprised of three proteins: Discs Large (hDlg), Scribble (hScrib) and Lethal giant larvae (Hug1-1). Due to the similarity in their mutant phenotypes and the genetic interactions observed between them, it has been surmised that hScrib, hDlg

and Hug1-1 function in a common pathway to regulate the establishment and maintenance of apicobasal polarity in epithelial cells (Bilder *et al*., 2000). An understanding of how hScrib, hDlg and Hug1-1 function in the establishment and maintenance of apicobasal cell polarity has largely come from studies in *Drosophila*. hDlg is a protein observed mainly at cell-cell junctions and maintains cell polarity by defining AJ. hDlg is also associated with the establishment of TJ and is hypothesised to act by recruiting tight junction proteins to sites of cell-cell contact in order to establish a non-permeable seal between epithelial cells (Stucke *et al*., 2007). Another member of the Scribble polarity complex, hScrib, is required for the definition of TJ and correct localisation of apical membrane complexes. hScrib is known to co-localise with hDlg at AJ (Gardiol *et al*., 2006; Massimi *et al*., 2008) (Figure 5.1). hScrib is also thought to have roles in signal transduction at points of cell-cell contact (Dow *et al*., 2003). The third component of the Scribble complex, Hugl-1 has not yet been implicated in the E6 disruption of cell polarity; however its expression is reduced in a number of non-HPV associated cancers including melanomas and cancers of the endometrium (Kuphal *et al*., 2006; Tsuruga *et al*., 2007).

The Par complex is restricted to the apical region of the cell membrane, and co-localises with the PDZ proteins MAGI-1, 2 and 3 (Yamanaka *et al*., 2003, 2006). It sits above the Scribble complex forming TJ (Figure 5.1). Although many of the proteins which make up the Par complex contain PDZ domains (such as Par3 and Par6) none have yet been implicated as targets for the high risk E6 PBM, however the MAGI-1,-2,-3 proteins have been shown to be targeted by high risk E6 proteins for degradation (Glaunsinger *et al*., 2000,Thomas *et al*., 2002).

The Crumbs complex, like the Par complex, is also localised to the apico-lateral membrane boundary (Figure 5.1). This complex is known to play a direct role in the establishment of cell polarity (Roh *et al*., 2003). Studies have shown that a change in the expression of any

component of the Crumbs complex (Crb3; Pals1 (protein associated with lin-7); or PAT-J (Pals-1 associated tight junction protein)) results in a loss of cell polarity (Lemmers *et al*., 2002, Roh *et al*., 2003). Members of this complex have been shown to be specific substrates for the E6 PBM. High risk HPV16 and HPV18 have been shown to target and degrade PAT-J, ultimately leading to the destabilisation of cell polarity complexes and a change in cell polarity (Storrs & Silverstein, 2007). Interestingly, the HPV18 E6* isoform is also able to degrade PATJ, highlighting the first evidence of a PDZ substrate which is degraded by both isoforms of HPV18 E6 (Storrs & Silverstein, 2007).

As previously mentioned, a large number of PDZ domain containing proteins, with roles in the regulation of cell polarity have been found to be targets of the HPV E6 PBM *in vitro*. Several of these targets are proteins found at regions of cell-cell contact, including the MAGI-1, 2, 3 proteins and MUPP1 (Glausinger *et al*., 2000; Thomas *et al*., 2002; Lee *et al*., 2000) which are found at subapical TJ (Ide *et al*., 1999; Hamazaki *et al*., 2001) and hDlg and hScrib proteins which are found at lateral AJ (Bilder & Perrimon, 2000; Firestein & Rongo, 2001).

The biochemical data supporting the identification of hDlg and hScrib as targets of high risk E6 is very strong; however it is still unclear what the natural target, or targets of the virus are *in vivo.* During the development of cervical cancer there is a marked progressive loss of both hScrib and hDlg protein expression (Watson *et al*., 2002; Cavatorta *et al*., 2004; Nakagawa *et al*., 2004; Lin *et al*., 2004). An analysis of hDlg levels from low grade and high grade squamous intraepithelial lesions revealed an increase in membrane and cytoplasmic staining of hDlg in cells of the superficial layer, in intraepithelial lesions from low grade patients, whilst the reverse staining pattern for hDlg is a feature of high grade intraepithelial lesions (Lin *et al*., 2004).

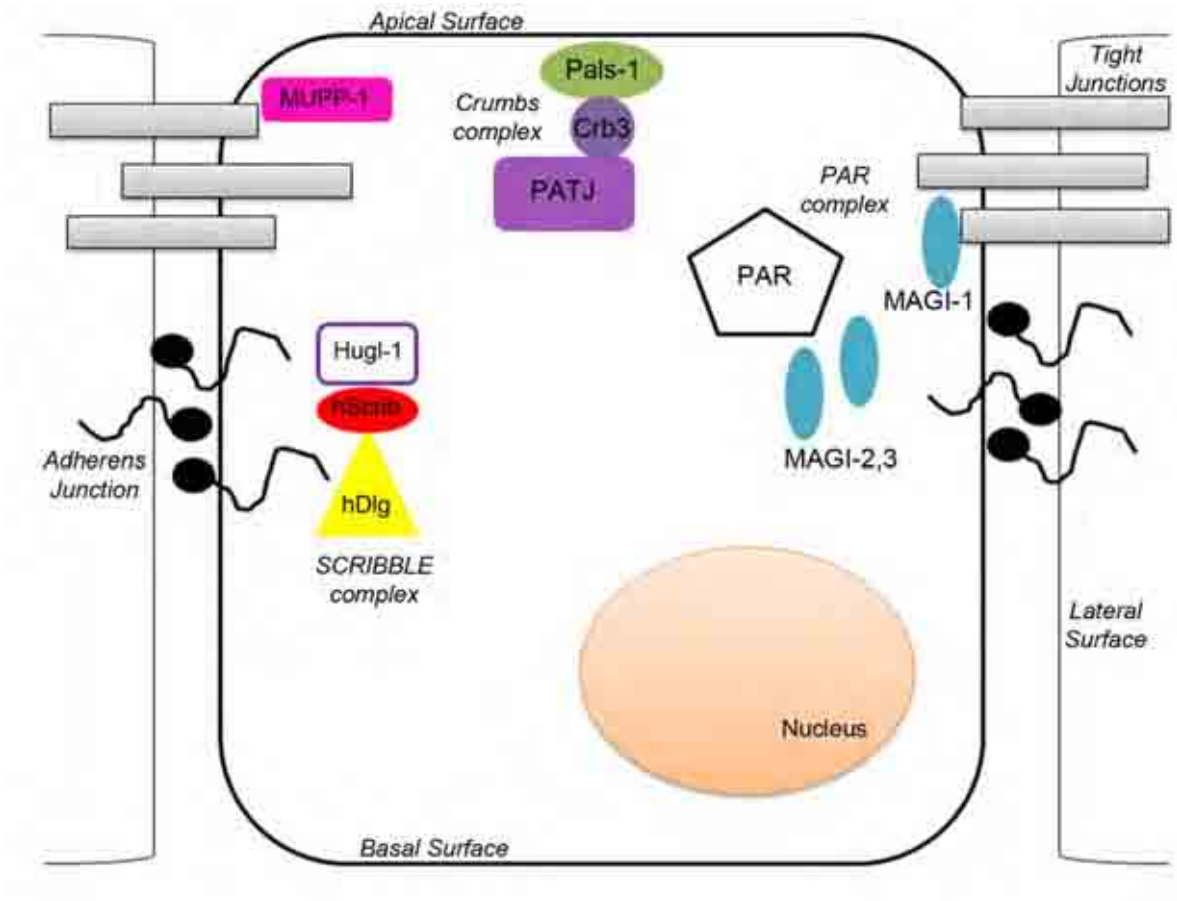


Figure 5.1 The Scrib, PAR and Crumbs polarity complex. The components of the Scribble complex are found at adherens junctions, just below the PAR complex which is localised to tight junctions. The Crumbs complex components are localised to the apical region of the cell membrane. Localisation of some of the E6 PDZ binding partners is shown. Figure adapted from Thaibault, 2011.

This part of the study forms part of an investigation to determine whether some of the known PDZ substrates such as hDlg and hScrib, are affected by the expression of E6 with altered PDZ activity in the context of whole HPV18 genomes. In addition, this study looks at whether changes to the PDZ binding activity affect the expression or localisation of components of polarity defining structures such as TJ and AJ.

5.2 Results

5.2.1 hDlg protein levels are reduced in HFKs harbouring wild type or E6ΔPKA genomes

By using heterologous over expression systems, high-risk HPV E6 proteins have been shown to target not only p53 but also PDZ proteins for degradation (Lee & Laimins, 2004). To assess the biological activity of the different E6 molecules, the protein levels of hDlg, one of the cellular targets of the PBM was examined by western blotting of protein extracts. hDlg is recognised as a protein triplet. The immune-reactive bands had apparent molecular weights of approximately 130 kDa and 120 kDa as previously reported (Lue *et al*., 1994; Wu *et al*., 1998). The lower band had a weight of approximately 110 kDa. The differences in band mobility and size may be due to post-translational modifications of hDlg, for example phosphorylation (Hanada *et al*., 1997; Mantovani *et al*., 2001). There is mounting evidence that hDlg exists in a number of different cellular locations. Predominantly it is found at sites of cell-cell contact, although nuclear and cytoplasmic forms have also been observed (McLaughlin *et al*., 2002; Massimi *et al*., 2003; Roberts *et al*., 2007).

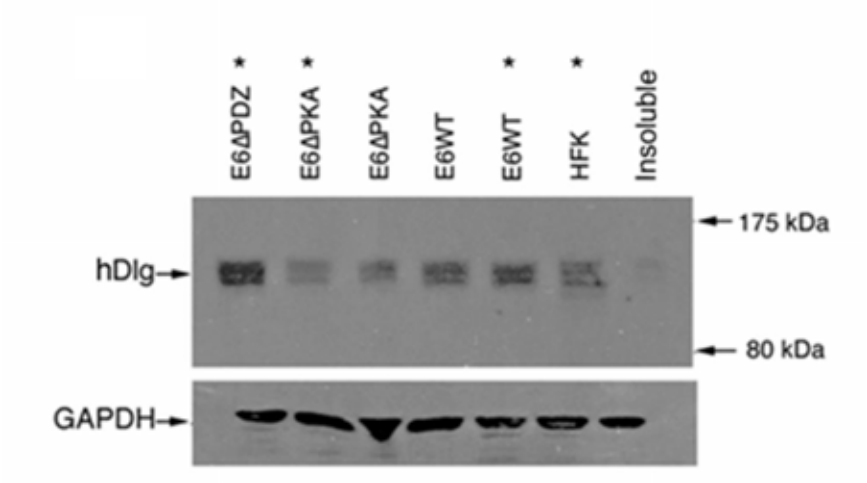
HFKs containing wild type, E6ΔPDZ and E6ΔPKA genomes were grown in monolayer cell culture to 80% confluency, cells were harvested and solubilised in 8 M urea. Western blots were probed with a 2D11 monoclonal antibody (anti-hDlg). As can be seen in Figure 5.2A, all donor lines, contained detectable levels of hDlg by western blot analysis. Typically

E6ΔPKA lines expressed levels of hDlg lower than those present in the wild type or E6ΔPDZ lines. In E6ΔPDZ lines, which are unable to target PDZ substrates, hDlg levels were significantly increased compared to wild type and untransfetced cells. Western blots from figure 5.2A are representative of two experimental repeats carried out in 3 separate donor lines. Signal intensities of hDlg levels detected from western blots from 3 separate donors were quantified and normalised to GAPDH levels. Densitometric analysis of hDlg protein bands confirmed the differences in hDlg protein levels seen by western blot analysis. Figure 5.2B shows the mean Optical Density (OD) readings of three separate hDlg western blots from 1 donor line. From figure 5.2B it can be seen that the hDlg densities of HFKs harbouring E6ΔPDZ genomes are comparable to normal HFKs. In HFKs containing wild type cells the mean OD reading is significantly lower than that of normal HFKs and those containing the E6ΔPDZ mutant genomes ($p=0.04$ and $p=0.03$ respectively). HFKs carrying the E6ΔPKA mutant genomes typically display the lowest overall OD readings. Moreover, the difference in density of hDlg bands observed from E6ΔPKA genomes is significantly less than those containing E6ΔPDZ genomes ($p=0.03$)

The subcellular distribution of hDlg in monolayer cultures was also examined. Normal HFKs as well as HFKs containing wild type and E6ΔPDZ or E6ΔPKA mutant genomes were grown over coverslips to 80% confluence and then fixed in 4% paraformaldehyde for analysis by immunofluorescence. hDlg levels were analysed by probing with a 2D11 (anti-hDlg) antibody. In monolayer cultures of normal HFKs, hDlg can be visualised at the cell-cell boundaries (Figure 5.2C). HFKs containing wild type genomes show less peripheral hDlg staining than normal HFKs with E6ΔPKA displaying the weakest hDlg staining at the cell boundaries (Figure 5.2C). In accordance with data from western blotting analysis, HFKs containing E6ΔPDZ genomes display a strong hDlg staining pattern, at levels comparable to normal HFKs (Figure 5.2C).

Taken together, these results indicate hDlg as a potential target for the HPV18 E6 PBM in the context of a productive HPV infection, since hDlg protein levels are altered in the presence of the HPV18 genome. The ability of HPV18 to degrade hDlg requires a functional E6 PBM since levels of the hDlg protein in HFKs containing the truncated PBM (E6ΔPDZ mutant) were at levels comparable to normal HFKs. Loss of the ability to negatively regulate the degradation, in the context of the E6ΔPKA mutant, results in a somewhat greater loss of the hDlg protein than seen in wild type containing HFKs and this is confirmed by western blotting and immunofluorescence analysis. In two of the donor lines, there is a shift in the hDlg protein band observed by western blot analysis (Figure 5.2A). This shift may be a result of post translational modifications of hDlg, for example phosphorylation (Hanada *et al*., 1997; Mantovani *et al*., 2001).

A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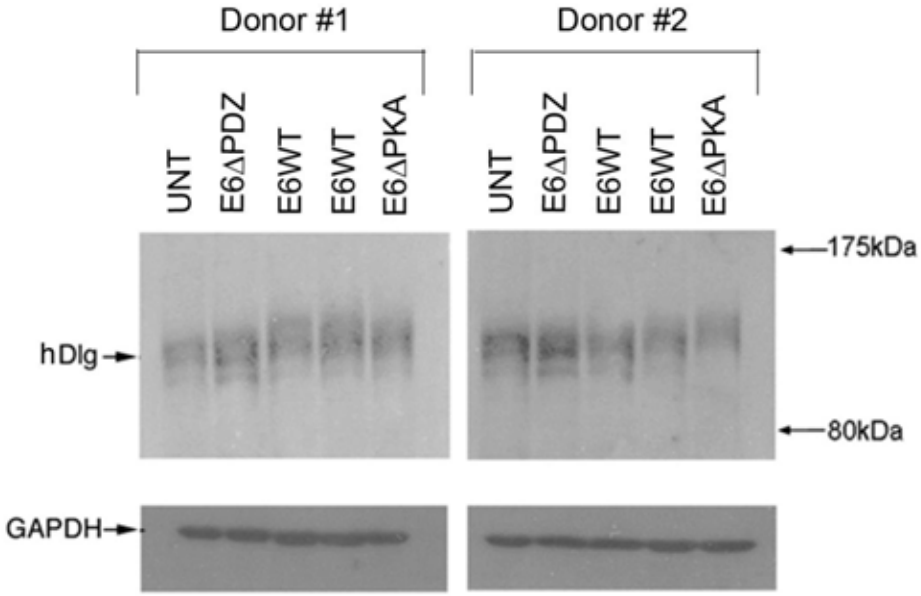
Donor #1
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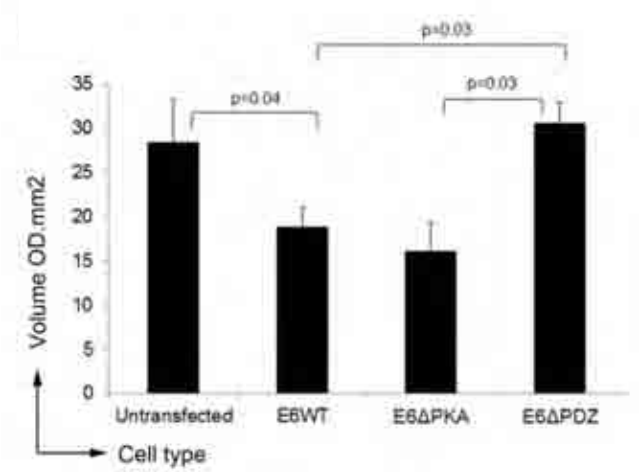
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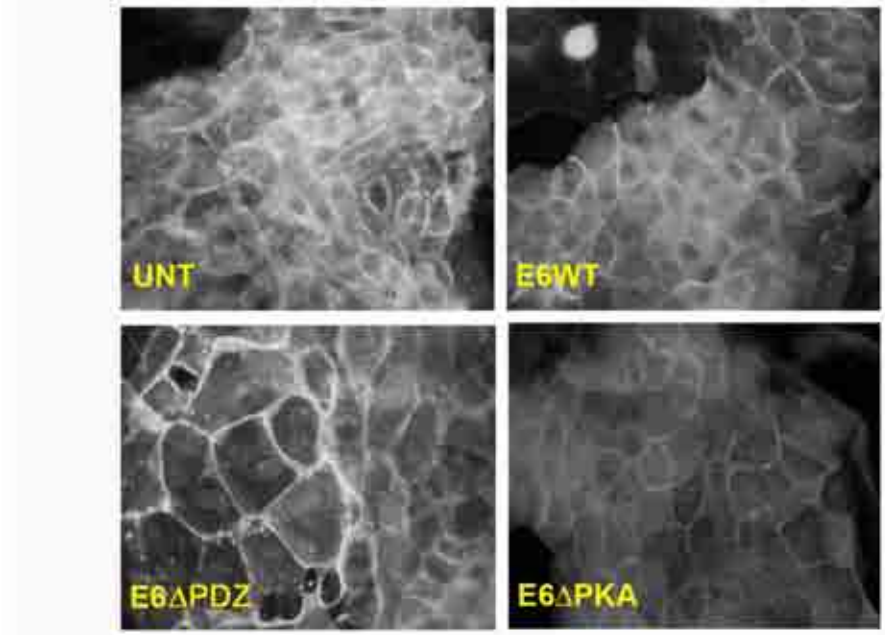


Figure 5.2 hDlg protein levels are affected by PDZ binding activity. (A) Normal HFKs and HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were grown to 80% confluency, harvested and lysates probed with an anti-hDlg antibody (2D11). Wild type containing cells contain levels of hDlg protein lower than untransfected cells and those containing E6ΔPDZ genomes, whilst mutants which are constitutively active for binding to PDZ substrates (E6ΔPKA) display the lowest levels of hDlg protein by western blot analysis. Western blots are representative of 3 independent experiments carried out in 3 donor lines. **(B)** The relative intensities of hDlg protein levels from normal HFKs as well as those containing wild type, E6ΔPDZ or E6ΔPKA genomes was measured by densitometry and normalised to GAPDH levels (Bio-Rad). The density of hDlg protein levels from normal HFKs was significantly greater than those containing wild type genomes ($p=0.04$). Moreover, densitometer analysis of hDlg protein levels from E6ΔPDZ and E6ΔPKA genomes revealed a significant reduction in density reading of hDlg protein in wild type containing cells and E6ΔPKA genome containing cells, compared with E6ΔPDZ lines ($p=0.03$ and $p=0.03$ respectively). The bar graph represents the optical density data from 3 independent experiments carried out in 1 donor line. **(C)** Immunofluorescence reflects the results seen by western blotting. Monolayer cultures of HFKs containing wild type genomes, E6ΔPDZ or E6ΔPKA genomes were grown on coverslips to 80% confluency, fixed in 4% paraformaldehyde and probed for immunofluorescence with anti-hDlg antibody (2D11). hDlg intensities are strongest for those cells containing E6ΔPDZ genomes whilst HFKs containing wild type or E6ΔPKA genomes display a reduced hDlg staining pattern. Images are representative of 3 independent experiments in 3 donor lines.

5.2.2 Changes to the E6 PBM do not affect the expression of hScrib, MAGI-1 or TIP-2 protein levels in HPV-immortalised keratinocytes

In addition to hDlg, other PDZ proteins such as hScrib (the human homologue of the Drosophila tumour suppressor protein Scribble, found localised with hDlg at AJ and MAGI-1 (localised at TJ) are also targets for degradation by the E6 PBM (Gardiol *et al*., 1999; Glaunsinger *et al*., 2000; Kiyono *et al*., 1997). The ability of E6 to bind and degrade these PDZ domain-containing proteins appears to vary, with HPV16 E6 showing a preference for hScrib and HPV18 E6 showing a preference for hDlg and MAGI-1 (Kranjec & Banks, 2010; Thomas *et al*., 2003).

To assess the ability of HPV18 wild type and mutant genomes to target other members of the PDZ binding domain family, HFKs containing wild type, or mutant (E6ΔPDZ, E6ΔPKA) genomes were grown to 80% confluency, lysed in a buffer containing 8M urea and the solubilized proteins were resolved by SDS-PAGE and subjected to western blot analysis with a number of anti-PDZ antibodies. As can be seen in Figure 5.3, there appears to be no significant changes in the expression levels of hScrib protein between wild type and mutant containing HFKs and this result is consistent across 2 separate donor lines. Moreover, no significant changes were observed in MAGI-1 levels in wild type or mutant HFK lines (Figure 5.3). Interestingly, the levels of TIP-2- a member of the PDZ family involved in TGFβ signalling (Favre-Bovin *et al*., 2005) appear to be somewhat stabilised in the presence of a productive HPV infection as levels of the protein increase in cells containing wild type, E6ΔPDZ or E6ΔPKA genomes. In line with other PDZ substrates such as hScrib and MAGI-1, there appears to be no significant differences in the expression levels of the TIP-2 protein between wild type and E6 mutant genome containing HFKs (Figure 5.3). Taken together, these data suggest that the HPV18 gene products from whole viral genomes do not significantly change the protein levels of the hScrib, MAGI-1 or TIP-2 in monolayer cultures.

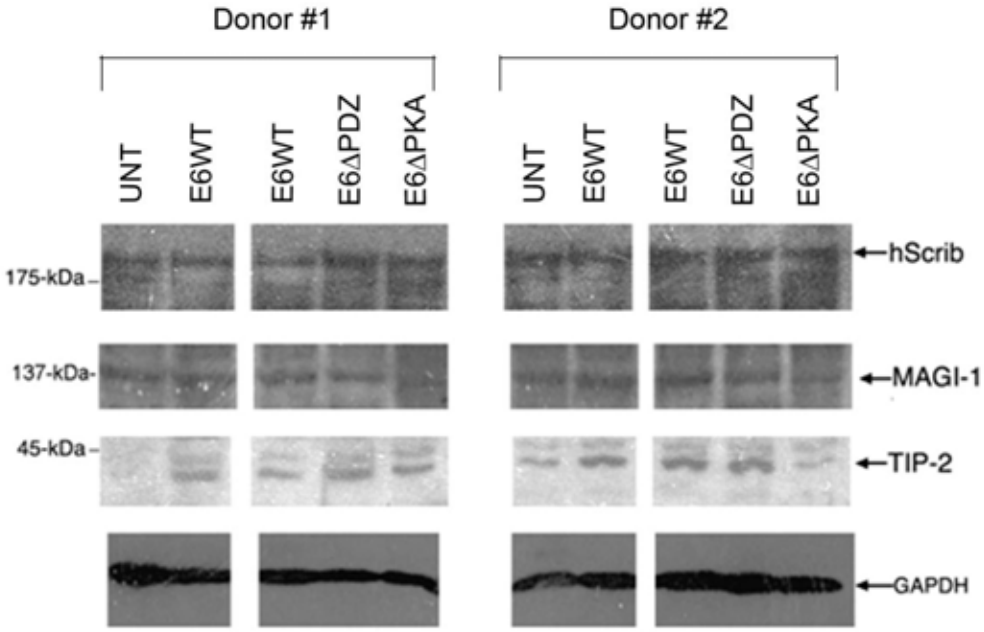


Figure 5.3 PDZ binding activity does not effect the expression of hScrib, MAGI-1 and TIP-2. Normal HFKs as well as HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were grown to 80% confluency, harvested and probed for immunoblotting with anti-hScrib, anti-MAGI-1 and anti-TIP-2 antibodies. Western blot analysis revealed no significant changes in the total protein levels of hScrib and MAGI-1 expressed from normal HFKs as well as those containing wild type or mutant genomes. Moreover, an analysis of TIP-2 expression levels revealed no significant changes in protein levels by western blot from HFKs containing wild type genomes and those containing either E6ΔPDZ or E6ΔPKA genomes, similar to that of hScrib and MAGI-1, however this protein appears to be stabilised in the presence of HPV18 genomes. Western blots are representative of 3 independent experiments carried out in 2 donor lines.

5.2.3 PTPN13 levels are not significantly changed in HPV18-transfected HFKs

PTPN13 is a member of the non-receptor phosphatases family which specifically fall into a class of phosphatases that contain FERM (four point, one ezrin, radixin, moesin) domains (Spanos *et al*., 2008). It is a highly modular 270 kDa protein with multiple interaction domains. The protein is known to contain five PDZ binding domains and has been implicated in several cellular pathways that play a role in cell survival. Previous reports have shown that HPV16 E6 interacts with and induces loss of the PTPN13 protein in a PDZ protein-binding manner (Spanos *et al*., 2008).

HFKs containing wild type, E6ΔPDZ and E6ΔPKA genomes were grown to 80% confluency, harvested and lysed in 8 M urea for subsequent analysis by western blotting with an anti-PTPN13 antibody. As can be seen in figure 5.4, there is no significant change in the protein expression of PTPN13 between normal HFK lysates and those which contain either wild type or mutant (E6ΔPDZ or E6ΔPKA) genomes. Interestingly, a band is observed in protein lysates from E6ΔPKA containing HFKs at approximately 175 kDa. This band is not visible in lysates from normal HFKs or those containing wild type or E6ΔPDZ genomes. The presence of this 175 kDa band is consistently present in E6ΔPKA lysates from three other donor lines suggesting that it is a common feature of HFKs expressing these genomes. The origin of the 175 kDa band is unknown however it could be a proteolytic breakdown product. Alternatively the band could be an alternatively spliced product which is stimulated in these cells.

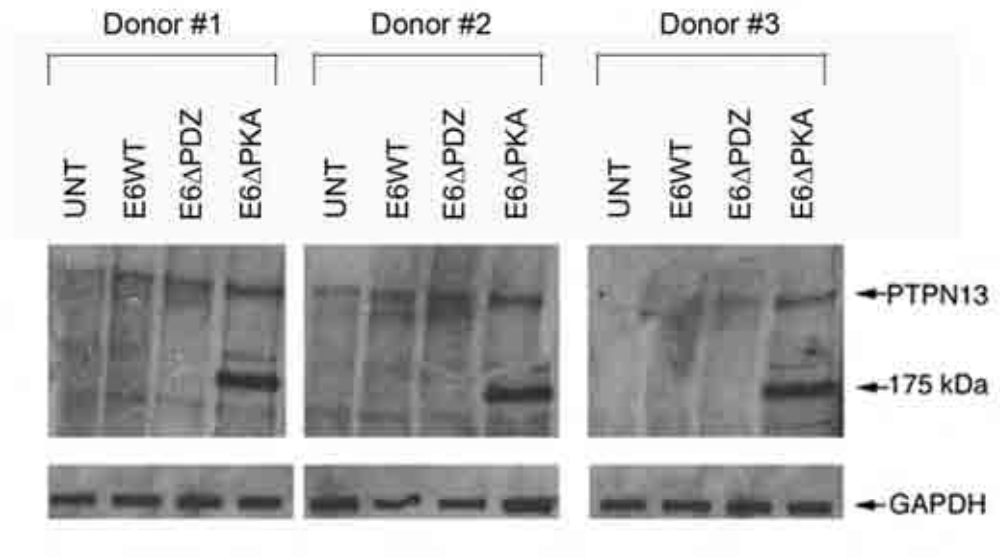


Figure 5.4 PTPN13 protein levels are not reduced in HFKs harbouring wild type, E6ΔPDZ or E6ΔPKA genomes. Monolayer cultures of cells containing either wild type or mutant genomes were grown to confluency, harvested and probed for immunoblotting with an anti-PTPN13 antibody. Western blot analysis of normal HFKs as well as HFKs carrying wild type, E6ΔPDZ and E6ΔPKA genomes revealed no significant changes in total protein levels of PTPN13 between normal HFKs and those containing wild type or E6ΔPDZ genomes. PTPN13 protein levels in HFKs containing E6ΔPKA genomes were similar to normal HFKs and those containing wild type or E6ΔPDZ genomes, however the presence of an additional protein band at approx. 175 k Da was a consistent feature of HFKs carrying this mutant genome. Western blots are representative of 2 independent experiments carried out in 3 donor lines.

5.2.4 Loss of negative regulation of the E6 PDZ-binding domain is associated with reduced hDlg protein staining in organotypic raft cultures

Whilst some changes in hDlg protein expression were noted in monolayer cell cultures containing wild type, E6ΔPDZ and E6ΔPKA genomes, the distribution of hDlg in HFKs containing wild type or mutant genomes stratified in organotypic raft cultures was also examined. Raft cultures of normal HFKs as well as HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were grown for 13 days, fixed, and paraffin embedded sections were probed by immunofluorescence with an anti-hDlg antibody (2D11). Images were taken on a Nikon Eclipse E600 microscope at the same exposure. As can be seen in figure 5.5A, a punctate pattern of localisation of hDlg protein can be seen at peripheral cell-cell contact regions. A minor population of hDlg proteins, distinct from the membrane-bound form can also be observed in the cytoplasm of normal HFKs (Figure 5.5A). Moreover, in the more differentiated suprabasal cell layers, hDlg protein is localised to the cell periphery at sites of cell: cell contact (Figure 5.5A). Raft cultures of HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes typically showed a similar pattern of hDlg localisation in basal cells. In HPV18 wild type transfected cells, a small reduction in the intensity of hDlg protein at the cell periphery was observed in suprabasal cell layers (Figure 5.5B). The relative amount of cytoplasmic hDlg also appears to be reduced in the differentiated suprabasal cell layers in these cultures (Figure 5.5B). Raft cultures containing E6ΔPDZ genomes cells displayed stronger hDlg staining than wild type cells, at levels comparable to untransfected HFKs in suprabasal cell layers and more differentiated cell layers (Figure 5.5C) with strong staining at cell membranes and sites of cell contact and these changes in hDlg protein intensity correlated with the total levels of hDlg protein detected by western blotting previously (Figure 5.5C; Figure 5.2A). Raft cultures of E6ΔPKA mutants, which have lost the ability to negatively

regulate the degradation of PDZ proteins, illustrate the most dramatic effect on hDlg protein levels. HFKs containing this mutant genome contain reduced levels of both cytoplasmic and membrane associated hDlg protein in more differentiated suprabasal cell layers (Figure 5.5D) when compared with other mutants of the PDZ binding motif (E6ΔPDZ) and wild type cells. These results indicate that changes to the PDZ binding domain affect the levels of hDlg protein in differentiating cells. Whilst a reduction in hDlg was observed in wild type raft cultures and those containing E6ΔPKA genomes, there is not complete loss of the protein. This is consistent with the retention of low levels of hDlg in cervical carcinoma cells such as Hela (Mantovani *et al*., 2001).

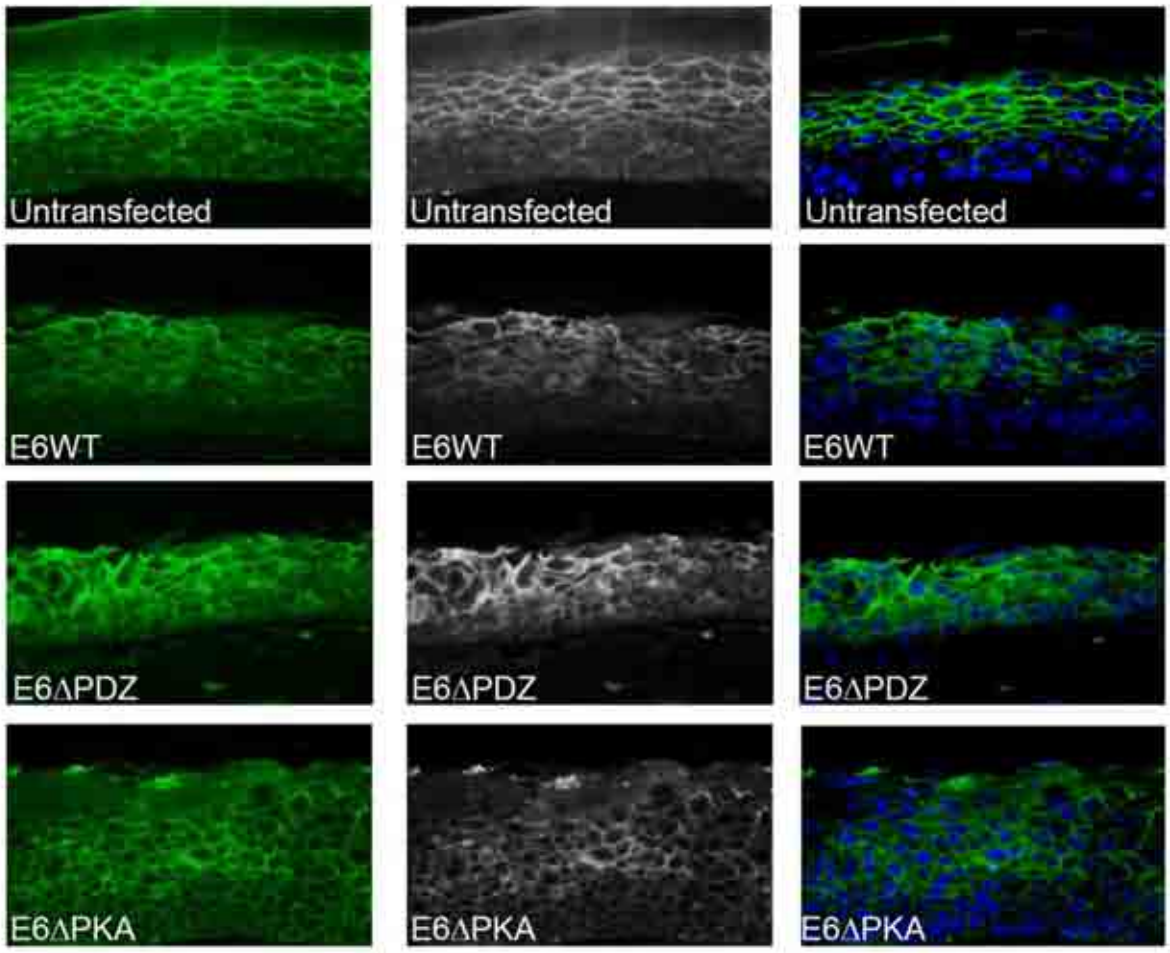


Figure 5.5 Constitutive expression of the E6 PBM correlates with a reduction in hDlg protein in organotypic raft cultures. Organotypic raft cultures of normal HFKs and HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were grown to 13 days, fixed in 4% paraformaldehyde and paraffin embedded sections were prepared for indirect immunofluorescence with anti-hDlg antibody (Green) and counterstained with DAPI (blue) for detection of nuclei. Images were taken on a Nikon Eclipse E600 microscope at the same exposure to avoid any bias. In normal HFKs the staining pattern of hDlg protein is strong and predominantly localised to the upper suprabasal cell layers in differentiating cells. The pattern of hDlg protein expression in E6ΔPDZ genome containing cells is similar to that of normal HFKs with hDlg protein expression at a similar level to that or normal HFKs. HFKs containing wild type genomes or the constitutively active E6ΔPKA mutation have diffuse pattern of staining in the basal and suprabasal cell layers. hDlg protein is detectable at the cell-cell junctions, similar to that of normal HFKs and E6ΔPDZ HFKs however the intensity of staining is reduced, particularly in the constitutively active E6ΔPKA mutant which display the weakest hDlg protein staining. These images are representative of 3 independent experiments carried out in 2 donor lines.

5.2.5 hScrib is not targeted for degradation by the E6 PBM in HPV18 transfected HFKs in organotypic raft cultures.

The results from hDlg staining of organotypic raft cultures revealed changes in the levels of hDlg protein between wild type and mutant genome containing HFKs. To assess whether the levels of other PDZ proteins are affected in a similar manner, the subcellular distribution of hScrib was assessed in organotypic raft cultures. Organotypic raft cultures were grown, fixed and paraffin embedded sections were subjected to immunofluorescence with an anti-hScrib antibody. As can be seen in Figure 5.6, there appears to be strong hScrib staining at the membrane cytoskeleton/sites of cell-cell contact in normal HFKs, especially in the more differentiated cell layers (Figure 5.6). The levels of hScrib are not changed significantly upon infection with HPV18 wild type genomes or HPV18 E6 mutant (E6ΔPKA or E6ΔPDZ) genomes (Figure 5.6). These data are in line with previous western blotting data which showed no significant changes in the expression of hScrib protein (Figure 5.3). Moreover, there appears to be no significant change in the localisation of the protein between normal HFKs and those containing wild type, E6ΔPDZ or E6ΔPKA genomes suggesting that hScrib does not act as a substrate for the HPV18 E6 PBM in differentiating cells. Failure to observed any changes in hScrib could be due to substrate specificity as HPV16 E6 has been shown to preferentially target hScrib over HPV18 (Thomas *et al*., 2003). Furthermore, the failure to observe any significant changes in hScrib protein expression in differentiating cells could be due to the fact that the E6 PBM induced subtle changes in the distribution of hScrib which were not readily discernible from this assay.

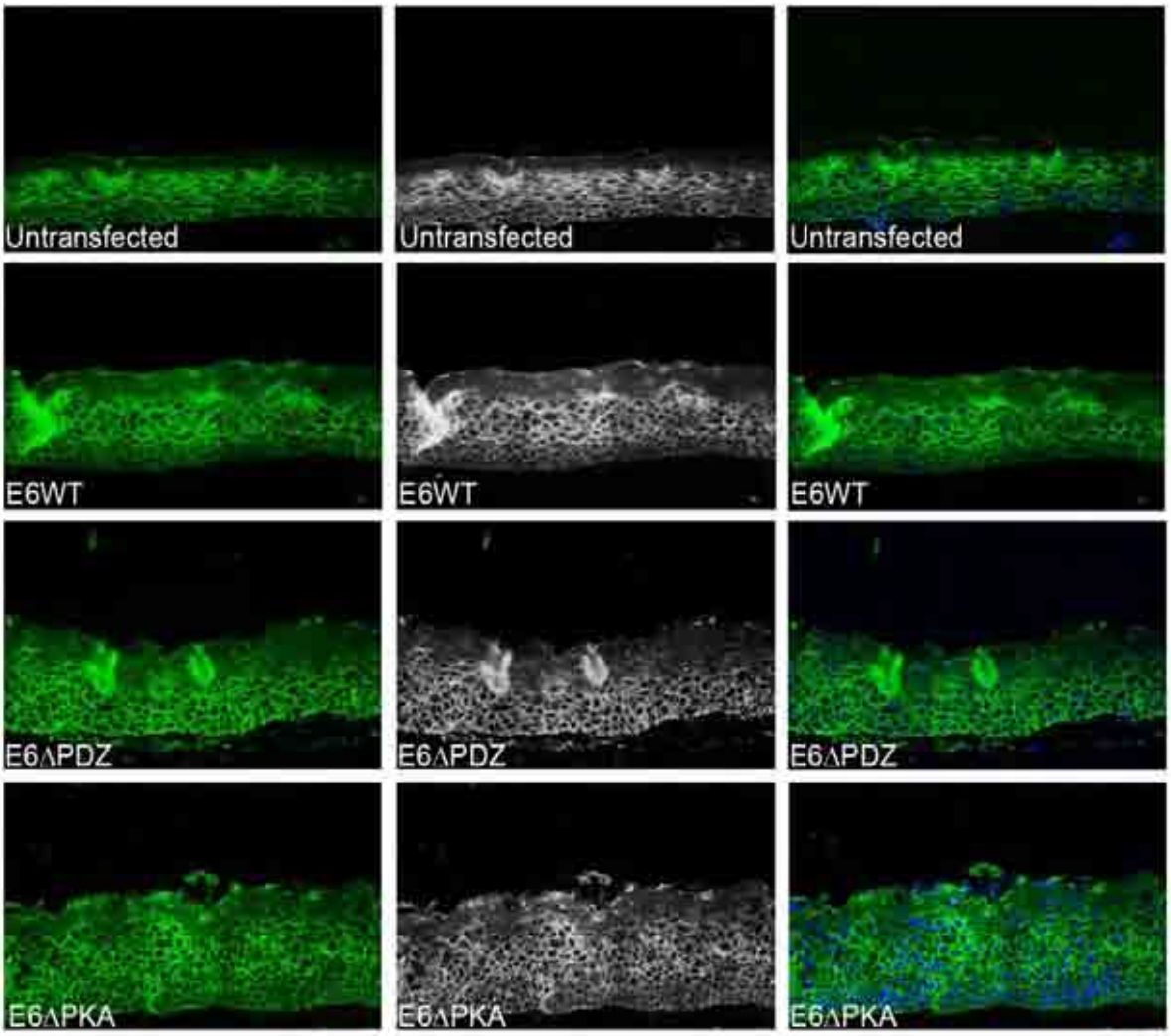


Figure 5.6 Changes to the E6PBM do not affect the expression of hScrib protein in differentiating cells. Paraffin embedded sections of normal HFKs and HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes were stained with anti-hScrib antibody (Green) and counterstained with DAPI (blue) for detection of nuclei by immunofluorescence microscopy. A similar pattern of hScrib staining was observed in normal HFKs and those carrying wild type genomes, with hScrib staining being predominantly found in the upper suprabasal cell layers of differentiating cells at areas of cell-cell contact/cell membranes. Expression of mutants of the E6 PBM do not significantly change the expression or localisation of hScrib protein staining in differentiating cells as a similar hScrib staining pattern was observed in HFKs containing E6ΔPDZ and E6ΔPKA genomes. These images are representative of 3 independent experiments carried out in 2 donor lines.

5.2.6 Abrogation of the E6 PBM does not effect connexin-43 expression in HPV-immortlaised keratinocytes

Connexin-43, also known as gap junction alpha-1 protein is a member of the connexin gene family and the encoded protein is a component of gap junctions, which are composed of arrays of intracellular channels that provide a route for the diffusion of low molecular weight materials from cell to cell (Kumar & Gilula, 1996). Disruption of gap junction proteins is frequently reported in malignant cell lines and tumours. Moreover, reduced connexin expression has been found in a number of different tumours (Holden *et al*., 1997; Saitoh *et al*., 1997; Saitoh *et al*., 2001). Immunohistochemistry of cervical biopsies showed reduced connexin-43 expression in dysplastic regions compared with normal epithelia (King *et al*., 2000). In the context of HPV, loss of connexin-43 expression has been shown to influence epithelial dysplasia (Aasen *et al*., 2003).

Previous experiments in connexin-43 null mice have shown changes in cell growth rate, loss of cell adhesion and altered cell morphology (Martyn *et al*., 1997; Naus *et al*., 1999). Data from this study has shown that changes in cell growth and morphology can be attributed to HFKs expressing a truncated E6 molecule lacking the PBM. To determine whether these changes in the E6 PBM activity are influenced by the levels of connexin-43 protein expression, normal HFKs and HFKs containing wild type and E6ΔPDZ genomes were grown to 80% confluency on coverslips and fixed in 4% paraformaldehyde in preparation for immunofluorescence with an anti-connexin-43 antibody. Immunofluorescence analysis of normal HFKs and HFKs containing wild type or E6ΔPDZ genomes show no changes in the expression level or distribution of connexin-43 within these cells. From figure 5.7 it can be seen that the levels of expression of protein remain consistent between normal HFKs and those containing either wild type HPV18 or the E6ΔPDZ mutant genomes (Figure 5.7).

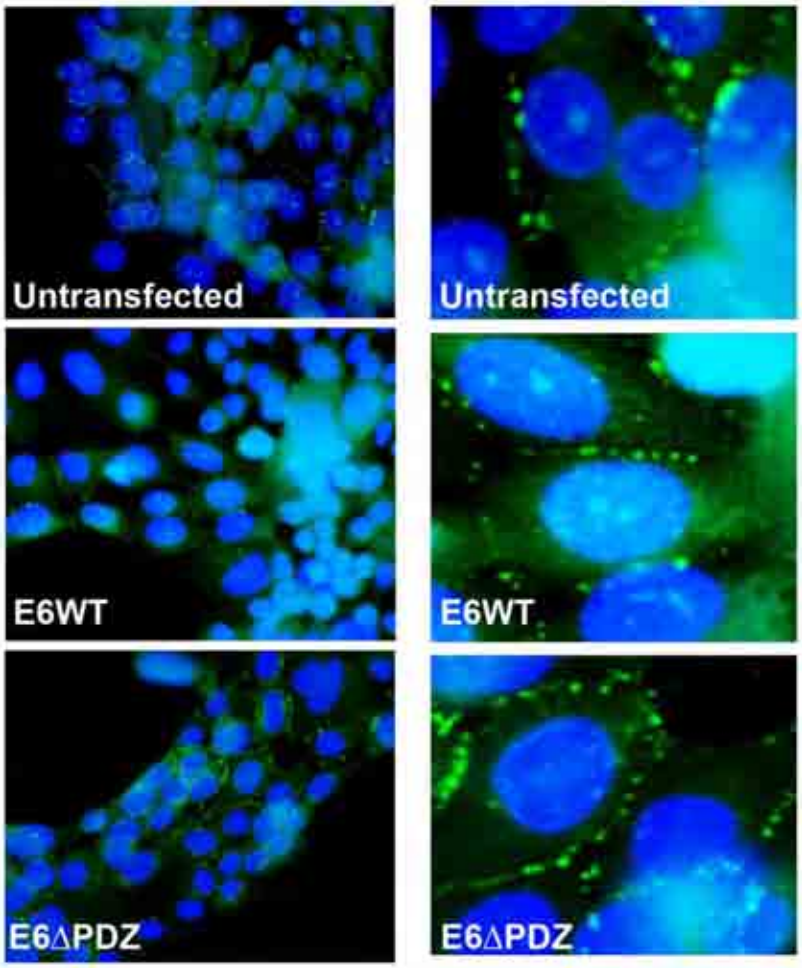


Figure 5.7 Abrogation of the E6 PBM does not affect the expression of Connexin-43. To assess whether changes in Connexin-43 expression are influenced by changes in E6 PBM activity, monolayer cultures of normal HFKs as well as those containing wild type or E6ΔPDZ genomes were grown on coverslips to 80% confluency, fixed in 4% paraformaldehyde and prepared for immunofluorescence with anti-Connexin-43 antibody (Green) and counterstained with DAPI (blue) for detection of nuclei. Immunofluorescence analysis of normal HFKs and HFKs containing wild type genomes showed a similar Connexin-43 expression pattern, with connexin-43 observed at the cell membrane. Expression of E6ΔPDZ genome containing cells does not significantly effect the expression of connexion-43 protein as the pattern of connexin-43 expression is similar to that of normal HFKs and HFKs containing wild type genomes. These images are representative of 3 independent experiments carried out in 2 donor lines.

Taken together, these results suggest that connexin-43 is not targeted by HPV18 as no changes were observed in connexin-43 levels between wild type containing and normal HFKs (Figure 5.7). In addition, the abrogation of the E6 PBM does not effect the expression or localisation of connexin-43 (Figure 5.7) suggesting that the phenotypes observed in HFKs expressing this E6 mutant may not be attributed to changes in connexin-43 expression.

5.2.7 Abrogation of the PBM does not effect expression of E-cadherin in HPV-immortalised keratinocytes

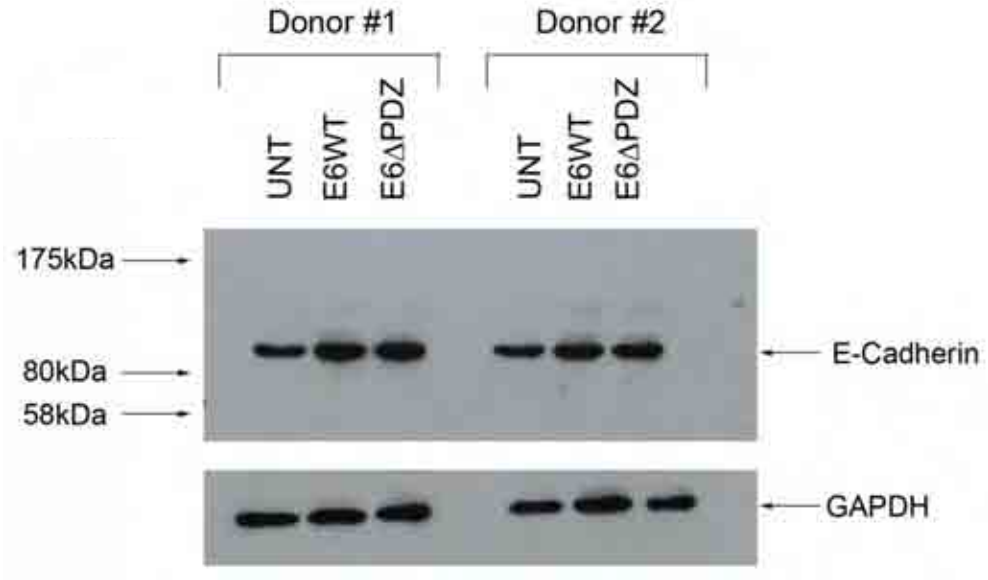
As previously discussed, in epithelial cells of vertebrates, hDlg is associated with the AJ. Adherens junctions are protein complexes which occur at cell-cell junctions in epithelial tissues and serve as a bridge connecting the actin cytoskeleton of neighbouring cells (Hartsock & Nelson, 2008). One of the major protein components of AJ is E-cadherin. E-cadherin is a calcium dependent cell-cell adhesion glycoprotein. In epithelial cells, E-cadherin is often found adjacent to actin containing filaments of the cytoskeleton. Mutations in this gene have implications in a number of different cancers including gastric, breast and ovarian cancers (Wijnhoven *et al*., 2000). E-cadherin down regulation decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility. In addition, the disruption of AJ's decreases the phosphorylation of E-cadherin by protein kinase CK2 and this process of down regulation is a common event in carcinogenesis (Serres *et al*., 2000).

To determine whether the changes in the phenotypes observed between wild type containing and E6ΔPDZ mutant genome containing cells could be attributed to changes in E-cadherin expression, normal HFKs as well as HFKs containing wild type or E6ΔPDZ genomes were grown on coverslips to 80% confluence and assessed by immunofluorescence as previously described with an anti-E-cadherin antibody (Chapter 2: Materials and Methods, Section 2.6.3). As can be seen in Figure 5.8A, E-cadherin can be seen at the cell periphery at sites of cell-cell contact. There appears to be no changes in intensity of E-cadherin staining at the cell

cytoskeleton between normal HFKs and HFKs containing HPV18 wild type genomes (Figure 5.8A). HFKs containing the E6ΔPDZ genomes have E-cadherin staining at levels comparable to normal HFKs and HFKs containing wild type genomes suggesting that the abrogation of the E6 PBM does not affect the levels of E-cadherin observed (Figure 5.8A).

To verify the results seen by immunofluorescence, monolayer cultures of normal HFKs and HFKs containing wild type or E6ΔPDZ genomes were grown to 80% confluence and lysates were extracted for immuunoblotting with an E-cadherin antibody. As can be seen in figure 5.8B, the levels of E-cadherin remain unchanged between normal HFKs and those harbouring wild type or E6ΔPDZ genomes (Figure 5.8B). Taken together these results suggest that E-cadherin levels are not significantly changed in monolayer cultures containing HPV18 genomes since no change was observed in E-cadherin levels by western blot analysis or immunofluorescence between normal HFKs and HFKs containing wild type genomes. In addition, the data from these experiments suggests that the abrogation of the E6 PBM has no significant effect on the levels of E-cadherin expression since no changes were observed in this study.

A



B

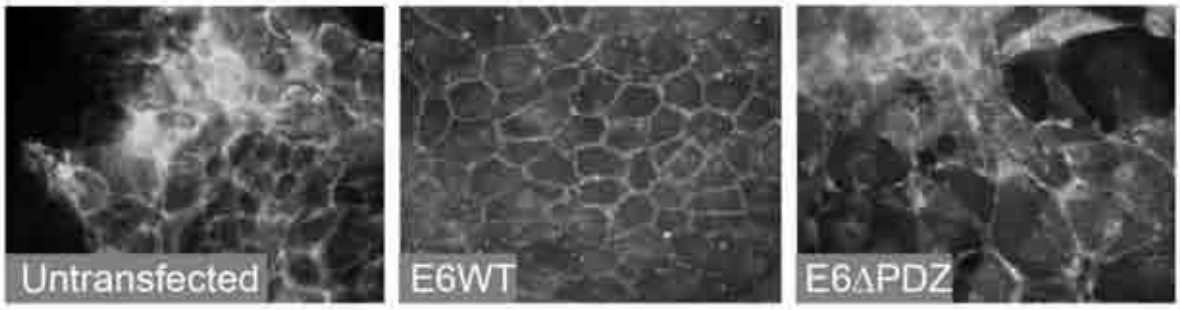


Figure 5.8 Abrogation of the E6 PBM does not effect the expression of E-cadherin. **(A)** Monolayer cultures of normal HFKs and HFKs containing wild type or E6ΔPDZ genomes were grown to 80% confluency, harvested and prepared for western blotting with anti-E-cadherin antibody. No significant changes were observed in E-cadherin protein expression between normal HFKs and those carrying either wild type or E6ΔPDZ genomes. GAPDH demonstrates equal loading of the protein samples. Western blot is representative of 3 independently performed experiments in 2 donor lines. **(B)** HFKs were also grown over coverslips, fixed in 4% paraformaldehyde and probed with an anti-E-cadherin antibody by immunofluorescence. These finding concur with the findings from (A). There are no significant differences in E-cadherin protein expression or localisation between normal HFKs and those carrying wild type or E6ΔPDZ genomes. Images are representative of 2 independently performed experiments in 2 donor lines.

5.3 Discussion

In this study the ability of HPV18 wild type, E6ΔPDZ and E6ΔPKA genomes to target known members of the PDZ domain containing family of proteins were examined. The differences in the behaviour between wild type and mutant genome containing HFKs correlated with the level of one of these targets, hDlg. hDlg has been previously shown to be important for regulating cell polarity and proliferation in response to cell-cell contact (Woods *et al*., 1996). E6ΔPDZ genomes express a non functional PDZ binding domain and show cellular levels of hDlg which are comparable to normal HFKs. Previous studies have shown that E6 processes hDlg for ubiquitin-mediated degradation and that this targeting is regulated by PKA (Kuhne *et al*., 2000). The E6ΔPKA mutation, which abrogates PKA's negative regulation, degrades hDlg in an E6-dependent constitutive manner, which is independent of cellular PKA levels. During the HPV replication cycle, PKA phosphorylation of E6 might be a means of regulating degradation of PDZ proteins and changes to mechanisms that regulate PKA activity. Indeed, a loss of PKA recognition of HPV18 E6 stimulates an increase in the loss of membrane-bound hDlg-an effect that correlates with enhanced morphological transformation of keratinocytes (Kuhne *et al*., 2000; Watson *et al*., 2003).

The decrease in hDlg protein levels exhibited by HFKs containing wild type and E6ΔPKA genomes may have been a result of a number of mechanisms. In addition to E6-directed degradation, hDlg is also controlled at the transcription level (Mantovani *et al*., 2001) so the reduction in hDlg levels could be attributed to proteosomal-mediated degradation or transcriptional down regulation. Interestingly, complete loss of the hDlg protein was never observed and only slight changes were seen in hDlg levels by immunofluorescence of organotypic raft cultures. This finding is consistent with the retention of the low levels of the hDlg protein in cervical carcinoma-derived cells like Hela (Mantovani *et al*., 2001). Furthermore, it is known that nuclear pools of hDlg are preferentially targeted by the E6

onocoprotein (Massimi *et al*., 2003) which may also explain why complete loss of hDlg was never observed as it could be a specific pool of the protein which is being degraded. Viral targeting of specific nuclear pools of hDlg provides strong evidence for hDlg having multiple functions within the cell (Roberts *et al*., 2007; McLaughlin *et al*., 2002) and suggests that hDlg may provide a number of functions essential for the survival in epithelial cells. Western blot analysis of HFKs containing E6ΔPDZ genomes revelaed a moderate increase in hDlg levels compared with normal HFKs. A recent study by Accardi and colleagues demonstrated that the E7 protein of HPV16 was involved in promoting the accumulation of phosphorylated forms of the PDZ protein NHERF-1. The accumulation of these phosphorylated forms of the protein were then preferetially targetted by E6 through its PDZ binding domain (Accardi *et al*., 2011). In the same respect, the E7 protein of HPV18 could be promoting the accumulation of phosphorylated forms of hDlg which are unable to be targetted by E6ΔPDZ genome containing cells (due to the lack of a PDZ binding motif) and may explain the increase in hDlg levels observed in these cell lines.

The presence of a class-1 PDZ binding motif on high risk E6 molecules would, in theory, render all cellular class 1 PDZ domain-containing proteins as putative proteloytic substrates for this viral oncoprotein. In the case of other potential PDZ substrates of E6 analysed in this study, no significant changes were observed in protein levels of these proteins in monolayer cultures of HFKs containing wild type, E6ΔPDZ or E6ΔPKA genomes by western blot analysis or by immunofluorescence. This is in agreement with a parallel study of HPV18 and HPV16 and their ability to target other members of the PDZ domain containing family for degradation, including FAP1, TIP2 and PTPN13. In each case, there was a failure to see any degradation of these targets by HPV18 or HPV16 (Kranjec & Banks, 2011). The failure to observe any significant changes in the expression of these proteins may also reflect the differences in the phosphorylation status of the target protein, which could influence

accessibility to the E6 PBM and subsequent targeting as shown in the case of hDlg (Massimi *et al*., 2006, Narayan *et al*., 2009). Moreover, it is possible that in binding proteins associated with cell polarity, high risk E6 proteins are actually targeting other functions of these proteins rather than simply mediating their degradation, such as the hDlg tumour suppressor function in its interaction with APC (Ishidate *et al*., 2000; Massimi *et al*., 2003) which may explain why no changes in the total levels of the protein were observed.

Western blot analysis of PTPN13 protein levels from HFKs containing wild type or mutant genomes revealed the presence of an additional 175 kDa band in cells containing E6ΔPKA genomes. This band was not present in lysates from wild type or E6ΔPDZ genomes. The origin of this band is unknown however it could be a proteolytic breakdown product or an alternatively spliced product which is stimulated in these cells.

In immortalised keratinocytes, E6 disrupts cell-cell association by a number of mechanisms including down regulation of expression levels of cell junction components or by interfering with pathways that assemble these structures at the cell membrane. Analysis of the epithelial junction marker protein E-cadherin and the gap junction protein connexin-43 were examined however no significant changes were observed in expression levels of either protein between normal HFKs and HFKs carrying a wild type or E6ΔPDZ genomes. It is possible that there may be some changes in the levels of these proteins; however the changes may be slight and not detectable by current the assays used. A more comprehensive analysis of TJ function is required to assess the contribution of wild type and mutant genomes to the degradation and localisation of cell junction components, in particular the ZO-1 protein which has been shown to be a critical regulator of tight junction assembly (Umeda *et al*., 2006; Fanning *et al*., 2007).

CHAPTER 6 FINAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Overview of findings

The E6 proteins of high risk HPV types have been shown to target a number of cellular proteins containing PDZ domains. The interaction between HPV and PDZ proteins is mediated by a class I PDZ binding motif (PBM) at the C-terminus of the E6 oncoprotein. The precise function of this domain in the infectious cycle of the virus or in virus-mediated carcinogenesis is not known although the E6 PBM has been shown to be important for E6 transformation of rodent cells (Kiyono *et al*., 1997) as well as anchorage independent cell growth (Spanos *et al*., 2008) and induction of EMT (Watson *et al*., 2003; Spanos *et al*., 2008), both of which are hallmarks of cancer and metastatic progression. Studies in HPV31 transfected primary HFKs have implicated this domain in the negative regulation of cellular proliferation and maintenance replication of viral episomes in undifferentiated cells (Lee & Laimins, 2004). More recent work by Nicolaides and colleagues in a transformed keratinocyte cell line transfected with HPV16 substantiate the requirement of the E6 PBM for episomal genome maintenance (Nicolaides *et al*., 2011).

The study described in this thesis has highlighted the importance of the E6 PBM function during both the early and late stages of the HPV18 viral life cycle. In addition, evidence from this study suggests that the function of the E6 PBM in the HPV life cycle and in carcinogenesis may be regulated by host cell signalling pathways.

6.2 Evaluating the loss of episomes in E6ΔPDZ genome containing cells

These studies have shown that primary human foreskin keratinocytes - the host cell of the virus- are able to support wild type genomes of the oncogenic virus HPV18. Moreover, these genomes are stably maintained upon extended passaging, in agreement with similar studies of HPV31 (Lee & Laimins, 2004). Interestingly, E6ΔPBM episomes are gradually lost from the cells and this effect on viral replication correlates with the appearance of integrated forms of the viral genome. These effects are independent of a constitutive E6 PBM function since HFKs containing E6ΔPKA genomes, which are constitutively active for PDZ binding, are stably maintained upon extended passaging. The molecular basis for this reduction is unknown; however the failure of viral episomes to persist in keratinocytes is a conserved feature of other high risk types including HPV16 and HPV31 (Nicolaides *et al*., 2011; Lee & Laimins, 2004). p53 has been shown to be a potential suppressor of the maintenance replication of HPV genomes following infection (Lepik *et al*., 1998). In this light, Nicolaides and colleagues suggest that the tight regulation of E6 is crucial for the maintenance replication in basal cells and that in the absence of an E6 PBM, levels of E6 are not stabilised, which may have implications on the ability of E6 to target other cellular proteins such as p53 (Nicolaides *et al*., 2011).

In this study no changes in the expression level of E6 proteins were observed between cells carrying the PBM deleted HPV18 genomes and the wild type genome containing cells in monolayer cultures or when stratified in organotypic raft cultures, suggesting that the episomal reduction observed is not attributed to significant changes in E6 stability. The differences in E6 stability observed between different HPV types could be attributed to differences in cell systems used to analyse the viral life cycle or HPV subtype. The ability to detect the E6 protein in *vitro* and *in vivo* has previously been notoriously difficult, however the development of antibodies which detect the E6 protein have been a major benefit to

understanding this protein. Future work may be to look at the subcellular distribution of the E6 protein in HFKs containing wild type, E6ΔPDZ and E6ΔPKA genomes.

As previously mentioned the maintenance replication of HPV genomes is not well tolerated in cells and requires the viral proteins to alter a number of cellular proteins including p53, Rb and PDZ proteins amongst others. Previous work has shown that these activities are tightly regulated in a way that loss of one function often influences the action of another (Park & Androphy, 2002). In a similar manner, the ability of E6 to bind to PDZ proteins may act to create a balance with another viral protein activity and a consequence of the inability of the E6 protein to target PDZ substrates may be episomal loss. The molecular basis for this episomal clearance is not understood and is an important area of future investigation.

Further analysis of the HPV18 genome containing cell lines indicated that cells harbouring HPV18 genomes unable to target PDZ substrates were defective in normal mitotic progression and this correlated with an increase in the number of atypic nuclei in cell cultures containing these E6ΔPDZ genomes. Previous studies have shown that during cytokinesis there is dramatic accumulation of hDlg within the midbody (Massimi *et al*., 2003; Unno *et al*., 2008). The presence of hDlg at the midbody suggests that hDlg may play a functional role in the process of cytokinesis. Centrosome abnormalities in response to HPV E6 expression have been reported previously and primarily lead to the development of multiple nuclei as well as micronuclei and large multi-lobed nuclei which may have formed as a consequence of a persistent block of cytokinesis (Duensing *et al*., 2000; Duensing *et al*., 2001). This may be explained in part by the ability of HPV E6 proteins to relax G_2/M checkpoint control, thereby allowing an increased proportion of cells to enter mitosis. In cells containing E6ΔPDZ genomes there is an increased frequency of atypic nuclei (particularly bi-nucleate and multi-nucleate cells) compared with matched cultures containing wild type or E6ΔPKA genomes. Overexpression studies of hDlg and hScrib have shown that when hDlg and hScrib are

overexpressed there is an inhibition of S-phase entry and proliferation (Hanada *et al*., 2000; Ishidate *et al*., 2000; Nagasaka *et al*., 2006). Furthermore, the studies from Unno and colleagues show that overexpression of hDlg constructs in Hela cells that contain HPV18 E6 cause defects in cytokinesis including cellular multinucleation (Unno *et al*., 2008). In this light it is tempting to speculate that targeting of hDlg by the E6 PBM is required to overcome a defect in cytokenesis in HPV containing cells. Moreover, since maintenance of HPV episomes is linked to mitotic tethering of the viral DNA (Sekhar *et al*., 2010), our observations may be relevant to the phenotype of these cells with regards episome persistence.

One experiment to consider would be to introduce a knockdown of the known PDZ targets e.g. silencing of hDlg in E6ΔPDZ lines which fail to target PDZ substrates, to evaluate the rescue of the episomal loss phenotype and determine which, if any of the PDZ substrates are responsible for the phenotypes observed. During this thesis, stable cell lines (in total 14 cell lines) were generated containing individual shRNAs to hDlg in cells containing E6ΔPDZ genomes. Upon analysis of hDlg protein expression by western blotting significant depletion of hDlg was not observed (data not shown); however due to time constraints these cell lines were not analysed further. In future analyses it will be important to extend the passage of these cell lines to determine if the mutant HPV18 genomes persist in the presence of the shRNA – it is possible that a small pool of hDlg has been targeted; also further knock-downs should be prepared using multiple pools of the shRNAs as well as shRNAs to other PDZ substrates such as hScrib and MAGI-1.

The reduction in episomes observed in HFKs containing E6ΔPDZ genomes also correlates with a loss of the *E2* gene in a PCR integration assay. Integration is a consequence of failure to maintain episomal forms of the genome. The mechanism behind the integration observed in HFKs containing E6ΔPDZ genomes is unclear however it could be a result of the positive

selection of cells with integrated copies of the genome over cells with episomes that are not maintained. A previous analysis in the HPV16-containing cervical keratinocyte cell line W12 has shown a selection of HPV16 integrants in cells with reduced numbers of episomes (Pett *et al*., 2006). In agreement with the results from this thesis, parallel studies in HPV16 and HPV31 of E6 mutants which are unable to bind to PDZ substrates also show cells containing these E6 mutants appear to integrated upon extended passaging (Nicolaides *et al*., 2011; Lee & Laimins, 2004). Previous studies have shown that cells containing integrated copies of the HPV genome acquire a selective growth advantage over cells harbouring episomal copies of the viral genome (Pett *et al*., 2004). Interestingly, although the mutant genome E6ΔPDZ has been shown integrate; HFKs containing E6ΔPDZ genomes still proliferate slower than wild type cells indicating that the E6 PBM does control growth in some manner. The mechanisms for this control have yet to be established.

In this study, HFKs containing E6ΔPDZ genomes showed a significant reduction in their growth, even when compared to normal HFKs. Conversely, abrogating the negative regulation of the PDZ domain by means of the PKA mutation results in a significantly faster growth than normal HFKs and HFKs containing wild type genomes. Previous work by Ishidate and colleagues has highlighted the importance of APC and hDlg in regulating cell proliferation. In this study they show that the C-terminal domain of APC interacts with the PDZ domain of hDlg to form a complex, and this complex is important in negatively regulating cell cycle progression (Ishidate *et al*., 2000). Moreover, inhibition of cell cycle progression was abolished when the PDZ domain of hDlg was mutated (Ishidate *et al*., 2000). Based on these data it is possible that constitutive targeting of PDZ substrates such as hDlg may disrupt the formation of a complex with APC and contribute to the phenotypes observed.

One of the major activities of the E6 oncoprotein is its ability to inhibit apoptosis, which is largely thought to occur through its interactions with p53, Bak and pro caspase 8 (Filippova *et*

al., 2007). A study by James and colleagues has shown that HPV16 E6 can also inhibit apoptosis in a PDZ-dependent manner (James *et al.*, 2006). A similar study by Zhan and colleagues identify Scribble as important in this process as a depletion of Scribble in mammary epithelia was shown to inhibit apoptosis (Zhan *et al.*, 2008). FACS analysis of HFKs containing wild type or mutant HPV genomes did not identify any significant sub G1 peaks, however as there is evidence that these PDZ proteins are important in regulating apoptosis, and so further examination should be considered.

On this basis of these findings, it is tempting to speculate that loss of PDZ targeting by phosphorylation of the E6 PBM could promote chromosomal integration of the viral DNA. If this is indeed the case then changes in the cellular signalling pathways such as PKA signalling may be a risk factor for malignant progression.

6.3 Signalling pathways implicated in the phenotypes observed

A number of the PDZ substrates targeted by the E6 oncoprotein have been implicated in the regulation of several signalling pathways in various systems including Wnt, Notch, NF-$_K$B and STAT signalling pathways. The precise mechanism by which the polarity regulators control signalling is still unclear. Moreover, how misregulation of these signalling pathways are associated with some of these phenotypes observed including loss of episomes, cell proliferation, differentiation and invasion is not fully explained.

NF-$_K$B activation has been implicated in the pathogenesis of many cancers (Dolcet *et al.*, 2005), with roles in a number of different processes related to transformation and oncogenesis including proliferation, migration, angiogenesis and prevention of apoptosis. Studies by James and colleagues revealed that HPV16 E6 was able to activate NF-$_K$B in airway epithelial cells (James *et al.*, 2006) and this was dependent upon the presence of the E6 PBM. NF-$_K$B activation in HPV-infected cells is likely to play a role in the proliferative capacity of cells by

protecting them from apoptosis, which is reflected in the diminished ability of HPV18 E6 mutants which are unable to bind to PDZ proteins (E6ΔPDZ) to proliferate and cause epithelial hyperplasia in organotypic raft cultures. An appreciation of which NF-$_K$B pathways are affected by the E6 PBM and what PDZ substrates are targeted is crucial to the understanding of how these PDZ dependent mechanisms contribute to the phenotypes observed.

Upregulation of Notch signalling has already been implicated in a number of different cancers including T-cell leukaemia (Clark *et al*., 2007; Roy *et al*., 2007). Studies in *Drosophila* have shown that the Lgl polarity protein is involved in the asymmetric distribution of Numb, an inhibitor of Notch signalling (Wirtz-Peitz *et al*., 2008). Moreover studies of Lgl null mice display enhanced hyperplasia which was associated with increased Notch signalling (Klezovitch *et al*., 2004). Haematoxylin and eosin staining of organotypic raft cultures revealed distinct differences in the morphology of HFKs containing wild type HPV18 genomes or mutants of the E6 PBM. E6ΔPKA containing HFKs which are constitutively active for binding and degrading PDZ substrates were associated with increased hyperplasia. Future work could focus on the contribution of Notch signalling to the phenotypes observed by silencing Numb in cells which are unable to bind to PDZ substrates (E6ΔPDZ) and see whether the same phenotypes are observed from HFKs containing E6ΔPKA genomes.

Alteration of Wnt signalling has been shown to be a major factor in the outcome of a number of different cancers including colorectal cancers (Clevers, 2006). hDlg was first implicated in Wnt signalling when it was shown that it could bind a negative regulator of Wnt signalling, APC (Matsumine *et al*., 1996). As previously mentioned, the binding of hDlg to APC has been shown to be important for the effects of hDlg on the cell cycle (Ishidate *et al*., 2000) and migration (Etienne-Manneville *et al*., 2005). This study has shown that changes to the HPV18 E6 PBM have consequences on cell growth. Constitutive binding of the E6 PBM was

associated with a significant increase in growth rates of keratinocytes compared with HFKs containing either wild type or E6ΔPDZ genomes and this correlated with lower levels of the hDlg protein by western blot analysis and immunofluresence microscopy of monolayer cultures and paraffin embedded sections. Based on these data, it is possible that the changes in growth and invasion observed in HFKs containing E6ΔPKA genomes could be attributed to a loss of binding of hDlg to APC, which may implicate Wnt signalling as a potential pathway for further studies.

As well as having roles in Wnt signalling, hDlg as well as other PDZ substrates have been shown to be important in the function of a number of G-protein coupled receptors (GPCR) including the serotonin 2A receptor, mGlu receptor and the tumour marker TEM5 (Xia *et al*., 2000; Yamamoto *et al*., 2004; Funke *et al*., 2005). The regulation of these GPCRs often requires direct binding of the PDZ substrate to the receptor. GPCR signalling is regulated by mPins (Sans *et al*., 2005; Yasumi *et al*., 2005) which has been shown to be a potent tumour suppressor in *Drosophila* neuroblasts (Lee *et al*., 2005). GPCR signalling has been shown to regulate a number of different pathways including the cAMP signalling pathway. GPCR signalling activates cAMP dependent protein kinase in a feedback loop which requires activated cAMP to phosphorylate the receptor. Loss of GPCR signalling has been shown to be associated with tumourigenesis *in vitro* and *in vivo* (Schafer *et al*., 2004). Data from this thesis has shown that changes in cell growth were attribute to changes in PKA signalling as treatment of HPV containing keratinocytes with a number of activators and inhibitors of PKA resulted in changes in the growth of HPV transfected keratinocytes, suggesting that this pathway could be important in determining a mechanism for the phenotypes observed.

The life cycle of HPV requires the establishment of a persistent infection with low copy episomes in infected basal cells. HPV genomes persist in infected cells by evading the host's innate and adaptive immune responses by a number of different mechanisms. The

JAK/STAT pathway is a major pathway which regulates the innate immune response. Recent studies by Hong and colleagues have shown that suppression of STAT-1 expression by HPV E6 was necessary for virus genome amplification and maintenance of episomes (Hong *et al*., 2011) and that E6AP binding was required for this suppression, indicating an important role for this activity in pathogenesis. Data from the study described in this thesis has shown that the HPV18 E6 PBM is required for the efficient establishment and maintenance replication of episomes in donor HFKs. Moreover, HPV genomes which are unable to bind to PDZ substrates (E6ΔPDZ) fail to amplify their genomes when suspended in 1.5% methylcellulose. Although STAT-1 suppression may not be involved in the phenotypes observed in this study, it is possible that other members of the IFN-stimulated gene factor 3 (ISGF-3) complex such as STAT-2 and IFN regulatory factor 9 could be selectively suppressed and that this suppression requires an E6-PDZ interaction.

The malignant potential of the high risk HPV types is largely attributed to the transforming potential of the HPV E6 and E7 oncoproteins. It is well established that these proteins work cooperatively. The E7 protein is responsible for the inactivation of Rb and activation of E2F which drives S phase genes. As a result of unscheduled DNA replication, apoptosis is triggered which is counteracted by the E6 oncoprotein by degradation of the tumour suppressor protein p53 (Scheffner *et al*., 1990; Huibregtse *et al*., 1991). Based on this observation, it is possible that the degradation of PDZ substrates may also be a consequence of the cooperative functions of E6 and E7. Recent studies have shown that the HPV16 E6 and E7 proteins work together to target the PDZ protein, NHERF-1 which is involved in signalling and transformation (Accardi *et al*., 2011). HPV16 E7 was shown to promote the accumulation of phosphorylated forms of NHERF-1, which was preferentially targeted by HPV16 E6. In the same manner, high risk E7 and/or other E6 functions may be responsible for the accumulation of other PDZ substrates which can be preferentially targeted by the E6

PBM, also the virus may induce these specific PDZ substrates for a specific part of the life cycle and they are then only targeted by E6 at times when not functional – hence the regulation of the E6 PBM by phosphorylation.

6.4 A physiological role for PKA phosphorylation in HPV infected cell

Loss of conditional regulation of the E6 PBM within HPV18 genomes (E6ΔPKA) was associated with a significant increase in cell growth compared to cells carrying the wild type genomes. This suggests that changes in PKA signalling in cells carrying wild type genomes may lead to changes in growth characteristics. Indeed, the treatment of HFKs harbouring wild type genomes with specific stimulators of PKA signalling (e.g dibutyryl-cAMP) impeded keratinocyte cell growth, whilst increased growth occurred upon inhibition of PKA signalling using the competitive inhibitor H89. The positive effects on cell growth were verified upon stratification of the E6ΔPKA cells in organotypic raft culture which showed significant increased hyperproliferation of suprabasal cells compared to wild type genome containing cells.

Conditional regulation of E6-PDZ targeting was also linked to cell invasion. Cells containing the E6ΔPKA genomes-which are unable to respond to PKA and thus constitutively active for PDZ targeting, showed increased invasion on collagen compared to wild type genomes that could still be regulated by PKA. A study by Watson and colleagues showed that using an identical mutation of E6, constitutive activation of the E6 PBM in cells which are unable to respond to PKA was associated with an increased EMT phenotype and actin cytoskeleton disorganisation (Watson *et al*., 2003). Cell invasion is the final step in the process of EMT which involves alteration of the epithelium structure and disruption of the basal lamina (Goode & Perrimon, 1997). In this light it is tempting to speculate that the phenotypes

observed may be due to an EMT. Moreover, it is unclear what effect the abrogation of the E6 PBM function has on cell invasion. In the same study by Watson and colleagues, changes to the epithelial phenotype of cells containing a mutant which is unable to bind to PDZ substrates (Thr156Glu) was less marked, however there was still evidence of some EMT characteristics such as actin stress fibre formation (Watson *et al*., 2003). Of course in this study a deletion of the E6 PBM was not examined and it is possible that the phosphomimic mutation Thr156Glu retains a degree of PDZ-binding. Future studies may focus on the requirement of an intact PBM to the invasive phenotypes observed.

This study has highlighted the importance of a regulated, functional PBM during the life cycle of HPV18 and link E6 PBM function to maintenance replication of viral episomes and growth regulation of episome-containing cells. Moreover, changes in activity of E6 PBM are associated with alteration in expression levels of some but not all known PDZ substrates including hDlg. It has been 15 years since hDlg was shown to be targeted by the E6 PBM of high risk papillomaviruses (Kiyono *et al*., 1997; Lee *et al*., 1997). Since then a number of other substrates of the E6 PBM have been identified including the most recent NHERF-1 protein (Accardi *et al.*, 2011). A comprehensive analysis of all known PDZ substrates of E6 is required to determine which PDZ substrates are responsible for the phenotypes observed in this study. It is worth bearing in mind that other potentially important PDZ targets may exist which as yet are undiscovered. Interestingly, western blot analysis of the tyrosine phosphatise, PTPN13 in cells containing a constitutively active PDZ domain revealed an additional band at approximately 175 kDa and this was present in multiple donor containing these mutant genomes. The origin of this band is unknown however it is of interest that it is present in all donor lines which express the constitutively active mutant. It is possible that the additional band represent an alternatively spliced product or proteotlytic breakdown product. Further investigation is required to determine the origin of this product.

An alignment of HPV sequences from high risk and low risk types, revealed that all of the HPV types within the alpha genus recognised as high risk types (Group 1 and 2, Figure 3.1) (Bouvard *et al*., 2009) contain a PBM at the extreme C-terminus. Moreover, of the types frequently found in cancer (Group 1, Figure 3.1) a PKA consensus motif (RXXT) overlapping the E6 PBM is a conserved feature, however this recognition motif is not found in viruses that contain an E6 PBM and not defined as fully carcinogenic. The conservation of this PKA recognition site within a distinct group of HPV types could therefore represent a molecular signature for carcinogenesis since mutation studies within the context of complete HPV18 genomes (E6ΔPKA) resulted in increased cell growth and invasion. Interestingly, HPV types defined as classified as probably, or possibly carcinogenic (Groups 2A and 2B respectively), do contain a PKA recognition motif within the extreme C-terminus of E6, however overlap with the PBM is infrequent; only HPV68 and HPV67 from Groups 2A and 2B respectively contain a PKA recognition motif which overlaps with the E6 PBM. Future studies may identify whether phosphorylation may alter the interactions between E6 and PDZ substrates in these HPV types and others.

Crystallization studies of E6 PBM-PDZ interactions have shown that the interaction between E6 PBM and its substrates occurs within the substrate groove of PDZ molecules between βB strand and the αβ helix (Zhang *et al*., 2007) with the main chain carboxyl group of the E6 peptide anchored within the motif of the PDZ protein (Zhang *et al*., 2007). Studies by Zhang and others have shown that arginine 153 contributes to the binding of MAGI-1 PDZ1 but not to the binding of hDlg or MAGI-3 PDZ1 (Thomas *et al*., 2008; Zhang *et al.*, 2007). Moreover, mutation of the R^{154}→G greatly reduced the ability of E6 to bind hDlg and MAGI-1 and induce their degradation (Zhang *et al*., 2007) suggesting that additional factors in the E6 sequence or structure can determine the specificity of binding and indicate that PDZ target selection may be influenced by other residues. A number of studies have shown that minor

differences in the E6 PBM can significantly affect PDZ domain targeting (Thomas *et al*., 2005; Zhang *et al*., 2007). It is possible that the changes made within the PKA-RM may have altered substrate selection and it will be important for future studies to verify that the mutant has a similar degradation profile of PDZ targets such as hDlg, MAGI-1, -2 , -3 and hScrib as the wild type E6 protein.

Like HPV E6, a number of other cellular proteins with PDZ binding motifs are negatively regulated by phosphorylation. A study by Chung and colleagues showed that casein kinase II is able to phosphorylate the serine residue within the PDZ motif of the NR2B subunit of NMDAR -a glutamate receptor which plays a critical role during neural development. Phosphorylation by CKII was shown to disrupt the interaction between PSD-95 or SAP 102 and NR2B, resulting in a decrease in the surface expression of NR2B in neurons (Chung *et al*., 2004). Moreover, studies from the Choi and Chetkovich laboratories have shown that PKA phosphorylation of the PDZ ligand of the transmembrane receptor regulatory protein STARGAZIN abrogates its binding to the PDZ substrate PSD-95 (Chetkovich *et al*., 2002; Choi *et al*., 2002). Similarly, phosphorylation of the PDZ motif on Kir2.3 ion channels disrupts the binding with PSD-95 at complexes in the hippocampus (Cohen *et al*., 1996). Other proteins with PDZ motifs which are negatively regulated by phosphorylation include the C terminal ligands of the GluR2 receptor which when phosphorylated by PKA and PKC *in vitro* and *in vivo*, abrogates the binding to the glutamate receptor-interacting protein GRIP (Matsuda *et al*., 1999; Chung *et al*., 2000). Other protein/PDZ interactions which are inhibited by phosphorylation are listed in Table 6.1.

Table 6.1 Phosphorylation of the PDZ domain modultes protein interactions

PDZ Containing Proteins	Binding Partners	PDZ Ligand	Kinase
PSD-95	Kir 2.3	ISYRREE**S**RI	PKA (RR/KX/**S/T**)
PSD-95	Stargazin	ANRRT**T**PV	PKA (RR/KX/**S/T**)
PSD-95/SAP102	NIR2B subunit of NMDAR	LSSIE**S**	CK2 (**S/T**XXE/D)
GRIP1	GluR2 subunit of AMPAR	YNYGIE**S**VKI	PKC (RXX**S/T**XR/K)
PSD-95/SAP97	LRP4	ERKL**S**SESQV	CamKII (RXX**S/T**)

Whilst many studies have reported that phosphorylation of the C-terminal PDZ domain of proteins, negatively regulates PDZ interactions, a number of studies have shown that phosphorylation can promote or have no effect upon PDZ interactions. A study by Von and colleagues demonstrated that calmodulin dependent kinase II and PKA phosphorylation of FATZ3 and FATZ1 respectively mediated the interaction with the PDZ proteins of ZASP, Cypher and other Enigma family protein members (Von *et al*., 2009). Conversely phosphorylation of the NR2C subunit of NMDAR by PKA and PKC did not affect binding to PSD-95 (Chen et al., 2006).

It is important to note that other viruses may also regulate their interactions by phosphorylation of the PDZ domain. Recent studies have shown that CKII phosphorylation of the HTLV-1 PDZ binding motif abolishes Tax-1 binding to hDlg indicating that phosphorylation of the PBM is an important mechanism which is shared amongst other virus types (Bidoia *et al*., 2010). Data on the phosphorylation sites of PDZ domains and the roles of phosphorylations of these domains will be useful to determine a regulatory mechanism for PDZ mediated interactions. Future studies may focus on how the phosphorylation of the E6 PBM affects the binding of E6 to all known PDZ substrates, i.e. does it enhance, suppress or have no effect on binding specific PDZ domain containing proteins. Moreover,

phosphorylation of the E6 PBM may result in the binding to other novel proteins which are not yet defined (Li *et al*., 2011).

6.5 Final Statement

These novel studies have shown that the E6 PBM, a conserved motif amongst high risk HPV types, has a crucial role in the early and late stages of the HPV18 life cycle. Interestingly, those HPV types that have a strong association with human cancers have evolved a strategy to regulate this domain by phosphorylation, and the data described in this thesis indicates that the conditional regulation of this domain is of physiological relevance to the HPV replication cycle and maintenance of viral episomes. Moreover, these studies provide evidence to suggest that changes in PKA signalling in cells infected with high risk HPV types has consequences on viral pathogenesis. Loss of PKA signalling in these studies using intact HPV genomes is associated with increased cell proliferation and cell invasion; both hallmarks of carcinogenesis. Thus changes in PKA signalling and phosphorylation of E6 during specific stages of the life cycle and/or malignancy can be expected to regulate this activity of E6. In addition, a change to the E6 PBM function in the viral genome-containing cells was associated with changes in expression of substrates of the E6 PBM including hDlg and PTPN13.

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