# Regulation of Growth Factor and Nutrient Sensing Pathways by Human Papillomavirus E6 Proteins

Karl Münger Jennifer Marie Spangle

**Regulation of Growth Factor and Nutrient Sensing Pathways by Human Papillomavirus E6 Proteins**

High-risk human papillomaviruses (HPVs) are associated with nearly all cases of cervical cancer and also contribute to other types of anogenital and oropharyngeal cancers. The high-risk HPV E6 oncoprotein contributes to malignant progression in part by the targeted degradation of the tumor suppressor p53. The activation of growth factor and nutrient sensing pathways including receptor protein tyrosine kinases (RPTKs) and mTORC1 may also support cellular transformation. Moreover, previous studies suggested that HPV16 E6 activates mTORC1. We are particularly interested in understanding the mechanisms by which HPV E6 activates mTORC1 and the function of mTORC1 activation in HPV infection.

Here we show that high-risk HPV16 E6 activates mTORC1 signaling and increases cap dependent translation through an increase in S6K signaling and an increase in 4E-BP1 phosphorylation. Mechanistically we found that HPV16 E6 activates AKT under conditions of nutrient deprivation. The combined approach of phospho-tyrosine immunoprecipitations and Western blot identified HPV16 E6 mediated activation of a subset of receptor protein tyrosine kinases. HPV16 E6 activates RPTKs at least in part by increasing the internalization of phosphorylated and activated receptor species. The signaling adaptor protein Grb2 associates with HPV16 E6, and Grb2 knockdown abrogated HPV16 E6 mediated mTORC1 activation. We hypothesize that Grb2 may be important in relaying E6 mediated RPTK activation to downstream signaling cascades.

In this dissertation we also evaluate mTORC1 signaling and cap dependent translation in cells expressing HPV16 E6 mutants and E6 proteins from other HPV types. Binding to p53 and the association with proteins that contain an LXXLL motif are important for HPV16 E6 mediated mTORC1 activation. An increase in mTORC1 activation and cap dependent translation is shared between high-and low-risk mucosal, but not cutaneous HPV E6 proteins. Association with proteins through their LXXLL binding motif is also important for low-risk mucosal HPV E6 activation of mTORC1 and cap dependent translation. Shared mucosal E6 activation of mTORC1 indicates that mTORC1 may be important for the viral lifecycle in mucosal epithelia. However, it does not rule out the possibility that together with other properties of high-risk HPV E6 proteins, mTORC1 activation may promote transformation.

## ACKNOWLEDGEMENTS

I would like to thank my PhD advisor and mentor Karl Munger. Karl has been my biggest academic champion and from him I have learned more than I can fit within the pages of this dissertation. As a supportive mentor, he has given me countless opportunities to test potentially ludicrous ideas in lab and try my hand at training and teaching others. His open mind and willingness to explore new areas has made my time as a student exciting, especially when we learned together. Beyond the bench I have learned and benefited from Karl's quick and exacting eye in designing experiments and compiling presentations and manuscripts. This is a skill that will never fail to be useful, and I am forever grateful. Most importantly Karl continues to encourage me to pursue a career as an academic scientist and has made it clear that I can always come back to talk. Thank you for this time and training in your lab and I certainly look forward to future discussions.

I would like to thank my dissertation advisory committee. Many thanks to Fred Wang for his critical view and concern over the bigger picture. He taught me how to see the forest through the trees - how to organize and prioritize experiments, and how to determine which questions are important, all with the model in mind. Sheila Thomas so generously shared reagents and protocols, and it was a pleasure having a signaling expert with whom I could discuss experimental nuances. I cannot thank my committee without thanking Nick Dyson for the support and encouragement he offered over the years, and for serving as the chair of my defense examination committee.

The Munger lab, both past and present, has fostered an exceptional environment for a growing scientist. Karl, for all of his features described above, but also for the unique skill of

selecting, developing and maintaining a network of lab mates that are supportive and considerate. The Mungers can be a critical bunch, but as a whole the lab has always been full of constructive and helpful coworkers that are also friends. Molly McLaughlin-Drubin has been there from the beginning, and has been a knowledgeable source for experimental design and manuscript organization. She is also a fantastic role model for women in science. From her I have learned what to expect on the long and arduous path ahead and how also to make the most of it and I know these lessons will go on beyond the writing of this dissertation. It is nothing short of a joy to have common interests of cats and crafting and I look forward to many years of friendship ahead.

Taking this path for my PhD has genuinely been the best decision that I have made – pushing boundaries and having those boundaries push right back. It was here that I met some of the most amazing people in my life, including life long friends and classmates, lab mates and role models, and my husband Andreas. I thank him for also committing to a PhD on the 8$^{th}$ floor, Channing labs. Thank you Andreas for eating all the baked goods I make, but also for listening to every practice talk and reading every manuscript including this one, multiple times. I cannot express enough gratitude for you challenging me to exceed my own personal expectations. Thank you for supporting me in times self-doubt and it is a pleasure to share the occasional small successes that come from unexpected experiments and places.

This experience would not be complete with thanking my parents for their encouragement from the beginning. Thank you for encouraging me to look within myself for strength and to be self sufficient. As a child, after I took a test or received a report card, my parents would ask me, “Did you do your best? Did you try your hardest? If so, you did all you could and for that we are proud of you.” Thank you both for motivating me to do “my” best but

still being proud when I wasn't "the" best –it turns out that has been excellent training for this career. I look forward to many more years of phone conversations on my way to and from work nearly every day, so we can experience the best of being a scientist together.

# TABLE OF CONTENTS

## TABLE OF FIGURES

# Chapter One

# Introduction

## 1.1 HUMAN PAPILLOMAVIRUSES

### 1.1.1 Classification

Papillomaviruses are a group of small, non-enveloped DNA viruses that comprise the *Papillomaviridae* virus family. They are species-specific viruses and are found in mammalian and non-mammalian vertebrates. Papillomaviruses are non-lytic viruses that infect epithelial cells and cause the formation of papillomas. The lesions can occur on either cutaneous or mucosal epithelia. A comparison of the sequence similarity of the L1 capsid protein is used to classify papillomaviruses as unique species within 29 distinct genera. Viruses with a sequence similarity of 60-70% belong to the same genera, whereas viruses sharing less than 90% homology within the L1 ORF are considered unique species (Bernard et al., 2010). The more than 200 identified human papillomavirus (HPV) types are classified within five genera – alpha, beta, gamma, mu and nu (Bernard et al., 2010; Schiffman et al., 2010) (Fig 1.1). Alpha-HPVs infect mucosal epithelia of the anogenital tract and oral cavity and are further classified as high-risk and low-risk based on the relative propensity of the lesions that they cause to undergo malignant progression. High-risk alpha-HPVs, such as HPV16 and HPV18, are associated with potentially premalignant lesions of the cervix, oropharynx, and other tissues found within the anogenital tract and oral cavity. Low-risk alpha-HPVs cause generally benign genital warts that only rarely undergo malignant progression (Moore et al., 1999). The ubiquitous beta-HPVs infect cutaneous epithelium, and are typically associated with benign warts that are readily cleared in non-immunocompromised individuals. However, infections with beta HPVs can contribute to the formation of squamous cell carcinomas in immunocompromised

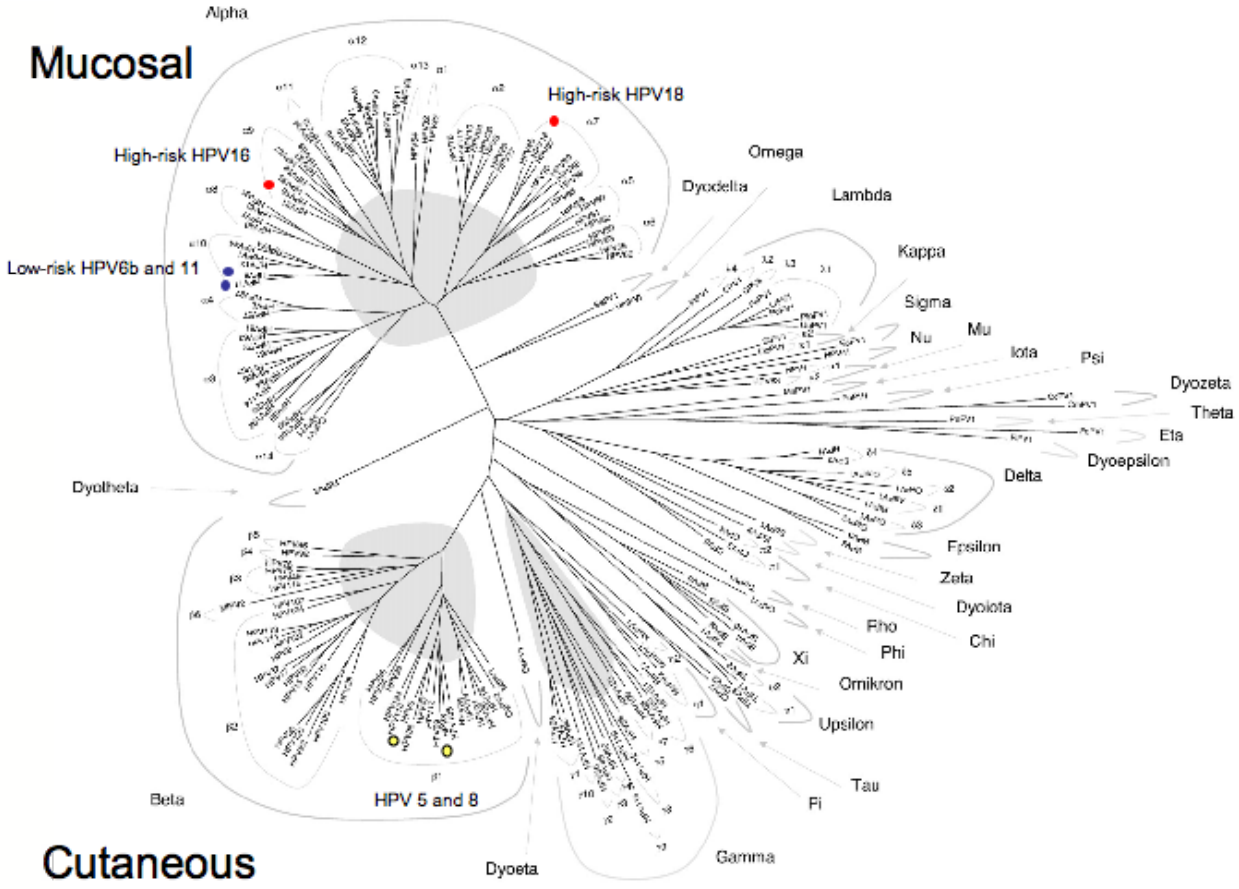


**Figure 1.1. Papillomavirus phylogenetic tree.** All 29 papillomavirus genera are represented by Greek letters, and, where applicable, Greek letters with the prefix "dyo." HPVs are classified based on the sequence similarity of their L1 capsid protein into the alpha, beta, gamma, mu and nu genera. Of the HPVs, alpha HPVs are associated with benign condylomas and malignant lesions of mucosal epithelia. Beta HPVs infect the cutaneous epithelia and are most frequently associated with benign skin warts. Prototypic HPVs used in this dissertation are designated accordingly: high-risk mucosal alpha, red; low-risk mucosal alpha, blue; cutaneous beta, yellow. (Used with permission from (Bernard et al., 2010)).

individuals or those with a rare genetic disorder, epidermodysplasia verruciformis (EV) (Lazarczyk et al., 2009).

### 1.1.2 Viral Life Cycle

**Viral Genome:**

The double-stranded circular DNA HPV genome is approximately 8 kb and encodes eight to nine open reading frames (ORFs) that are transcribed from a single DNA strand and are encoded from each of the three reading frames. The genome consists of three functional regions: the long control region (LCR), also known as the non-coding region (NCR) or the upstream regulatory region (URR), the early (E) region, and the late (L) region. The approximately 1 kb non-coding LCR contains the viral origin of replication as well as DNA elements that regulate early viral transcription and contribute to the tissue tropism of HPVs (Mistry et al., 2007). The early region contains the E1, E2, E4, E5, E6, E7, and E8 ORFs, although not all HPVs encode E5 or E8. For example, HPV Type 16, the predominant virus type described here, encodes E1, E2, E4, E5, E6, E7, and E8. The late gene region encodes two ORFs, encoding the L1 and L2 major and minor capsid proteins, respectively (Fig. 1.2). E1, E2, E6, and E7 are transcribed from the early promoter ($P_{97}$ in HPV16) during the non-productive phase of the viral life cycle. The late promoter ($P_{670}$ in HPV16) is active during productive infection, causing the elevated expression of the E1, E2, E4, E5 early proteins and the L1 and L2 capsid proteins (reviewed in (Doorbar, 2006).

**Infection**

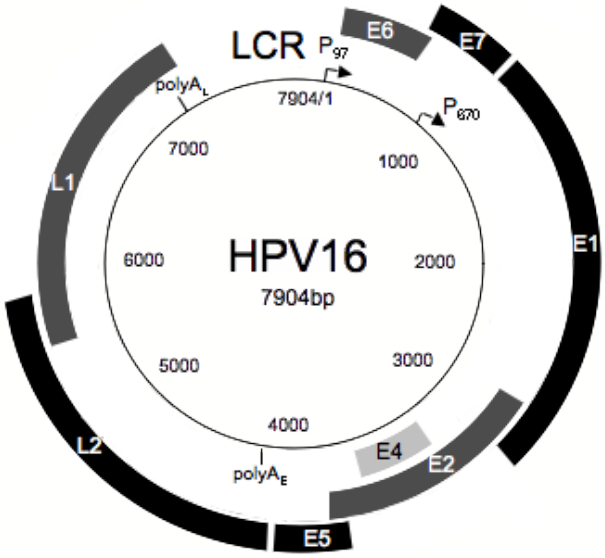


**Figure 1.2. The HPV16 Genome.** The HPV16 genome is approximately 7900bp in length and encodes the early (E) proteins E6, E7, E1, E2, E4, E5, and the late (L) proteins L1 and L2. The early $P_{97}$ promoter is active in the undifferentiated basal epithelia, whereas the late $P_{670}$ promoter becomes active in the differentiated epithelia. The encoded genes are translated from each of the three open reading frames (ORFs), as indicated by color (light grey, ORF1; dark grey, ORF2; black, ORF3). L1 and L2 protein expression is also regulated by mRNA transcript polyadenylation, indicated as $polyA_E$ or $polyA_L$.

HPVs infect epithelial cells, with alpha HPVs infecting mucosal epithelia and beta HPVs infecting cutaneous epithelia. Because HPV relies on the host for viral genome replication, HPV must initially infect the proliferating cells of the basal epithelium in order to establish a persistent infection. The virus typically gains access to the basal layer through a microabrasion in the skin (reviewed in (Moody and Laimins, 2010)). Squamocolumnar junctions, including the transformation zone in the cervix, contain reserve cells that can give rise to either squamous or columnar epithelia. It is thought that these reserve cells are relevant targets for HPV infection (reviewed in (Schiffman et al., 2007)).

Initial attachment of the virus to cells in the basement membrane is mediated through an association between the L1 capsid protein and cellular heparan sulfate proteoglycans (HSPGs) (Giroglou et al., 2001). Association with HSPGs induces a conformational change in the capsid, exposing L2 for cleavage by furin or a furin-related protease (Richards et al., 2006). It is generally thought that the conformational change and L2 cleavage exposes a domain that binds the as yet-unidentified cellular receptor. An α-6-integrin containing complex has been proposed as a receptor for HPV, but subsequent studies showed that α-6-integrin deficient cell lines were still susceptible to infection (Evander et al., 1997; Sibbet et al., 2000). The intracellular trafficking of the viral capsid, as well as genome entry into the nucleus, is poorly defined.

**Productive Viral Infection**

Viral genome replication is tightly associated with the differentiation state of infected cells. The early promoter is active in non-terminally differentiated epithelial cells, whereas upon differentiation the late promoter becomes active, at which point the late genes are transcribed

(reviewed in (Knipe, 2006)). Elements in the LCR also play an important role in the coordination of viral transcription with the differentiation state of the infected cell. The LCR contains enhancer elements that are responsive to cellular factors and also contains a number of transcription factor-binding sites.

Following infection of basal epithelial cells, the viral genome is amplified to 50-100 copies per cell and is then maintained as an episome (reviewed in (Venuti et al., 2011)). During this non-productive phase of the viral life cycle the viral DNA is replicated bi-directionally via theta structure intermediates (Yang and Botchan, 1990). The productive phase of viral life cycle begins when the basal cells divide. During this phase the replication factors, E1 and E2, are among the first viral proteins expressed. As the basal cells divide, the E2 protein tethers the genome of host chromosomal DNA such that it is segregated to the nuclei of the daughter cells (Ilves et al., 1999; Oliveira et al., 2006). In addition to viral genome segregation, the E2 protein also regulates viral genome replication. E2 binds to the palindromic ACC-$N_6$-GGT sequences within the LCR, leading to the recruitment of the viral helicase, E1. The origin of replication is flanked by E2 binding sequences, and E2 associates with the viral helicase, E1, and recruits it to the A/T rich origin of replication. Once bound to the origin, E1 forms a double hexameric ring complex similar in structure and function to cellular MCM, and recruits cellular DNA replication factors (Gloss et al., 1987). The ATPase and DNA helicase activity of E1 is required for the initiation and elongation phases of DNA replication of the viral genome (Titolo et al., 1999). Some investigators have suggested that the endoplasmic reticulum associated E5 transmembrane protein may also contribute to viral genome replication, as E5 mutant viruses exhibit impaired genome amplification, but the mechanism for this is unclear (Hwang et al., 1995; Straight et al., 1995).

Proteins encoded by the E2 ORF also play a major role as modulators of viral gene expression and can function as transcriptional activators or repressors. At low levels E2 activates the $P_{97}$ early promoter. At high levels E2 represses early gene transcription, possibly by displacing the Sp1 transcription factor from a site adjacent to the $P_{97}$ promoter (Dong et al., 1994).

The basal epithelial cells divide asymmetrically, producing one basal daughter cell and one suprabasal daughter cell that moves upwards and begins to differentiate. As mentioned previously, viral genome replication, late gene expression and viral progeny production is restricted to terminally differentiated epithelial cells. In order for viral genome replication to occur in these differentiated epithelial cells, a cellular environment suitable for DNA replication must be maintained. The HPV proteins E6 and E7 are necessary for this function. E7 proteins from cutaneous, low-risk mucosal, and high-risk mucosal HPV types associate with the retinoblastoma tumor suppressor protein (pRb) and related p107/p130 pocket proteins with varying efficiencies. The HPV E7 LXCXE motif mediates the association with the pocket proteins pRb, p107 and p130 (reviewed in (McLaughlin-Drubin and Munger, 2009b)). Only high-risk HPV E7 proteins target pRb and other pocket proteins for degradation. HPV16 E7 does so by associating with and reprogramming the cullin 2 ubiquitin ligase complex such that pRb, p107, and 130 are substrates for proteasome mediated degradation (Huh et al., 2007). The ubiquitin ligase that participates in targeting pRb for degradation by other high-risk HPV E7 proteins has not yet been identified. High-risk HPV E7 mediated pRb degradation relieves pRb mediated E2F repression, enabling E2F transactivation of genes that encode proteins involved in DNA replication. HPV E6 contributes to aberrant cell cycle activation and cell proliferation through the formation of a tripartite complex with p53 and the E3 ubiquitin ligase E6 associated

protein (E6AP, UBE3A), also leading to the destabilization of p53 (Scheffner et al., 1993). High-risk HPV E6 has other biological properties that contribute to immortalization, transformation and oncogenesis, which are discussed in greater detail below. In brief, the E6 and E7 oncoproteins are responsible for inducing and/or maintaining a DNA synthesis competent state in differentiated epithelia that is necessary for robust genome replication and production of viral progeny during the late, productive stage of the viral life cycle.

The late stages of the viral life cycle are restricted to the differentiated epithelia where viral genomes are replicated to high copy number, the viral late promoter is activated and the viral L1 and L2 capsid proteins are produced. The major capsid protein, L1, forms a pentameric structure, to which one molecule of the minor L2 capsid protein centrally associates (reviewed in (Sapp and Bienkowska-Haba, 2009)). Assembly of L1 into capsomeres occurs in the cytoplasm, followed by transport into the nucleus. Following nuclear transport, PML body localized L2 contributes to efficient virion packaging (Stauffer et al., 1998). L2 is also required for viral genome encapsidation. The E4 protein may be involved in viral egress, as it disrupts the keratin network in the differentiated epithelium and fragments the cornified cellular envelope (Doorbar et al., 1991). Following encapsidation, infectious virus is released as the outermost layers of the dermis are sloughed off, for tissue reinfection or infection of a new host.

### 1.1.3 HPV associated disease: Prevalence, Diagnosis, Treatment and Prevention

HPV infection has been linked to a number of diseases, most notably cervical cancer. High-risk HPV infection is the number one risk factor for developing cervical cancer. HPV infections represent one of the most common sexually transmitted diseases and high-risk HPV associated cervical cancer remains a leading cause of cancer death in women worldwide despite

advances in cancer screening and prevention. According to the World Health Organization 2010 Annual Report, there are an estimated 2.4 billion women aged 15 years or older who are at risk of developing cervical cancer. Each year approximately 530,000 new cases of HPV positive cervical cancer are diagnosed. Cervical cancer is the second most frequent cancer amongst women worldwide, causing 275,000 deaths annually. HPV infections account for nearly 100% of all invasive cervical cancer cases, with over 70% of these cases attributed to infections with HPV16 and HPV18 (WHO 2010 Annual Report).

Progress in HPV screening and diagnosis has been critical in the reduction of the rates of HPV-associated cervical carcinogenesis and mortality worldwide. The most widely used and available screening procedure that is used to identify HPV associated premalignant and malignant lesions is the Pap smear, or Pap test, named after its inventor Georgios Papanicolau. A swab of exfoliated cells from the endo-and ectocervical areas is collected. Cells are fixed, prepared as a monolayer on a microscope slide, stained, and analyzed for the presence of cytological abnormalities. Characteristic abnormalities include the presence of cells with enlarged nuclei and hyperchromasia. Cells with such abnormalities are referred to as koilocytes. There are currently no specific treatments for HPV positive cervical lesions, short of surgical removal. Benign skin lesions caused by cutaneous HPV infection or low-risk HPV associated genital warts can be removed if they do not spontaneously regress. Typical procedures include cryotherapy, surgical or laser removal, or application of chemical agents, which can also be used for removal of high-risk HPV associated lesions (Hellner and Munger, 2011).

Two prophylactic HPV vaccines that reduce HPV infection and the long-term risk of cervical cancer are currently available (reviewed in (Moody and Laimins, 2010)). Gardasil® is a quadrivalent vaccine that offers protection against the two low-risk HPVs (HPV6 and HPV11)

that are most commonly associated with genital warts and the two high-risk HPVs (HPV16 and HPV18) that are associated with the majority of cervical cancers in many regions of the world. Gardasil® is now FDA approved for use in young women as well as men ages 9 to 26, with a recommended three doses over the course of a six month period. Cervarix® is a bivalent vaccine protecting recipients from HPV16 and HPV18 infection. Both vaccines are made of L1 capsid protein assembled into virus like particles (VLPs) (Day et al., 2010). The long-term protection offered by these vaccines remains to be determined, although both vaccines are highly efficacious and have been shown to offer protection for five or more years (Romanowski, 2011).

### 1.1.4 HPV Associated Carcinogenesis and Oncogenic Activities of the HPV E6 Oncoprotein

During carcinogenesis, integration of the viral genome or subgenomic fragments into the host genome occurs. Integration is a nonessential and accidental event, as it terminates the viral life cycle. Integration results in the dysregulated expression of HPV E6 and E7 because expression of the E2 regulatory protein is lost. Both HPV E6 and E7 contribute to the initiation and maintenance of the transformed phenotype (reviewed in (Doorbar, 2006)). High-risk HPV E5 proteins have been shown to increase anchorage independent growth, suggesting that they may also function in cellular transformation. Co-expression of HPV E5 with HPV E6 causes the induction of cellular morphological changes such as increased nuclear size and an increase in koilocyte detection (reviewed in (Venuti et al., 2011)). It has also been suggested that high-risk HPV E5 contains hyperproliferative activity, as E5 has been reported to activate EGFR signaling (Pim et al., 1992).

**The High-Risk HPV E6 Oncoprotein**

The HPV E6 proteins are small proteins of approximately 150 amino acids in size that contain two CXXC-$X_{29}$-CXXC zinc binding domains (reviewed in (Howie et al., 2009)) (Fig 1.3A). Zinc binding through the CXXC motifs is important for the structural integrity of the HPV E6 protein. HPV E6 proteins lack enzymatic activities and do not directly associate with specific nucleic acid sequences, and therefore modulate cellular processes through the association with and modification of host cellular protein complexes. Some of the protein-protein interactions that drive HPV E6 activities are described in detail below. Antibodies for detection of some HPV E6 proteins have only recently been generated and therefore most studies have been performed with epitope tagged versions of E6. Such studies suggest that high-risk HPV E6 proteins may localize to both the nucleus and the cytoplasm (Barbosa et al., 1989; Cole and Danos, 1987).

Alternative splicing of the HPV16 E6 transcript results in the expression of at least two truncated HPV E6 mRNAs, termed E6* and E6** (Fig1.3B, C). Similar splice variants have also been reported for HPV 18E6 and other high-risk HPV E6 ORFs. Alternative splicing of HPV E6 may occur in order to generate mRNAs from which HPV E7 is efficiently translated, since HPV E6 and HPV E7 ORFs mRNAs are expressed from the same transcript in different reading frames, with the E6 stop and the E7 start codons separated by only a few nucleotides. The majority of HPV transcripts identified in cervical cancer lines encode spliced E6 sequences. However, the biological activities of HPV E6* splice variants are poorly understood. It has been proposed that HPV E6* possesses dominant negative activity by binding and repressing full length HPV E6 and also reducing the activity of the HPVE6-E6AP complex (Pim and Banks, 1999; Pim et al., 1997). HPV16 E6* has also been proposed to associate with procaspase 8

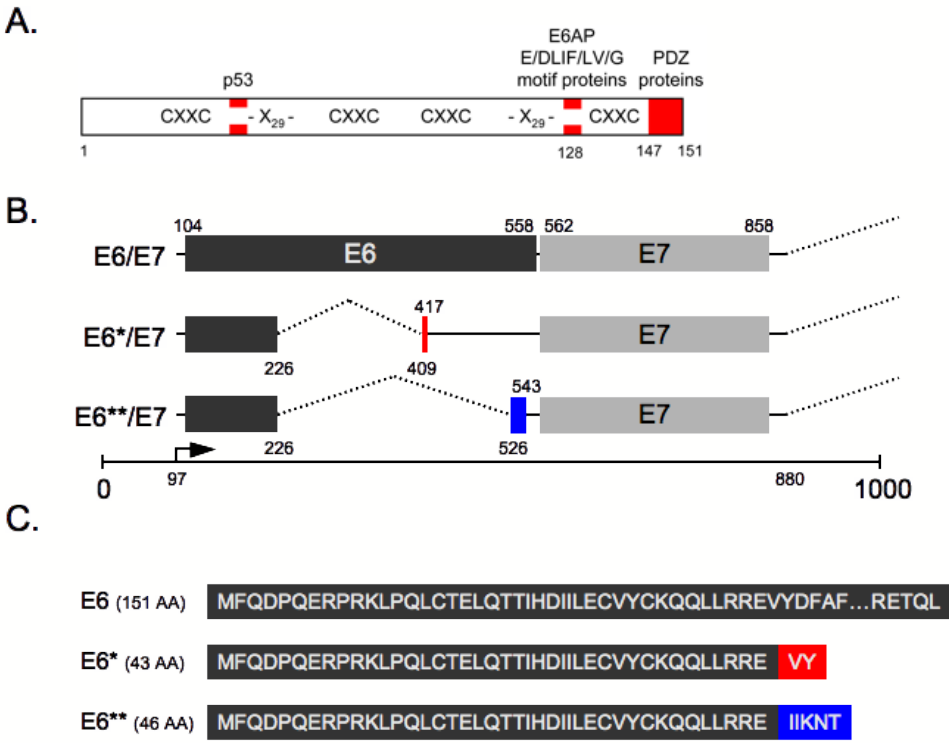


**Figure 1.3. The HPV16 E6 protein.** (A) HPV16 E6 is 151 amino acids in length, and has two zinc motifs for stability. High-risk HPV E6 proteins have several biological functions, including the association with the tumor suppressor p53, the targeted degradation of p53 and other proteins through the association with proteins through their LXXLL motif, and binding PDZ proteins. Three previously characterized mutations that abrogate these described functions are utilized in this dissertation. The HPV16 E6 Y54D mutant does not associate with p53, the I128T mutant no longer associates with proteins that have LXXLL motifs, such as E6AP, and the ΔPDZ mutant has a truncated carboxyl-terminus and no longer binds proteins that have PDZ domains. (B) High-risk HPV E6 proteins exist in at least three forms as a result of internal splicing events. The ORFs separating HPV16 E6 and HPV16 E7 are separated by two nucleotides in overlapping reading frames. Translation of full length HPV16 E6 excludes the translation of HPV16 E7 (top) HPV16 E6* (middle) and HPV16 E6** transcripts are generated from RNA splicing in which the donor splice site is identical but separate acceptor sites are utilized. The amino acid sequences of full length HPV16 E6, HPV16 E6* and HPV16 E6** are compared in (C).

and enhance its stability, inhibiting apoptosis. Full length HPV16 E6, on the other hand, has been reported to associate with procaspase 8 and to target it for ubiquitin mediated degradation (Tungteakkhun et al., 2010). My own proteomic analyses do not provide any evidence that the HPV16 E6 splice variants bind or target p53 for degradation, associate with E6AP, or associate with PDZ proteins (Appendix 5).

**E6 targets p53 for proteasomal degradation through an E6AP dependent mechanism**

High-risk mucosal HPV E6 proteins associate with p53 and interfere with its tumor suppressor activities. The p53 tumor suppressor engages G1 cell cycle arrest and/or apoptosis in response to DNA damage and other cellular stresses. The association of HPV E6 with p53 prohibits p53 from binding DNA, abrogating its functions as a transcription factor (Lechner and Laimins, 1994). To overcome p53 induced apoptosis and G1arrest, high-risk HPV E6 proteins target p53 for ubiquitin mediated degradation through the formation of a complex with the HECT family E3 ubiquitin ligase E6 associated protein (E6AP, UBE3A) (Scheffner et al., 1993). HPV E6 mediated p53 degradation causes the dysregulation of the normal p53 transcriptional program, eliminating p53 induced growth arrest and apoptosis as well as other p53 activities. High-risk HPV E6 proteins have also been described to target associated proteins for degradation through a ubiquitin-independent pathway, presumably by direct association with the proteasome (Camus et al., 2007). Low-risk HPV E6 proteins do not target p53 for degradation. HPV E6 associates with E6AP through an LXXLL motif present in E6AP. This association is conserved amongst high- and low-risk mucosal HPV E6 proteins. Therefore, the low-risk HPV E6-E6AP complex may also target cellular proteins for ubiquitin-mediated degradation, although only one cellular substrate, the proapoptotic protein Bak, has been tentatively characterized (Thomas and

Banks, 1999). HPV E6 proteins also bind other proteins that contain LXXLL motifs, including paxillin and E6BP (E6 binding protein) (Elston et al., 1998; Tong and Howley, 1997; Vande Pol et al., 1998).

**High-risk E6 proteins contribute to cellular immortalization by activating hTERT**

High-risk HPV E6 has many functions that are independent of p53 that may contribute to the transforming potential of high-risk HPV. HPV16 E6 increases telomerase activity through the transcriptional activation of the human telomerase reverse transcriptase (hTERT) gene, independent of the association with p53 or PDZ proteins (Klingelhutz et al., 1996). hTERT activity is required for efficient replication of telomeres and the maintenance of telomerase activity is essential for the immortalization of primary cells grown in culture. HPV16 E6 has been reported to activate hTERT transcription by interacting with transcription factors cMyc and NFX-123 (Gewin et al., 2004; Liu et al., 2005; Veldman et al., 2003). The NFX1 isoform NFX-91 transcriptionally represses hTERT, and is degraded by the HPV16 E6-E6AP complex to promote hTERT transcription activation (Gewin et al., 2004). More recently, HPV16 E6 was shown to directly bind to active hTERT, causing an indirect association between HPV16 E6 and chromosomal DNA (Liu et al., 2009). This suggests that HPV16 E6 regulates hTERT at the transcriptional and post transcriptional levels (Liu et al., 2009). Low-risk mucosal or cutaneous HPV E6 proteins do not transcriptionally activate or bind hTERT.

**Interaction of high-risk HPV E6 proteins with PDZ proteins mediate multiple biological activities**

The carboxyl termini of high-risk HPV E6 proteins contain a conserved binding motif, (S/T)-X-V-I-L, that mediates the association with cellular proteins that have one or multiple PDZ domains. Low-risk HPV E6 proteins do not contain a PDZ binding domain. The PDZ (PSD-95; Discs Large; Zonula-occludens -1) motif is a structural domain of approximately 80 to 90 amino acids that forms a β-sandwich and two α-helices. Proteins that contain PDZ domains are functionally diverse, with many serving as scaffolding proteins for signal transduction and cell polarity. They are frequently localized to specific subcellular compartments, such as epithelial junctions or neuronal synapses (Tonikian et al., 2008). Many PDZ proteins associate with high-risk HPV E6 proteins and may be substrates for ubiquitination by the E6/E6AP complex, leading to their proteasomal degradation (Massimi et al., 2004; Spanos et al., 2008; Thomas et al., 2005). The interaction between high-risk HPV E6 and PDZ proteins is important for HPV E6 associated transformation and oncogenic potential. Transgenic mice that express HPV E6 that lack the PDZ binding domain are less susceptible to developing cancers in comparison to wild type HPV E6 expressing mice (Nguyen et al., 2003). Considering the PDZ domain is present in more than 100 cellular proteins, with more than 250 individual PDZ domains represented in the genome, the opportunities for HPV E6 mediated regulation are numerous (Tonikian et al., 2008). Some of the high-risk HPV E6 associated PDZ proteins that may be relevant to transformation and oncogenesis are discussed below.

MAGUK and LAP proteins: hDlg and hScrib

Membrane Associated GUanylate Kinase (MAGUK) proteins are a family of proteins classified by containing multiple PDZ domains, and include MAGI-1, MAGI-2, MAGI-3, hDlg, and others. hDlg is important for the maintenance of cellular polarity and adherens junctions. Dlg

localization to adherens junctions also promotes PI3K localization to the cellular periphery. This may be the result of hDlg binding the p85 regulatory subunit of class I PI3Ks (Laprise et al., 2004). hScrib is a member of the Leucine-Rich and PDZ domain (LAP) protein family that localizes to adherens junctions and the basolateral region of epithelial cells (Humbert et al., 2008). HPV16 E6 mediated hScrib degradation disrupts tight junctions, as detected by reduced localization of ZO-1, a component of tight junctions (Nakagawa and Huibregtse, 2000). hScrib has also been implicated in signal transduction, inhibiting Ras/Raf downstream signaling and inhibiting Ras induced cellular migration and invasion (Dow et al., 2008).

Non-receptor tyrosine phosphatases: PTPN13 and PTPN3

HPV16 E6 binds the putative tumor suppressors PTPN13/PTPL1 and PTPN3/PTPH1 and targets them for E6AP mediated degradation (Spanos et al., 2008). PTPN13 has been implicated in several signaling pathways including directly inactivating Src and negatively regulating ErbB2 signaling (Glondu-Lassis et al., 2010; Zhu et al., 2008). HPV16 E6 mediated loss of PTPN13 promotes anchorage independent growth and synergizes with Ras to support tumorigenic growth in mice (Spanos et al., 2008). HPV16 E6 mediated degradation of the membrane bound and growth factor signaling associated phosphatase PTPN3 promotes cell growth under conditions of restricted nutrients and growth factors, including reduced serum, without supplementary EGF or insulin (Spanos et al., 2008).

## 1.2 ACTIVATION OF GROWTH FACTOR RESPONSIVE RECEPTOR PROTEIN TYROSINE KINASES

Receptor protein tyrosine kinases (RPTKs) are transmembrane proteins that initiate mitogenic signaling pathways and promote cellular processes such as cell growth, size, adhesion, and migration in response to activation by growth factors. Following protein synthesis, RPTKs are post-translationally modified in the Golgi by N-linked glycosylation (Lane et al., 1985; Slieker et al., 1986), after which they are translocated to the plasma membrane. Here the receptors bind specific extracellular or cell associated ligands. Ligand binding stimulates receptor homo- and heterodimerization. This triggers activation of kinase activity and trans-phosphorylation of intracellular tyrosine residues. These phosphorylated tyrosine residues then act as binding sites for intracellular signal transduction proteins resulting in activation and the initiation of mitogen signaling pathways including PI3K/AKT/mTORC1. RPTK activation by growth factors also causes receptor internalization, which may occur via clathrin-mediated endocytosis or through a clathrin-independent mechanism, depending on the receptor. RPTK internalization is important for their activation as the affinity of some ligands for their receptor is high enough such that the RPTK signaling lifespan is extended following clathrin-mediated endocytosis.

### 1.2.1 ErbB/HER activation and signal transduction – EGFR

Over 20 different ligands have been described for ErbB RPTKs, including epidermal growth factor (EGF). Ligand binding and dimerization stimulate ErbB tyrosine kinase activity and induce autophosphorylation at multiple tyrosine residues (Guy et al., 1994). Since no ErbB2

ligands have been identified to date (reviewed in (Yarden and Sliwkowski, 2001)), it is generally accepted that ErbB2 is activated by heterodimerization with other ligand activated ErbB family members. Ligand binding to EGFR induces the trans-phosphorylation of at least 7 carboxyl-terminal, intracellular tyrosine residues (Fernandes et al., 2001). EGFR tyrosine phosphorylation serves as a binding site for adaptor proteins through their Src homology (SH2) domains, causing the activation of downstream signaling cascades. The adaptor protein Grb2 directly associates with EGFR following Y1068 autophosphorylation, activating both the PI3K and Ras/MAPK signaling pathways (Rojas et al., 1996). Shc binds EGFR following Y1148 and Y1173 autophosphorylation, activating MAPK signaling, and EGFR autophosphorylation at Y992 promotes PLCγ binding and signal transduction (Emlet et al., 1997; Zwick et al., 1999). Tyrosine phosphorylation also mediates EGFR stability; Y1045 phosphorylation causes c-Cbl binding and subsequent targeting of EGFR for ubiquitin mediated degradation (Levkowitz et al., 1999) (Fig 1.4). EGFR dimerization-dependent internalization through clathrin-mediated endocytosis is associated with receptor activation and can either lead to receptor recycling to the cell surface or receptor degradation (Wang et al., 2005). Robust EGFR activation is only obtained upon receptor internalization, although clathrin mediated internalization is biased towards receptor recycling rather than degradation, sustaining receptor activation (Sigismund et al., 2008).

### 1.2.2 IR/ IGFR1-R activation and signal transduction

Insulin receptors (IR) and insulin-like growth factor receptors (IGFR) are also activated upon dimerization followed by ligand binding. While insulin and IGF-I/IGF-II are the physiological ligands for IR and IGFR, respectively, IR/IGFR heterodimers bind both insulin and IGF with reduced affinity for insulin and with comparable affinity for IGF (reviewed in

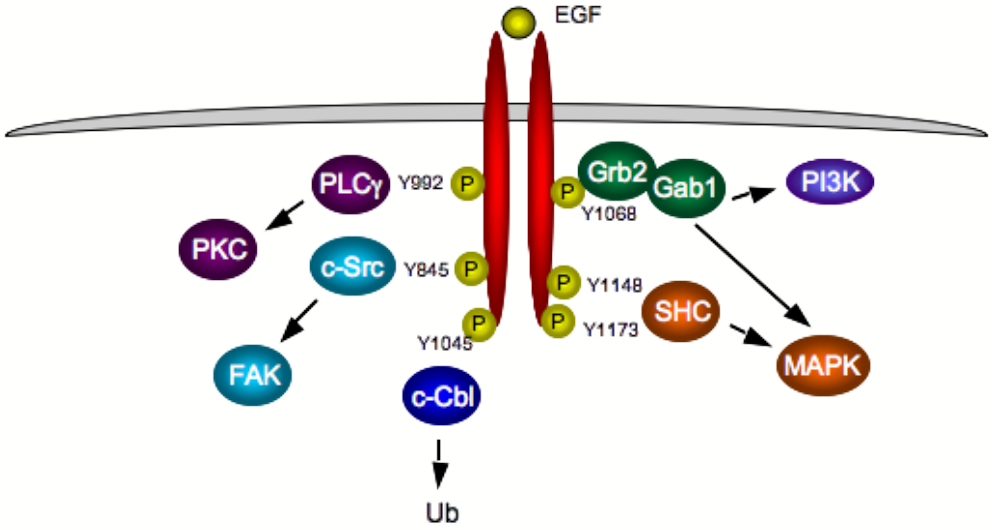


**Figure 1.4. EGFR Activation and Regulation.** EGFR is activated by ligand binding and dimerization, which induces the autophosphorylation of several carboxyl-terminal tyrosine residues. Autophosphorylation promote the association of adaptor proteins via SH2 domains with the receptor and activate downstream signaling cascades. SHC associates with EGFR following phosphorylation of Y1148 and Y1173, activating MAPK signaling; Grb2 binds after EGFR Y1068 phosphorylation and causes the activation of MAPK and PI3K signaling; EGFR Y992 phosphorylation promotes PLCγ association and the activation of PKC; C-Src binds EGFR following Y845 phosphorylation, which activates FAK signaling. EGFR stability is regulated by the phosphorylation of Y1045, promoting the association with c-Cbl and the targeted EGFR degradation. EGFR activation is abrogated by protein tyrosine phosphatases including PTP1B and SHP2.

(Belfiore et al., 2009)). Following ligand binding, IRβ and IGFR1β-R are autophosphorylated in a conserved kinase activation loop at residues Y1146/1150/1151 or Y1131/1135/1136, respectively (Hernandez-Sanchez et al., 1995; White et al., 1988) (Fig 1.5). Autophosphorylation causes full receptor activation and initiates downstream effector signaling. Similar to ErbB RPTKs, autophosphorylation induces clathrin mediated receptor internalization. There is also evidence for caveolin dependent internalization. IR/IGFR1-R phosphorylation is regulated by the adaptor proteins Grb10 and Grb14. Grb10 and Grb14 may maintain IR/IGFR1-R activation by protecting the receptors from dephosphorylation by tyrosine phosphatases. Alternatively, Grb10 regulates the stability of IGFR1-R by targeting it for NEDD4 mediated ubiquitination and proteasomal degradation (Vecchione et al., 2003). Grb10 is also a downstream target of mTORC1. mTORC1 mediated Grb10 phosphorylation increases its stability, causing feedback inhibition with the lipid kinase phosphoinositide 3-kinase (PI3K) , AKT, and MAPK/ERK signaling pathways. As a result, Grb10 may also participate in IR/IGFR1-R negative feedback inhibition (Hsu et al., 2011; Yu et al., 2011). The major downstream effectors of IR and IGFR1-R signaling are the PI3K and Ras/MAPK signaling pathways. PI3K and Ras/MAPK activation is dependent on IR/ IGFR1-R mediated phosphorylation of insulin receptor substrates 1 and 2 (IRS) at YxxM motifs. Phosphorylation of IRS1/2 at this motif allows for recognition and association of adaptor proteins Grb2 and Shc, or class Ia PI3K regulatory subunits, which in turn activate PI3K and Ras/MAPK signaling pathways. Protein phosphatases also regulate IR and IGFR1-R activity, including SHP2/PTPN11, which associates with, dephosphorylates, and inactivates IRS1/2 through its SH2 domain (Goldstein et al., 2000). The protein tyrosine phosphatase PTPN1/PTP1b dephosphorylates EGFR and IR (Haj et al., 2003; Haj et al., 2002).

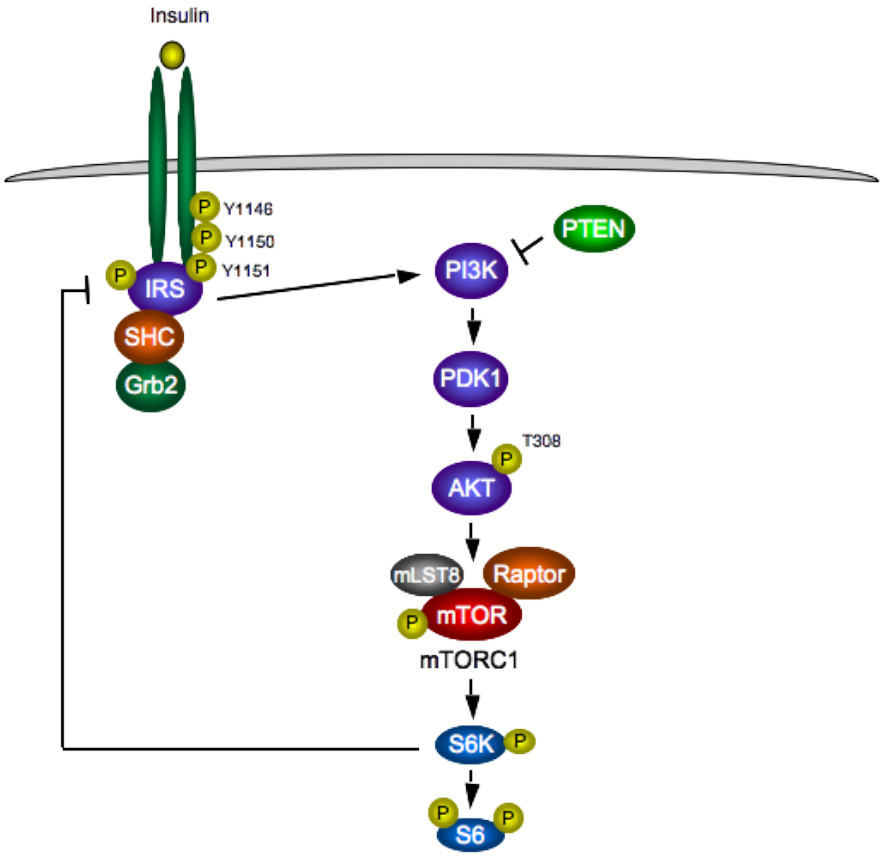


**Figure 1.5. IR Activation and Regulation.** IR is activated by ligand binding and dimerization, which induces the autophosphorylation of the carboxyl-terminus at several tyrosine residues. IR is phosphorylated at Y1146/50/51, with the phosphorylation of analogous residues occurring for IGFR. Phosphorylation promotes the association of IR and insulin receptor substrate 1 (IRS1). This predominantly results in the activation of PI3K signaling, although other adaptor proteins including Grb2 and SHC can associate indirectly with the receptor through binding IRS-1 and activate downstream signaling networks. IR activation is abrogated by protein tyrosine phosphatases including PTP1B and SHP2.

## 1.3 mTOR SIGNALING: REGULATION AND THE ROLE OF mTORC1 SIGNALING IN THE INITITATION OF CAP DEPENDENT TRANSLATION

The mammalian target of rapamycin (mTOR) is a protein serine-threonine kinase that is the catalytic subunit of at least two different complexes with distinct biological functions (Fig 1.6). TOR was identified through genetic and biochemical screens based on sensitivity to the drug rapamycin. Screening yeast for genes that developed mutations that enabled them to grow in the presence of rapamycin initially identified TOR (Heitman et al., 1991). Crosslinking and affinity purification of the small Rapamycin associated protein FKBP12 from Rapamycin treated cells later identified mammalian TOR (Heitman et al., 1991; Sabatini et al., 1994). The mTOR complex 1 (mTORC1) contains the mTOR kinase, Raptor, mLST8, and PRAS40. mTORC1 serves as a major regulator of cell growth, proliferation, and metabolism by integrating upstream growth factor associated signals, as well as energy status and nutrient availability. These signals are relayed to downstream effectors S6K and 4E-BP1, and canonical cap dependent translation is regulated. The specific role(s) of the mTOR complex 2 (mTORC2), which consists of the mTOR kinase, Rictor, mLST8 and SIN1, are less well defined. Current data suggest that unlike mTORC1, mTORC2 kinase activity is regulated by growth factors only (reviewed in (Zoncu et al., 2011)). The mTORC2 complex has been implicated in regulating cytoskeletal organization, and phosphorylates members of the AGC kinase family, including AKT at S473 and serum and glucocorticoid kinase 1 (SGK1) at S422 (Garcia-Martinez and Alessi, 2008; Sarbassov et al., 2005). The mTORC2 complex also associates with actively translating ribosomes and phosphorylates nascent AKT peptides at T450 to promote AKT stability and prevent

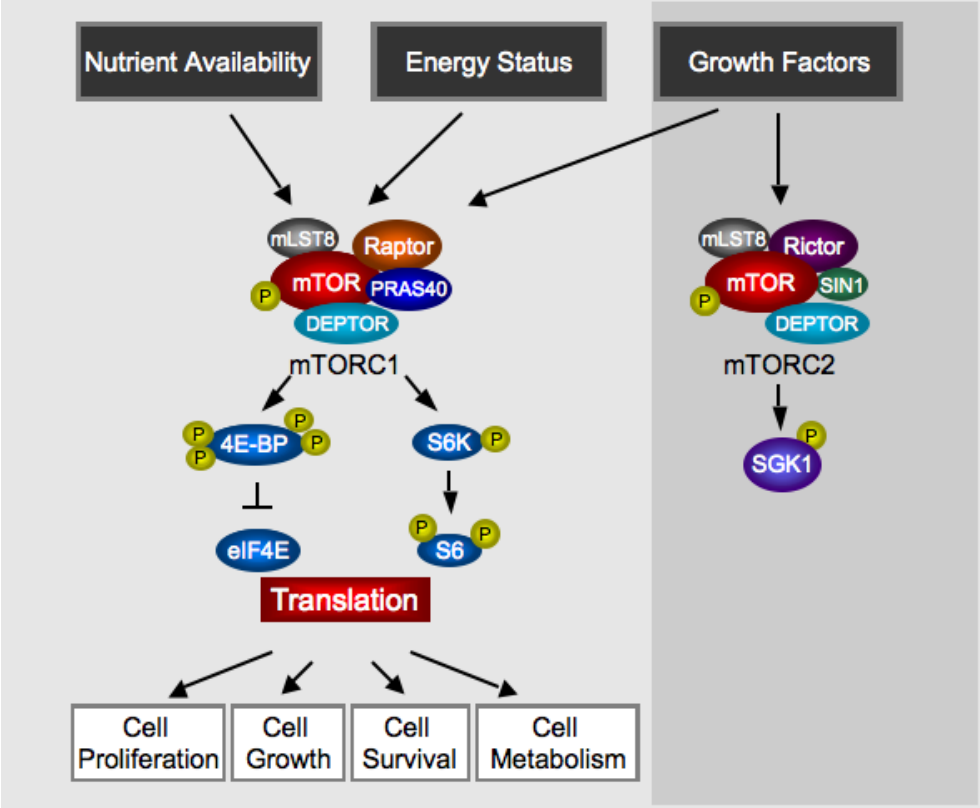


**Figure 1.6. mTOR functions in two biologically distinct complexes.** The mTOR kinase is part of two complexes, mTORC1 and mTORC2. mTORC1 contains the mTOR kinase, raptor, mLST8, PRAS40, and DEPTOR, and is responsive to growth factors, energy status, and nutrient availability. mTORC1 integrates these environmental cues and regulates cell growth and proliferation by increasing protein synthesis through activating S6K and 4E-BP1 and increasing cap dependent translation. mTORC2 is comprised of the mTOR kinase, rictor, mLST8, Sin1, and deptor. mTORC2 is only responsive to growth factors, and phosphorylates several substrates including AKT and SGK1.

ubiquitination (Oh et al., 2010). These functions of mTORC2 connect mTORC2 activity to mTORC1, and the mTORC2 component Rictor is a S6K1 substrate, which is a downstream effector of mTORC1 (Dibble et al., 2009; Julien et al., 2010). This suggests that the two mTOR complexes may function in an intricate and highly connected signaling network.

Despite overlapping regulation by growth factors, mTORC1 and mTORC2 were initially characterized based on their differential sensitivity to Rapamycin. While the mTORC1 complex is sensitive to inhibition to Rapamycin at low concentrations on the order of minutes, the mTORC2 complex remains resistant to Rapamycin on the order of hours. Prolonged Rapamycin exposure, however, inhibits the assembly of mTORC2 complexes, reducing AKT S473 phosphorylation and activation (Sarbassov et al., 2006). Since the discovery of Rapamycin and its antiproliferative and immunosuppressive properties, Rapamycin and its derivatives are currently under evaluation in many clinical trials for various kinds of cancers (Dowling et al., 2010).

Insulin or other growth factor associated signaling at the cellular membrane have been most tightly linked to mTOR activation, although G protein coupled receptors have also been implicated. Insulin binding to dimerized insulin receptors causes receptor activation and subsequent phosphorylation and activation of substrates including the adaptor proteins IRS1/2. As a result PI3K is recruited to the plasma membrane and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$] is phosphorylated to generate PtdIns(3,4,5)$P_3$ (reviewed in (Cantley, 2002)). Association of phosphoinositide-dependent kinase -1 (PDK1) and PtdIns(3,4,5)$P_3$ at the plasma membrane causes PDK1 activation and phosphorylation of PDK1 substrates including AKT, SGK1 and other AGC kinases.

The serine-threonine kinase AKT (PKB) also promotes cell growth and proliferation, through numerous downstream effectors. AKT reaches full activation at the plasma membrane through two sequential phosphorylation events. The initial phosphorylation event occurs at S473 by mTORC2, which serves as a priming event for PDK1 mediated AKT phosphorylation at T308. AKT activates mTORC1 signaling through several mechanisms.

### 1.3.1 Upstream regulators of mTORC1: AKT phosphorylation and inhibition of TSC2 relieves mTORC1 inhibition

TSC1 and TSC2 (Tuberous Sclerosis Complex 1 and 2) form a heterodimeric complex and negatively regulate mTORC1 activity. TSC2 has GTPase-activating protein (GAP) activity, and when associated with the GTPase Rheb, stimulates the conversion of Rheb to the inactive, GDP-bound form that inhibits mTORC1. Activated AKT inhibits TSC2 by phosphorylating it on at least four residues (Inoki et al., 2002; Manning et al., 2002) (Fig 1.7). Mutation of TSC2 residues that are phosphorylated by AKT cause marked reduction of phosphorylation of the mTORC1 downstream effectors S6K and 4E-BP1. TSC2 is also regulated by the energy sensing AMP-activated protein kinase (AMPK) pathway. Regulated by LKB1, the heterotrimeric AMPK complex responds to the cellular AMP/ATP ratio. Conditions of limited nutrient availability stimulate AMPK to phosphorylate TSC2 at S1387. This promotes TSC2 activation and subsequent mTORC1 inhibition (Inoki et al., 2003). mTORC1 is phosphorylated at several sites, including S2448 and S2481. The kinase that phosphorylates S2448 is controversial; AKT was initially proposed to phosphorylate S2448, and PI3K inhibition with Wortmannin abrogated this event (Nave et al., 1999). S2448 phosphorylation was later shown to be Rapamycin dependent.

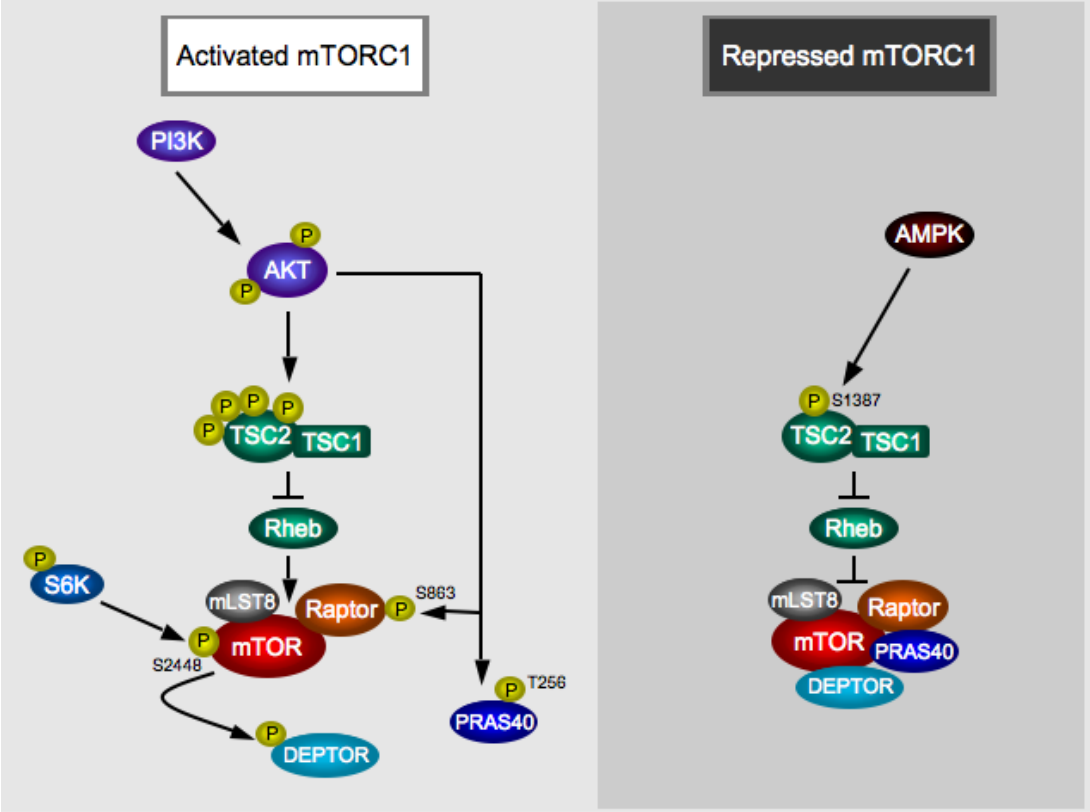


**Figure 1.7. Multiple kinases regulate mTORC1 activation and inhibition**. mTORC1 is activated upon the following events: (1) PI3K/AKT is activated, inhibiting TSC2 by phosphorylating it at four residues. (2) PI3K/AKT is activated, causing the phosphorylation and dissociation of PRAS40 from the mTORC1 kinase complex. (3) PI3K/AKT activation phosphorylates and activates raptor. (4) Low levels of mTORC1 activity promote S6K activation, which phosphorylates mTORC1. (5) Activated mTORC1 phosphorylates DEPTOR, targeting DEPTOR for ubiquitin-mediated degradation. mTORC1 activity is repressed following AMPK phosphorylation and activation of TSC2, which in turn inhibits mTORC1. The association of mTORC1 with PRAS40 and DEPTOR are also inhibitory.

Moreover, siRNA against S6K abrogated S2448 phosphorylation, demonstrating that S6K is likely involved in the phosphorylation of this site (Holz and Blenis, 2005).

### 1.3.2 Additional mechanisms of mTORC1 regulation

The PI3K/AKT/TSC2 signaling pathway can also activate mTORC1 through alternative mechanisms. The mTORC1 component Raptor is phosphorylated in a PI3K/AKT/TSC2 dependent manner. Insulin stimulates phosphorylation of Raptor at S863, and treatment with the PI3K inhibitor Wortmannin inhibits this phosphorylation event. Moreover, there is a marked reduction in S863 Raptor phosphorylation in TSC2 -/- mouse embryonic fibroblasts (MEFs) (Foster et al., 2010). AKT also phosphorylates PRAS40 (Fig 1.6). PRAS40 is a Raptor interacting protein that inhibits mTORC1 under conditions of growth factor deprivation. Alternatively, insulin stimulates AKT- mediated phosphorylation of PRAS40 at T246. Phosphorylation at this site promotes the dissociation of PRAS40 from the mTORC1 complex and relieves PRAS40 induced mTORC1 repression (Sancak et al., 2007). Other regulatory proteins of the mTORC1/2 complexes have been identified using biochemical purification of these complexes. DEPTOR, a PDZ protein with dysregulated expression in many cancers, was identified as an mTORC1/2 interacting protein and inhibitor. DEPTOR expression is regulated by mTORC1/2, with reduced or loss of expression causing mTORC1/2 activation and the activation of S6K, SGK1, and AKT. However, DEPTOR over-expression causes S6K inhibition. This relieves the S6K negative feedback loop on insulin signaling and enables activation of AKT (Peterson et al., 2009). mTORC1 also regulates its own activation by directly phosphorylating DEPTOR (Fig 1.6), an event that targets DEPTOR for βTRCP mediated proteasomal

degradation (Gao et al., 2011). Collectively, loss of DEPTOR or PRAS40 expression causes increased cell growth and proliferation.

The dephosphorylation of proteins involved in mTORC1 signaling negatively regulates mTOR activation. The lipid phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is an inhibitor of PI3K signaling dephosphorylates PtdIns(3,4,5)$P_3$ and represses PI3K mediated signaling to PDK1. Other protein phosphatases also negatively regulate PI3K/mTORC1 signaling, including SHP2 (Zito et al., 2007).

### 1.3.3 The role of mTOR signaling in translation initiation: mTORC1 effectors

The activation of mTOR signaling promotes the initiation of cap dependent translation through at least two downstream effectors: S6K and 4E-BP1.

S6K

A series of at least three phosphorylation events by three distinct kinases activate the 70-kDa ribosomal subunit 6 kinase 1 (S6K1) (Keshwani et al., 2011). GSK3β first phosphorylates S6K1 at S371 in the turn-helix regulatory motif in the linker region. This event is critical for S6K conformational stability and is necessary for S6K T389 phosphorylation. (Shin et al., 2011). (Fig 1.8) S6K is thought to be phosphorylated next in the activation loop of the catalytic domain by PDK1 at T229 (Pullen et al., 1998). The final phosphorylation of the S6K hydrophobic motif in the linker region at T389 by mTORC1 results in fully activated S6K. S6K1 also participates in a negative feedback inhibitory loop that causes attenuated mTORC1 signaling. Insulin resistance and inhibition of downstream signal transduction can occur when S6K1 phosphorylates IRS-1, whereas S6K inhibition can restore signaling and insulin responsiveness

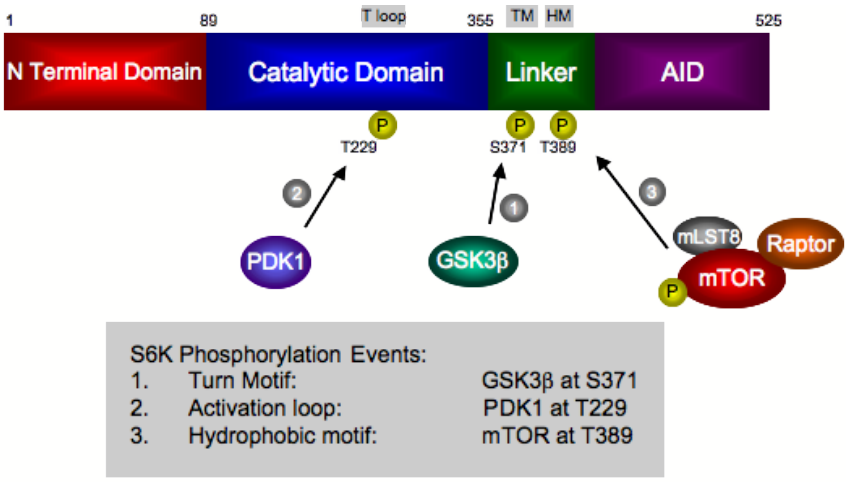


**Figure 1.8. Sequential phosphorylation events activate S6K.** S6K1, shown here, has an amino-terminal nuclear localization signal (NLS) and several domains that regulate its activity. Residues within the catalytic domain and linker region are phosphorylated by kinases that regulate S6K activity, and the autoinhibitory domain (AID) also can be phosphorylated. S6K activation is stepwise. First, GSK3β phosphorylates S6K in a turn motif within the linker region. This stabilizes S6K for later phosphorylation in the hydrophobic motif also in the linker region. PDK1 next phosphorylates S6K in the catalytic domain. Lastly, the mTORC kinase complex phosphorylates the hydrophobic motif of S6K.

(Harrington et al., 2004; Um et al., 2004) (Fig 1.5). S6K also associates with the protein phosphatase 2A (PP2A), and under conditions of mTOR inhibition, PP2A can dephosphorylate and inactivate S6K. Under conditions of mTORC1 activation, mTORC1 phosphorylates and inactivates PP2A, maintaining downstream S6K activation (Peterson et al., 1999).

S6K1 phosphorylation activates numerous downstream effectors, including those associated with ribosome biogenesis and translation elongation. S6K1 is one of several kinases that phosphorylates 40S ribosomal protein S6 on residues S235/36 and S240/44. S6 phosphorylation promotes the translation of mRNAs through several mechanisms. S6K may increase the translation of mRNAs containing the 5' terminal oligopyrimidine tract (5' TOP), or a stretch of four to 15 CU rich nucleotides located in a relatively short and unstructured 5' UTR, under insulin rich conditions. Many genes involved in translation have been reported to contain a 5' TOP, including ribosomal genes, supporting ribosome biogenesis (reviewed in (Meyuhas, 2000)). Translation of many 5'TOP mRNAs is Rapamycin sensitive, although an increase in the translation of some 5'TOP mRNAs has been detected in S6K1 and S6K2 double knock out MEFs (Pende et al., 2004). Other targets of S6K1 include eukaryotic translation factors such as initiation factor eIF4B and elongation factor eEF2K (Raught et al., 2004; Wang et al., 2001) S6K functions to generally increase cap dependent translation through the phosphorylation of eIF4B, which in turn activates the eIF4A helicase and unwinds highly structured and long mRNA 5'UTRs for efficient translation initiation (Raught et al., 2004; Shahbazian et al., 2006). Although not all mRNA 5' UTRs are highly structured, a subset encode proteins with critical functions including HIF1$\alpha$, Cyclin D1, and MYC. (Fig 1.9)

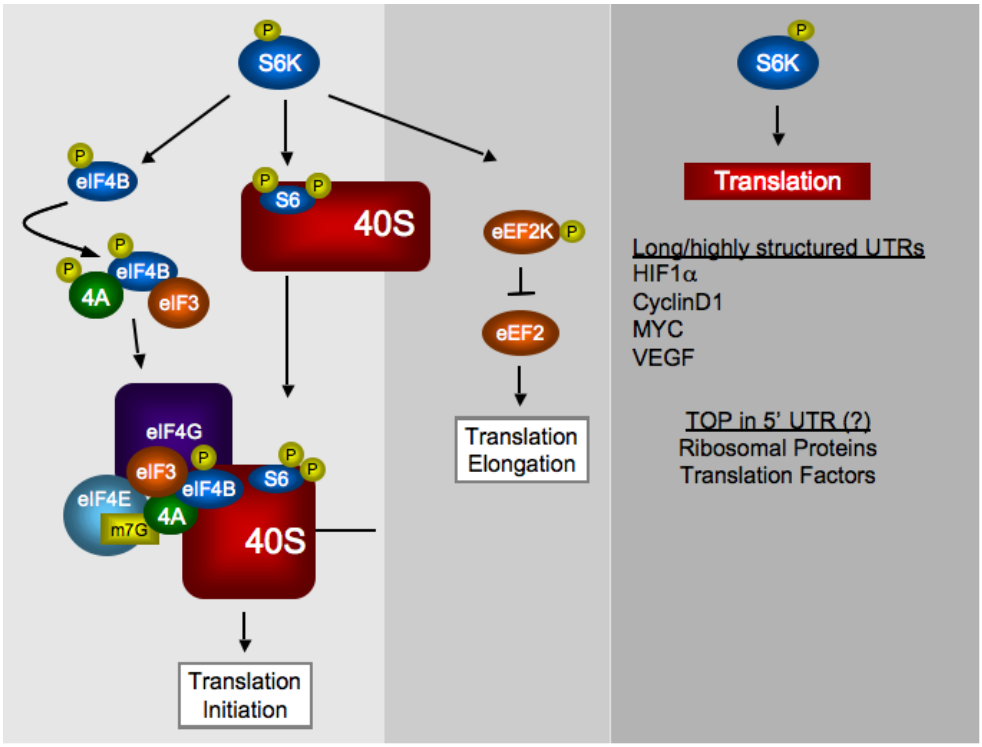


**Figure 1.9. S6K regulates the translation of several types of mRNAs.** Substrates of activated S6K are involved in translation initiation and translation elongation. The ribosomal protein S6 is the best-studied S6K substrate, and is phosphorylated at several residues by S6K. Upon S6 phosphorylation, S6K also phosphorylates eIF4B, recruiting it to eIF4A, enhancing eIF4A helicase activity. Phosphorylation of eIF4B also increases its recruitment to the scaffolding complex eIF3, to which the 40S ribosome later binds. S6K regulates translation elongation by phosphorylating the elongation factor eEF2K. This phosphorylation event promotes translation by facilitating the dephosphorylation and activation of eEF2. mTORC1/S6K regulates both general cap dependent translation, but has also been shown to increase the translation of specific mRNA species. The translation of mRNAs that have 5'UTRs much longer than the average length of 150 base pairs, and thus potentially are more structured are increased. The translation of 5'TOP mRNAs are increased, although more recent data suggest that this is mTORC1 dependent but independent of S6K.

4E-BP1

The eukaryotic translation initiation factor 4E binding protein-1 (4E-BP1), is another downstream effector of mTORC1 signaling. 4E-BP1 negatively regulates the association of the eukaryotic translation initiation factor eIF4E with the 5' mRNA cap (Fig 1.10). eIF4E is present in rate limiting quantities within the cell. Thus, eIF4E association with the 5' mRNA cap and initiating the recruitment of the remainder of the eIF3 complex is considered a key regulatory event in translation initiation (Jackson et al., 2010). 4E-BP1 can be phosphorylated at multiple sites. Hypophosphorylated 4E-BP1 binds eIF4E tightly, preventing its association with the 5' mRNA cap. Sequential phosphorylation at multiple residues leads to hyperphosphorylated 4E-BP1 and eIF4E dissociation, relieving eIF4E repression and freeing eIF4E to associate with the 5' mRNA cap and participate in translation initiation. 4E-BP1 is basally phosphorylated at T37/46, which occurs in vitro by mTORC1, priming it for subsequent phosphorylation (Gingras et al., 1999; Mothe-Satney et al., 2000a). Subsequent serum-induced phosphorylation at T70 and S65 promote the 4E-BP1 dissociation from eIF4E, both of which are regulated by PI3K and mTORC1 activity (Gingras et al., 2001; Mothe-Satney et al., 2000b).

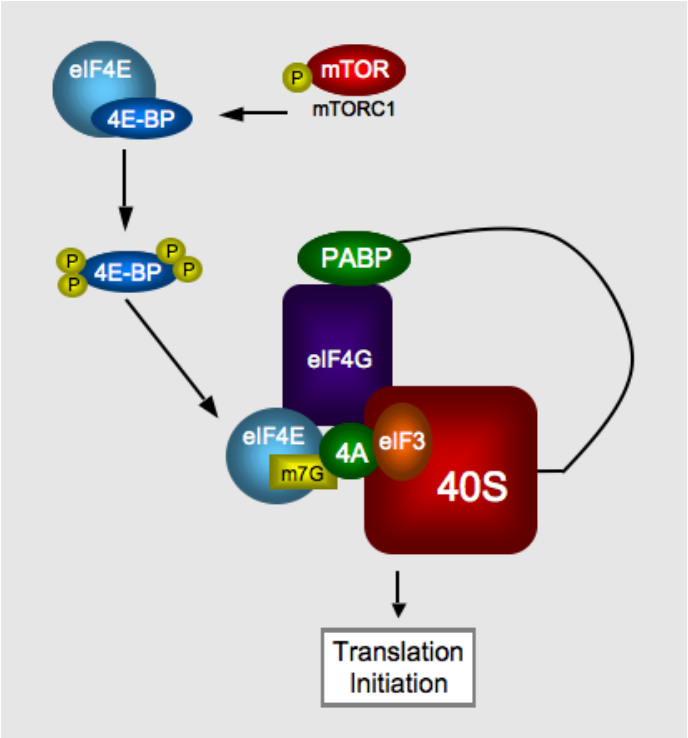


**Figure 1.10. Phosphorylation of the eukaryotic translation initiation factor 4E binding protein (4E-BP1) increases cap dependent translation initiation by relieving eIF4E repression.** mTORC1 hyperphosphorylates 4E-BP1 at four residues. Hypophosphorylated 4E-BP1 associates with the translation initiation factor eIF4E such that it cannot bind the 5' mRNA cap. 4E-BP1 T37/46 phosphorylation are priming events. mTORC1 next phosphorylates T70, and lastly S65, fully activating 4E-BP1. Hyperphosphorylated 4E-BP1 is no longer able to associate with eIF4E. eIF4E then binds the 5'mRNA cap and recruits eIF4G and the eIF3 complex to initiate translation.

## 1.4 DNA VIRUSES AND THE REGULATION OF THE RPTK/PI3K/AKT/mTORC1 SIGNALING AXIS

Multiple DNA tumor viruses dysregulate the RPTK-AKT-mTORC1 signaling axis. Polyomaviruses, including simian virus 40 (SV40) and murine polyomavirus, activate signaling cascades that are important for translational regulation. The plasma membrane bound polyomavirus middle T antigen (mT) activates AKT and other downstream mitogenic pathways through association with and subsequent recruitment of the Class I PI3K p85 regulatory subunit (Ichaso and Dilworth, 2001; Kaplan et al., 1987; Summers et al., 1998; Whitman et al., 1985). Additionally, polyomavirus mT physically associates with insulin receptor and IGF-1R and recruits Src, increasing AKT and ERK1/2 activity (Novosyadlyy et al., 2009). Polyomavirus small T (sT) antigens relieve repression of mitogenic signaling by functioning as a subunit of PP2A (Rodriguez-Viciana et al., 2006). Recently the first human cancer-associated polyomavirus, Merkel cell polyomavirus (MCPyV) was identified from Merkel cell carcinoma, a rare but highly lethal form of cancer. Like SV40 sT and polyomavirus mT proteins, MCPyV sT activates signaling downstream of AKT and mTORC1 (Shuda et al., 2011). MCPyV St activates 4E-BP1, contributing to its transforming capacity, although the mechanism of activation is unclear (Shuda et al., 2011).

Adenoviruses also activate growth factor associated signaling cascades that increase cellular translational output. The adenovirus early proteins E4Orf1 and E4Orf4 have been reported to activate PI3K and mTORC1 signaling as indicated by AKT and S6K phosphorylation, respectively (O'Shea et al., 2005). In combination, E4Orf1 and E4Orf4 can activate signaling downstream of mTORC1 independent of growth factors and nutrients, which

is similar to what we have described in HPV16 E6 expressing cells in subsequent chapters of this dissertation (O'Shea et al., 2005; Spangle and Munger, 2010). Although it appears that adenovirus E4Orf1 and E4Orf4 have some overlapping functions including promoting assembly of the 5' mRNA cap to enhance cap dependent translation initiation, only E4Orf4 binds and relocalizes PP2A, causing S6K phosphorylation and activation (O'Shea et al., 2005). Additionally, the adenoviral early protein E1A has been reported to regulate translation by increasing 4E-BP1 hyperphosphorylation (Gingras and Sonenberg, 1997)

Within the Herpesviridae family, proteins with similar functions in activating metabolic signaling pathways have been identified. During infection, human cytomegalovirus engages cellular receptors, causing AKT activation, which is sustained by the expression of immediate early genes IE1 or IE2 (Yu and Alwine, 2002). Similarly, the Epstein-Barr virus encoded oncogenes LMP1 and LMP2A activate AKT. LMP2A also activates mTORC1 and increases the phosphorylation and activation of 4E-BP1 but not S6K (Fukuda and Longnecker, 2007; Moody et al., 2005; Scholle et al., 2000; Shair et al., 2007; Swart et al., 2000). LMP1 may also phosphorylate STAT3, promoting the formation of p50/Bcl-3 complexes and increasing EGFR transcription (Kung and Raab-Traub, 2008).

RNA viruses also engage in activities that increase viral RNA translation and may contribute to the shut off of host mRNA translation. Such examples include viruses belonging to the picornaviridae and the orthomyxoviridae families. The genome of Polioviruses (Picornaviridae) encodes and utilizes an internal ribosomal site (IRES) for translation initiation, eliminating the need to initiate canonical cap-dependent translation in the absence of (1) a 5' mRNA cap; and (2) appropriate upstream signals from mTORC1/4E-BP1. Alternatively, each genome segment from the Influenzavirus (orthomyxoviridae) lacks a 5' mRNA cap and cleaves

the 5'mRNA cap from host transcripts and the subsequently fuses the displaced 5'mRNA cap onto the viral RNA genome segment, causing enhanced viral RNA translation and a reduction in the translation of host transcripts.

One cause of viral induced mTORC1 activation is the increase in cap dependent translation. The maintenance of cellular translation is of general importance for a successful viral replication and progeny virion production. It is beneficial for viruses to encode proteins that maintain or dysregulate the activation of cellular pathways that control protein synthesis. Keeping these pathways activated is beneficial for cellular and viral protein translation. This may promote viral genome replication and support the translation of viral proteins necessary for virion packing and encapsidation. Further, the sustained activation of translation may also enable the translation of viral proteins with more diverse functions such as virally encoded proteins important for host immune evasion or shutoff. The cellular immune response to viral infection emphasizes the importance of protein synthesis on infection, as the innate immune system can also respond by immune system shutoff and activating PKR.

Maintaining a replication competent cellular milieu with abundant amino acids and energy is likely very important for human papillomaviruses given infection is initiated in the basal epithelium with nutrient rich conditions, but viral genome amplification and virion packaging only occurs in the outer layers of the differentiated epithelium. Under normal uninfected conditions, these cells are removed from the nutrient rich basal epithelium, with few opportunities for nutrient and gas exchange. Successful production of progeny virus is therefore dependent on the maintenance or temporary activation of metabolic pathways that promote translation and cell growth while minimizing apoptosis, and tightly regulating autophagy. The HPV oncoproteins may contribute independently to the coordinate regulation of these pathways.

HPV16 E7 has been reported to increase the formation of autophagic puncta in primary human foreskin keratinocytes (Zhou and Munger, 2009). HPV16 E7 has been reported to cause a shift in energy production from oxidative phosphorylation to anaerobic fermentation, i.e. the conversion of pyruvate to lactate. HPV16 E7 has reported to increase the dissociation of the tetrameric form of pyruvate kinase M2 (M2-PK) to a dimeric form. Dimeric M2-PK has a reduced affinity for phosphoenolpyruvate, which reduces its conversion to pyruvate for entrance into the citric acid cycle (Mazurek et al., 2001a; Zwerschke et al., 1999). As a result, following glycolysis, dimeric M2-PK shifts the equilibrium away from oxidative phosphorylation to fermentation i.e., generation of lactate from pyruvate, altogether a much less efficient mechanism to generate ATP. This phenomenon, first described by Otto Warburg, is commonly observed in oncogenesis. Thus, HPV16 E7 may cause metabolic reprogramming and reduce available ATP levels. It is tempting to speculate that HPV16 E6 overrides HPV E7 induced metabolic effects by turning on mTORC1 signaling. HPV16 E6 has been reported to activate mTORC1 signaling through the E6AP dependent degradation of the mTORC1 negative regulator TSC2 (Lu et al., 2004). Cervical carcinomas and high grade squamous intraepithelial lesions, which express high-risk HPV E6 and HPV E7 proteins, have increased activation of mTORC1 and S6K as indicated by immunohistochemistry (Feng et al., 2009; Oh et al., 2006). Moreover, the activation of 4E-BP1, mTORC1, and S6K in relevant HPV positive cervical cancer cell lines and patient derived specimens supports HPV16 E6 mediated activation of metabolic signaling pathways. Chapters in this dissertation suggest novel mechanisms for HPV16 E6 to activate mTORC1 through receptor protein tyrosine kinases and increasing cap dependent translation (Spangle and Munger, 2010; Zhou and Munger, 2009).

## SUMMARY AND SIGNIFICANCE

HPV is associated with over 99 percent of all cases of cervical cancer, worldwide. HPV is also associated with other malignancies of the anogenital tract and with head and neck carcinomas most prominently oropharyngeal cancers. Unlike other types of solid human tumors, the genetic factors that contribute to HPV associated carcinogenesis are well defined. The HPV E6 and E7 oncoproteins are necessary and sufficient for the induction and maintenance of transformation. High-risk HPVs infect the nutrient rich cells of the epithelial basement membrane, where HPV genomes are maintained as an episome. The basal epithelial cells undergo asymmetric cell division, giving rise to the more differentiated, but also nutrient deprived layers of the outer squamous epithelium. Viral genome replication and progeny virion assembly is confined to the differentiated cells of the epithelium. It is well understood that the destabilization of pRb by high-risk HPV E7 maintains S phase competence and drives the cell cycle while the HPV E6 mediated destabilization of p53 eliminates cell cycle checkpoints enabling aberrant DNA replication. It is, however, unclear how completion of the viral life cycle can occur in the presumed absence of abundant nutrients and energy sources that are required for active DNA replication and then also stimulate protein synthesis.

HPV16 E7 has been reported to cause metabolic stress by switching energy generation from oxidative phosphorylation to anaerobic fermentation. Moreover, HPV16 E7 induces autophagy (Zhou and Munger, 2009). In the following chapters I first describe HPV16 E6 mediated activation of mTORC1 and subsequent increase in protein synthesis. Initial efforts to delineate the mechanism of mTORC1 activation suggested that HPV16 E6 sustains AKT activation under conditions of nutrient deprivation. HPV16 E6 mediated AKT activation is attributed to the activation of PDK1 and mTORC2. A comparative analysis of cutaneous and

high- and low-risk mucosal HPV E6 demonstrated that the ability to activate mTORC1 and increase cap dependent translation is shared amongst mucosal HPV E6 proteins. Mutational analysis mapped HPV E6 mediated activation of these pathways to the LXXLL binding motif. Several proteins associate with HPVE6 through an LXXLL motif, including the E3 ubiquitin ligase E6AP (UBE3A). Lastly I evaluated upstream signaling events that may cause mTORC1 activation and increase cap dependent translation. I show that HPV16 E6 increases the activation of receptor protein tyrosine kinases including EGFR, IR, and IGFR. Receptor activation appears to be mediated by the increased internalization and degradation of phosphorylated receptor species. HPV16 E6 also associates with the signaling adaptor protein Grb2, which may be contributing to increased receptor activation, internalization, and degradation. These data have lead to the model that the biological properties of high-risk HPV E6 and HPV E7 are balanced to meet the metabolic needs during successful viral infection and/or genome replication (Zhou et al., 2009). Ultimately HPV E6 stimulates signaling cascades that promote protein synthesis and generates the cellular machinery that may be necessary for DNA replication as well as viral capsid proteins necessary for packaging.

# CHAPTER TWO

## The human papillomavirus type 16 E6 oncoprotein activates mTORC1 signaling and increases protein synthesis

**The human papillomavirus type 16 E6 oncoprotein activates mTORC1 signaling and increases protein synthesis**

Jennifer Spangle[1,2] and Karl Munger[1,2]*

[1]Division of Infectious Diseases, Brigham and Women's Hospital [2]Committee on Virology, Harvard Medical School, Boston, Massachusetts, 02115

* Corresponding author. Mailing Address: Channing Laboratories 861, 181 Longwood Ave., Boston, MA 02215. Phone (617) 525-4282, Fax (617) 525-4283, E-mail: kmunger@rics.bwh.harvard.edu

This chapter is adapted from a previously published manuscript (see Appendix 4):

Spangle, JM., and K. Munger. 2010. The human papillomavirus type 16 E6 oncoprotein activates mTORC1 signaling and increases protein synthesis. J. Virol *84*: 9398-9407.

Contributions: I performed all of the experiments described in this manuscript. Karl Munger and I wrote the manuscript together.

**Abstract**

The mammalian target of Rapamycin (mTOR) kinase acts as a cellular rheostat that integrates signals from a variety of cellular signal transduction pathways that sense growth factor and nutrient availability as well as intracellular energy status. It was previously reported that the human papillomavirus type 16 (HPV16) E6 oncoprotein may activate the S6 protein kinase (S6K) through binding and E6AP-mediated degradation of the mTOR inhibitor tuberous sclerosis complex 2 (TSC2) (Lu et al., 2004; Zheng et al., 2008). Our results confirmed that HPV16 E6 expression causes an increase in mTORC1 activity through enhanced phosphorylation of mTOR and activation of downstream signaling pathways S6K and eukaryotic initiation factor binding protein 1 (4E-BP1). However, we did not detect a decrease in TSC2 levels in HPV16 E6-expressing cells. We discovered, however, that HPV16 E6 expression causes AKT activation through the upstream kinases PDK1 and mTORC2 under conditions of nutrient deprivation. We show that HPV16 E6 expression causes an increase in protein synthesis by enhancing translation initiation complex assembly at the 5' mRNA cap and an increase in cap-dependent translation. The increase in cap-dependent translation likely results from HPV16 E6-induced AKT/mTORC1 activation, as the assembly of the translation initiation complex and cap-dependent translation are Rapamycin sensitive. Lastly, coexpression of the HPV16 E6 and E7 oncoproteins does not affect HPV16 E6-induced activation of mTORC1 and cap-dependent translation. HPV16 E6-mediated activation of mTORC1 signaling and cap-dependent translation may be a mechanism to promote viral replication under conditions of limited nutrient supply in differentiated, HPV oncoprotein-expressing proliferating cells.

## Introduction

HPVs initially infect basal epithelial cells, where the viral episome is maintained extrachromosomally at a low copy number. High-level viral genome replication and production of progeny virus is confined to the outer, terminally differentiated layers of the infected squamous epithelium, where metabolic activity of the host cells and available nutrients are presumably more limited. Moreover, it has been reported that HPV16 E7 expression induces the "Warburg effect", a switch from an oxidative phosphorylation-based to a glycolytic mode of glucose metabolism (Mazurek et al., 2001a, b). This may trigger an autophagy-like process when HPV16 E7 is expressed in human keratinocytes (Zhou and Munger, 2009) to generate metabolites that can be used for energy consuming processes including viral replication.

The mammalian target of Rapamycin complex 1 (mTORC1) signaling cascade serves as a metabolic sensor, integrating a diverse array of signals, including nutrient and growth factor availability. mTORC1 signaling regulates a variety of cellular processes including cell growth, viability, and proliferation, at least in part through the activation of protein translation (reviewed in (Ma and Blenis, 2009). mTORC1 kinase activity is negatively regulated through TSC1/TSC2 mediated inhibition of the Ras homologue and mTOR activator Rheb (Zhang et al., 2003). TSC2 itself is regulated through phosphorylation at eight or more sites by a diverse set of kinases including AKT (reviewed in reference (Ma and Blenis, 2009)). TSC2 phosphorylation by AKT at multiple sites inhibits TSC2, releasing mTOR from repression for subsequent activation of downstream signaling cascades that regulate protein translation; the ribosomal S6 protein kinase (S6K) and the eukaryotic initiation factor binding protein 1 (4E-BP1) pathways ((Inoki et al., 2002), reviewed in (Ma and Blenis, 2009; Mamane et al., 2006))

Previous studies have suggested that HPV16 E6 may activate mTORC1 signaling. A yeast two-hybrid screen with E6 as the bait initially identified peptides corresponding to TSC2 and the homologous protein E6TP1 (Elston et al., 1998; Gao et al., 1999). Subsequent studies suggested that HPV16 E6 may bind and degrade TSC2 through an E6AP dependent mechanism, thereby activating mTORC1 (Lu et al., 2004; Zheng et al., 2008). These latter studies, however, were performed by transient transfection, and it remained unclear whether in cells with stable expression of E6, TSC2 levels are decreased, whether increased mTORC1 activity can be detected, whether this is relevant in the context of a nutrient deprived state, and if the increase in mTORC1 signaling in E6 expressing cells results in a corresponding increase in cap-dependent translation.

Here we report that HPV16 E6 expression does not reduce the steady state levels of TSC2, but instead HPV16 E6 activates mTORC1 as a result of increased AKT activity through the PDK1 and mTORC2 pathways. Moreover, mTORC1 activity is sustained in HPV16 E6 expressing primary human foreskin keratinocyte populations under conditions of nutrient deprivation. Furthermore, HPV16 E6 expression causes activation of the S6K and 4E-BP1 translation regulatory pathways, causes enhanced binding of translation initiation factors to a synthetic cap structure and increases cap dependent translation as measured by luciferase reporter assays. The HPV16 E6 mediated increases in binding of translation initiation factors to the cap and cap-dependent translation are Rapamycin sensitive, suggesting a connection between HPV16 E6 mediated increase in mTORC1 activation and enhanced cap dependent translation. Lastly, co-expression the HPV E7 oncoprotein does not affect these processes, suggesting that the ability of E6 to activate mTORC1 signaling and cap-dependent translation may be relevant in the context of an HPV infection.

## Materials and Methods

**Plasmids.** Plasmids used in this study include the retroviral vectors pLXSN (control), pLXSN HPV16 E6, pLXSN HPV16 E7, pLXSN HPV16 E6/E7 (Halbert et al., 1992), a set of human ß-actin promoter driven expression vectors, p1318 (control), p1435 (HPV16 E7), p1436 (HPV16 E6), p1321 (HPV16 E6/E7), and p1319 (HPV16 early coding region) (Munger et al., 1989), a pCMV Bam Neo based vector (Baker et al., 1990; Munger et al., 1989). The pFR_CrPV_xb bicistronic firefly/Renilla luciferase reporter plasmid (Petersen et al., 2006) was used for translation reporter assays and was obtained from Phil Sharp through Addgene (plasmid 11509).

**Cells lines and Culture.** 293, 293T and U2OS cells (ATCC) were maintained in Dulbecco's modified Eagle Media (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50U/ml penicillin, and 50µg/ml streptomycin. RKO pC cells (pCMV control cells) and RKO 10.2 (HPV16 E6-expressing) cells (Kessis et al., 1993), were generously provided by Kathleen Cho (University of Michigan, Ann Arbor, MI) and maintained in modified McCoy's medium (Invitrogen) supplemented with 10% newborn calf serum (NCS), 50 U/ml penicillin, 50 µg/ml streptomycin, and 500 µg/ml G418. Primary human foreskin keratinocytes (HFKs) were isolated from anonymous newborn circumcisions as previously described (McLaughlin-Drubin et al., 2008) and maintained in Keratinocyte Serum Free Media (KSFM) supplemented with human recombinant epidermal growth factor 1-53, bovine pituitary extract (Invitrogen), 50 U/ml penicillin, 50 µg/ml streptomycin, 20 µg/ml gentamycin, and 1 µg/ml amphotericin B. All experiments were performed with HFKs passaged less than ten times. For growth factor withdrawal experiments, HFKs were seeded onto poly-d-lysine coated plates (BD). For nutrient

deprivation assays, 90% confluent HFKs were washed twice with phosphate buffered saline (PBS), followed by incubation in PBS for 15-30 minutes prior to lysis.

**Western blotting and Antibodies.** Cell lysates were prepared by incubating the cells in ML buffer (McLaughlin-Drubin et al., 2008) (300 mM NaCl, 0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA supplemented with one Complete EDTA-free protease inhibitor cocktail tablet (Roche) per 25 ml lysis buffer and one PhosSTOP phosphatase inhibitor cocktail tablet (Roche) per 5 ml lysis buffer. Cells were then scraped and lysates cleared by centrifugation at 16,110 x g for 10 minutes at 4°C. Protein concentrations were determined using the Bradford method (Bio-Rad). Proteins were separated by SDS-polyacrylamide electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were blocked in 5% nonfat dry milk in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris [pH 7.4], 0.1% Tween-20) and probed with the appropriate antibody. Primary antibodies were used at a 1:1000 dilution, unless otherwise specified: ß-actin (#1501; Chemicon), p53 (Ab-6; Calbiochem), SGK1 (ab43606; Abcam), Firefly Luciferase (ab498; Abcam), Renilla Luciferase (PM047; MBL), SGK1 S422 (1:500, sc-16745-R, Santa Cruz), Flag (#3165, Sigma), mTOR (#2972), mTOR S2448 (#2971), S6K (#9202), S6K T389 (#9206), S6 (#2317), S6 S235/36 (#4858), S6 240/44 (#4838), TSC2 (#3635), 4E-BP1 (#9644), 4E-BP1 T37/46 (#2855), 4E-BP1 S65 (#9451), 4E-BP1 T70 (#9455), Akt (#9272), Akt S473 (#4060), Akt T308 (#9275), eIF4G (#2498), and SGK1 T256 (#2939), all from Cell Signaling Technology. Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham) were used at 1:10,000 and 1:15,000 dilutions, respectively. Proteins were visualized by enhanced chemiluminescence (Perkin Elmer) and exposed on Kodak BioMax

XAR film or electronically acquired with a Kodak Image Station 4000R equipped with Kodak Imaging Software, version 4.0.

**7-Methyl GTP binding assays.** Proteins that interact with a synthetic 7-Methyl-GTP RNA cap structure were purified as previously described (Kumar et al., 2000). In brief, 250 µg aliquots of cell lysates were precleared with 25 µl sepharose pre-washed in Buffer D for one hour and combined with 30 µl of a 50% slurry of 7-methyl-GTP-Sepharose (GE Healthcare, UK) pre-washed in Buffer D (50 mM HEPES pH 7.4, 40 mM NaCl, 2 mM EDTA, 0.1% Triton X100), and incubated for one hour at 4°C. After washing the resin three times with Buffer D, samples were analyzed by SDS PAGE and immunoblotting.

**Transfections and luciferase assays**. U2OS cells were transfected in six-well plates in triplicate for luciferase reporter assays using FuGene6 reagent (Roche). One µg pFR_CrPV_xb was cotransfected with two µg p1318, p1435, p1436, p1319, or p1321. HFKs were transfected in six-well plates in triplicate (seeded at 300,000 cells/well) using FuGene6 reagent (Roche) with 0.5 µg pFR_CrPV_xb and 1.5 µg of the previously described plasmids. Both U2OS cells and HFKs were lysed forty-eight hrs post transfection in 450 µl passive lysis buffer (dual luciferase reporter kit; Promega) per well. The supernatants were subjected to the dual luciferase reporter assay. The fold change in activity was determined by calculating the ratio of firefly luciferase activity to renilla luciferase activity as compared to control vector transfected cells. At least three independent experiments were performed.

## Results

**mTORC1 signaling is increased in HPV16 E6 expressing RKO cells.** The tuberous sclerosis tumor suppressor 2 (TSC2), sometimes also referred to as tuberin, is a negative regulator of mTORC1 activity (Figure 1A). Previous studies suggested the HPV16 E6 oncoprotein may be able to associate with TSC2 (Elston et al., 1998). Moreover, transient expression studies in HEK 293 cells suggested that HPV16 E6 not only binds to but can also enhance E6AP mediated TSC2 degradation, thereby activating mTORC1 signaling (Lu et al., 2004). Based on these findings we evaluated mTORC1 signaling in RKO human colon cancer cells with stable expression of HPV16 E6 (RKO E6) (Kessis et al., 1993) as well as control RKO cells. RKO cells have intact p53 and pRB tumor suppressor pathways and previous work has shown that p53 activities are lost upon E6 expression (Havre et al., 1995). Consistent with the published results, RKO E6 cells showed evidence of increased mTORC1 activity as evaluated by phosphorylation of the mTOR kinase at serine residue (S) 2448 (Figure 1B). TSC2 steady state levels, however, were not decreased in RKO E6 cells as compared to control cells. In contrast, p53 tumor suppressor levels were dramatically decreased in RKO E6 cells, indicating that there are no defects in E6/E6AP induced proteasomal degradation in these cells (Fig 2.1A). Similarly, HPV16 E6 expression did not reduce TSC2 levels in multiple experiments with several different primary human foreskin keratinocyte (HFK) populations (Figure 2.1F) or upon transient transfection of HPV16 E6 in HEK293 or U2OS cells (data not shown). Moreover, we did not detect association of HPV16 E6 with TSC2 by immunoprecipitation experiments (data not shown).

To determine whether the observed increased mTOR S2448 phosphorylation causes increased mTORC1 activity we evaluated the phosphorylation status of downstream phosphorylation targets in RKO E6 and control RKO cells. The eukaryotic translation

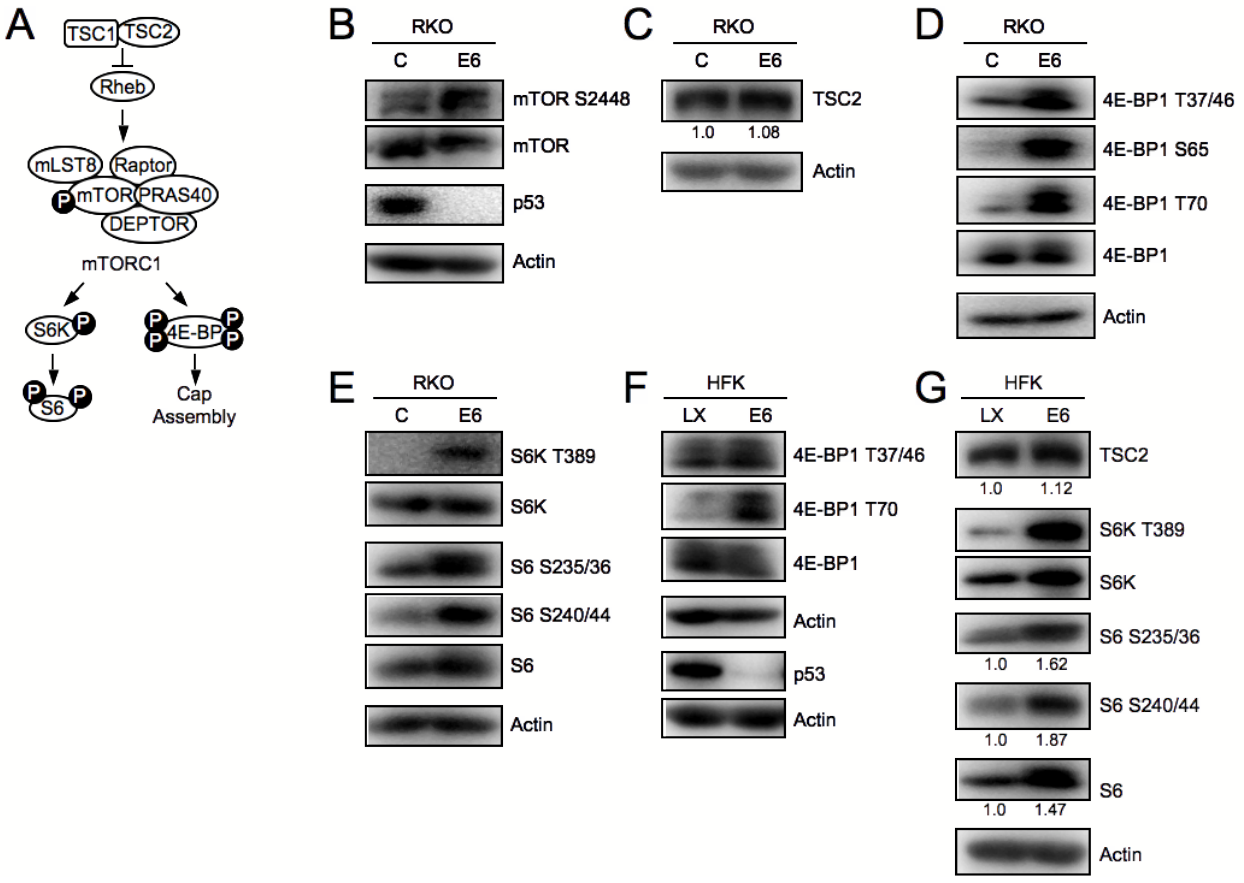


**Figure 2.1 HPV16 E6 expression activates mTOR1, 4E-BP1, S6K, and S6 phosphorylation through a TSC2-independent mechanism.** (A) Schematic diagram of mTORC1 signaling. See text for details. (B to E) Western blot analysis of mTOR phosphorylation (B), TSC2 expression (with quantifications shown below) (C), 4E-BP1 phosphorylation (D), and S6K and S6 phosphorylation (E) in HPV16 E6-expressing and control RKO cells. A p53 blot is shown in panel B to document HPV16 E6 expression, and actin blots are shown as loading controls. Also shown are results from Western blot analysis of 4E-BP1 (F) and TSC2 expression and S6K and S6 phosphorylation (G) (with quantifications shown below) in HPV16 E6-expressing and control (LX) primary human foreskin keratinocyte cultures (HFKs). A p53 blot is shown in panel F to document HPV16 E6 expression, and actin blots are shown as loading controls.

initiation factor 4E binding protein-1 (4E-BP1) regulates formation of a functional mRNA cap structure. Hypophosphorylated 4E-BP1 inhibits functional interaction of eukaryotic translation initiation factor 4E (eIF4E) with the 5' mRNA cap structure (reviewed in (Jackson et al., 2010)). Upon mTORC1 activation, 4E-BP1 is sequentially phosphorylated by mTOR at at least four residues. 4E-BP1 phosphorylation at threonine (T)37 and T46 serve as priming phosphorylation events that are required for subsequent phosphorylation and activation at T70 and S65. Hyperphosphorylated 4E-BP1 is released from the cap, allowing for recruitment of eIF4E and other translational initiation factors to the 5' mRNA cap (Gingras et al., 2001). Consistent with increased mTORC1 activity in RKO E6 cells, phosphorylation of 4E-BP1 at T37/46, S65 and T70 was strikingly increased in these cells (Fig 2.1C).

The S6 kinase (S6K) is another well-established mTORC1 substrate. Once phosphorylated at T389 by mTORC1, S6K activates and phosphorylates the ribosomal subunit 6 (S6), an important factor in ribosome biogenesis and a subunit of the 40S ribosome, at serines 235, 236, 240 and 244 (Ferrari et al., 1991). Phosphorylated S6 is incorporated into the 40S ribosome at the mRNA binding site and has been correlated with an increase in protein synthesis (reviewed in (Jastrzebski et al., 2007)). Phosphorylation of S6K at T389 and its substrate S6 at S235/236 and S240/244 was strikingly increased in RKO E6 cells as compared to control RKO cells (Fig. 2.1D). These results further support the notion that HPV16 E6 expression in RKO cells causes increased mTORC1 signaling.

To ensure that the observed activation of mTORC1 by HPV16 E6 is not specific to the RKO cell line, we performed similar experiments in HPV16 E6 expressing primary HFK populations. As compared to control vector transfected HFKs, HFK E6 cells showed increased phosphorylation of 4E-BP1 (Fig. 2.1E) as well as S6K and its substrate S6, while TSC2 steady

state levels were unchanged (Fig 2.1F). Similar results were obtained with a second independently derived set of HFK E6 and HFK control populations. Of note, we also consistently found evidence for increased S6K and S6 steady state levels in HKF E6 populations, which in combination with mTORC1 activation (as evidenced by increased 4E-BP1 phosphorylation) may contribute to the increased detection of S6 phosphorylation at S235/36 and S240/44 (Fig. 2.1E, F).

In order to confirm that the observed effects on 4E-BP1, S6K and S6 phosphorylation are a result of mTORC1 activation, we treated RKO E6 and RKO control cells with 100 nM Rapamycin for one hour. Phosphorylation of S6K, S6 and 4E-BP1 was decreased in RKO E6 cells, consistent with our model that these phosphorylation events are a result of mTORC1 activation (Figure 2.2).

In combination these results show that HPV16 E6 expression causes increases mTORC1 activity through a mechanism that does not appear to involve TSC2 degradation.

**HPV16 E6 mediated mTORC1 activation is mediated by PDK1 and mTORC2 activation.** Since we found no evidence for decreases in TSC2 steady state levels in HPV16 E6 expressing cells (Figs 2.1B, F and data not shown) and we did not detect an association of HPV16 E6 with TSC2 by immunoprecipitation experiments (data not shown), we evaluated alternative signaling events upstream of mTORC1 activation.

Members of the AKT serine/threonine kinase family are important activators of mTORC1 signaling (Pearce et al., 2010). 3-Phosphoinositide-dependent kinase 1 (PDK1) is downstream of Phosphoinositide 3-kinase (PI3K) and activates AKT by T308 phosphorylation, which in turn causes mTORC1 activation (Alessi et al., 1997) and, the mTORC2 kinase complex

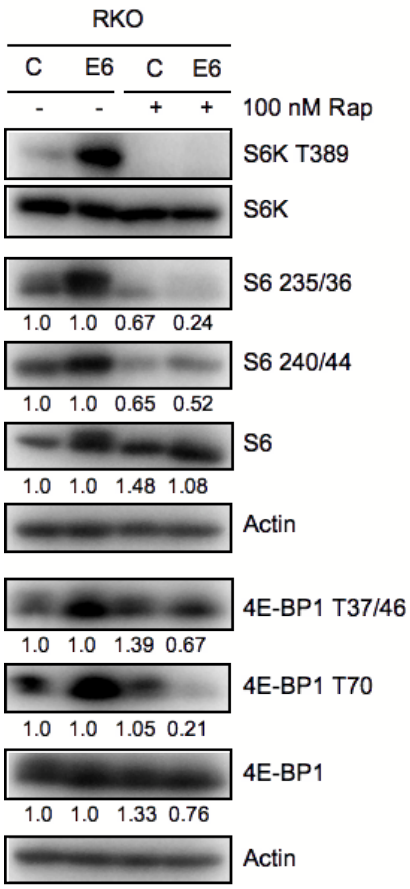


**Figure 2.2. HPV16 E6 expression causes increased S6K, S6, and 4EBP1 phosphorylation through mTORC1 activation**. Western blot analysis of mTORC1 downstream signaling components in RKO control and HPV16 E6-expressing RKO cells, treated with dimethyl sulfoxide (DMSO) or 100 nM Rapamycin (Rap) for 1 h prior to lysis. Relative levels of unphosphorylated and phosphorylated species of S6 and 4EBP1 are indicated. Actin blots are shown as loading controls.

activates AKT by S473 phosphorylation (Fig 2.3A). Hence we assessed AKT T308 and S422 phosphorylation in RKO E6 and RKO control cells. RKO E6 cells showed increased AKT T308 phosphorylation as compared to control RKO cells. In contrast, RKO E6 and RKO control cells each showed high levels of AKT S473 phosphorylation. To confirm that PDK1 activity is increased in RKO E6 cells, we also evaluated T256 phosphorylation of the PDK1 substrate serum- and glucocorticoid-inducible kinase 1 (SGK1). Consistent with increased PDK1 activity, SGK1 T256 phosphorylation was increased in RKO E6 as compared to RKO control cells (Fig 2.3B).

Given that RKO cells are a colon cancer derived line that may harbor mutations, which may cause aberrant AKT phosphorylation, we next evaluated AKT T308 and S473 phosphorylation in HPV16 E6 expressing primary HFK and control HFK populations. When grown in growth factor containing keratinocyte serum free medium, AKT was phosphorylated at T308 and S473 even in control HFKs (data not shown). To assess AKT phosphorylation of cells in a nutrient deprived state, we incubated the cells in phosphate buffered saline (PBS) for 15 or 30 minutes. Under these conditions of nutrient deprivation, we detected increased AKT S473 and T308 phosphorylation in HFK E6 as compared to control HFKs (Fig 2.3C). Similar results were obtained when HFKs were treated with Earle's balanced salt solution containing 1 mg/ml glucose (data not shown). These results suggest that AKT S473 and T308 phosphorylation is maintained in HPV16 E6 expressing HFKs under conditions of limited growth factor availability

To assess whether sustained AKT S473 and T308 phosphorylation in HPV16 E6 expressing HFKs is a result of sustained PDK1 and mTORC2 activity, respectively, we assessed SGK1 phosphorylation. PDK1 phosphorylates SGK1 at T256, whereas mTORC2 phosphorylates SGK1 at S422. Consistent with our model, SGK1 S256 and S422 phosphorylation was sustained

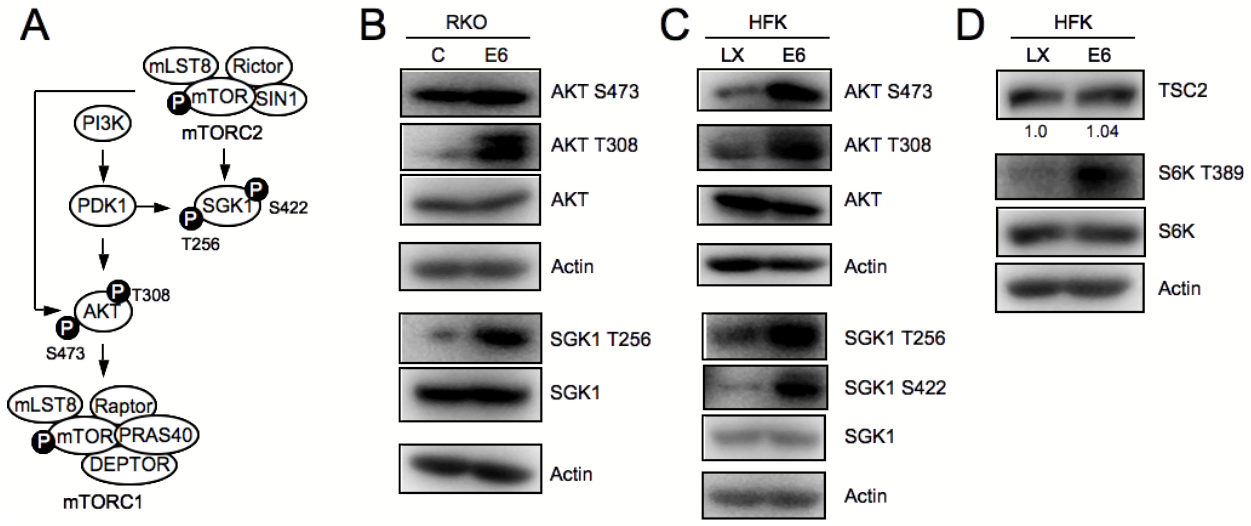


**Figure 2.3. HPV16 E6 expression causes AKT activation**. (A) Schematic diagram of AKT phosphorylation through PDK1 and mTORC2 pathways. (B) Western blot analysis of AKT phosphorylation in control and HPV16 E6-expressing RKO cells. SGK1 is phosphorylated by PDK1 at T256 and is included as a control for PDK1 activation in HPV16 E6-expressing RKO cells. Actin blots are shown as loading controls. (C) Sustained AKT activation in control (LX) and HPV16 E6-expressing HFK populations under conditions of nutrient deprivation. SGK1 is phosphorylated by PDK1 at T256 and by mTORC2 at S422 and is included as a control for PDK1 and mTORC2 activation in HPV16 E6-expressing HFKs. Actin blots are shown as loading controls. (D) Sustained S6K activation in control (LX) and HPV16 E6-expressing HFK populations under conditions of nutrient deprivation. A TSC2 blot with quantification is shown to document similar expression in the two cell populations after nutrient deprivation; an actin blot is shown as a loading control.

in HFK E6 cells under conditions of nutrient deprivation (Fig. 2.3C). Moreover, S6K T389 phosphorylation was detected in HFK E6 but not in HFK control cells under conditions of nutrient deprivation. Of note, TSC2 levels were not decreased in HFK E6 cells undergoing growth factor restriction as a result of PBS treatment (Fig. 2.3D).

These results suggest that HPV16 E6 expression activates mTORC1 at least in part through PDK1 and mTORC2 mediated AKT phosphorylation and that this activation is sustained during conditions of nutrient deprivation.

**HPV16 E6 expression increases the assembly of the translation initiation complex at the mRNA cap.** 4E-BP1 phosphorylation by mTORC1 allows association of translation initiation factors to the 5' mRNA cap structure thereby activating cap-dependent translation (Jackson et al., 2010). To evaluate whether HPV16 E6 expression enhances the assembly of the translation initiation complex at the mRNA cap, we performed *in vitro* cap-binding assays. Lysates from RKO E6 and RKO control cells were incubated with 7-Methyl GTP Sepharose and association of initiation factor eIF4G was assessed by Western blotting. As expected, we detected increased eIF4G binding to the synthetic cap structure with lysates from RKO E6 cells as compared to RKO control cells (Fig 2.4). To determine whether the observed increase in eIF4G binding observed with RKO E6 cell lysates is caused by increased mTORC1 activity, we also performed experiments with cell lysates prepared from RKO E6 and RKO control cells that were treated with the mTORC1 inhibitor Rapamycin for 1 hour prior to harvesting. Inhibition of mTORC1 abrogated eIF4G binding to the cap structure in RKO E6 cells (Fig. 2.4).

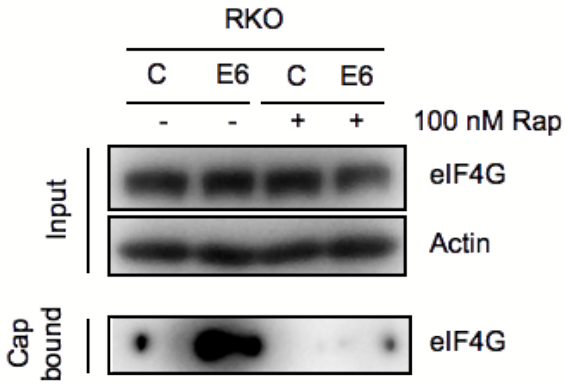


**Figure 2.4. Increased binding of the translation initiation factor eIF4G to a synthetic 7-methyl-GTP (7MeGTP) mRNA cap structure in HPV16 E6-expressing RKO cell lysates, which is sensitive to Rapamycin treatment.** Control and HPV16 E6-expressing RKO cells were treated with dimethyl sulfoxide (DMSO) or 100 nM Rapamycin (Rap) for 1 h prior to lysis. Cap binding assays were performed as described in Materials and Methods. Levels of eIF4G in a 50-μg sample, representing 25% of the cap-binding reaction, together with an actin blot, are shown in the top panel (Input). Blot results for cap-bound eIF4G are shown in the bottom panel.

These results show that binding of translation initiation factors to the 5' mRNA cap is increased in HPV16 E6 expressing cells and that this most likely represents a consequence of mTORC1 activation.

**HPV16 E6 expression causes increased translation of capped mRNA.** Given the observed increased binding of eIF4G to a synthetic cap in vitro with RKO E6 cells we next determined if HPV16 E6 expression might increase cap dependent translation. The U2OS human osteosarcoma line was used for the initial experiments because it is contains wild type p53 and is highly transfectable. We performed dual luciferase reporter assays utilizing a bicistronic reporter vector, pFR_CrPV_xb (Petersen et al., 2006), that drives expression of the firefly and renilla luciferase genes from a minimal thymidine kinase promoter. Firefly luciferase is translated by a cap dependent mechanism, whereas translation of renilla luciferase is through a cap-independent mechanism from a cricket paralysis virus (CrPV) internal ribosomal entry site (IRES) (Fig. 2.5A, upper panel). Co-expression of HPV16 E6 caused a 3.56±0.68 fold increase in firefly luciferase activity compared to control vector cotransfection. In contrast renilla luciferase activity was only increased 1.22±0.24 fold compared to vector cotransfection. When normalized to Renilla luciferase activity, HPV16 E6 co-transfection caused a statistically significant 2.92±0.33 fold ($p < 0.0001$) increase in firefly luciferase activity (Fig 2.5A, lower panels) as compared to vector transfected cells. To confirm that the HPV16 E6 mediated increase in cap dependent translation is not a result of transcriptional regulation or aberrant splicing of the bicistronic mRNA, we performed quantitative real time reverse transcription PCR for firefly and Renilla luciferases. These experiments showed that the mRNA levels of firefly and Renilla luciferase were unchanged (data not shown). Moreover, we also directly assessed steady state levels of firefly

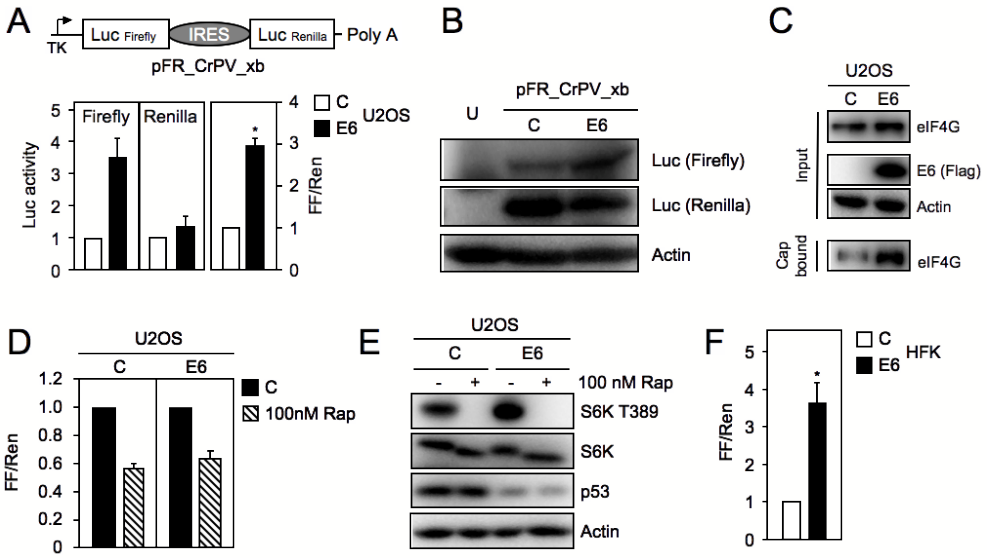


**Figure 2.5. HPV16 E6 expression causes as increase in cap-dependent translation, which is sensitive to Rapamycin treatment.** (A) Diagram of bicistronic firefly Renilla reporter plasmid, pFR_CrPV_xb, used for these experiments. Firefly luciferase is translated through a cap-dependent mechanism, whereas Renilla luciferase is expressed from an internal ribosomal entry site (IRES) through a cap-independent mechanism (top). HPV16 E6 expression causes an increase in firefly but not Renilla luciferase activity (bottom). U2OS cells were transfected with control or HPV16 E6 expression vector, and lysates were processed for Renilla and firefly luciferase assays at 48 h posttransfection. The data are presented as the change of firefly and Renilla luciferase activities normalized to control vector-transfected cells (left and middle) and the fold change of normalized firefly compared to normalized Renilla luciferase activity (FF/Ren) (right). The bar graphs represent averages and standard deviations of four experiments, each performed in triplicate. The asterisk denotes statistical significance ($P < 0.0001$). (B) Western blot analysis of firefly and Renilla luciferase expression in U2OS cells transiently transfected with the indicated plasmids. U, untransfected cells. (C) Western blot analysis of eIF4G binding to a synthetic 7-methyl-GTP (7MeGTP) mRNA cap upon transient transfection of HPV16 E6 or control vector in U2OS cells. (D) HPV16 E6-mediated increase in cap-dependent translation is Rapamycin sensitive. U2OS cells were transfected with pFR_CrPV_xb and the indicated plasmids; 18 h prior to lysis, cells were treated with dimethyl sulfoxide (DMSO) or 100 nM Rapamycin (Rap). The graph represents averages and standard deviations of four experiments, each performed in triplicate. (E) Western blot analysis of S6K phosphorylation in U2OS cells transiently transfected with HPV16 E6 or control vector. One hour prior to lysis, cells were treated with DMSO or 100 nM Rapamycin (Rap). Decreases in p53 levels are shown to document HPV16 E6 expression, and an actin blot is included as a loading control. (F) Transient transfection of HPV16 E6 activates cap-dependent translation in primary HFKs. Cells were transfected with pFR_CrPV_xb and the indicated plasmids and processed for Renilla and firefly luciferase assays at 48 h posttransfection. Firefly and Renilla luciferase activities were normalized to control vector-transfected cells and are presented as fold changes of normalized firefly relative to normalized Renilla luciferase activity. The bar graph represents the average and standard deviation of four experiments, each performed in triplicate; asterisks indicate statistical significance ($P = 0.0001$).

and Renilla luciferase proteins by Western blotting in U2OS cells that were transiently co-transfected with the reporter plasmid and HPV16 E6 or the control vector. Consistent with the enzyme activity results, expression of HPV16 E6 caused an increase in firefly but not Renilla luciferase levels (Fig 2.5B). We also performed cap binding experiments and similar to what we observed in RKO cells with stable expression of HPV16 E6 (Fig 2.4), transient expression of HPV16 E6 in U2OS cells caused increased association of eIF4G with a synthetic mRNA cap structure (Fig 2.5C). To confirm that mTORC1 signaling is necessary for the HPV16 E6 mediated increase in cap dependent translation, dual luciferase reporter assays were performed with cells that were treated with 100 nM Rapamycin for 24 hours prior to harvesting. These experiments results show that cap dependent translation is reduced in Rapamycin treated HPV16 E6 as well as control vector transfected U2OS cells (Fig. 2..5D). To further confirm that HPV16 E6 expression increases mTORC1 activity in U2OS cells and that this is inhibited by Rapamycin treatment, we also evaluated mTORC1 dependent S6K phosphorylation at T389. As expected, transient expression of HPV16 E6 caused S6K T389 phosphorylation that was reduced upon treatment with Rapamycin (Fig 2.5E).

To assess whether HPV16 E6 expression can cause increased cap-dependent translation in biologically relevant cells, we performed dual luciferase reporter assays in primary HFKs. Similar to what we observed with U2OS cells, co-transfection of HPV16 E6 caused a statistically significant 3.49±0.56 fold ($p = 0.0001$) increase in firefly luciferase as compared to control vector transfected cells.

Hence, HPV16 E6 expression can increase cap dependent translation and that mTORC1 signaling is necessary for HPV16 E6 to modulate this process.

**HPV16 E7 co-expression does not affect E6 induced activation of mTORC1 and cap dependent translation.** Since HPV E6 and E7 oncoproteins are co-expressed in high-risk HPV associated lesions and cancers, we also evaluated mTORC1 signaling and cap dependent translation in HPV16 E6/E7 co-expressing cells. Phosphorylation of S6K at T389 by mTORC1 was similarly increased in HFK populations with co-expression of HPV16 E6/E7 as in HPV16 E6 expressing HFKs (Fig 2.6A). While expression of HPV16 E7 alone did not affect cap dependent, expression of HPV16 E6/E7 or the entire HPV16 early coding region in U2OS cells caused statistically significant (2.40±0.30 fold; $p = 0.0013$) and (2.36 ±0.23 fold; $p = 0.0005$) increases in firefly luciferase activity, respectively, similar to E6 co-transfection (2.97±0.31 fold; $p < 0.0001$) (Fig 2.6B).

Hence, HPV16 E7 co-expression does not markedly affect the ability of HPV16 E6 to activate mTORC1 activity and to augment cap dependent translation.

**Discussion**

Previous reports have suggested that mTOR is activated in cells transiently expressing HPV16 E6, as indicated by an increase in S6K phosphorylation (Lu et al., 2004). This activity was attributed to the ability of HPV16 E6 to interact with and accelerate TSC2 degradation through an E6AP-dependent pathway (Zheng et al., 2008). In our experiments, TSC2 steady-state levels were unaltered in HPV16 E6-expressing RKO cells and HFKs relative to that in control cells (Fig. 2.1C and G and Fig. 2.3D) and upon transient transfection of HPV16 E6 in HEK293 or U2OS cells (data not shown). Moreover, we did not detect association of HPV16 E6 with TSC2 by immunoprecipitation experiments (data not shown). Hence, the reported E6AP-mediated TSC2 degradation by HPV16 E6 is not a rate-limiting mechanism by which HPV16 E6

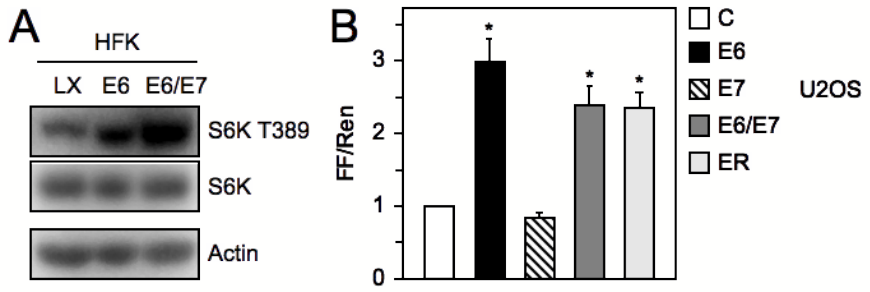


**Figure 2.6. HPV16 E7 coexpression does not affect E6-induced S6K T389 phosphorylation or cap-dependent translation.** (A) Western blot analysis of S6K T389 phosphorylation in HFK populations with stable expression of HPV16 E6 or HPV16 E6/E7 or control vector (LX)-transduced HFKs. An actin blot is shown as a loading control. (B) U2OS cells were transiently transfected with pFR_CrPV_xb and human _-actin-promoter-driven expression vectors for HPV16 E6, E7, E6/E7, the entire HPV16 early coding region (ER), or empty vector as a control and processed for Renilla and firefly luciferase assays at 48 h posttransfection. Firefly and Renilla luciferase activities were normalized to control vector-transfected cells and are presented as fold changes of normalized firefly relative to normalized Renilla luciferase activity. The bar represents the average and standard deviation of four experiments, each performed in triplicate; asterisks indicate statistical significance ($P \leq 0.0013$).

expression causes mTORC1 activation in our experimental systems.

Here we report that cells with stable HPV16 E6 expression show evidence of active mTORC1 signaling, as evidenced by activation of the S6K and 4E-BP1 downstream cascades (Fig. 2.1). Most importantly, mTORC1 activity is sustained in HPV16 E6-expressing HFKs under conditions of nutrient deprivation (Fig. 2.3). In contrast to the previously published studies, we did not find any evidence for HPV16 E6 binding to TSC2 and/or lowering its steady-state levels in the cells that we studied (Fig. 2.1C and G and Fig. 2.3D). Our results, however, suggest that HPV16 E6 expression causes mTORC1 activation, at least in part, through an AKT-dependent mechanism. HPV16 E6 expression in primary human epithelial cells caused AKT activation through at least two distinct pathways, PDK1 and mTORC2 (Fig. 2.3C). As with mTORC1, our results show that AKT remains active in HPV16 E6-expressing HFKs under conditions of nutrient deprivation. HPV16 E6 expression also caused an increase in cap-dependent translation (Fig. 2.5 and 2.6). This effect correlated with increased binding of translation initiation factors to a synthetic cap (Fig. 2.4 and 2.5C) and was inhibited by the mTORC1 inhibitor Rapamycin (Fig. 2.4 and Fig. 2.5D and E), suggesting that HPV16 E6-mediated activation of translation may represent a consequence of mTORC1 activation.

The HPV16 E6 and E7 oncoproteins play important functions during the viral life cycle (Flores et al., 2000; Thomas et al., 1999). Whereas HPVs initially infect proliferative basal epithelial cells, high-level viral genome replication and synthesis of viral progeny is restricted to terminally differentiated epithelial cells. The HPV E6 and E7 proteins contribute to the viral life cycle by uncoupling the process of epithelial cell differentiation from cell cycle withdrawal. The HPV E7 protein, in particular, through degradation of the retinoblastoma tumor suppressor pRB and the

related family members p107 and p130, causes increased transcription of E2F-responsive genes, many of which encode enzymes that are rate limiting for cellular DNA synthesis (reviewed in references (Havre et al., 1995; Longworth and Laimins, 2004; McLaughlin-Drubin and Munger, 2009a). Since HPV genome replication is acutely dependent on expression of host cellular replication proteins, one might envision that the ability of HPV16 E6 to activate translation of capped mRNAs represents an additional facet of this strategy in order to ensure adequate expression of cellular proteins that are necessary for viral genome replication. In addition, or alternatively, the ability of HPV16 E6 to activate protein synthesis may also contribute to high-level synthesis of viral proteins, particularly the L1 and L2 capsid proteins that need to be abundantly expressed during productive viral replication. While there is no direct evidence for such a mechanism, translational control of the L1 capsid protein synthesis has been suggested by results from experiments where HPV31 episome-containing human epithelial cells were induced to undergo differentiation by suspension in methylcellulose-containing medium. Under these conditions, the authors observed abundant expression of L1-encoding mRNAs; however, there was no evidence for L1 protein synthesis (Ruesch et al., 1998). There is also evidence for translational regulation of early protein synthesis during epithelial cell differentiation in HPV-positive cells. When HPV16-positive CaSki cervical carcinoma cells were cultured in methylcellulose- or $CaCl_2$-containing medium to induce differentiation, increased expression of the E7 oncoprotein was observed. This increase was not at the level of transcription or protein stability, but rather the authors observed an increase in association of E7-encoding mRNAs to polysomes. These authors also observed sustained phosphorylation of 4E-BP1 upon differentiation of CaSki cells but not with HPV-negative HaCaT cells or primary HFKs. Moreover, mTORC1 inhibition by Rapamycin treatment reduced 4E-BP1 phosphorylation and

HPV16 E7 oncoprotein expression in these cells (Oh et al., 2006).

Increased mTOR S2448 and S6K T389 phosphorylation was also observed in HPV-positive high-grade cervical squamous intraepithelial lesions (Feng et al., 2009), and there is also evidence for increased AKT phosphorylation in HPV-positive high-grade cervical squamous intraepithelial lesions (Menges et al., 2006). Given our results, it is tempting to speculate that these effects may at least in part represent a consequence of HPV16 E6 expression.

There are several reports that have shown that HPV16 E7 expression may also cause AKT activation (Menges et al., 2006; Pim et al., 2005). Several mechanisms have been proposed. HPV16 E7 may activate AKT by a pRB-dependent process, causing p27kip1 cytoplasmic accumulation and induction of cellular migration (Charette and McCance, 2007; Menges et al., 2006). It has also been reported that HPV16 E7 can activate AKT independently of the pRB pathway through binding and inhibition of protein phosphatase 2A (Pim et al., 2005). In another study, however, cells that ectopically expressed HPV16 E7 and activated AKT showed a significantly higher rate of cellular proliferation and migration than either AKT or HPV16 E7-expressing cells (Dow et al., 2008). These results would suggest that HPV16 E7 expression is not sufficient to fully activate AKT. While our experiments did not directly address the possible contribution of HPV16 E7 in AKT phosphorylation, there was no evidence that coexpression of HPV16 E6 and E7 caused an increase in mTORC1 signaling compared to HPV16 E6-expressing cells (Fig. 2.6A). Moreover, HPV16 E7 expression did not increase cap-dependent translation in our reporter assays (Fig. 2.6B).

Aberrant activation of AKT and mTORC1/2 is frequently observed in human cancers, and mTORC1 inhibitors have been evaluated as antineoplastic agents (Dowling et al., 2010; Menon and Manning, 2008; Shor et al., 2009). As the regulation of mTORC2 and its

downstream signaling pathways are increasingly understood, it is becoming apparent that the development of mTORC2-specific rictor inhibitors may also limit aberrant cellular growth and proliferation associated with human cancers. Hence it is conceivable that HPV16 E6-mediated AKT and mTORC1 and mTORC2 activation may also contribute to the transforming activities of HPV16 E6. If that was the case, inhibition of AKT and mTORC1 and/or mTORC2 should be evaluated as a therapeutic modality for HPV-associated lesions and cancers.

Our studies presented here were focused on AKT, but they do not exclude the possibility that HPV16 E6 expression may also affect mTORC1 activity through other pathways. Activation of the p53 tumor suppressor inhibits mTORC1 activity through sestrin 1 and sestrin 2. These two proteins are transcriptional targets of p53 and activate the AMP-responsive protein kinase (AMPK). AMPK phosphorylates and activates the mTOR inhibitor TSC2, thereby inhibiting mTOR (Budanov and Karin, 2008). E6/E6AP-mediated p53 degradation may therefore be predicted to short-circuit this regulatory loop and may contribute to sustained mTORC1 activity.

In addition, several PDZ proteins have been implicated in mTOR signaling. Inactivation of hScribble, which is targeted for degradation by HPV16 E6 (Nakagawa and Huibregtse, 2000), was shown to dysregulate MAP kinase signaling (Dow et al., 2008), which is predicted to activate mTORC1. More recently, Sabatini's group identified a novel mTORC1/mTORC2-associated inhibitor, DEPTOR, which contains a PDZ domain (Peterson et al., 2009) and thus may be a potential candidate for HPV16 E6 association and degradation.

Our results show that HPV16 E6 expression in primary epithelial cells activates AKT through at least two pathways, PDK1 and mTORC2, but the exact mechanism remains unknown. PDK1 is downstream of PI3K signaling. Several transforming viral proteins have been reported to activate PI3K, including SV40 small tumor antigen and the mouse polyomavirus middle tumor

antigen (reviewed in reference (Cheng et al., 2009)). Our future experiments will explore whether PI3K is activated by HPV16 E6 expression. A number of scenarios are possible, including activation of upstream signaling events, direct activation of PI3K, or inhibition of the phosphatase and tumor suppressor PTEN. Alternatively, HPV16 E6 may activate PDK1 by a PI3K-independent mechanism. Importantly, PDK1 also activates kinases other than AKT, including SGK1 (Fig. 2.3) and the Rho/Rac effector target PKN (Dong et al., 2000), a serine/threonine protein kinase, with a catalytic domain that is similar to that of protein kinase C. Interestingly, PKN has been reported to associate with high-risk HPV E6 proteins (Gao et al., 2000). It will be interesting to determine the biological consequences of PDK1-mediated activation of kinases other than AKT.

Our results also suggest that HPV16 E6 activates mTORC2 signaling. Recent reports suggest that rictor expression is critical to the activation of mTORC2, with rictor overexpression activating the kinase complex and resulting in increased cell growth and motility in gliomas, and rictor short hairpin RNA (shRNA) knockdown inhibiting cellular proliferation in colon cancer cell lines (Masri et al., 2007; Roulin et al., 2010). Interestingly, the FOXO1 transcription factor regulates rictor transcription, which is in turn regulated by AKT (Chen et al., 2010). These authors suggest that FOXO1 balances mTORC1 inhibition and mTORC2 activation through two separable transcriptional activities of FOXO1: direct inhibition of mTORC1 through sestrin-3 gene transcription and activation of mTORC2 through rictor gene transcription as a coactivator of a distinct transcriptional activating complex. Collectively this results in the maintenance of cellular energy homoeostasis even under conditions of nutrient stress. It is possible that the HPV16 E6 oncoprotein expression uncouples these processes through independent activation of mTORC1 and mTORC2. Alternatively, the PDZ protein and mTOR inhibitor DEPTOR

described above inhibits both mTORC1 and mTORC2 and thus should be evaluated as a potential candidate for HPV16 E6-mediated mTORC2 regulation.

We initiated these studies after we discovered that HPV16 E7 expression in normal human epithelial cells triggers an autophagy-like response (Zhou and Munger, 2009). Autophagy is a survival pathway that allows survival of cells under conditions of metabolic stress (reviewed in reference (Levine and Kroemer, 2008)). While we do not know the exact mechanism by which E7 expression may trigger such a response, it has been reported that HPV16 E7 expression causes the "Warburg effect," a metabolic switch from an oxidative phosphorylation-based pathway to a glycolytic pathway (Zwerschke et al., 1999). While such a switch may offer a number of advantages for a rapidly proliferating cell, including efficient growth under conditions of lower oxygen concentrations and increased synthesis of metabolic precursors (Vander Heiden et al., 2009), conversion of glucose to lactate generates far less energy in the form of ATP than conversion to $CO_2$ through oxidative phosphorylation. Particularly under conditions of limiting supply of nutrients, as may be the case in terminally differentiated cells in a squamous epithelium, autophagy may eventually lead to the demise of the cell. It is thus tempting to speculate that the ability of HPV16 E6 to activate mTORC1 signaling, a major regulator of autophagy, may function to dampen the autophagy response to HPV16 E7 expression and limited availability to nutrients. In such a model, expression of the HPV16 E6 protein would induce a cellular state of "blissful ignorance" and allow metabolically stressed, HPV-infected cells to survive long enough to support synthesis of viral progeny (Zhou et al., 2009).

# CHAPTER THREE

## The ability of mucosal Human Papillomavirus E6 proteins to increase protein synthesis is dependent on the integrity of the LXXLL binding motif

# The ability of mucosal Human Papillomavirus E6 proteins to increase protein synthesis is dependent on the integrity of the LXXLL binding motif

Jennifer Spangle[1,2], Nayana Ghosh-Choudhury[1], and Karl Munger[1,2]*

[1]Division of Infectious Diseases, Brigham and Women's Hospital [2]Committee on Virology, Harvard Medical School, Boston, Massachusetts, 02115

* Corresponding author. Mailing Address: Channing Laboratories 861, 181 Longwood Ave., Boston, MA 02215. Phone (617) 525-4282, Fax (617) 525-4283, E-mail: kmunger@rics.bwh.harvard.edu

This chapter is based on a manuscript that submitted to the Journal of Virology.

Contributions: I wrote this manuscript and performed all of the experiments described in it aside from those described below. I trained Nayana Ghosh-Choudhury, a summer student. She cloned HPV18, HPV6b, and HPV11 E6 mutants with primers I designed and performed the bicistronic luciferase reporter assays with the HPV18, HPV6b and HPV11 E6 mutants. Karl Munger helped design the research and edited the manuscript.

**Abstract**:

The HPV16 E6 protein was previously shown to activate mTORC1 signaling and increase protein synthesis. It remains unclear whether HPV E6-mediated mTORC1 activation and subsequent events are important for viral replication and progeny virion production. Here we report that mucosal HPV E6 proteins from high- and low-risk HPV types activate protein synthesis by increasing cap dependent translation. In contrast, however, the E6 proteins encoded by the cutaneous beta HPV5 and 8 do not. Utilizing previously characterized HPV16 E6 mutants, we identified the LXXLL binding motif as a contributing factor to cap dependent translation activation. The LXXLL binding motif mediates the association with cellular binding partners including the ubiquitin ligase E6AP and others through their LXXLL motif. Mutational analysis of HPV6b and HPV11 E6 identified analogous HPV6b E6 and HPV11 E6 LXXLL binding mutants that are also important in the low-risk HPV E6-mediated increase in protein synthesis. Moreover, high- and low-risk HPV E6 LXXLL binding mutants have reduced mTORC1 activation. This shared function amongst mucosal HPV types suggests that activation of upstream metabolic signaling cascades such as mTORC1 may be important for the viral lifecycle in specific epithelial tissue types. Alternatively, in conjunction with other functions of high-risk HPV E6 that are absent in low-risk HPV E6, mTORC1 activation may contribute to transformation.

**Introduction**:

Human papillomaviruses (HPVs) are small double stranded DNA viruses with a tropism for mucosal and cutaneous epithelial cells. Over 200 HPV types have been identified, of which approximately 30 infect the mucosal epithelium. Mucosal HPV types are further categorized by their propensity to cause lesions that can progress to carcinogenesis. Low-risk mucosal HPV types including HPV6b and HPV11 are most frequently associated with benign genital warts, whereas high-risk mucosal HPV types such as HPV16 and HPV18 cause squamous intraepithelial lesions that can progress to cancer. High-risk HPVs are associated with over 99% of cervical cancers and also with other anogenital cancers at a reduced prevalence. High-risk HPV infection is also associated with head and neck cancer, accounting for approximately 25% of all oral cancers, including those of the oropharynx and tonsil (reviewed in references ((McLaughlin-Drubin and Munger, 2009a) and (Schiffman et al., 2007)). HPV-induced carcinogenesis is often associated with the integration of the viral genome into host chromosomal DNA. This results in the dysregulated expression of the HPV E6 and E7 proteins. The high-risk E6 and E7 proteins are sufficient for the induction and maintenance of transformation of cervical epithelial cells in culture. High-risk HPV E6 and E7 also sufficient for carcinogenesis in transgenic mouse models (reviewed in reference (McLaughlin-Drubin and Munger, 2009a)).The high-risk HPV E6 and E7 onocoproteins lack enzymatic and nucleic acid binding activities, and therefore modulate cellular processes through the association with and modification of cellular protein complexes. The most well characterized cellular targets of high-risk HPV E6 and E7 oncoproteins are the p53 and retinoblastoma (pRb) tumor suppressors, respectively (reviewed in references (Howie et al., 2009; McLaughlin-Drubin and Munger, 2009b)).

High-risk HPV E6 proteins form a tripartite complex with p53 and the cellular ubiquitin ligase E6AP, targeting p53 for ubiquitination and proteasome-mediated degradation (Scheffner et al., 1993). High-risk HPV E6 oncoproteins also associate with cellular PDZ proteins including MUPP1, Dlg, hScrib, PTPN13, and PTPN3 through the HPV E6 carboxyl-terminal PDZ binding domain (Gardiol et al., 1999; Glaunsinger et al., 2000; Jing et al., 2007; Kiyono et al., 1997; Lee et al., 2000; Lee et al., 1997; Nakagawa and Huibregtse, 2000; Spanos et al., 2008). Associated cellular PDZ proteins may also be targeted for proteasomal-mediated degradation via the E6/E6AP complex. High-risk HPV E6 proteins also contribute to cellular transformation and immortalization through transcriptionally activating hTERT, the catalytic protein subunit for human telomerase (Klingelhutz et al., 1996). A large number of additional potential cellular targets of E6 proteins have been reported. Yeast two-hybrid screens using HPV16 E6 as bait separately identified the mTORC1 associated GTPase activating protein (GAP) E6TP, and ERC-55/E6BP, two proteins that are putatively involved in HPV16 E6 associated transformation (Chen et al., 1995; Gao et al., 1999).

Despite differences in the lesions they are associated with, low-risk and high-risk HPV E6 proteins share several cellular targets. For example, HPV16 E6 and E7 independently stabilize HIF1α under hypoxic conditions (Nakamura et al., 2009). This stabilization is also observed in HPV11 genome expressing keratinocytes, although it is unclear if this is attributed to HPV11 E6 and/or E7 (Nakamura et al., 2009).The most extensively studied shared biological activity of high- and low-risk mucosal HPV E6 proteins is the association with the E3 ubiquitin ligase E6AP via the LXXLL motif on E6AP (Brimer et al., 2007). LXXLL motifs are defined as leucine rich amphipathic helices with limited leucine substitution for hydrophobic residues and at least one negatively charged amino acid in an ‘X’ position (reviewed in (Howie et al., 2009)).

Many high-risk HPV E6-E6AP substrates have been identified, including the aforementioned tumor suppressor p53 and a subset of PDZ proteins, whereas very few have been proposed for low-risk HPV E6 proteins. The pro-apoptotic protein Bak associates with high- and low-risk mucosal HPV E6 proteins and is a substrate for the HPVE6/E6AP complex (Thomas and Banks, 1999). Cutaneous HPVE6 proteins have also been shown to associate with and target Bak for degradation, although the mechanism remains unclear (Underbrink et al., 2008). A yeast-two hybrid screen using HPV18 and HPV6 E6 as the bait identified GPS-2, a suppressor of G-protein and MAPK activation, as a putative binding partner of high- and low-risk HPV E6 proteins. Over-expression of high- and low-risk HPV E6 proteins reduced GPS-2 detection, suggesting that HPV E6 expression may target GPS-2 for degradation, although additional experiments are necessary to confirm this (Degenhardt and Silverstein, 2001). Mucosal E6 proteins have been reported to associate with other proteins aside from E6AP through the LXXLL binding motif, including paxillin and E6BP (Elston et al., 1998; Tong and Howley, 1997). Despite the conserved LXXLL binding motif, paxillin was shown to associate with the bovine papillomavirus-1 (BPV1) and high-risk HPV16 E6 proteins but not low-risk HPV6b or HPV11 E6 proteins (Tong and Howley, 1997). E6-E6BP association was only tested with BPV1 E6 (Chen et al., 1995; Tong and Howley, 1997).

The mammalian target of rapamycin complex 1 (mTORC1) signaling pathway has been established as a major regulator of cellular metabolism. mTORC1 responds to a variety of cellular signals including, but not limited to, nutrient and growth factor availability, and cellular ATP and amino acid levels. Upstream signals converge upon the mTORC1 kinase complex, which consequently regulates downstream cellular processes including cell proliferation, growth, and size. Mechanistically, mTORC1-mediated regulation of cellular anabolic processes is

dependent at least in part on the activation of protein synthesis. mTORC1 regulates cap dependent translation by phosphorylating the mitogen-activated p70S6 Kinase (S6K), which in turn phosphorylates and activates the ribosomal protein S6, which is involved in translation initiation. mTORC1 also phosphorylates the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). 4E-BP1 hyperphosphorylation relieves repression of the translation initiation factor eIF4E. (reviewed in (Ma and Blenis, 2009)). mTORC1 is negatively regulated by the tuberous sclerosis complex 1 and 2 (TSC1/TSC2) complex. When activated, the GTPase activating protein (GAP) TSC2 inhibits the Ras homologue and GTPase Rheb. Consequently mTORC1 is inhibited as is the phosphorylation and activation of downstream pathways S6K and 4E-BP1.

Previous studies have suggested that high-risk HPV E6 proteins increase protein synthesis and activate upstream mTORC1 signaling. Yeast-two hybrid screening using HPV 16 E6 as bait identified peptides corresponding to TSC2 and E6TP1 (Elston et al., 1998; Gao et al., 1999). HPV16 E6 was also reported to bind TSC2 and target it for E6AP dependent proteasome mediated degradation (Lu et al., 2004). Previous data suggested that E6 mediated TSC2 degradation was not conserved amongst high-risk HPV E6 proteins, but rather restricted to HPV16 E6 (Lu et al., 2004). We have previously reported that HPV16 E6 activates mTORC1 signaling and increases protein synthesis independently of TSC2. We found that HPV16 E6 activates mTORC1 and downstream signaling cascades S6K and 4E-BP1 in primary human foreskin keratinocytes (HFKs), RKO, and U2OS cells under transient and stable expression systems. Mechanistically, we found that HPV16 E6 increases the phosphorylation and activation of at least two upstream kinases PDK1 and mTORC2 (Spangle and Munger, 2010).

Here we report that high-risk HPV16, HPV18 and low-risk mucosal HPV6b and HPV11 E6 proteins share the ability to activate mTORC1 and increase cap dependent translation, whereas cutaneous HPV5 and HPV8 E6 proteins do not. Utilizing previously characterized HPV16 E6 mutants, we show that the LXXLL binding motif, as well as the p53-binding motif, are important for the HPV16 E6 mediated increase in protein synthesis.

**Materials and Methods:**

**Plasmids**:

Plasmids used in this study include a set of human β-actin promoter driven expression vectors, p1318 (control), p1436no* (HPV16 E6), HPV18 E6, HPV6b E6, HPV11 E6, HPV5 E6, HPV8 E6 (Munger et al., 1989); a set of pCMV BamNeo N vectors (with Flag-hemagglutinin fused to the amino terminus of the HPV E6 protein) pNCMV, pNCMV HPV16 E6no*, pNCMV HPV18 E6no*, pNCMV HPV6b E6, pNCMV HPV11 E6; a set of lentiviral vectors pLentiN (control), pLenti HPV16 NE6no*, pLenti HPV18 NE6no*, pLenti HPV6b NE6, pLenti HPV11 NE6, pLenti HPV5 NE6, pLenti HPV8 NE6, and were generated by Gateway cloning into the pLenti6.3/V5 TOPO gateway compatible vector (Invitrogen). Additional HPV E6 mutants were generated using site directed mutagenesis (Quikchange, Stratagene). For the purposes of this study, the HPV16 and HPV18 E6 expression vectors were mutagenized such that they do not splice to form the previously characterized '*' or '**' major splice variants (Sedman et al., 1991). Site directed mutagenesis was used to eliminate two donor splice site within HPV16 E6, with the resulting HPV16 E6 termed HPV16 E6no*. Mutagenesis at this site causes a coding mutation in E6 (V42L and V44L, for HPV16 and 18, respectively) that does not interfere with the ability of HPV16 E6 to contribute to epithelial cell immortalization. Site directed

mutagenesis was also used to introduce mutations into HPV6b, 11, and 18 E6 proteins such that E6AP binding may be compromised, based on original data that that implicated HPV16 residues L110, I128, and G130 in efficient E6AP binding. These mutants were made in the β-actin and pLentiN6.3 E6 background. Site directed mutagenesis was also used to generate a HPV16 E6 mutant that no longer associated with PDZ proteins, based on previous studies that implicated HPV18 E6 carboxyl-terminus in associating with PDZ proteins (Gardiol et al., 1999). This mutant was made in the β-actin and pLentiN6.3 E6 background

**Primers**:

HPV16 E6no* (V42L)

F: 5'-

TACTGCAAGCAACAGTTACTGCGACGCGAGCTATATGACTTTGCTTTTCGGGA-3'

R: 5'-

TCCCGAAAAGCAAAGTCATATAGCTCGCGTCGCAGTAACTGTTGCTTGCAGTA-3'

HPV18 E6no* (V44L)

F: 5'-

CAAGACAGTATTGGAACTTACAGAGGCATTTGAATTTGCATTTAAAGATTTAT-3'

R: 5'-

ATAAATCTTTAAATGCAAATTCAAATGCCTCTGTAAGGTCCAATACTGTCTTG-3'

HPV18 E6I130T

F: 5'-TGAAAAACGACGATTCCACAACACAGCTGGGCACTA-3'

R: 5'-TAGTGCCCAGCTGTGTTGTGGAATCGTCGTTTTTCA-3'

HPV6b E6L111Q

F: 5’-CTGTGTCACAAACCGCAGTGTGAAGTAGAAAAGG-3’

R: 5’-CCTTTTCTACTTCACACTGCGGTTTGTGACACA-3’

HPV6b E6I127T

F: 5’-TAACCAAGGCGCGGTTCACAAAGCTAAATTGTACGTG-3’

R: 5’-CACGTACAATTTAGCTTTGTGAACCGCGCCTGGGTTA-3’

HPV6b E6L129T

F: 5’-CAAGGCGCGGTTCATAAAGACAAATTGTACGTGGAAGGGT-3’

R: 5’-ACCCTTCCACGTACAATTTGTCTTTATGAACCGCGCCTTG-3’

HPV11 E6L111Q

F: 5’-TTGTTACCTGTGTCACAAGCCGCAGTGTGAAATAGAAAAACTAAAGC-3’

R: 5’-GCTTTAGTTTTTCTATTTCACACTGCGGCTTGTGACACAGGTAACAA-3’

HPV11 E6I127T

F: 5’-TTGGGAAAGGCACGCTTCACAAAACTAAATAACCAGTGG-3’

R: 5’-CCACTGGTTATTTAGTTTTGTGAAGCGTGCCTTTCCCAA-3’

HPV11 E6L129T

F: 5’-GGGAAAGGCACGCTTCATAAAAACAAATAACCAGTGGAAGGG-3’

R: 5’-CCCTTCCACTGGTTATTTGTTTTTATGAAGCGTGCCTTTCCC-3’

HPV16 E6 ΔPDZ

F: 5’-GTCTTGTTGCAGATCATCAAGAACATGAAGAGAAACCCAGC-3’

R: 5’-GCTGGGTTTCTCTTCATGTTCTTGATGATCTGCAACAAGAC-3’

The pFR_CrPV_xb bicistronic firefly/Renilla luciferase vector was used for luciferase reporter assays and was obtained from Phil Sharp through Addgene (plasmid 11509) (Petersen et al., 2006).

**Cell lines and Culture:**

293T and U2OS cells (ATCC) were maintained in Dulbecco's modified eagle medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. Primary human foreskin keratinocytes were isolated from anonymous newborn circumcisions as previously described (McLaughlin-Drubin et al., 2008), and maintained in keratinocyte serum-free medium (KSFM) supplemented with human recombinant epidermal growth factor 1-53, bovine pituitary extract (Invitrogen), 50 U/ml penicillin and 50 μg/ml streptomycin, 20 μg/ml gentamicin, and 1 μg/ml amphotericin B. HPV onocogene expressing HFKs were generated by lentiviral infection with the corresponding pLenti6.3N vectors. pLenti6.3N vector expressing cells were maintained following blasticidin selection (3 μg/ml). All experiments were performed with HFKs passaged less than ten times. For nutrient deprivation assays, HFKs were grown to 90% confluence, at which point they were washed twice with phosphate buffered saline (PBS), followed by an incubation in either PBS for 15 minutes or starved of EGF for 2 hrs prior to lysis. Poly-D-lysine coated plates (BD Biosciences) were used for experiments in which HFKs were starved in PBS for 15 minutes. Cells were then scraped and cleared by centrifugation at 16,110 x g for 10 min at 4$^{o}$C.

**Western blotting and antibodies:**

Cell lysates unless otherwise indicated were prepared by incubating the cells in ML buffer (300 mM NaCl, 0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) supplemented with one complete EDTA-free protease inhibitor cocktail tablet (Roche) per 25 ml lysis buffer and one PhosSTOP phosphatase inhibitor cocktail tablet (Roche) per 7.5 ml lysis buffer (McLaughlin-Drubin et al., 2008). Lysates intended for HA immunoprecipitation were prepared by incubating the cells in MC lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40), supplemented with one complete EDTA phosphatase inhibitor cocktail tablet (Roche). Cells were then scraped and lysates cleared by centrifugation at 16,110 x g for 10 min at 4$^{o}$C. Protein concentrations were determined using the Bradford method (Bio-Rad). Proteins were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Unless otherwise noted, membranes were blocked in 5% nonfat dry milk in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris [pH 7.4], 0.1% Tween 20) and probed with the appropriate antibody. The following antibodies were used at a 1:1000 dilution unless otherwise specified: β-Actin (1501; Chemicon), p53 (Ab-6, Calbiochem), Flag (4 μg/ml, F3165, Sigma), UBE3A/E6AP (1:500, H00007337-M01, Novus Biologicals), S6K (9202), S6K T389 (9206), S6 (2317), S6 S235/36 (4858), S6 S240/44 (4838), all from Cell Signaling Technology. Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were used at dilutions of 1:10,000 or 1:15,000, respectively. Proteins were visualized by enhanced chemiluminescence (Perkin Elmer, Millipore) and exposed on Kodak BioMax XAR film, or electronically acquired and quantified with a Kodak Image Station 4000R equipped with Kodak Imaging Software, version 4.0, or with a Carestream Gel Logic 4000.

**Immunoprecipitation**

For HA immunoprecipitations, one 15cm plate of 293T cells was seeded and $CaCl_2$ transfected with the appropriate pLenti6.3N vector (N, 16E6no*, 16E6no* I128T, 18E6 no*, 18E6no* I130T, 6bE6, 6bE6 L111Q, 6bE6 I127T, 6bE6 L129T, 11E6, 11E6 L111Q, 11E6 I127T, or 11E6 L129T. 72 hours post transfection the cells were lysed as described above in MC buffer. Lysates were cleared with low-binding durapore PVDF 0.45 μM membrane spin filters (Millipore) and protein concentration was subsequently measured using the Bradford Method (Bio-rad). Prewashed HA antibody-agarose conjugate (Sigma) was then incubated with lysate for 2 hrs, washed, and sample buffer added.

**Transfections and Luciferase Assays**

Primary human foreskin keratinocytes were transfected as described (Spangle and Munger, 2010). In brief, cells were transfected in triplicate in 6-well plates for luciferase reporter assays using FuGene 6 (Roche). One microgram of pFR_CrPV_xb was co-transfected with two μg of the appropriate β-actin promoter driven vector. Forty-eight hours post transfection, cells were lysed and scraped in 125 μl passive lysis buffer (dual luciferase reporter kit; Promega) per well. The supernatants were subjected to the dual luciferase reporter assay. The fold change in activity was determined by calculating the ratio of firefly activity to Renilla luciferase activity compared to the control vector-transfected cells. At least three independent experiments were performed and the Student's T test was used to calculate statistical significance.

**Results**:

**Mucosal but not cutaneous HPV E6 proteins increase cap dependent translation.** Our previous studies suggested that HPV16 E6 increases cap dependent translation (Spangle and

Munger, 2010). Based on these findings, we evaluated the ability of other HPV types to activate cap dependent translation in U2OS osteosarcoma cells and primary human foreskin keratinocytes (HFKs). U2OS cells were selected for these experiments because of their high transfection efficiency, while HFKs are the more cumbersome but physiologically more relevant cell culture model. We utilized a bicistronic luciferase reporter construct pFR_CrPV_xb, expressing firefly and Renilla luciferase as a single transcript from the minimal TK promoter. They are translated independently because they are separated by an internal ribosomal entry site (IRES). Firefly luciferase is translated by a cap dependent mechanism whereas Renilla luciferase is translated by a cap independent mechanism that is dependent on the IRES of Cricket Paralysis Virus (Petersen et al., 2006). Transient co-expression of high-risk mucosal HPV16 and HPV18 E6 proteins robustly activated cap dependent translation in U2OS cells (4.32 ± 1.04, $p < 0.001$, and 3.24 ± 0.58 fold, $p < 0.001$, respectively and relative to control). Low-risk mucosal HPV6b and HPV11 E6 proteins activated cap dependent translation but not as efficiently as high-risk HPV E6 proteins (2.94 ± 0.91, $p < 0.001$, and 2.19 ± 0.47 fold, $p < 0.003$, respectively and relative to control), whereas cotransfection of the cutaneous HPV5 and HPV 8 E6 proteins had no effect (1.21 ± 0.18, $p = 0.059$ and 1.16 ± 0.19 fold, $p = 0.16$, respectively) (Fig 3.1, left). Luciferase reporter assays performed in primary HFKs yielded similar results despite their comparatively low transfection efficiency (Fig 3.1, right).

These results show that the ability of HPV E6 proteins to activate cap dependent translation and increase protein synthesis is conserved amongst high- and low-risk mucosal HPVs.

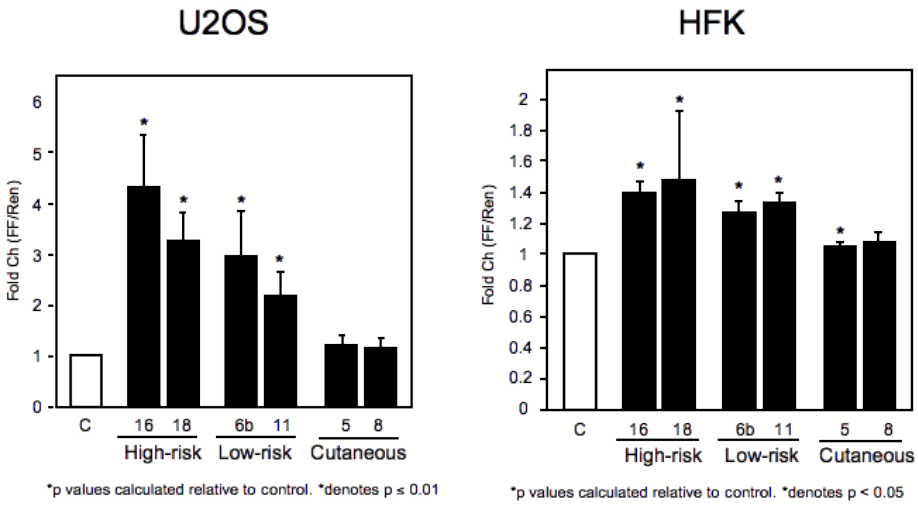


**Figure 3.1. Mucosal but not cutaneous HPV E6 proteins increase cap dependent translation.** U2OS cells (left) were transiently co-transfected with the pFR_CrPV_xb bicistronic reporter construct and the CMV promoter driven expression vectors for high-risk mucosal E6 proteins (16 or 18), low-risk mucosal E6 proteins (6b or 11), cutaneous E6 proteins (5 or 8), or empty vector as a control. HFKs (right) were transiently co-transfected with the pFR_CrPV_xb construct and the human β-actin promoter driven E6 expression vectors. Cells were lysed and Renilla and firefly luciferase were measured 48h post transfection. Firefly and Renilla luciferase values were normalized to control vector-transfected cells and are presented as the fold change of normalized firefly luciferase relative to normalized Renilla luciferase. The bars represent the average and one standard deviation from four independent experiments for U2OS and five independent experiments for HFKs.

**The LXXLL binding motif and p53 binding are important for the HPV16 E6 mediated increase in cap dependent translation.** Utilizing previously characterized HPV16 E6 mutants, we evaluated which sequences in HPV16 E6 are important for its ability to activate cap dependent translation. We tested HPV16 E6 mutants deficient in p53 binding (Y54D) and LXXLL motif binding (I128T) (Liu et al., 1999). Truncation of the carboxyl-terminal six amino acids in HPV18 E6 yields a mutant that is defective for binding and degradation of cellular PDZ proteins (Gardiol et al., 1999). Thus, we also generated an equivalent carboxyl-terminal truncation HPV16 E6 mutant, ΔPDZ (Fig 3.2). Bicistronic luciferase reporter assays in primary HFKs suggest that all tested HPV16 E6 mutants do not activate cap dependent translation as efficiently as wild type HPV16 E6 (Fig 3.3). However, the HPV16 E6 Y54D and I128T mutants that affect p53 binding and LXXLL motif binding, respectively, significantly decreased the HPV16 E6 mediated increase in protein synthesis (wild type HPV16 E6 1.80 ± 0.08 fold. HPV16 E6 Y54D 1.26 ± 0.23, $p = 0.125$ relative to control and $p = 0.0177$ relative to HPV16 E6. HPV16 E6 I128T 1.16 ± 0.11 fold, $p = 0.0873$ relative to control and $p = 0.0013$ relative to HPV16 E6). In contrast, the HPV16 E6 ΔPDZ mutant had an intermediate phenotype (1.47 ± 0.07 fold, $p = 0.003$ relative to control with a reduced fold change in comparison to wild type HPV16 E6 ($p = 0.0066$ relative to HPV16 E6).

Therefore, these results suggest that more than one biological activity may contribute to the ability of E6 mediated to activate cap dependent translation. Combinatorial LXXLL binding motif/PDZ binding HPV16 E6 mutants have also been tested for activation of cap dependent translation, with no additive inhibitory effect (data not shown).

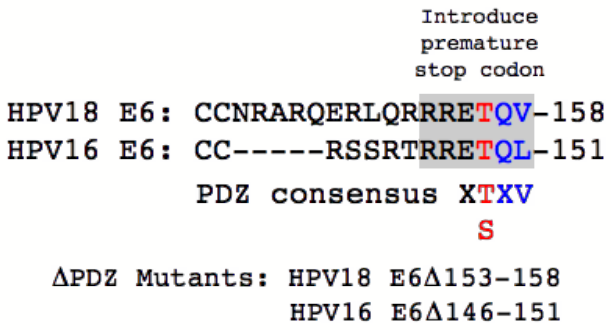


**Figure 3.2. HPV16 and 18E6 PDZ binding mutants.** High-risk mucosal HPV16 and 18E6 proteins are aligned from the C terminus. The PDZ binding consensus, XT/SXV, is shown in red and blue. The grey box indicates the residues that were eliminated with the introduction of the premature stop codon. These mutants were generated based on the mutants described by (Gardiol et al., 1999).

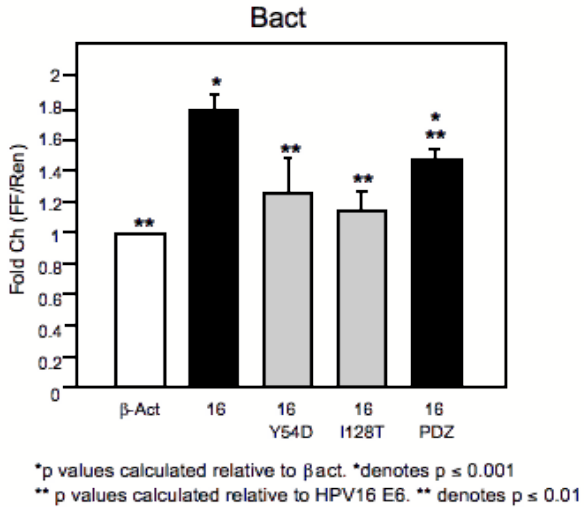


**Figure 3.3. The LXXLL binding motif and p53 binding are important for HPV16 E6 mediated increase in cap dependent translation.** HFKs were transiently co-transfected with the pFR_CrPV_xb reporter construct and the human β-actin promoter driven expression vectors for wild type HPV16 E6, p53 binding mutant (HPV16E6 Y54D), LXXLL binding motif mutant (HPV16E6 I128T, PDZ binding mutant (HPV16E6 ΔPDZ), or empty vector as a control. Cells were lysed and firefly and Renilla luciferase measured 48h post transfection. Firefly and Renilla luciferase values were normalized to control vector-transfected cells and are presented as the fold change of normalized firefly luciferase relative to normalized Renilla luciferase. The bars represent the average and one standard deviation from three independent experiments.

**High-and low-risk HPV E6 proteins increase protein synthesis through overlapping mechanisms.** High- and low-risk HPV E6 proteins activate cap dependent translation. The LXXLL binding motif is conserved amongst all mucosal HPV E6 proteins whereas p53 binding and the carboxyl-terminal PDZ binding domain are not. We therefore utilized low-risk HPV E6 proteins to determine the shared mechanism by which mucosal HPV E6 proteins increase cap dependent translation. Only E6AP has thus far been identified to associate with both high- and low-risk HPV E6 proteins through the LXXLL binding motif. Therefore, we generated previously characterized and novel putative LXXLL binding motif defective HPV6b and HPV11 E6 proteins and tested their ability to (1) bind E6AP, and (2) activate cap dependent translation by luciferase reporter assays. Previous studies identified leucine residue 111 (L111) in HPV11 E6 as important for E6AP binding, which was also established for the analogous residue in HPV16 E6 (Brimer et al., 2007; Liu et al., 1999). Similarly, previous work also revealed that in addition to I128, glycine residue 130 (G130) in HPV16 E6 was also important for E6AP binding. Therefore, we generated three putative LXXLL/E6AP binding motif defective HPV6b and HPV11 E6 mutants: L111Q, I127T, and L129T (Fig 3.4A). Transient transfection of 293T cells with Flag-HA tagged HPV E6 proteins followed by HA immunoprecipitation and E6AP Western blot confirms that HPV16 E6 binds E6AP, whereas E6AP binding by the HPV16 E6 I128T mutant is abrogated (Fig 3.4B, Left). Similar results were obtained for HPV18 E6 and the previously characterized E6AP binding mutant HPV18 I130T (Fig 3.4B, Middle). Consistent with previously published results (Brimer et al., 2007), the HPV11 E6 L111Q mutant is defective for E6AP association. The HPV11 E6 I127T mutant exhibits reduced E6AP binding whereas the HPV111 E6 L129T mutation only has a minor effect on E6AP binding (Fig 3.4B, right). Using this set of mutants we next determined whether an intact LXXLL binding motif, as assessed by

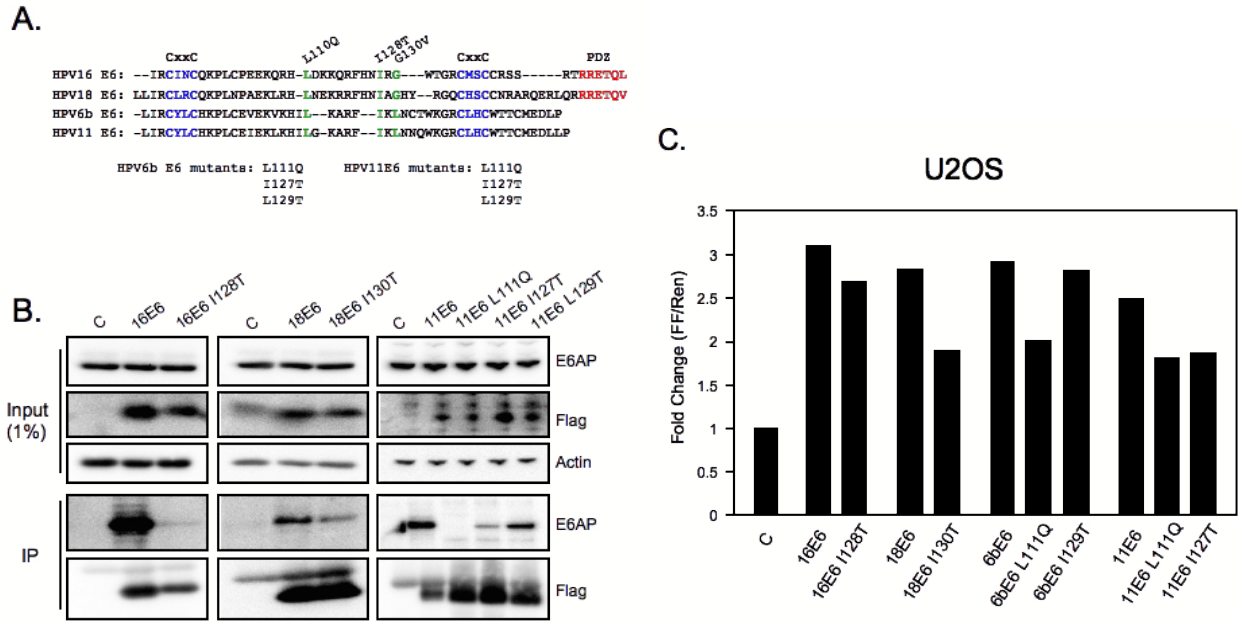


**Figure 3.4. High and low-risk HPV E6 proteins increase cap dependent translation through overlapping mechanisms**. (A) Mucosal E6 protein carboxyl-terminal sequence homology. Sequence alignment based on the conserved CxxC zinc binding sites, shown in blue. The HPV16 E6 L110Q, I128T, G130V, and HPV11E6 L111Q residues were previously implicated in the association with cellular proteins via an LXXLL binding motif. These mutants and analogous mutants are shown in green (HPV18E6 I130T, HPV6b and 11E6 L111Q, I127T, L129T). The carxboxyl-terminal high-risk HPV E6 PDZ binding domain is shown in red. (B) Western blot analysis of HA immunoprecipitations from 293T cells transiently transfected with N-terminally Flag-HA tagged CMV (NCMV) promoter driven E6 expression vectors (HPV16 E6 and mutant, left; HPV18 E6 and mutant; middle, HPV11E6 and mutants, right) into 293T cells. Proteins immunoprecipitated by anti-HA agarose were identified by Western blotting for HPV E6 proteins (anti-Flag) and E6AP. Input represents 1% of IP, and actin is shown as a loading control. (C) U2OS cells were transiently co-transfected with the pFR_CrPV_xb bicistronic reporter construct and the human β-actin promoter driven E6 expression vectors or empty vector as a control. Cells were lysed and Renilla and firefly luciferase were measured 48h post transfection. Firefly and Renilla luciferase values were normalized to control vector-transfected cells and are presented as the fold change of normalized firefly luciferase relative to normalized Renilla luciferase. The bars represent the average and one standard deviation from three independent experiments.

E6AP binding, is important for HPV E6 proteins to increase cap dependent translation. We transiently co-transfected primary HFKs with a panel of high- and low-risk HPV E6 proteins and their respective LXXLL binding motif mutants and the bicistronic luciferase reporter and evaluated cap dependent translation. The results suggest that the LXXLL binding motif is important for high- and low-risk HPV E6 proteins to activate cap dependent translation, as it is reduced upon expression of the HPV16 I128T and HPV18 I130T mutants (Fig 3.4C). The HPV6b and HPV11 E6 L111Q and I127T mutants exhibit reduced activation of cap dependent translation whereas the HPV6b and HPV11 E6 L129T mutants activated cap dependent translation comparably to wild type HPV6b and HPV11 E6 proteins (Fig 3.4C).

Hence, these experiments are consistent with a model that the integrity of the LXXLL binding motif is important for high-risk as well as low-risk mucosal HPVE6 proteins to activate cap dependent translation.

**Multiple sequences, including the LXXLL binding motif contribute to the ability of high-risk mucosal HPV E6 proteins to activate mTORC1 signaling.** We tested the previously characterized HPV16 E6 mutants deficient in p53 binding (Y54D), LXXLL motif binding (I128T) (Liu et al., 1999), and PDZ binding (ΔPDZ) for mTORC1 activation, using S6K phosphorylation as a surrogate marker. HFKs with stable expression of these HPV16 E6 mutants were generated. Expression of HPV16 E6 I128T and Y54D mutants, which target the LXXLL motif and p53 binding, respectively, exhibited reduced S6K phosphorylation (Fig 3.5A). The expression of the HPV16 E6 ΔPDZ mutant showed S6K phosphorylation similar to wild type HPV16 E6 expressing cells (Fig 3.5A). These results are similar to what we observed with the bicistronic luciferase reporter assays (Fig 3.3). This suggests that the ability of HPV16 E6 to

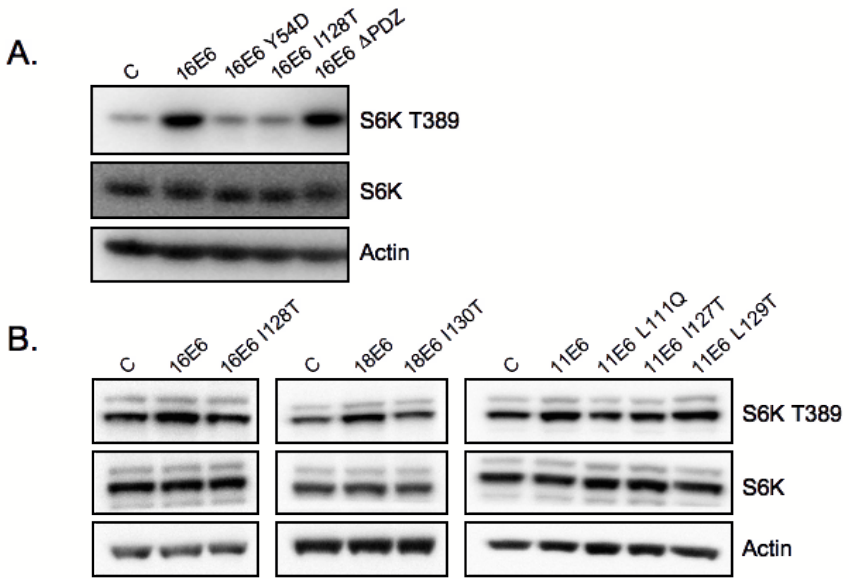


**Figure 3.5. The LXXLL binding motif is important for mucosal HPV E6 mediated mTORC1 activation.** (A) Western blot analysis of S6K T389 phosphorylation in HPV16 E6, Y54D, I128T, and ΔPDZ expressing and control vector (LXSN) HFKs. (B) Western blot analysis of S6K T389 phosphorylation in HPV16 E6, 16E6 I128T (left); HPV18 E6, 18E6 I130T (middle); HPV11 E6, 11E6 L111Q, 11E6 I127T, 11E6 I129T (right) expressing and control vector (pLentiN6.3) HFKs. Actin is shown as a loading control.

activate mTORC1 signaling correlates with the ability of HPV16 E6 to activate cap-dependent translation. Given that the ability to associate with LXXLL motif containing proteins such as E6AP is conserved with low-risk and high-risk HPV E6 proteins, we next investigated whether the integrity of the LXXLL motif binding sequence is also important for the ability of low-risk HPVE6 proteins to activate mTORC1 signaling using S6K phosphorylation as surrogate marker. As expected expression of the HPV16 and 18 E6 LXXLL motif binding mutants 128T and I130T, respectively, in HFKs do not cause increased S6K phosphorylation (Fig 3.5B, left and middle, respectively). Similarly, expression of the HPV11 L111 and I127 E6 mutant showed low levels of S6K phosphorylation (Fig 3.5B, right), suggesting that similar to what we observed with HPV16 E6, the integrity of the LXXLL motif in the high-risk HPV18 E6 protein as well as the low-risk HPV11 E6 protein is important for their ability to activate mTORC1 signaling and that this correlated with their ability to increase cap-dependent translation as determined by luciferase reporter assays (Fig 3.4C).

Taken together our results show that the integrity of LXXLL binding motif that is conserved in low-risk as well as high-risk mucosal HPV E6 proteins, but not in cutaneous HPV E6 proteins importantly contributes to the ability of mucosal HPV E6 proteins to activate mTORC1 signaling and cap dependent translation.

**Discussion:**

It was previously shown that HPV16 E6 activates mTORC1 signaling. Several mechanisms have been proposed, including association with and subsequent destabilization of the mTORC1 negative regulator TSC2 (Lu et al., 2004). Our own experiments suggest that in primary HFKs stable HPV16 E6 expression does not activate mTORC1 through TSC2

destabilization but rather through the activation of at least two kinases upstream of AKT; PDK1 and mTORC2 (Spangle and Munger). We also showed that HPV16 E6 increases cap dependent translation by increasing cap dependent translation and that this is, at least in part dependent on mTORC1 activation (Spangle and Munger, 2010).

Here we show that the ability to activate mTORC1 and increase cap dependent translation is conserved amongst high- and low-risk mucosal HPV E6 proteins (Fig 3.1). High-risk HPV16 and HPV18 E6 proteins increase cap dependent translation whereas low-risk mucosal HPV E6 proteins from type 6b and 11 do so to a lesser extent. All mucosal HPV E6 proteins tested activate mTORC1 signaling to a similar level. In contrast, cutaneous HPV E6 proteins do not activate cap dependent translation. Since infection with low-risk mucosal HPV types are rarely associated with carcinogenesis, it is likely that the ability of mucosal HPV E6 proteins to activate mTORC1 and enhance cap-dependent translation are related to a common requirement during the viral life cycle. Given that all HPVs, including those that infect the cutaneous epithelia, require adequate production of viral and cellular proteins necessary for viral genome replication and progeny virion production it is surprising that cutaneous HPV E6 proteins do not detectably activate mTORC1 signaling or increase cap-dependent translation. One might hypothesize that mucosal HPV E6 proteins evolved a distinct repertoire of biological properties as a result of tissue tropism, explaining the specificity of mucosal HPV E6 mediated activation of mTORC1 and cap dependent translation. One cannot rule out the possibility that successful viral genome replication or progeny virion production in the mucosal epithelium involves unique requirements as a result of different gene expression profiles. Transcriptional regulation is also different between mucosal and cutaneous HPVs. Introduction of HPV16 and HPV5 long control region (LCR) reporter constructs into cutaneous and mucosal epithelial cells

demonstrated that appropriate cellular tropism is important for robust transcriptional activation (Mistry et al., 2007). This cell type dependent promoter activation may be caused by the differential expression and participation of transcription factors. Given these apparent differences in the cellular milieus of cutaneous and mucosal epithelia, it is thus tempting to speculate that corresponding HPV types may have evolved distinct molecular strategies to optimally exploit the available host cellular environment.

The bicistronic reporter assays utilizing high-risk HPV16 E6 mutants indicated that more than one biochemical activity is important for cap dependent translation, including LXXLL motif binding. We therefore evaluated the contribution of the LXXLL binding motif in a simpler model – the low-risk mucosal HPV E6 protein. Low-risk HPV E6 proteins lack the transforming potential of high-risk HPV E6 proteins as they do not bind and degrade p53 or associate with PDZ proteins through a carboxyl-terminal PDZ binding domain. The LXXLL binding motif, however, while absent in cutaneous HPV E6 proteins, is conserved between high-risk and low-risk mucosal HPV E6 proteins. We generated known and novel HPV6b and HPV11 E6 LXXLL binding motif mutants and confirmed the loss of E6AP binding for these mutants. Bicistronic luciferase reporter assays in primary HFKs indicated that the LXXLL binding motif is required for mucosal HPV E6 proteins, including the low-risk HPV6b and HPV11 E6 proteins, to activate cap dependent translation (Fig 3.4). Considering that the E3 ubiquitin ligase E6AP is the only currently identified LXXLL motif partner shared by low-risk and high-risk mucosal HPV E6 proteins, E6AP is an attractive candidate to mediate mTORC1 activation and enhance cap dependent translation. However, to date only the pro-apoptotic protein Bak has been identified as a low-risk HPV E6/E6AP substrate and it is difficult to envision how Bak degradation might cause increased cap dependent translation. It is possible, however, that E6AP binding is required

for HPV E6 mediated increase in mTORC1 and protein synthesis, but the ubiquitin ligase activity of E6AP may be dispensable. It has been reported that E6AP may serve as a transcriptional coactivator of estrogen receptor alpha (ERα), and that the ubiquitin ligase activity is dispensable for this activity (Nawaz et al., 1999). Several ERα target genes have been implicated in upstream signaling events of mTORC1 and cap dependent translation, including insulin growth factor binding protein 4 (IGFBP4), ErbB4, as well as other growth factor and metabolism associated genes such as NDRG1, a downstream effector of mTORC2 signaling (Lin et al., 2004). Future experiments will be focused on directly addressing the potential contributions of E6AP in mucosal HPV E6 mediated activation of mTORC1 signaling and enhancement of cap-dependent translation.

It is conceivable that the LXXLL binding motif may mediate the association of additional cellular proteins with mucosal HPV E6 proteins. Our group and others have been utilizing large-scale proteomics to identify high- and low-risk HPV E6 associated proteins. Proteomics studies in our group (Appendix 2) have putatively identified another E3 ubiquitin ligase that may associate with high- and low-risk HPV E6 proteins, HUWE1/MULE/ARFBP1. HUWE1 has 14 putative LXXLL motifs, one being the prototypic amphipathic helix with at least one negatively charged amino acid in the "X" position. We are currently in the process of validating association of mucosal HPV E6 proteins with HUWE1. Interestingly, HUWE1 regulates processes such as cell proliferation and apoptosis, through the targeted ubiquitination of substrates including the apoptotic protein Mcl-1, N-Myc, and also p53. It is possible that a currently unidentified substrate(s) of either or both the E6/E6AP or E6/HUWE1 complex may contribute to mTORC1 activation or cap dependent translation.

Using previously characterized HPV16 E6 mutants, we show that the E6 mediated activation of cap dependent translation correlates with an intact LXXLL binding motif as well as p53 binding. There was also a small but statistically significant contribution of the carboxyl-terminal PDZ binding domain to HPV16 E6 mediated cap dependent translation. The HPV16 E6 mutants that we used for our studies are well characterized and each maintain separable biological functions, e.g., the HPV16 E6 LXXLL motif binding I128T mutant has been reported to associate with p53 by immunoprecipitation, but does not degrade p53 as it can no longer associate with E6AP. These results suggest that multiple biochemical activities of high-risk HPV E6 proteins contribute to full activation of cap dependent translation, some of which may be dependent of binding p53, PDZ proteins, or association with proteins such as E6AP through their LXXLL binding motif.

We have recently identified that high-risk HPV16 E6 activates growth factor sensitive receptor protein tyrosine kinases through the association with the signaling adaptor protein Grb2 (see chapter four). E6 expression also causes increased internalization of activated receptor species, increased receptor degradation and decreased EGFR half-life. It is possible that HPV16 E6 associates with and functionally modifies the Grb2 complex through the targeted ubiquitination of a Grb2 associated protein, in turn regulating Grb2 function. This is reasonable, as Grb2 has been implicated in both in receptor activation and internalization (Sigismund et al., 2008). We are therefore actively pursuing additional proteomics studies of high-and low-risk HPV E6 associated proteins under normal growth conditions and proteasomal inhibition.

Given that the activation of cap dependent translation is dependent at least in part on the upstream activation of mTORC1 (Spangle and Munger, 2010), we are currently comparing the ability of cutaneous and mucosal HPV E6 proteins to activate mTORC1, as indicated by an

increase in S6K phosphorylation. It is expected that cutaneous HPV E6 proteins will not activate mTORC1, as they do not activate cap dependent translation in the bicistronic luciferase reporter assays. We will also be evaluating signaling events upstream of mTORC1, and expect that mucosal HPV E6 proteins will activate mTORC1 through PDK1 and mTORC2, under conditions of nutrient deprivation.

# CHAPTER FOUR

# The HPV16 E6 oncoprotein promotes the phosphorylation of receptor protein tyrosine kinases and increases internalization of phosphorylated receptor species

# The HPV16 E6 oncoprotein promotes the phosphorylation of receptor protein tyrosine kinases and increases internalization of phosphorylated receptor species

Jennifer M. Spangle[1,2] and Karl Munger[1,2*]

[1]Division of Infectious Diseases, Brigham and Women's Hospital [2]Committee on Virology, Harvard Medical School, Boston, Massachusetts, 02115 [3]Dana Farber Cancer Institute, Boston, Massachusetts, 02115

* Corresponding author. Mailing Address: Channing Laboratories 861, 181 Longwood Ave., Boston, MA 02215. Phone (617) 525-4282, Fax (617) 525-4283, E-mail: kmunger@rics.bwh.harvard.edu

This chapter is the basis of a manuscript to be submitted.

Contributions: I wrote this manuscript and performed all of the experiments described in it and Karl Munger helped design the research and edited the manuscript.

**Abstract**:

The high-risk human papillomavirus E6 proteins promote transformation and oncogenesis. HPV16 E6 sustains the activity of the mTORC1 and mTORC2 signaling cascades under conditions of growth factor withdrawal. This may be relevant during viral infection as the viral life cycle is completed in nutrient poor, terminally differentiated, and non-dividing host epithelial tissue. Phosphotyrosine immunoprecipitations and Western blots demonstrated that the HPV16 E6 oncoprotein increases the phosphorylation of receptor protein tyrosine kinase effector signaling pathways and we determined the origin of the activation to be at the level of growth factor sensitive receptor protein tyrosine kinases ErbB2/EGFR/IGFR/IR. HPV16 E6 also increased the internalization of phosphorylated receptor species, which is suggestive of enhanced receptor activity. The receptor protein tyrosine kinase signaling adaptor protein Grb2 was identified as a binding partner of HPV16 E6, and Grb2 knockdown ablated HPV16 E6 mediated activation of mTORC1. Lastly HPV16 E6 expression increased cellular migration in wound healing and transwell migration assays through an EGFR/IR/IGFR dependent mechanism. This work identifies a novel mechanism of perturbing the host signaling machinery to promote energy consuming processes that may support cellular transformation and carcinogenesis by the HPV16 E6 oncoprotein.

**Introduction**:

Human papillomaviruses (HPVs) are small viruses with double stranded DNA genomes that infect squamous epithelial tissue. To date, over HPV 200 types have been identified and categorized based on the type of host epithelial tissue they infect. The majority of HPVs infect the cutaneous epithelium where they cause generally benign warts. Approximately 30 HPVs infect the mucosal epithelium and these HPVs are classified as "low-risk" or "high-risk" based on the propensity for malignant progression of the lesions that they cause. Low-risk mucosal HPVs, such as HPV types 6 (HPV6) or 11 (HPV11), can cause genital warts that are typically cleared and do not progress to malignancy. In contrast, infection with high-risk mucosal HPV type 16 (HPV16) or 18 (HPV18) can cause squamous intraepithelial lesions that can progress to cancer. High-risk HPVs, such as HPV16 and HPV18, are commonly associated with anogenital tract carcinomas, and are the causative agent for nearly 100% of all diagnosed cervical cancers worldwide (WHO Annual Report 2010). High-risk HPVs are also associated with other anogenital tract cancers and oropharyngeal carcinomas. A frequent hallmark of HPV-associated carcinogenesis is the integration of the HPV genome or a portion thereof into a host chromosome. This results in the dysregulated expression of the high-risk HPV E6 and HPV E7 oncoproteins. The HPV E6 and E7 oncoproteins together are sufficient for the initiation and maintenance of the transformed phenotype of cervical cancer cells. HPV E6 and E7 oncoprotein expression has also been shown to cause cancer in a transgenic mouse model (reviewed in reference (McLaughlin-Drubin and Munger, 2009a)). The high-risk HPV E6 and HPV E7 oncoproteins each have many described biological activities, with the most predominant being the association with and subsequent targeting of the p53 and pRb tumor suppressors for degradation, respectively (reviewed in references (Howie et al., 2009) and (McLaughlin-Drubin

and Munger, 2009b)). High-risk HPV E6 proteins associate with the E3 ubiquitin ligase E6AP (UBE3A) through the LXXLL binding motif in E6. High-risk HPV E6 proteins form a tripartite complex with E6AP and p53, targeting p53 for ubiquitination and subsequent degradation through the proteasome (Scheffner et al., 1993). The E6/E6AP complex has been reported to target other HPV E6 binding partners for degradation, including members of a diverse group of PDZ proteins that have been reported to associate with high-risk mucosal HPV E6 proteins through a carboxyl-terminal PDZ binding domain. Reported high-risk HPV E6 binding partners or substrates include the PDZ proteins hDlg, hScribble, MUPP1, MAGI1, and the non-receptor protein phosphatases PTPN3 and PTPN13 (Gardiol et al., 1999; Glaunsinger et al., 2000; Jing et al., 2007; Kiyono et al., 1997; Lee et al., 2000; Lee et al., 1997; Nakagawa and Huibregtse, 2000; Spanos et al., 2008). High-risk HPV E6 proteins also contribute to the immortalization of the host cell through activating hTERT, the catalytic component of the human telomerase enzyme (Klingelhutz et al., 1996).

High-risk HPVs infect the basal epithelial cells, which presumably occupy a nutrient rich environment. The basal epithelial cells undergo asymmetric cell division, in which one daughter cell remains a basal cell and the other becomes a cell that is poised to differentiate as part of the nutrient deprived squamous epithelium. Viral genome replication and progeny virion assembly is confined to the otherwise non-dividing differentiated cells of the epithelium. High-risk HPV E7 maintains S phase competence by destabilizing pRb. The simultaneous HPV E6 mediated destabilization of p53 eliminates cell cycle checkpoints enabling aberrant DNA replication. It is, however, much less characterized how completion of the viral life cycle can occur in an environment with presumed restricted nutrients and energy sources that are required for DNA

replication and protein synthesis. HPV16 E7 expression has been reported to cause a metabolic switch from oxidative phosphorylation to glycolysis and anaerobic fermentation, a phenomenon commonly referred to as the "Warburg effect" (Zwerschke et al., 1999). Further, serum starvation of HPV16 E7 expressing cells induces the trophic sentinel response. This is a response to oncogene expression sending signals of growth despite a shortage of nutrients and growth factors that causes caspase independent cell death without cytochrome C release (Eichten et al., 2004). Co-expression of HPV16 E6 was shown to abrogate the trophic sentinel response triggered by E7. Our group reported that HPV16 E7 induces autophagy, which is the catabolic process of recycling cellular organelles through the lysosomal machinery. HPV E7-induced autophagy increased the number of LC3b positive vesicles and increased the conversion of LC3bI to LC3bII (Zhou and Munger, 2009). The HPV16 E7-mediated increase in autophagy is likely balanced by activities of the high-risk HPV E6 oncoprotein, namely E6 mediated activation of mTORC1. Our group and others have reported that HPV16 E6 activates mTORC1 signaling under normal growth and nutrient deprived conditions (Lu et al., 2004; Spangle and Munger, 2010). mTORC1 functions as a cellular rheostat, integrating environmental cues of energy status, growth factors, amino acids, and nutrient availability. We found that HPV16 E6 mediated mTORC1 activation causes an increase in cap dependent translation, which is at least in part a consequence of mTORC1 activation (Spangle and Munger, 2010). mTORC1 activation directly inhibits autophagy, suggesting that during HPV infection, HPV16 E6 expression may suppress HPV E7-induced autophagy. The combined effects of pRb and p53 inactivation coupled with mTORC1 activation and autophagy suppression support the model that the biological properties of high-risk HPV E6 and HPV E7 are balanced to meet the metabolic needs during successful viral infection and/or genome replication (Zhou et al., 2009). HPV16 E6

counterbalances the E7 induced effects of reduced energy availability and increased autophagy by activating mTORC1 and thus inhibiting autophagy. This results in the short-term increase in protein synthesis that may be used to generate the cellular machinery necessary for DNA replication as well as viral capsid proteins necessary for packaging.

HPV16 E6 was previously shown to activate mTORC1 through the upstream kinases PDK1 and mTORC2 under conditions of nutrient derivation. AKT and mTORC1 are activated independently of TSC2 destabilization (Spangle and Munger, 2010). PDK1 is downstream of PI3K and can be activated through multiple membrane-associated signaling events including the activation of G coupled protein receptors (GPCRs) and receptor protein tyrosine kinases (RPTKs). Hence, we addressed the hypothesis that HPV16 E6 may activate mTORC1 through RPTKs. RPTKs are transmembrane proteins with cytoplasmic tyrosine kinase activity and initiate mitogenic signaling pathways, including the PI3K/AKT/mTORC1 signaling axis. The biological processes that are regulated by RPTKs are diverse and include cell growth, cell size, adhesion, migration, invasion and others. Here we focus on the ErbB family and the related insulin receptor (IR) and insulin-like growth factor receptor (IGFR). Ligand binding and subsequent dimerization initiates RPTK activation through receptor autophosphorylation. Signaling adaptor proteins are then recruited to the receptors via their SH2 domain and activation downstream effector cascades. EGFR activation is well characterized, with distinct autophosphorylation events triggering the activation of multiple downstream effectors, including Grb2, Shc, and PLCγ (Fernandes et al., 2001; Levkowitz et al., 1999; Rojas et al., 1996). IR and IGFR are autophosphorylated in a conserved kinase activation loop that also activates downstream effector signaling (Hernandez-Sanchez et al., 1995; White et al., 1988). ErbB and

IR/IGFR autophosphorylation induces receptor internalization through clathrin mediated endocytosis, an event that is also associated with receptor activation and can either lead to receptor recycling to the cell surface or receptor degradation (Sigismund et al., 2008; Wang et al., 2005). The consequences of either ErbB or IR/IGFR receptor activation have the shared effect of activating downstream signaling cascades including AKT and mTORC1, which has been shown previously to be activated by HPV16 E6.

Here we report that HPV16 E6 activates RPTK signal transduction under conditions of nutrient deprivation. We show that HPV16 E6 increases the phosphorylation of IR, IGFR, and ErbB RPTKs. RPTK activation causes the activation of downstream signaling pathways under conditions that simulate metabolic stress and increased viral oncoprotein expression as would occur naturally during HPV induced infection. We also show that HPV16 E6 causes an increase in the internalization of phosphorylated receptor species. We identify the association of HPV16 E6 with Grb2 and demonstrate that Grb2 knockdown abrogates HPV16 E6 mediated mTORC1 activation. We also show that the HPV16 E6 mediated increase in RPTK activation causes an increase in cellular migration, and that HPV16 E6 can maintain an increase in cellular migration in the absence of growth factors, which may be relevant to HPV associated carcinogenesis. Together these results show that HPV16 E6 activates RPTKs and the RPTK PI3K/AKT/mTORC1 and Ras/MEK/ERK effector signaling pathways in the absence of growth factor ligands.

**Materials and Methods**

**Plasmids**:

Plasmids used in this study include the retroviral vectors pLXSN (control) and pLXSN HPV16 E6 (Halbert et al., 1992); and pCMV N (control) and pCMV HPV16 NE6no* (Baker et al., 1990; Munger et al., 1989). Lentiviral vectors including pLentiN (control) and pLenti HPV16 NE6no* were generated by Gateway cloning into the pLenti6.3 gateway compatible vector (Invitrogen). For the purposes of this study, the HPV16 E6 expression vectors were mutagenized such that they no longer have the capacity to generate the major splice variants (Sedman et al., 1991). Site directed mutagenesis was used to eliminate two donor splice site within HPV16 E6, with the resulting HPV16 E6 V42L mutant termed HPV16 E6no*. Mutagenesis at this site yields a coding in E6 that does not interfere with the ability of HPV16 E6 to contribute to epithelial cell immortalization. The pFR_CrPV_xb bicistronic firefly/Renilla luciferase vector (Petersen et al., 2006) was used for luciferase reporter assays and was obtained from Phil Sharp through Addgene (plasmid 11509).

**Cell lines and Culture:**

293T and U2OS cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. Primary human foreskin keratinocytes were isolated from anonymous newborn circumcisions as previously described (McLaughlin-Drubin et al., 2008), and maintained in keratinocyte serum-free medium (KSFM) supplemented with human recombinant epidermal growth factor 1-53, bovine pituitary extract (Invitrogen), 50 U/ml penicillin and 50 μg/ml streptomycin, 20 μg/ml gentamicin, and 1 μg/ml amphotericin B. HPV onocogene expressing HFKs were generated by either retroviral infection with the corresponding pLXSN based vectors

or lentiviral infection with the corresponding pLenti6.3N based vectors. pLXSN or pLenti6.3N vector expressing cells were maintained following neomycin selection (250 μg/ml) or blasticidin selection (3 μg/ml), respectively. All experiments were performed with HFKs passaged less than ten times. For nutrient deprivation assays, HFKs were grown to 90% confluence, at which point they were washed twice with phosphate buffered saline (PBS), followed by incubation in either PBS for 15 minutes or KSFM lacking EGF for 2 hours prior to analysis. Poly-D-lysine coated plates (BD Biosciences) were used for experiments in which HFKs were starved in PBS for 15 minutes. Cells were then scraped and cleared by centrifugation at 16,110 x g for 10 min at 4°C.

**Western blotting and antibodies:**

Unless otherwise indicated, protein lysates were prepared by incubating the cells in ML buffer (300 mM NaCl, 0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) supplemented with one complete EDTA-free protease inhibitor cocktail tablet (Roche) per 25 ml lysis buffer and one PhosSTOP phosphatase inhibitor cocktail tablet (Roche) per 7.5 ml lysis buffer) (McLaughlin-Drubin et al., 2008). Cell lysates intended for global phosphotyrosine Western blots were prepared by incubating the cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholic acid [DOC], 0.1% SDS, 50 mM Tris-HCl [pH 7.5]), supplemented as described above for ML Buffer with the addition of 50 mM Pervanadate). Cell lysates intended for HA immunoprecipitation and immunoaffinity purification were prepared by incubating the cells in MC lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, supplemented with one complete EDTA protease inhibitor cocktail tablet (Roche). Cells were then scraped and lysates cleared by centrifugation at 16,110 x g for 10 min at 4$^{o}$C. Protein concentrations were determined using the Bradford method (Bio-Rad). Proteins were separated by SDS-PAGE and

electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Unless otherwise noted, membranes were blocked in 5% nonfat dry milk in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris [pH 7.4], 0.1% Tween 20) and probed with the appropriate antibody. The following antibodies were used at a 1:1000 dilution unless otherwise specified: β-Actin (1501; Chemicon), p53 (Ab-6, Calbiochem), Grb2 (ab86713, Abcam), Flag (4 μg/ml, F3165, Sigma), anti-phosphotyrosine (05-1050X, Millipore), EphRA2 (NBP1-47400, Novus Biologicals), PDGFR (3174), ErbB2 (2165), EGFR (4267), EGFR Y992 (2235), EGFR Y1068 (3777), EGFR Y1173 (4407), S6K (9202), S6K T389 (9206), RSK (9333), RSK S380 (9335), ERK (9102), ERK T202/Y204 (4370), IGF-1Rβ (3027), IGF-1Rβ Y1135/36/IRβ Y1150/51 (3024), PI3K p110α (4249), PI3K p110β (3011), PI3K Class III (3358), PTEN (9188), all from Cell Signaling Technology. Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were used at dilutions of 1:10,000 or 1:15,000, respectively. Proteins were visualized by enhanced chemiluminescence (Perkin Elmer, Millipore) and exposed on Kodak BioMax XAR film, or electronically acquired and quantified with a Kodak Image Station 4000R equipped with Kodak Imaging Software, version 4.0, or with a Carestream Gel Logic 4000 pro, equipped with Kodak Imaging Software, version 4.0.

**Immunoaffinity purification**

For HA immunoaffinity purifications, four 15cm plates of 293T cells were seeded at a density of 40% and $CaCl_2$ transfected with the appropriate NCMV vector (NCMV, HPV16 E6no*, HPV16 E6no* I128T, HPV16 E6no*PDZ, HPV18 E6 no*, HPV6b E6, HPV11 E6, HPV5 E6, or HPV8 E6) at 24 hours after seeding. 72 hours post transfection the cells were lysed in MC buffer and lysates were subsequently cleared by centrifugation at 16,110 x g for 10 min at 4$^{o}$C. Protein

concentrations were determined using the Bradford method (Bio-Rad). Lysates were cleared with low-binding Durapore PVDF 0.45 μM membrane spin filters (Millipore) and protein concentration was subsequently measured using the Bradford method (Bio-rad). 60μl of prewashed HA antibody-agarose conjugate (Sigma) was then incubated with 3 ml lysate for 2 hrs, washed, and eluted three times for 30 min with 250 μg/ml HA peptide (Sigma) in PBS. Ten percent of total eluate was separated by SDS PAGE and silver stained using the Silverquest staining kit (Invitrogen). The remainder was concentrated by precipitation with 20% trichloroacetic acid (TCA) and analyzed mass spectrometry at the Taplin Mass Spectrometry Core facility (Harvard Medical School).

**Phosphotyrosine Immunoprecipitations**

For phosphotyrosine immunoprecipitations, cells were grown under normal growth conditions or starved of EGF in KSFM lacking EGF for 2 hrs, at 37$^{o}$C, prior to lysis. Cell lysates were prepared in ML buffer. Lysates were pre-cleared in 25 μl prewashed Sepharose (Sigma) for 1 hour, rocking at 4$^{o}$C and then incubated with 25 μl prewashed anti-phosphotyrosine antibody-agarose conjugate (Millipore) for 3 hrs, rocking at 4$^{o}$C. Following immunoprecipitation, the beads were washed and sample buffer added, and proteins separated by SDS PAGE and transferred onto PVDF membrane for Western blotting against ERBB2, EPHA2, PDGFR, and actin.

**Receptor Internalization Assay**

*Internalization Assay*: Primary HFKs were seeded into 15cm dishes and internalization and degradation assays were performed when the cells reached 90% confluence. Cells were washed

twice in ice cold PBS-CM buffer (PBS, 1 mM $MgCl_2$, 0.l mM $CaCl_2$) followed by a 40 min incubation in 5 ml PBS-CM with the addition of 0.5 mg/ml sulfo NHS-SS-Biotin (Pierce), rocking at 4$^o$C. Cells were then washed two additional times in PBS-CM and incubated with PBS containing 50 mM $NH_4Cl$ for 10 min, rocking at 4$^o$C, followed by two washes in PBS-CM. Considering HFKs are sensitive to brief changes in nutrient availability, cells were at this point considered "starved" (Spangle and Munger, 2010). KSFM without any growth factors and supplements was added to one plate in order to investigate ligand independent receptor internalization over 180 minutes. A true generalized ligand independent internalization experiment would have involved incubation in PBS over the full 180 min time course, but that is not feasible in HFKs due to their loss of adherence in the absence of growth media at 37$^o$C. Total cell surface receptors were measured by directly lysing one plate without stimulation or subsequent glutathione reduction. Following stimulation, cells were washed twice with PBS-CM and reduced by washing twice for 15 min each with Glutathione Buffer (50 mM reduced glutathione, 90 mM NaCl, 1 mM $MgC1_2$, 0.1 mM $CaC1_2$, 60 mM NaOH) rocking, at 4°C. Cells were then washed twice with PBS-CM followed by one 15 min wash with PBS-IAA (PBS containing 50mM Iodoacetamide), rocking at 4$^o$C. Lysates were then prepared by incubating cells with ID lysis buffer (200 mM NaCl, 75 mM Tris [pH 7.5], 2.5 mM EDTA, 2.5 mM EGTA, 1.5% Triton X-100, 0.75% NP40, 0.1% SDS), supplemented with one complete EDTA protease inhibitor cocktail tablet (Roche) and one PhosStop phosphatase inhibitor cocktail tablet (Roche). Receptor internalization assays were performed by pre-clearing lysate with 40 µl Pansorbin (Calbiochem) for 45 min, after which the Pansorbin was removed by centrifugation at 16,110 x g for 10 min at 4$^o$C. The pre-cleared lysate was then incubated with 40 µl of washed Streptavidin-agarose resin (Thermo Scientific) for 16 hrs, rocking at 4$^o$C, after which the beads were washed

four times in ID buffer, 2X sample buffer was added, and proteins separated by SDS PAGE and electrotransferred onto PVDF for Western blotting with EGFR, IR/IGFR, and actin antibodies.

**Wound Healing Assay**

Primary HFKs were seeded into 6cm dishes and a wound was introduced into the monolayer with a p200 pipette tip when the cells reached 90% confluence. Just prior to wounding the monolayer, the cells were washed two times with PBS and the media was replaced with KSFM lacking EGF for the minus EGF condition or with complete KSFM for the plus EGF condition, plus inhibitors when applicable. The cells were then incubated over a time course at 37$^{o}$C. Images of the wounded monolayer were captured at t = 0, t = 13h, and t = 25h for the minus EGF condition and t = 0, t = 7h, t = 13h, and t = 25hr for the plus EGF condition using a Zeiss light microscope equipped with the Axiovision Release 4.4 SP2 software package. The surface area of each resulting image was calculated using Image J software (NIH). The surface area relative to the samples at t = 0 was determined. A total of 3 independent experiments were performed and statistical significance was calculated using the Student's T test.

**Transwell Migration Assay**

Primary HFKs were trypsinized and resuspended in KSFM minus EGF. 30,000 HFKs were resuspended in 150 μl of KSFM minus EGF and placed in the upper chamber of a transwell permeable support membrane insert (8.0 μM, Costar® product 3422) pre-wetted with 50 μl KSFM minus EGF. The bottom chamber was filled with 600 μl KSFM minus EGF, and the cells were incubated at 37$^{o}$C for 30 minutes or one hour. Cells were then scraped from the upper chamber of the transwell membrane and the membrane was fixed with 100% methanol for 30

minutes and stained with Crystal Violet. Cells remaining on the underside of the membrane were then counted in three separate fields of view per experiment. A total of three independent experiments were performed and statistical significance calculated using the Student's T test.

**Transfections and luciferase Assays**

U2OS cells were transfected as described in (Spangle and Munger, 2010). In brief, cells were transfected in triplicate in 6-well plates for luciferase reporter assays using FuGene 6 (Roche). One microgram of pFR_CrPV_xb was co-transfected with two μg NCMV or NCMV 16E6no*. Forty-eight hours post transfection, cells were lysed in 450 μl passive lysis buffer (dual luciferase reporter kit; Promega) per well. The supernatants were subjected to the dual luciferase reporter assay. The fold change in activity was determined by calculating the ratio of firefly activity to Renilla luciferase activity compared to the control vector-transfected cells. Three independent experiments were performed and the Student's T test was used to calculate statistical significance.

**siRNA Transfections**

HFKs were seeded in 6 well dishes in triplicate and siRNA against Grb2 (smart pool, Dharmacon) or scrambled siRNA was transfected with Lipofecamine 2000 (Invitrogen) to a final concentration of 40 nM. Lysates were prepared 72 hrs post transfection.

**Results:**

**Receptor protein tyrosine kinase signaling pathways remain activated in HPV16 E6 expressing cells subjected to EGF deprivation.** We previously published that HPV16 E6 activates S6K and 4E-BP1 through mTORC1. We determined that persistent AKT phosphorylation under conditions of nutrient deprivation resulted from PDK1 and mTORC2 activation (Spangle and Munger, 2010). To determine the origin of activation, we first evaluated the phosphorylation of receptor protein tyrosine kinases (RPTKs). Activation of RPTK signaling is initiated by the autophosphorylation of tyrosine residues on the intracellular domains of these proteins. We therefore determined if HPV16 E6 expression increases the phosphorylation of cellular proteins at tyrosine residues in primary HFKs under conditions of nutrient deprivation. Cells were starved either by EGF withdrawal for two hours or with PBS treatment for 15 minutes, followed by evaluation of tyrosine phosphorylation. HPV16 E6 expression sustains the phosphorylation of tyrosine residues in HFKs starved of EGF for two hours (Fig 4.1). Tyrosine phosphorylation is also sustained at a higher level in HPV16 E6 expressing cells than in control cells upon more stringent PBS starvation.

We next specifically evaluated ErbB2 tyrosine phosphorylation. Phosphotyrosine immunoprecipitation followed by Western blotting demonstrates that HPV16 E6 increases the phosphorylation of ErbB2 growing normally in keratinocyte serum free media (Figure 4.2A, left). ErbB2 tyrosine phosphorylation is also higher in HPV16 E6 expressing cells as compared to control cells when the cells were grown for 2 hrs in the absence of EGF in the growth medium (Figure 4.2A, right). In contrast, the tyrosine phosphorylation of the unrelated EphRA2 and PDGFR was comparable in HPV16 E6 and control cells grown under conditions of normal growth media and in the absence of EGF (Fig 4.2A). EGFR autophosphorylation and

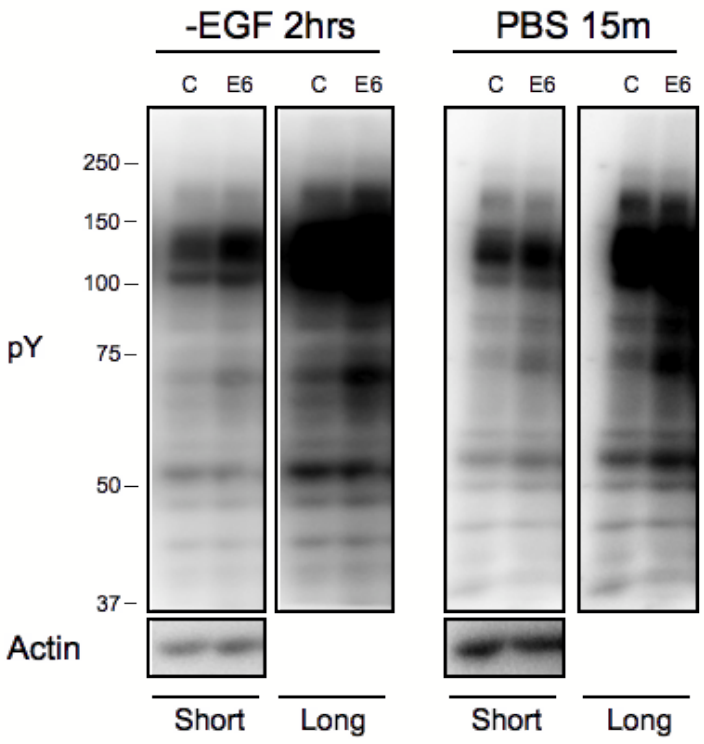


**Figure 4.1. HPV16 E6 increases global phosphorylation of cellular proteins at tyrosine residues in nutrient deprived HFKs.** Western blot analysis of proteins phosphorylated at tyrosine residues in HFK populations with stable expression of a control vector or HPV16 E6 experiencing nutrient deprivation by EGF withdrawal for two hours (left) or PBS starvation for 15 minutes (right). Actin is shown as a loading control

activation is well described, with the phosphorylation of specific tyrosine residues linked to the subsequent recruitment of adaptor proteins and the activation of specific effector signaling pathways. We next evaluated EGFR autophosphorylation at specific tyrosine residues. Under conditions of EGF starvation, Western blot experiments with phosphospecific antibodies revealed that HPV16 E6 maintains the autophosphorylation and presumably activation of EGFR at least three identified residues: Y992, Y1068, and Y1173, which are associated with PLCγ activation, Grb2 binding and MAPK/PI3K signaling, and Shc binding and MAPK signaling, respectively (Figure 4.2B). It should be noted that we did not detect a change in the phosphorylation of EGFR Y1045 in HPV16 expressing HFKs, which is associated with c-Cbl mediated targeting of EGFR for degradation (data not shown). We next considered the possibility that HPV16 E6 may be activating multiple RPTKs. We evaluated the phosphorylation status and activation of insulin receptor-β (IRβ) and insulin-like growth factor receptor-β (IGFI-Rβ) utilizing an antibody that recognizes specific phosphorylated tyrosine resides on both receptors: Y1135/36 on IRβ and Y1150/51 on IGFI-Rβ HPV16 E6 maintains the autophosphorylation of IRβ/IGF-IRβ under conditions of nutrient deprivation (Figure 4.2C).

Thus, immunoprecipitation and Western blot experiments confirm the activation of ErbB, IR, and IGFR by HPV16 E6 under normal growth conditions as well as after EGF starvation.

**HPV16 E6 causes the activation of RPTK effector signaling.** Our group and others have shown that HPV16 E6 activates mTORC1 (Lu et al., 2004; Spangle and Munger, 2010), and that E6 mediated mTORC1 activation causes an increase in translation of capped mRNAs (Spangle and Munger, 2010). HPV16 E6 has also been reported to activate FAK signaling, which is also

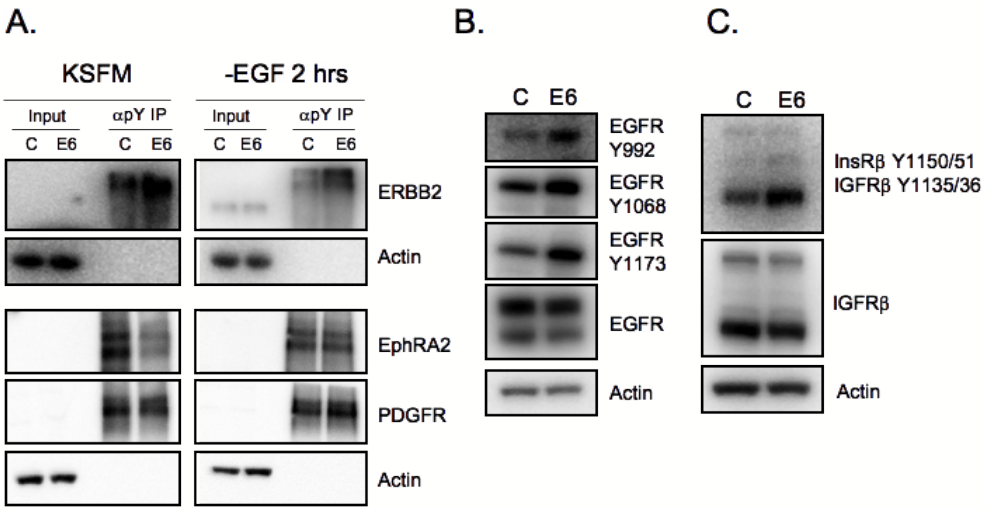


**Figure 4.2. ERBB2 phosphorylation is increased in HPV16 E6 expressing primary HFKs**. (A) Western blot analysis of ERBB2, EPHRA2, and PDGFR following phospho-tyrosine immunoprecipitation (IP) of HFK lysates stably expressing HPV16 E6 or the pLentiN6.3 control vector under two separate growth treatments (KSFM, left; EGF withdrawal for 2 hours, right). For each IP, 10% input is on the left of the IP. (B) and (C) Western blot analysis indicating the phosphorylation status of EGFR(B) or IR/IGFR (C) in HPV16 E6 or LXSN control vector expressing stable HFKs.

a RPTK effector (McCormack et al., 1997; Vande Pol et al., 1998). Given that we detected a maintenance of EGFR Y1068 and Y1173 phosphorylation, and IRβ/IGFI-Rβ phosphorylation, all of which are associated with RAS/MAPK signaling, we evaluated MAPK activity under conditions of EGF withdrawal. HPV16 E6 expression sustains the phosphorylation and activation of MAPK effectors ERK1/2, and the ERK substrate RSK in comparison to control cells (Figure 4.3A). In addition to mTORC1, Ras/MAPK signaling can activate cap dependent translation through the RSK mediated phosphorylation of the ribosomal protein S6 (S6 S235/36). To determine if MAPK signaling is required for HPV16 E6 to activate cap dependent translation, we utilized a bicistronic luciferase reporter construct (Petersen et al., 2006). In brief, U2OS cells were transiently transfected with the reporter construct and a vector expressing HPV16 E6 or empty vector, and MAPK activity was inhibited with the MEK inhibitor U0126 or DMSO. DMSO treatment indicates that HPV16 E6 increases cap dependent translation, as previously described (Figure 4.3C, black bars) (Spangle and Munger, 2010). However, the HPV16 E6 mediated increase in cap dependent translation is sensitive to MEK inhibition (Figure 4.3C, dark grey bars). It was previously shown that mTORC1 activity is partially required for HPV16 E6 to activate cap dependent translation (Spangle and Munger, 2010). To determine the relative contribution of MAPK and mTORC1 activation in HPV16 E6 mediated increase of protein synthesis, Rapamycin and U0126 were used to simultaneously inhibit mTORC1 and MAPK. The sensitivity of HPV16 E6 mediated increase in cap dependent translation to MAPK and mTORC1 co-inhibition is additive (Figure 4.3C, light grey bars). Western blots demonstrate that U0126 treatment effectively inhibits MEK signaling and are shown in Figure 4.3B.

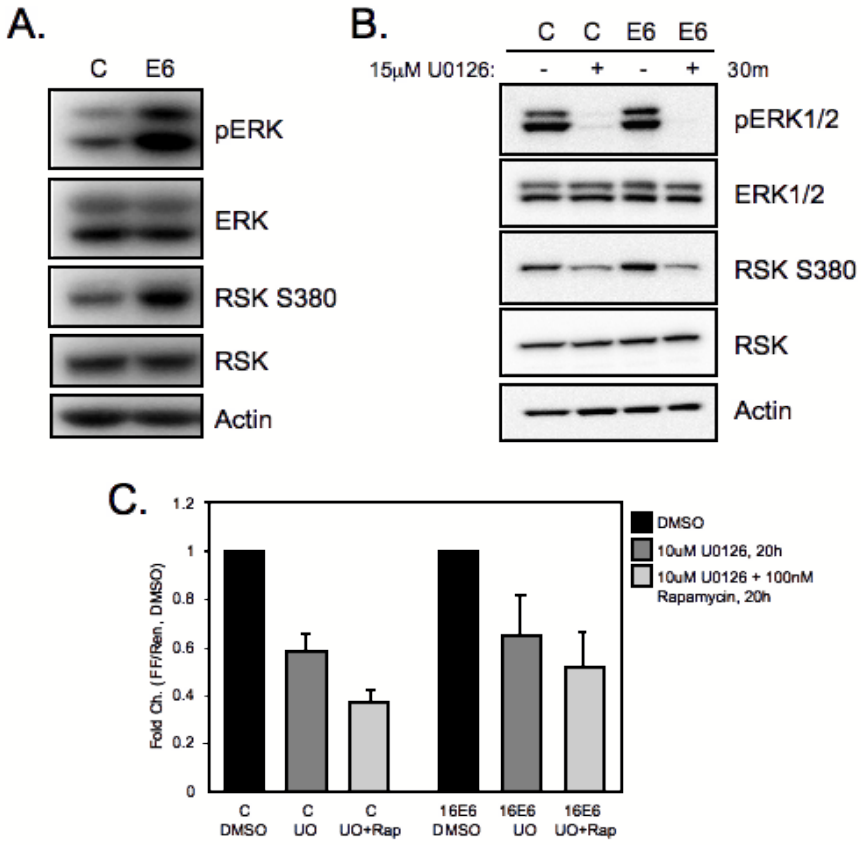


**Figure 4.3. Signaling pathways downstream of RPTKs are activated by HPV16 E6.** (A) Western blot analysis of MAPK signaling in primary HFKs stably expressing HPV16 E6 or LSXN control vector and experiencing nutrient withdrawal. (B) Western blot analysis of MAPK signaling in primary HFKs stably expressing HPV16 E6 or LXSN control vector under conditions of MAPK inhibition. 30 minutes prior to lysis, cells were treated with DMSO or the MEK inhibitor U0126 (15 μM). (C) HPV16 E6 mediated increase in cap dependent translation is dependent on MEK and mTORC1 signaling. U2OS cells were transfected with pFR_CrPV_xb and the indicated plasmids and processed for firefly and Renilla luciferase activity at 48 hours post transfection. 20 hours prior to lysis, cells were treated with DMSO, 10 μM U0126, or a combination of 10 μM U0126 and 100 nM rapamycin. Firefly and Renilla luciferase were normalized to DMSO treated cells and presented as fold changes of normalized firefly relative to normalized Renilla activity. The bar graph represents the average and standard deviation of three experiments, each performed in triplicate.

**EGFR or IR/IGFR inhibition reduces mTORC1 activation in HPV16 E6 expressing primary HFKs.** We next hypothesized that if RPTK signal transduction is required for HPV16 E6 mediated mTORC1 activation, RPTK inhibition should abrogate the E6 mediated mTORC1 activation. Treatment of cells with Gefitinib reduced EGFR autophosphorylation on Y1068 in control and HPV16 E6 expressing cells (Fig 4.4A). EGFR inhibition with Gefitinib partly inhibited the E6 mediated increase in S6K phosphorylation. There was some increase in S6K phosphorylation over the time course of inhibitor treatment in both control and E6 expressing cells. This may be due to a compensatory activation of other RPTKs that activate mTORC1, such as IRβ/IGFI-Rβ. IRβ/IGFI-Rβ inhibition with OSI-906 had similar effects with efficient receptor inhibition in both E6 and control cells, although receptor inhibition in E6 expressing HFKs was not as complete as in control HFKs at early time points (Fig 4.4B). Shortly after IR/IGFR inhibition S6K phosphorylation increased in both control and HPV16 E6 expressing cells, potentially suggesting compensatory S6K phosphorylation, perhaps via EGFR signaling. Indeed, AKT activation has been shown to occur upon MAPK inhibition (Yu et al., 2002).

These results demonstrate that EGFR and IRβ/IGFI-Rβ RPTKs may independently activate mTORC1 in HPV16 E6 expressing HFKs.

**HPV16 E6 increases internalization of activated receptor species.** HPV16 E6 may activate RPTK and RPTK effector signaling through several different mechanisms. HPV16 E6 may be targeting protein or lipid phosphatases for degradation. We evaluated PTEN, SHP1 and PTP1b by Western blot and found no evidence for destabilization under normal growth conditions (Fig 4.5A and data not shown) or after nutrient deprivation by PBS starvation (Fig 4.5B). It has also been reported that PTEN activity may be dependent on subcellular localization, since NEDD4.1

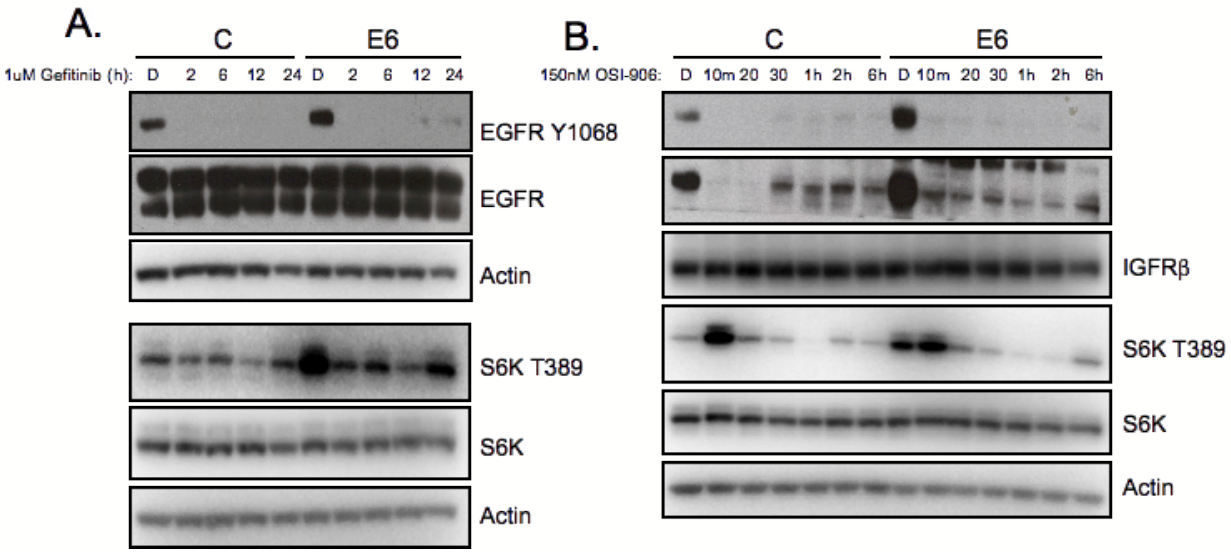


**Figure 4.4. RPTK inhibition reduces mTORC1 activation in HPV16 E6 expressing HFKs.** (A) Western blot analysis of EGFR and mTORC1 activation in HFKs stably expressing HPV16 E6 or pLentiN6.3 control vector following EGFR chemical inhibition with Gefitinib. Cells were treated with DMSO or 1 μM Gefitinib over a 24 hour time course. Actin is shown as a loading control. (B) Western blot analysis of IRβ/IGFRβ and mTORC1 activation in HFKs stably expressing HPV16 E6 or pLentiN6.3 control vector following IRβ/IGFRβ chemical inhibition with OSI-906. Cells were treated with DMSO or 150 nM OSI-906 over a 6 hour time course. Actin is shown as a loading control.

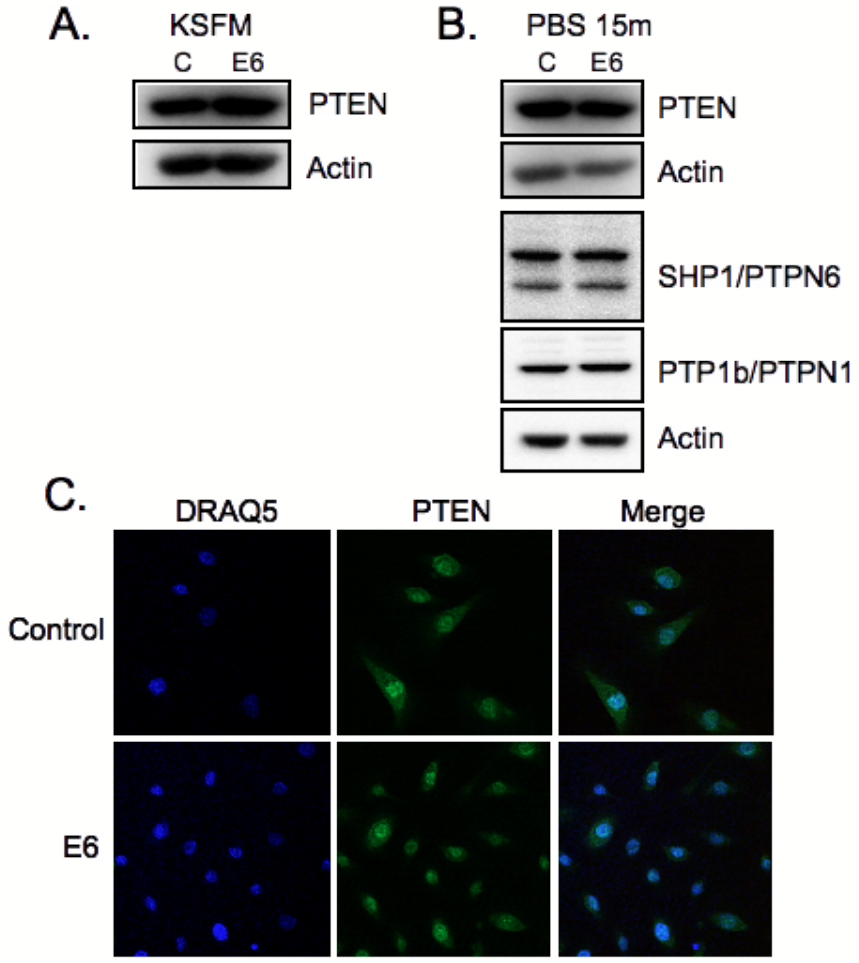


**Figure 4.5. HPV16 E6 mediated activation of AKT/MAPK is not due to the destabilization or change in localization of phosphatases**. For (A) and (B), actin is shown as a loading control. (A) Western blot analysis of the dual specificity phosphatase PTEN in HFKs with stable expression of a control vector of HPV16 E6 under normal growth conditions (KSFM). (B) Western blot analysis of PTEN, PTP1b and SHP1 in HFKs with stable expression of a control vector of HPV16 E6 under conditions of nutrient deprivation (PBS, 15 minutes). (C) Confocal Immunofluorescence imaging of PTEN (green) subcellular localization in control or HPV16 E6 expressing HFKs. The nuclei are stained with DRAQ5 (blue).

mediated PTEN monoubiquitination increases PTEN localization to the nucleus (Wang et al., 2007). It has also been shown that PTEN nuclear localization is increased when cells are deficient in ATP (Lobo et al., 2008). We found no detectable change in PTEN subcellular localization in HPV16 E6 expressing HFKs (Fig 4.5C). We next considered the possibility that HPV16 E6 may affect receptor internalization or degradation. Since HPV16 E6 causes RPTK and AKT activation under conditions of nutrient deprivation, the internalization of cell surface receptors was evaluated in the absence of ligand in KSFM lacking EGF and other supplements. HPV16 E6 increases the internalization of phosphorylated EGFR, IR and IGFR receptor species in the absence of growth factors (Fig 4.6A, lanes without supplements). HPV16 E6 also reduces EGFR half-life from 11.5 hrs to 8.3 hrs, which supports an E6 mediated increase in receptor degradation (Fig 4.6B).

These results suggest that HPV16 E6 mediated activation of RPTK signaling and downstream effector cascades is the result of a direct effect on the receptors, increasing the internalization and subsequent degradation of activated receptors.

**HPV16 E6 association with the signaling adaptor protein Grb2 causes mTORC1 activation.** To determine if HPV16 E6 activates RPTKs through protein-protein interactions, we performed affinity purification/mass spectrometry experiments to identify HPV E6 interacting proteins. We transiently transfected 293T cells with amino terminally Flag-HA epitope tagged HPV16 E6 and performed HA affinity purification followed by LC-MS-MS (Fig 4.7). We confirmed known binding partners including E6AP (UBE3A), p53 and multiple PDZ proteins including LIN7C, Dlg, and hScrib (Appendix 1). We also identified novel potential binding partners including Grb2, a signaling adaptor protein that associates with autophosphorylated receptors,

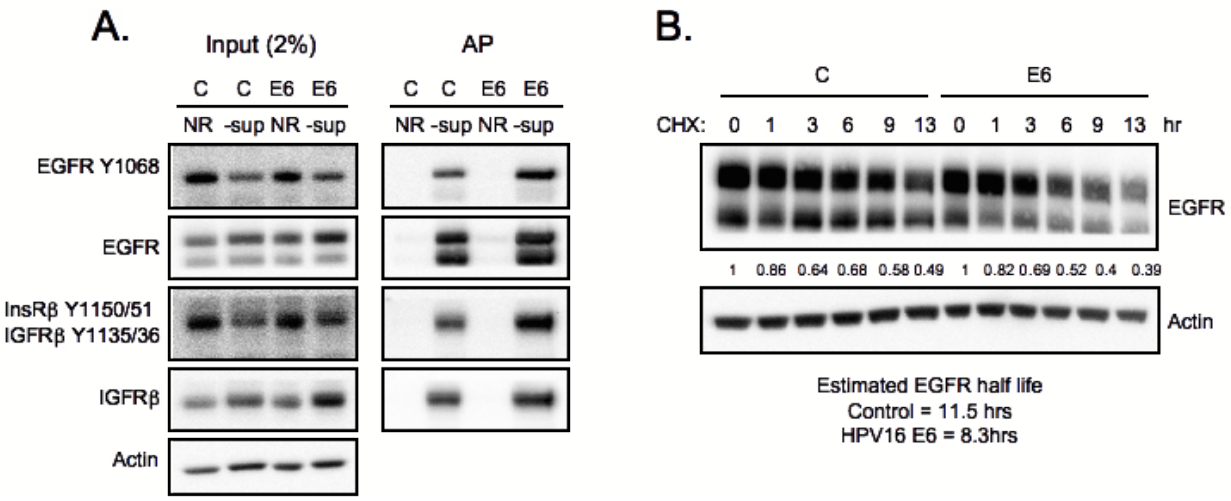


**Figure 4.6. HPV16 E6 increases the internalization and degradation of activated receptor species.** (A). Western blot analysis of EGFR (middle) and IR/IGFR (bottom) following receptor internalization, with non-reduced (NR) measuring surface-bound receptors, starved (ST) measuring ligand independent internalization, and the time course representing time (in minutes) of keratinocyte growth media stimulation to promote receptor internalization prior to harvesting and immunoprecipitation. Internalization assays were performed as described in the Materials and Methods. Levels of EGFR and IR/IGFR in a 5 μg sample, representing 1% of the internalization assay/resulting streptavidin immunoprecipitation, together with actin, are shown in the top panel (Input). (B) Western blot analysis of EGFR half-life following 10 μg/ml cycloheximide treatment. Actin is shown as a loading control.

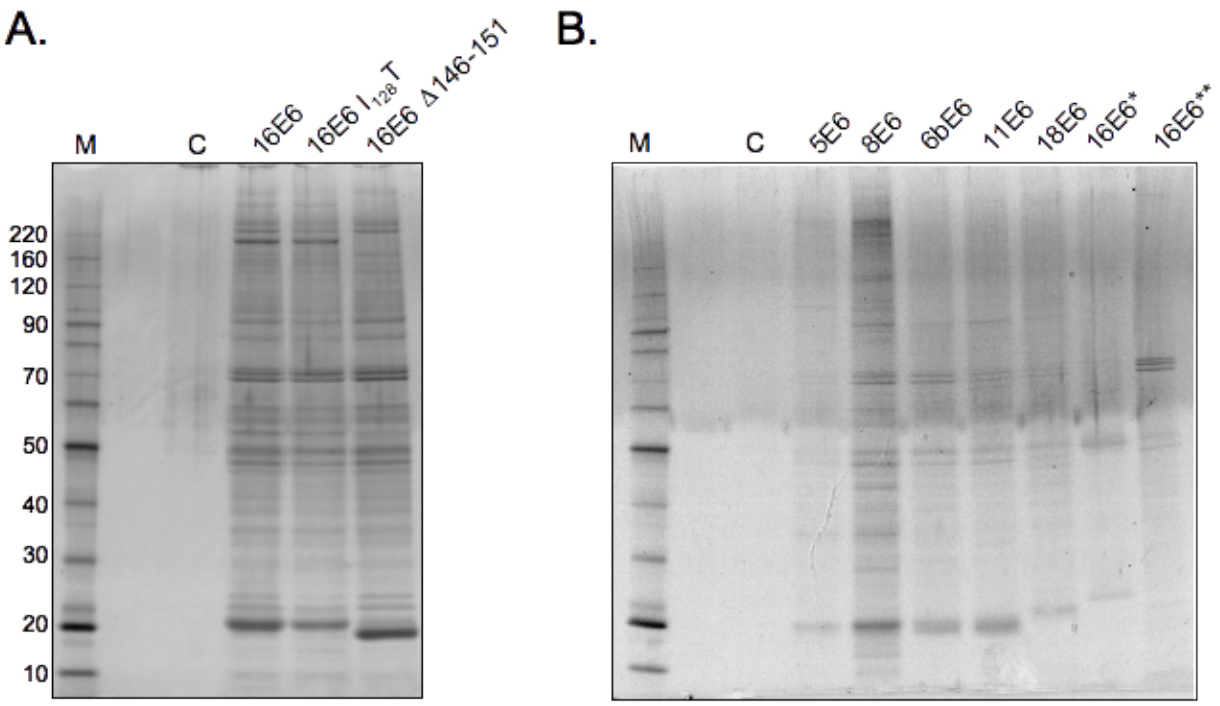


**Figure 4.7. Identification of HPV E6 associated cellular proteins.** (A) Silver stain of HA-Immunoprecipitation eluate (10% of total IP) of 293T cells transiently transfected with Flag-HA epitope tagged HPV16 E6 or HPV16 E6 mutants (I128T, PDZ). All HPV16 E6 vectors lack the splice donor site and therefore do not produce the internally spliced '*' or '**' transcripts. 48 hours post transfection, cells were lysed and HA affinity purification was performed. (B) Silver stain of HA-Immunoprecipitation eluate (10% of total reaction) of 293T cells transiently transfected with amino terminally Flag-HA epitope tagged HPV E6 proteins (Cutaneous: 5, 8; low-risk mucosal: 6b, 11; high-risk mucosal: 18; high-risk HPV16 E6 splice variants: 16E6*, 16E6**). 48 hours post transfection, cells were lysed and the IP was performed.

specifically EGFR at phosphotyrosine 1068 and activates PI3K and MAPK signaling. This is consistent with our results that suggest that HVP16 E6 increases the phosphorylation of Y1068, an event that promotes the association of Grb2 with EGFR (Figure 4.2B). The association of HPV16 E6 and Grb2 was confirmed by immunoprecipitation/Western blot experiments under standard conditions as well as under conditions of *in vivo* crosslinking (Figure 4.8A). We next determined if Grb2 is important in the HPV16 E6 mediated activation of mTORC1 by depleting Grb2 and evaluating mTORC1 activation. Grb2 knockdown reduced the phosphorylation of the mTORC1 surrogate marker S6K in HPV16 E6 expressing HFKs but not in control HFKs (Figure 4.8B).

Together these results demonstrate that HPV16 E6 associates with the signaling adaptor protein, Grb2, and that Grb2 is important for HPV16 E6 mediated mTORC1 activation.

**HPV16 E6 increases cellular migration through a RPTK dependent mechanism**

Our group has shown that HPV16 E6 activates two RPTK effector signaling pathways: mTORC1 (Spangle and Munger, 2010) and MAPK (Fig 4.3). The activation of mTORC1 and MAPK signaling pathways promote cellular events that are widely associated with carcinogenesis including cellular migration. Therefore, we hypothesized that HPV16 E6 mediated RPTK activation increases cellular migration by wound healing and transwell migration assays. First, wounds were introduced into the primary HFK monolayer in the presence or absence of EGF. HFKs with stable HPV16 E6 expression efficiently migrated to close the wounded area over a 25 hour time course in the presence of EGF with greater efficiency when compared to HFKs expressing empty vector (Fig 4.9, relative reduction in

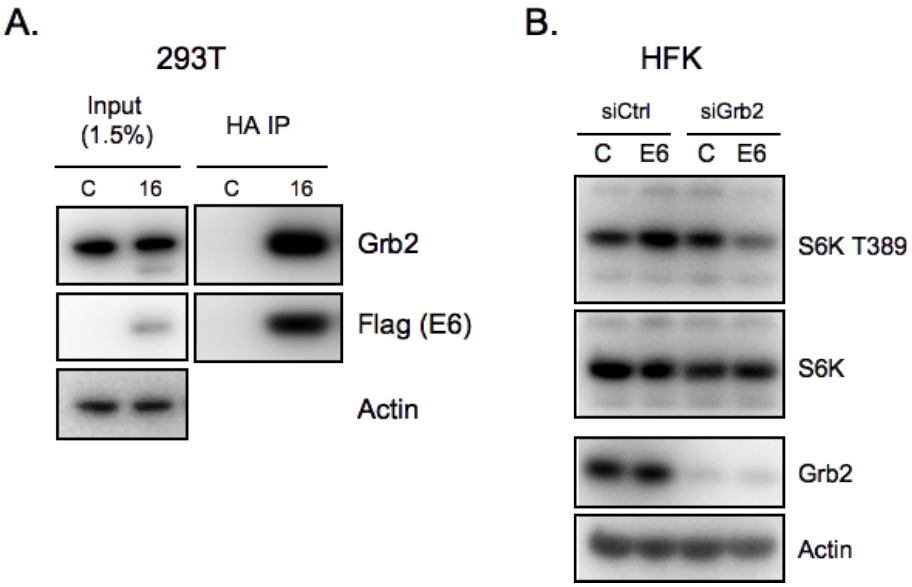


**Figure 4.8. HPV16 E6 associates with signaling adaptor protein Grb2, which is important for HPV16 E6 mediated mTORC1 activation**. (A) Western blot analysis of Grb2 and Flag-HA epitope tagged HPV16 E6 following HA immunoprecipitation of Grb2 in NCMV HPV16 E6 or control vector transfected 293T cells (right panel). Immunoprecipitation was performed under native DSP crosslinking conditions. Levels of HPV16 E6 (Flag) and Grb2 in a 35 μg sample, representing 1.5% of the IP are shown on the left, with actin as a loading control (Input). (B). Western blot analysis of S6K T389 in Grb2 siRNA transfected HFK populations with stable expression of a control vector or HPV16 E6. 48 hours prior to lysis, cells were transiently transfected with a siRNA pool specific to Grb2. An actin blot is shown as a loading control.

surface area at t = 25h, 0.25 ± 0.03 and 0.66 ± 0.04, respectively; p = 0.0065). Interestingly, HPV16 E6 expression caused an even more rapid and significant decrease in the surface area of the wound in the absence of EGF over the same time course in comparison to empty vector (Fig 4.10, relative reduction in surface area at t = 13h, 0.2 ± 0.06 and 0.87 ± 0.09, respectively; p < 0.0001). We next determined if the inhibition of RPTKs may inhibit the ability of HPV16 E6 to increase cellular migration in wound healing assays. Treatment with the EGFR inhibitor Gefitinib or the IR/IGFR inhibitor OSI-906 impaired HPV16 E6 mediated cellular migration in the presence and absence of EGF such that no statistical difference between HPV16 E6 and empty vector expressing cells were observed (Fig 4.9, plus EGF relative reduction in surface area at t = 25h, 0.80 ± 0.02 and 0.90 ± 0.16 (Gefitinib), 0.60 ± 0.40 and 0.82 ± 0.12 (OSI-906), respectively; Fig 4.10 minus EGF relative reduction in surface area at t = 13h, 0.63 ± 0.01 and 0.96 ± 0.07 (Gefitinib), 0.83 ± 0.05 and 0.84 ± 0.17 (OSI-906), respectively). As expected, the inhibition of either mTORC1 or MEK signaling also inhibit the ability of HPV16 E6 to increase cellular migration in wound healing assays in the presence and absence of EGF (Fig 4.9, plus EGF relative reduction in surface area at t = 25h, 0.57 ±0.22 and 0.71 ± 0.21 (Rapamycin), 0.78 ± 0.07 and 0.84 ±0.17 (U0126), respectively; Fig 4.10 minus EGF relative reduction in surface area at t = 13h, 0.67 ±0.01 and 0.85 ± 0.22 (Rapamycin), 0.86 ± 0.1 and 0.87 ± 0.04 (U0126), respectively.

We also evaluated if HPV16 E6 increases the migration of primary HFKs through a size restricting membrane in the transwell migration assay in the absence of EGF over a short time course to eliminate cell division. Stable expression of HPV16 E6 causes an increase in cellular migration through the membrane as early as 1 hour, in the absence of EGF (Fig 4.10, fold change in migration relative to control, 1.75 ± 0.4, p = 0.0319).

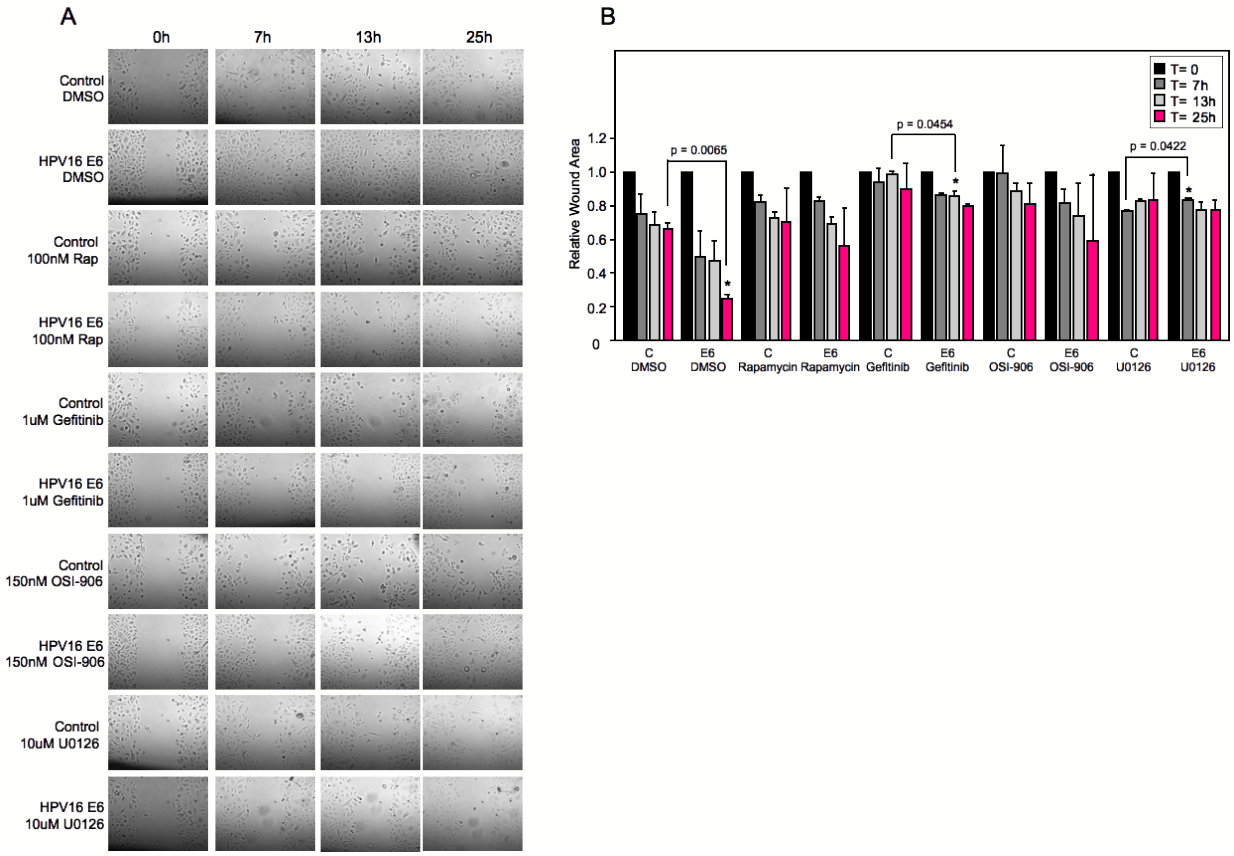


**Figure 4.9. HPV16 E6 increases cellular migration in the presence of EGF.** Wound healing assay with HFKs stably expressing HPV16 E6 or pLentiN6.3 control vector following wounding of the cellular monolayer under normal growth conditions following RPTK and effector pathway inhibition with Gefitinib, OSI-906, Rapamycin, or U0126. Cells were treated with DMSO or 100 nM Rapamycin, 1 μM Gefitinib, 150 nM OSI-906, or 10 μM U0126 and closure of the monolayer was measured over a 25h time course. (B). Quantification of wound closure as shown in (A). Surface area of wounds were calculated relative to the surface area of the wound at t = 0h. The bar represents the average and standard deviation of four experiments for DMSO treated samples and two experiments for drug treated samples; asterisks indicate statistical significance ($P < 0.05$).

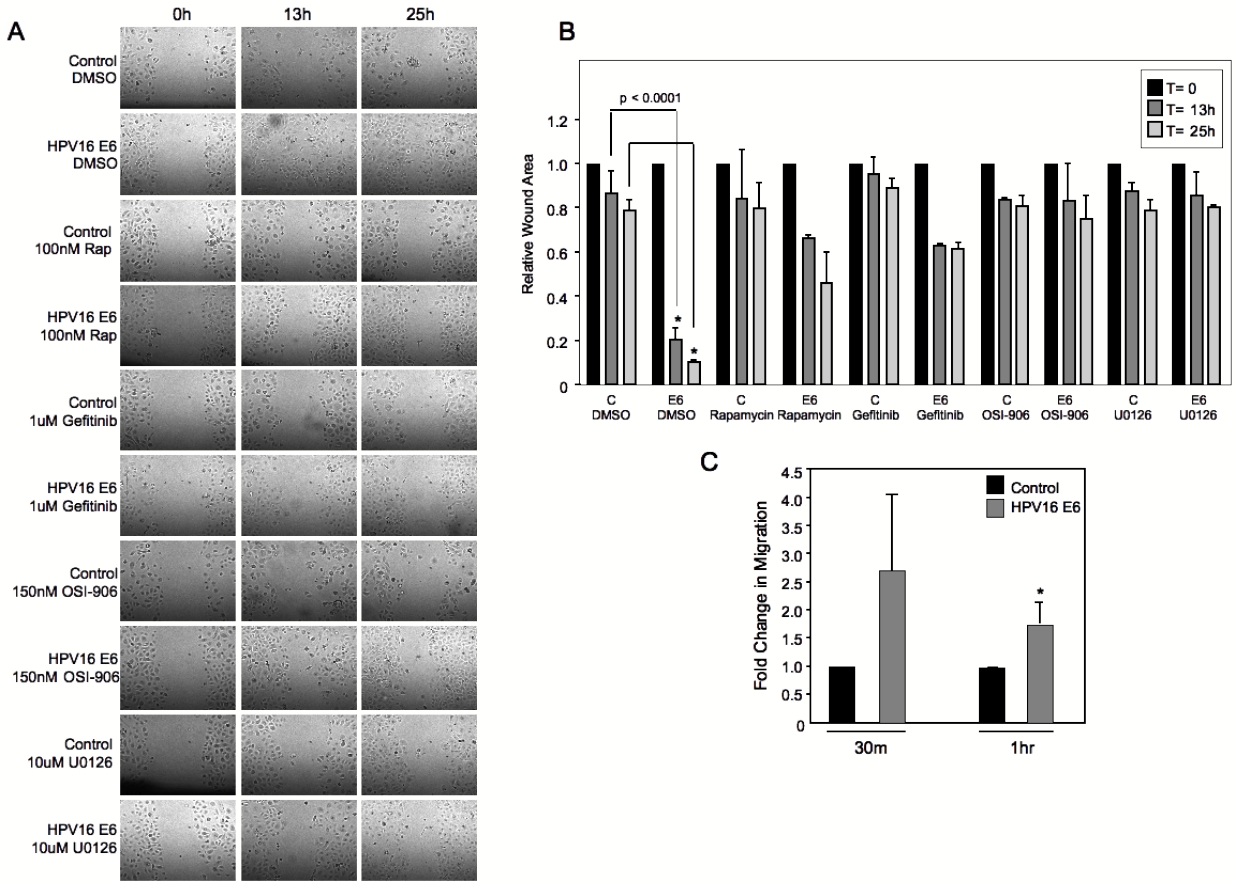


**Figure 4.10. HPV16 E6 increases cellular migration in the absence of EGF.** (A). Wound healing assay with HFKs stably expressing HPV16 E6 or pLentiN6.3 control vector following wounding of the cellular monolayer in the absence of EGF following RPTK and effector pathway inhibition with Gefitinib, OSI-906, Rapamycin, or U0126. Cells were treated with DMSO or 100 nM Rapamycin, 1 μM Gefitinib, 150 nM OSI-906, or 10 μM U0126 and closure of the monolayer was measured over a 25h time course. (B). Quantification of wound closure as shown in (A). Surface area of wounds were calculated relative to the surface area of the wound at t = 0h. The bar represents the average and standard deviation of three experiments; asterisks indicate statistical significance ($P < 0.001$). (C). Transwell migration assay with HFKs stably expressing HPV16 E6 or pLentiN6.3 control vector following the seeding of cells in the absence of EGF on the top chamber of a transwell insert and determining migration to the bottom chamber in the absence of EGF. The bars represent the average and the standard deviation of four experiments for DMSO treated samples and two experiments for drug treated samples; the asterisk indicates statistical significance ($P < 0.05$).

These results suggest that under both normal growth conditions (+EGF) and in the absence of RPTK ligand (-EGF) HPV16 E6 increases the migration of primary HFKs, and that EGFR/IR/IGFR effector signaling pathways are required for efficient HPV16 E6 mediated cellular migration.

**Discussion:**

A combined approach of phosphotyrosine immunoprecipitations and Western blotting for activated receptor species under normal growth conditions and EGF withdrawal revealed that HPV16 E6 expression causes sustained activation of RPTKs including ErbB2, EGFR, IRβ and IGFR1β-R (Fig 4.2) under normal growth conditions as well as in the absence of their growth factor ligands. Activation of downstream signaling cascades including the RAS/MAPK pathway was also demonstrated. Previous studies demonstrated that HPV16 E6 activates mTORC1 and increases cap dependent translation through a PDK1 and mTORC2 dependent mechanism. Cap dependent translation is reduced in U0126 treated HPV16 E6 cells as well as in control vector-transfected cells. The combined inhibition of MEK and mTORC1 further reduces cap dependent translation. This may suggest that HPV16 E6 activates cap dependent translation through two at least partially independent signaling pathways that are both downstream of RPTKs. This further supports HPV16 E6 mediated activation of RPTKs. We show that EGFR or IRβ/IGFR1β-R inhibition abrogates HPV16 E6 mediated mTORC1 activation as indicated by reduced phosphorylation of the surrogate marker S6K. We are in the process of testing the effect of EGFR and/or IRβ/IGFR1β-R inhibition on E6 mediated increase of cap dependent translation. We hypothesize that EGFR and/or IRβ/IGFR1β-R inhibition will have similar effects on the HPV16 E6 mediated increase of cap dependent translation as MEK and/or mTORC1 inhibition,

further implicating RPTK activation in downstream signal activation driven by the HPV16 E6 oncoprotein. These results support previous studies that show that HPV16 E6 activates signaling cascades downstream of RPTKs. For example, HPV16 E6 has been reported to activate FAK signaling, causing disruption of the cellular cytoskeletal structure (McCormack et al., 1997). HPV16 E6 and the Bovine Papillomavirus type 1 E6 protein have been shown to associate with paxillin and disrupt actin cytoskeletal structure, which may have implications on the transformed cell phenotype through anchorage independent growth (Neary and DiMaio, 1989; Tong and Howley, 1997; Vande Pol et al., 1998). It has also been shown that HPV16 E6 targets TAp63β for E6AP independent proteasomal degradation, which promotes anchorage independent growth (Khalifa et al., 2011). Further, high-risk HPV16 and HPV18 E6 proteins have been reported to associate with and target scaffolding PDZ domain containing proteins including MAGI-1 for proteasome-mediated degradation, disrupting tight junctions and also promoting anchorage independent growth (Kranjec and Banks, 2011). It is therefore possible that some or all of these reported activities are regulated by the HPV16 E6 mediated activation of RPTKs.

Our findings that HPV16 E6 activates RPTK signaling are in accordance with previously published data. It has been suggested that HPV oncoproteins activate EGFR signaling. HPV16 E6 and E7 have been reported to transcriptionally activate EGFR (Akerman et al., 2001; Sizemore et al., 1998). However, the HPV16 E6/E7 mediated EGFR mRNA increase is not universal, as it was only observed in approximately one half of HFK populations evaluated (Akerman et al., 2001). Here we have not identified any HPV16 E6 specific increase in EGFR protein levels. EGFR activation has also been studied in the benign respiratory papillomas caused by low-risk mucosal HPV infection (Johnston et al., 1999). HPV types 6b and 11 are associated with respiratory papillomas, and the E6 proteins from HPV6b and HPV11 have been

implicated in increased EGFR expression. EGFR expression was not due to gene amplification event, nor was it associated with an increase in mRNA expression. Instead low-risk mucosal HPV E6 proteins increase EGFR recycling to the cell surface by approximately 20% (Johnston et al., 1999). Low-risk HPV E6 associated increase in EGFR expression was shown to increase EGFR tyrosine phosphorylation and cause increased MAPK activity. In contrast to the studies on respiratory papillomas, we have not detected an increase in total receptor levels in HPV16 E6 expressing HFKs, but rather a specific increase in phosphorylated receptor species.

We demonstrate that HPV16 E6 increases the internalization (Fig 4.6) of a subset of activated growth factor sensitive RPTKs, including EGFR, IRβ and IGFR1β-R in the absence of growth factors. These receptors are considered activated because they are phosphorylated at known residues that promote receptor association with signaling adaptor proteins and the activation of downstream signaling cascades. It is well established that receptor activation causes increased receptor internalization, and internalized receptors maintain signaling potential. This is consistent with our observation of increased phosphorylation of internalized receptor species. Following internalization, receptors are then recycled to the cell surface or are degraded through endosomal fusion with the lysosome. Thus, increased activated receptor internalization causes increased receptor turnover and degradation. EGFR half-life is shorter in HPV16 E6 expressing cells, which is consistent with increased activated receptor degradation.

The signaling adaptor protein Grb2 was identified as an E6 associated protein, and Grb2 knockdown reduced HPV16 E6 mediated mTORC1 activation, suggesting that Grb2 is important in relaying EGFR, IRβ and IGFR1β-R activation to downstream effectors (Fig 4.8). It is possible that HPV16 E6 mediated receptor internalization following autophosphorylation and activation is mediated by Grb2 and it is tempting to speculate that the HPV16 E6-Grb2 association promotes

receptor internalization. Moreover, EGFR receptor internalization is required for the maintenance of AKT activation (Goh et al.; Sigismund et al., 2008). Indeed, Grb2 has been implicated in receptor internalization as it is reported to interact with dynamin, an exchange factor that is important for inclusion of receptors into vesicles during endocytosis (Wang and Moran, 1996). The mechanics of the Grb2-receptor association and activation is, however, different. Grb2 associates with EGFR directly following receptor autophosphorylation (Batzer et al., 1994). IRβ and IGFR1β-R form indirect interactions with Grb2 via the IRS-1 and Shc adaptor proteins (Skolnik et al., 1993). The PDGFR and Grb2 association is indirect, with SHP-2 mediating the association between Grb2 and PDGFR (Bazenet et al., 1996). Similarly, the EphRA2-Grb2 association is indirect, requiring Shc to form a complex that then leads to the activation of downstream signaling cascades including MAPK activation (Pratt and Kinch, 2002). Thus, we favor a model in which Grb2 and a specific set of adaptor proteins are required that cause receptor internalization. HPV16 E6 may associate with and functionally modify the complex that Grb2 participates in that contributes to receptor activation or internalization. Potential modifications include increasing the stability or activity of the complex or targeting a Grb2 inhibitor for proteasome mediated degradation. Targeting an inhibitor of Grb2 for degradation is a particularly attractive model given our recent data suggesting that the LXXLL binding motif of HPV E6 proteins is important for HPV E6 mediated activation of mTORC1 signaling and cap dependent translation (see chapter three). HPV E6 proteins associate with several proteins via the LXXLL binding motif, including the E3 ubiquitin ligase E6AP. It is also possible that the HPV16 E6/Grb2 association may increase Grb2 localization to the cellular membrane, increasing direct RPTK association and the activation of downstream signaling

networks. Experiments are currently underway to address the subcellular distribution of Grb2 in relation to HPV16 E6, EGFR, and IRβ/IGFR1β-R.

The HPV E6 and HPV E7 oncoproteins share biological functions with proteins encoded by other DNA tumor viruses, including polyomaviruses SV40, Merkel cell polyomavirus (MCPyV), and murine polyomavirus, and adenoviruses. The PI3K/AKT/mTORC1 signaling axis is targeted for activation by many DNA tumor viruses. The ability of plasma membrane bound mouse polyoma Middle T antigen to activate AKT and other downstream mitogenic pathways through association and subsequent recruitment of the Class I PI3K p85 regulatory subunit has been well documented (Ichaso and Dilworth, 2001; Kaplan et al., 1987; Summers et al., 1998; Whitman et al., 1985). It was recently reported that the MCPyV small T antigen causes aberrant hyperphosphorylation and activation of eukaryotic translation initiation factor 4E binding protein (4E-BP1). This study claimed that 4E-BP1 activation is independent of mTORC1 or mTORC2, but no actual mechanism was proposed (Shuda et al., 2011). It is possible that DNA tumor viruses may be implicated in one or multiple of the described mechanisms to activate PI3K/AKT/mTORC1. These mechanisms are not mutually exclusive and may account for widespread activation of a variety of signaling pathways including activation of MAPK, mTORC1 and FAK signaling. It is therefore tempting to speculate that oncoprotein mediated RPTK activation through engaging Grb2 and promoting the internalization of activated receptors is conserved amongst multiple DNA tumor viruses. We are currently addressing this question.

# CHAPTER FIVE

## Summary, Discussion, and Future Directions

## Summary

Together with the high-risk HPV E7 oncoprotein, HPV16 E6 contributes to the transformed phenotype. High-risk HPV E6 and E7 reprogram the cell, driving S-phase entry and cellular proliferation through the targeted degradation of p53 and pRb, respectively. Other functions of HPV16 E6 may contribute cellular reprogramming. Previous studies implicated HPV16 E6 in the activation of insulin signaling through mTORC1 activation. The biological consequences of HPV16 E6 mediated mTORC1 activation had not been investigated. It was unknown if mTORC1 activation is shared amongst other HPV types, and therefore if mTORC1 activation is associated with the transformed phenotype observed in high-risk HPV types. Therefore, through the studies described in this dissertation, we aimed to confirm that HPV16 E6 activates mTORC1, to investigate whether this activation affects known mTOR-regulated cellular processes, such as translation, and to identify the mechanism by which HPV16 E6 activates mTORC1. The results from each data chapter are summarized as follows:

### Chapter 2: The human papillomavirus type 16 E6 oncoprotein activates mTORC1 signaling and increases protein synthesis

HPV16 E6 was previously shown to activate mTORC1 signaling with an increase in S6K and S6 phosphorylation (Lu et al., 2004). It was proposed that HPV16 E6 activates mTORC1 through the E6AP dependent degradation of the mTORC1 negative regulator TSC2. Interestingly, targeting TSC2 for degradation was restricted to HPV16 E6 and was therefore not degraded by other high-risk E6 proteins such as HPV18 E6. Our initial results confirmed that stable expression of HPV16 E6 phosphorylates and activates S6K and S6 in primary cells and other cell types. We found that HPV16 E6 activates also increases 4E-BP1

hyperphosphorylation, further supporting the model that HPV16 E6 causes general activation of mTORC1 signaling. We evaluated cellular processes downstream of mTORC1 and demonstrated that HPV16 E6 increases the association of translation initiation factors with a synthetic mRNA cap. Moreover, HPV16 E6 increased cap dependent translation in bicistronic luciferase reporter assays. Concomitant HPV16 E7 expression did not reduce the ability of HPV16 E6 to activate cap dependent translation, indicating that mTORC1 activation and subsequent increase in cap dependent translation is relevant in the context of viral infection. Mechanistically, HPV16 E6 did not associate with TSC2, nor did HPV16 E6 cause TSC2 destabilization. To dissect the mechanism by which HPV16 E6 activates mTORC1, we focused on growth factor associated signaling and tested AKT and mTORC1 activation under conditions of nutrient deprivation. Both short term PBS and EBSS starvation maintained phosphorylation of AKT S473 and T308, suggesting that the activation of PDK1 and mTORC2 is sustained under conditions of nutrient deprivation in HPV16 E6 expressing primary HFKs.

**Chapter 3: The mechanism by which mucosal human papillomavirus E6 proteins activate mTORC1 and increase protein synthesis is dependent on an intact LXXLL binding motif**

To narrow down the biochemical activities of HPV16 E6 that are responsible for activation of mTORC1 and cap dependent translation, we investigated whether different mucosal and cutaneous E6 proteins share this function with HPV16 E6. While mucosal HPV E6 proteins increased protein synthesis, cutaneous E6 proteins did not. To complement these studies, we utilized HPV16 E6 mutants that do not degrade p53 (Y54D), associate with proteins via their LXXLL motifs less efficiently (I128T), and cannot associate with PDZ proteins (ΔPDZ). Although all three of the HPV16 E6 mutants did not increase cap dependent translation in

bicistronic luciferase reporter assays as efficiently as wild type HPV16 E6, the Y54D and I128T mutants were statistically similar to the empty vector. These results suggest that more than one biological property of high-risk HPV E6 proteins contribute to activating mTORC1 and increasing cap dependent translation, including p53 binding and association with proteins that contain a LXXLL motif. Multiple proteins have been reported to associate with HPV16 E6 through an LXXLL motif, including Paxillin and E6BP, but the best characterized is the E3 ubiquitin ligase E6AP (UBE3A). The increase in cap dependent translation was shared between high- and low-risk HPV E6 proteins, and one of the only known biological properties that are shared amongst all mucosal E6 proteins is the association of E6AP. To test the hypothesis that a functional LXXLL binding motif is required for the ability of mucosal HPVE6 proteins to activate mTOR and translation, we generated LXXLL binding motif mutants of mucosal HPV E6 proteins that had lost the association with E6AP and tested them for activation of cap dependent translation and mTORC1. Upregulation of mTORC1 activity and cap dependent translation were abrogated by mutation of the LXXLL binding motif, suggesting that mucosal HPV E6 proteins mediate these events by associating with LXXLL-containing binding partners.

**Chapter 4: The HPV16 E6 oncoprotein phosphorylates receptor protein tyrosine kinases and increases internalization of phosphorylated receptor species**

In order to address whether HPV16 E6 expression activates signaling cascades upstream of PDK1 and mTORC2, we evaluated the tyrosine phosphorylation of receptor protein tyrosine kinases (RPTKs). Phospho-tyrosine immunoprecipitation demonstrated an increase in tyrosine phosphorylated ErbB family members but not EphRA2 or PDGFR RPTKs under normal growth conditions and a maintenance of tyrosine phosphorylated ErbB family members upon EGF

withdrawal. Moreover, HPV16 E6 maintained the tyrosine phosphorylation of EGFR and IRβ/IGF1β-R when starved of EGF. HPV16 E6 expression shortened EGFR half-life, suggesting an HPV E6 mediated effect on receptor internalization. HPV16 E6 indeed increased internalization of phosphorylated and activated EGFR/IRβ/IGF1β-R receptor species as determined by internalization assays. To identify HPV E6 associated proteins that may be contributing to RPTK activation, we performed large scale HA affinity purification followed by mass spectrometry. HPV16 E6 associated complexes contained both known and novel binding partners, including the signaling adaptor protein Grb2. Association of Grb2 with HPV16 E6 was confirmed, and subsequent Grb2 siRNA knockdown demonstrated that Grb2 is required for efficient E6 mediated mTORC1 activation.

**General Discussion and Future Directions**

The results presented in this thesis demonstrate that HPV16 E6 activates mTORC1 and downstream signaling cascades in primary human foreskin keratinocytes. The HPV16 E6 mediated mTORC1 activation causes an increase in cap dependent translation. To determine the mechanism of mTORC1 activation, we evaluated growth factor associated AKT signaling and found that HPV16 E6 expressing primary HFKs maintain increased AKT activation during nutrient deprivation through PBS starvation. Although nutrient deprivation is not necessary to detect HPV16 E6 mediated activation of S6K, S6, or 4E-BP1, it is required to unmask the effects of HPV16 E6 expression on AKT activity. Under normal growth conditions, HFKs expressing control vector have a high basal phosphorylation of AKT at both S473 and T308. It is possible that primary HFKs maintain high basal AKT phosphorylation because their growth in a monolayer culture is different from the growth in normal stratified epithelia, where only cells in the basal epithelia are dividing. The monolayer culture might mimic the basal epithelia and thus stimulate proliferation in part through the activation of AKT. Starving the HFKs in PBS reduced AKT activation in control cells because the stimulus resulting in AKT phosphorylation had been removed. In contrast, HPV16 E6 expressing HFKs maintained AKT phosphorylation because the signal promoting AKT phosphorylation is associated with HPV16 E6 expression rather than nutrient availability. PDK1 and mTORC2 remained active in HPV16 E6 expressing HFKs experiencing PBS starvation, suggesting that PDK1 and mTORC2 are involved in HPV16 E6 mediated AKT activation. Since this work was published, it was demonstrated that AKT may be phosphorylated by a third kinase, IKKε/TBK1 (Ou et al., 2011; Xie et al., 2011) at residues S473 and T308. The potential role of IKKε/TBK1 in HPV16 E6 mediated AKT activation was not investigated in this thesis. It should be noted that HPV16 E6 had previously been reported to

activate mTORC1 (Lu et al., 2004). The reported mechanism for HPV16 E6 mediated mTORC1 activation was different from what we observed. The authors showed that S6K and S6 phosphorylation is increased in HEK293 cells with transient HPV16 E6 expression. GST pulldowns were presented that showed that only HPV16 E6, and not E6 proteins from other HPV types, associates with TSC2. We were unable to reproduce these experiments in model cells.

Mutational analysis of HPV16 E6 suggests that multiple biological properties of E6 contribute to the activation of mTORC1 and cap dependent translation. Inactivation of the HPV16 E6 LXXLL binding motif decreased activation of mTORC1 and cap dependent translation. This may be the result of the association with the ubiquitin ligase E6AP/UBE3A with HPV16 E6 through its LXXLL motif. Alternatively, the association of HPV16 E6 with other proteins via the LXXLL binding motif may play a role in the activation of mTORC1 and cap dependent translation. Although the LXXLL binding motif is important for high-risk HPV E6 mediated increase in cap dependent translation, mutation of this motif is not sufficient to abrogate the increase of cap dependent translation. The association of HPV16 E6 with p53 additionally contributes to the activation of cap dependent translation, whereas mutation of the HPV16 E6 PDZ binding domain had only minor effects. Moreover, low-risk mucosal HPV E6 proteins that lack PDZ binding domains also increase mTORC1 activity and cap dependent translation. Therefore, it is clear that HPV16 E6 and other HPV E6 proteins activate mTORC1 and cap dependent translation independent of the association with PDZ proteins. Thus, multiple biological functions of high-risk HPV E6 proteins, including an intact LXXLL binding motif and the association with p53, play a role in these processes. These functions may be linked to the known biological activities of HPV16 E6. For example, the association of HPV16 E6 with E6AP

through the E6AP LXXLL motif targets many proteins for degradation, some of which may be important in RPTK or mTORC1 signal transduction.

Receptor protein tyrosine kinases (RPTKs) are one of several mechanisms that can initiate AKT activation. Since HPV16 E6 mediated AKT activation was revealed through nutrient deprivation, we evaluated RPTK phosphorylation and activation in HPV16 E6 expressing primary HFKs under normal growth conditions and after EGF withdrawal. Indeed, HPV16 E6 caused an increase in tyrosine phosphorylation of a subset of RPTKs, as identified by a combination of immunoprecipitations and Western blotting. These receptors are considered activated because they are phosphorylated at known residues that promote receptor association with signaling adaptor proteins and the activation of downstream signaling cascades. HPV16 E6 increased the internalization and degradation of phosphorylated receptor species, which suggests that HPV16 E6 causes increased receptor turnover. It has been shown that EGFR internalization is required for the maintenance of signaling events downstream of EGFR (Goh et al., 2010; Sigismund et al., 2008). It is possible that HPV16 E6 causes an increase in receptor activation and internalization, and increased receptor degradation is a bystander effect of increased activation. Interestingly, EGFR activation has been studied in the benign respiratory papillomas caused by low-risk mucosal HPV infection (Johnston et al., 1999). HPV types 6b and 11 are associated with respiratory papillomas, and the E6 proteins from HPV6b and HPV11 have been implicated in increased EGFR expression. EGFR expression was not due to a gene amplification event, nor was it associated with an increase in mRNA expression. Instead low-risk mucosal HPV E6 proteins upregulated EGFR recycling to the cell surface by approximately 20% (Johnston et al., 1999). Low-risk HPV E6 associated increase in EGFR expression was shown to promote EGFR tyrosine phosphorylation and MAPK activity. In contrast to the studies on

respiratory papillomas, we have not detected an increase in total receptor levels in HPV16 E6 expressing HFKs, but rather a specific increase in phosphorylated receptor species.

In order to investigate how HPV16 E6 activates RPTK signaling, we aimed to identify potential binding partners of E6 that may mediate its effects on RPTK and/or mTORC1 signaling. Therefore, we performed affinity purifications and mass spectrometry with E6 proteins from multiple HPV types (high-risk HPV16 and HPV18; low-risk HPV6b and HPV11; cutaneous HPV5 and HPV8; HPV16 E6 mutants deficient in E6AP binding and association with PDZ proteins). This approach yielded multiple previously known and novel candidate interactors (Appendices 1 and 2). Several of the HPV16 E6 associated proteins that we identified in these studies have been implicated in mTORC1 signaling. We validated that HPV16 E6 associates with the signaling adaptor protein Grb2, and showed that Grb2 knockdown reduces HPV16 E6 mediated mTORC1 activation. Grb2 is important for the activation of EGFR, IGFR and IR signaling pathways and has been implicated in receptor internalization (Wang and Moran, 1996). Given these data we have developed the model that HPV16 E6 induces the internalization and activation of several RPTKs and causes the activation of mTORC1 and cap dependent translation (Fig 5.1). Moreover, the association of HPV16 E6 with Grb2 could potentially contribute to relaying EGFR, IRβ and IGFR1β-R activation to downstream effectors. The association of HPV16 E6 with Grb2 may functionally modify the Grb2 complex and in turn enhance Grb2 function. This could be manifested as an increase in Grb2 association with endosomes, which would increase receptor internalization similar to what was reported in this dissertation. For example, HPV16 E6 may promote the association of additional proteins with Grb2, and enhance Grb2 function. This may be through the targeted degradation of a Grb2 repressor. Regardless of the specific mechanism, knockdown of Grb2 indicates its importance in HPV16 E6 mediated

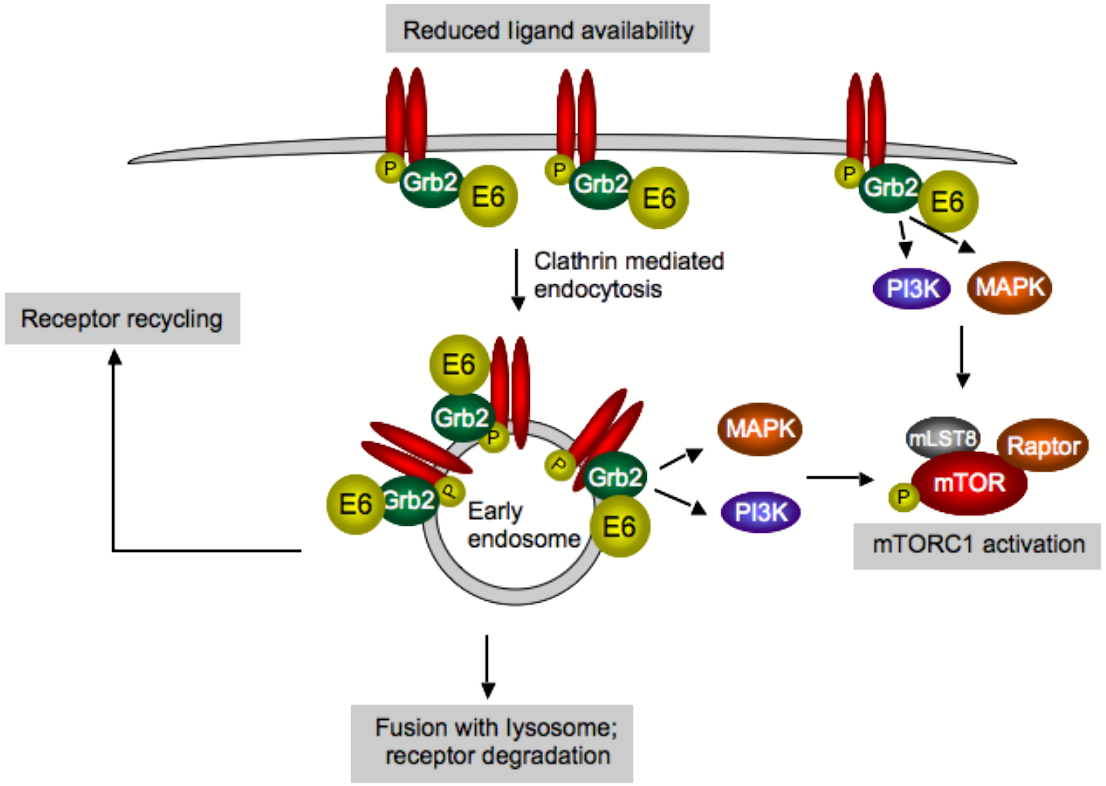


**Figure 5.1. Model: The ability of HPV16 E6 to increase the internalization of activated RPTKs causes mTORC1 activation and an increase in cap dependent translation.** HPV16 E6 expressing cells maintain activation of RPTKs and downstream signaling pathways in the absence of ligand. We hypothesize that the association between Grb2 and HPV16 E6 is necessary to increase the internalization of activated receptor species, which may cause the activation of both MAPK and mTORC1 signaling cascades described throughout this dissertation.

mTORC1 activation. Further experiments to test the above mechanisms, such as evaluating RPTK and Grb2 subcellular localization in the presence of HPV16 E6 are underway. We also intend to address whether Grb2 is important in the HPV16 E6 mediated increase in cap dependent translation.

The combination of affinity purification and mass spectrometry indicated additional known and novel HPV16 E6 associated proteins that have previously been implicated in RPTK or mTORC1 signaling. All three members of the CASK/Dlg/Lin7C complex were detected, which has previously been described to associate with HPV16 or HPV18 E6 proteins. This complex has been shown to recognize RPTKs through their PDZ domains, as EGF receptors, for instance, have carboxyl-terminal PDZ binding domains (reviewed in (Nourry et al., 2003). Moreover, this complex is involved in the biosynthetic trafficking and stability of ErbB RPTKs at the basolateral plasma membrane (Shelly et al., 2003). Dlg is targeted for ubiquitin mediated degradation by high-risk HPV E6 proteins (Thomas et al., 2005). It is possible that HPV16 E6 may bind and functionally modify this complex, increasing the activation of downstream signaling cascades. This may be the result of targeted degradation of complex components, the association of new proteins with this complex, or both. Further, the affinity purification suggested that HPV16 E6 associates with the GIGYF2 protein. GIGYF2 interacts with Grb10, which is phosphorylated by mTORC1 and participates in the negative feedback regulation of insulin signaling (Hsu et al., 2011; Yu et al., 2011). The association between HPV16 E6 and GIGYF2 may disrupt this negative feedback loop, and thus result in hyperactive mTORC1 signaling. In addition, the TIPRL protein was identified as putative E6 interactor. TIPRL negatively regulates the PP2A phosphatase that can dephosphorylate multiple components of the mTORC1 signaling pathway, including S6K and AKT (Peterson et al., 1999; Schalm et al.,

2005; Ugi et al., 2004). We favor a general model in which multiple HPV16 E6 binding partners activate RPTK and mTORC1 signaling. While Grb2 knockdown is sufficient to abrogate mTORC1 activity as seen by a reduction in S6K phosphorylation, other HPV16 E6 associated proteins, including those described above, may activate MAPK signaling and subsequently mTOR (Figure 5.2). Future studies should deplete cellular Grb2 in combination with other putative E6 binding partners and evaluate S6K as well as ERK phosphorylation.

We also evaluated the ability of E6 proteins from other HPV types to activate mTORC1. E6 proteins from high- and low-risk mucosal HPV types increase mTORC1 activity, while cutaneous HPV E6 proteins do not activate mTORC1. This is in accordance with the regulation of cap-dependent translation by HPV E6 proteins, as only mucosal, but not cutaneous, HPV E6 proteins upregulate cap-dependent translation and establishes a strong correlation between mTOR activity and cap-dependent translation in HPV E6 expressing keratinocytes. High-risk HPV16 and HPV18 E6 proteins increase cap dependent translation the most in primary HFKs, whereas low-risk mucosal HPV E6 proteins from type 6b and 11 do so to a lesser extent. We hypothesize that the observed higher levels of high-risk HPV E6 mediated cap dependent translation are caused by more than one biological function of the high-risk HPV E6 protein. This is supported by reporter assays using HPV16 E6 mutants and the identification of several putative E6 binding partners that are involved in RPTK and/or mTORC signaling. Although the LXXLL binding motif is important for high-risk HPV E6 mediated increase in cap dependent translation, mutation of this motif is not sufficient to abrogate this effect. In comparison, the LXXLL binding motif of low-risk mucosal HPV E6 proteins is required for them to activate cap dependent translation. RPTK signaling and internalization in low-risk mucosal HPV E6 proteins

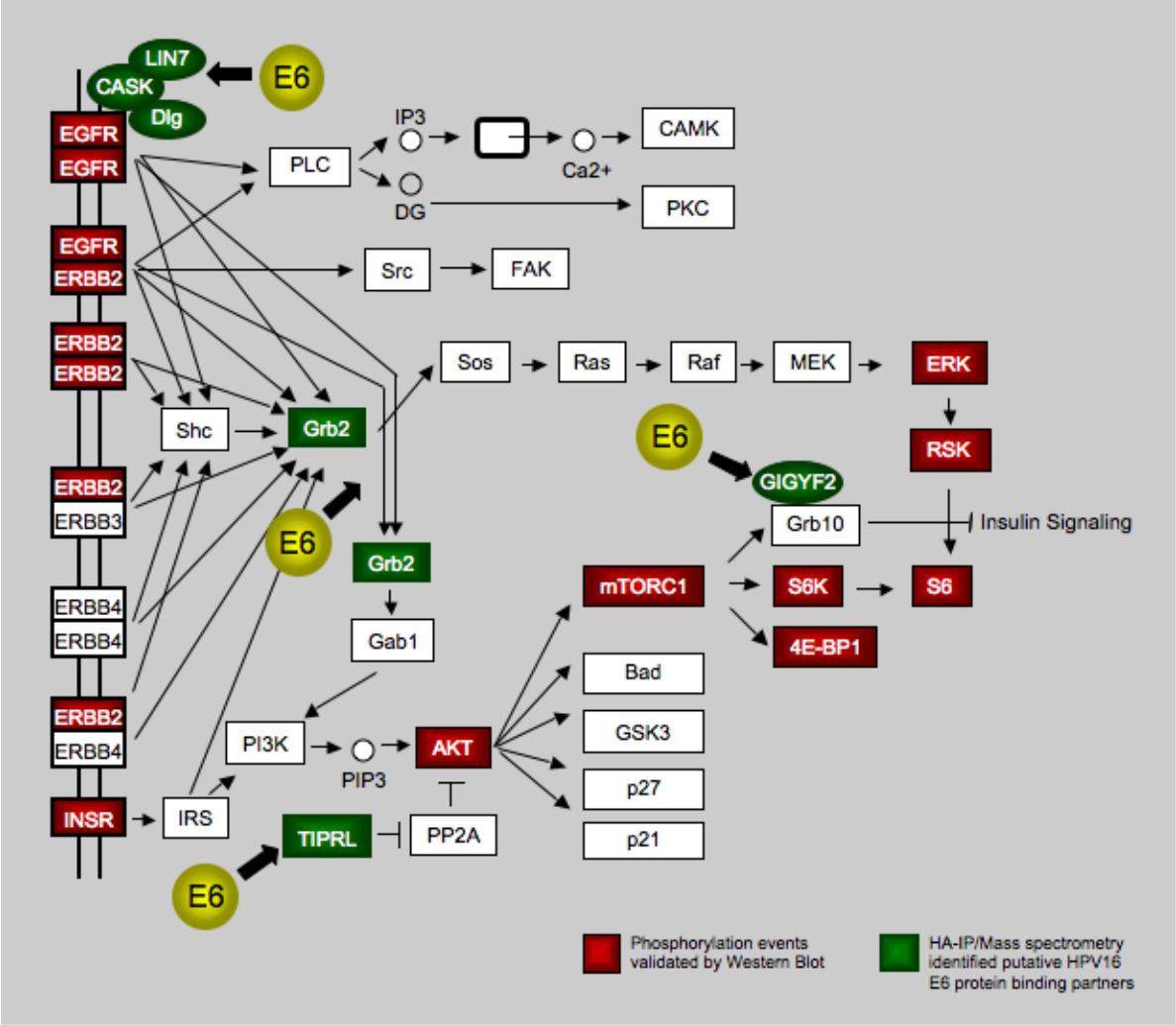


**Figure 5.2. The association of HPV16 E6 with multiple proteins may activate the RPTK signaling network.** HA- affinity purification and mass spectrometry identified several associated proteins involved in RPTK signaling. These proteins, including Grb2, GIGYF2, TIPRL, and members of the scaffolding complex with CASK-Dlg-Lin7C are shown in green. Also depicted are the signaling networks that HPV16 E6 has been shown to activate by phosphotyrosine immunoprecipitation and Western blot, which are shown in red. Diagram modified from KEGG pathways.

expressing HFKs remains to be investigated. Activated RPTKs increase mTORC1 activity, which in turn increases cap dependent translation. We found that all mucosal HPV E6 proteins tested activate mTORC1 and increase cap dependent translation. Given the causal relationship between RPTK activation and downstream signaling events, we hypothesize that low-risk mucosal HPV E6 proteins will also increase the phosphorylation and internalization of RPTKs.

In high-risk HPV associated malignancies, the two viral oncoproteins E6 and E7 are coexpressed. HPV16 E7 was previously described to trigger the trophic sentinel response in serum starved cells (Eichten et al., 2004). The trophic sentinel response is a form of cell death that arises from conflicting signals of cellular growth in the absence of available nutrients and growth factors. This type of cell death is independent of caspase activation, and cellular mitochondria remain functional and do not release cytochrome C (Eichten et al., 2004). HPV16 E7 expressing HFKs induce autophagy at low levels in normal growth conditions and higher levels in serum starved cells (Zhou and Munger, 2009). Additionally, HPV16 E7 associates with pyruvate kinase M2 (PKM2), causing a shift from the oxidative phosphorylation based metabolism to the less efficient anaerobic fermentation that is commonly observed in transformed cells (Zwerschke et al., 1999). By activating autophagy in the absence of serum, E7 expressing cells survived, likely short term, on the brink of cell death. Although LC3B positive autophagic puncta were not evaluated in cells co-expressing E6 and E7, the ability of E7 to induce the tropic sentinel response was abrogated by HPV16 E6 or dominant negative p53 co-expression. The HPV16 E7 associated trophic sentinel response was dependent on the targeted degradation of pRb and the presence of functional p53. These data suggested that HPV16 E6 mediated p53 degradation keeps cells alive and growing even under conditions of limited nutrient availability, underscoring the importance of p53 binding in E6 mediated mTORC1

activation. Under conditions of abundant nutrients, mTORC1 phosphorylates the autophagy regulatory kinase ULK1, inhibiting autophagy (Kim et al., 2011). Indeed, HPV16 E7 activates low levels of autophagy under normal growth conditions and enhanced autophagy when cells are serum deprived. However, HPV16 E6 activates mTORC1 in the absence of nutrients and growth factors, conditions in which mTORC1 would normally be inhibited and autophagy activated. In this dissertation, we show that that activation of mTORC1 and cap dependent translation is maintained in which the co-expression of the HPV16 E6 and E7 oncoproteins (Fig 2.7). Our data support a model that HPV16 E6 mediated mTORC1 activation counterbalances the HPV16 E7 induction of autophagy, instead increasing protein synthesis (Fig 5.3 and Appendix 7). One might envision that disrupting this balance by inhibiting mTOR activity may re-activate the trophic sentinel pathway and may be used therapeutically for HPV associated lesions and cancer. Consistent with this model a recent study in a mouse model of HPV associated anal cancer suggested efficacy of rapamycin in the treatment of such lesions (Stelzer et al., 2010).

There may be many reasons why HPVs cause RPTK and mTORC1 activation. Activation of growth factor associated signaling is likely to play a role in the viral life cycle. Since infection with low-risk mucosal HPV types are rarely associated with carcinogenesis, the ability of mucosal HPV E6 proteins to activate mTORC1 and to enhance cap-dependent translation are probably related to a common requirement during the viral life cycle. Moreover, HPV mediated activation of mTORC1 and cap dependent translation may support translation of viral mRNA. mTORC1 has been shown to specifically increase the translation of several types of mRNAs. mTORC1 activation increases the translation of mRNAs that contain a 5' terminal oligopyrimidine tract (5' TOP) in a short and unstructured 5' untranslated region (5'UTR). This includes genes encoding ribosomal RNAs. mTORC1 activation also increases the translation of

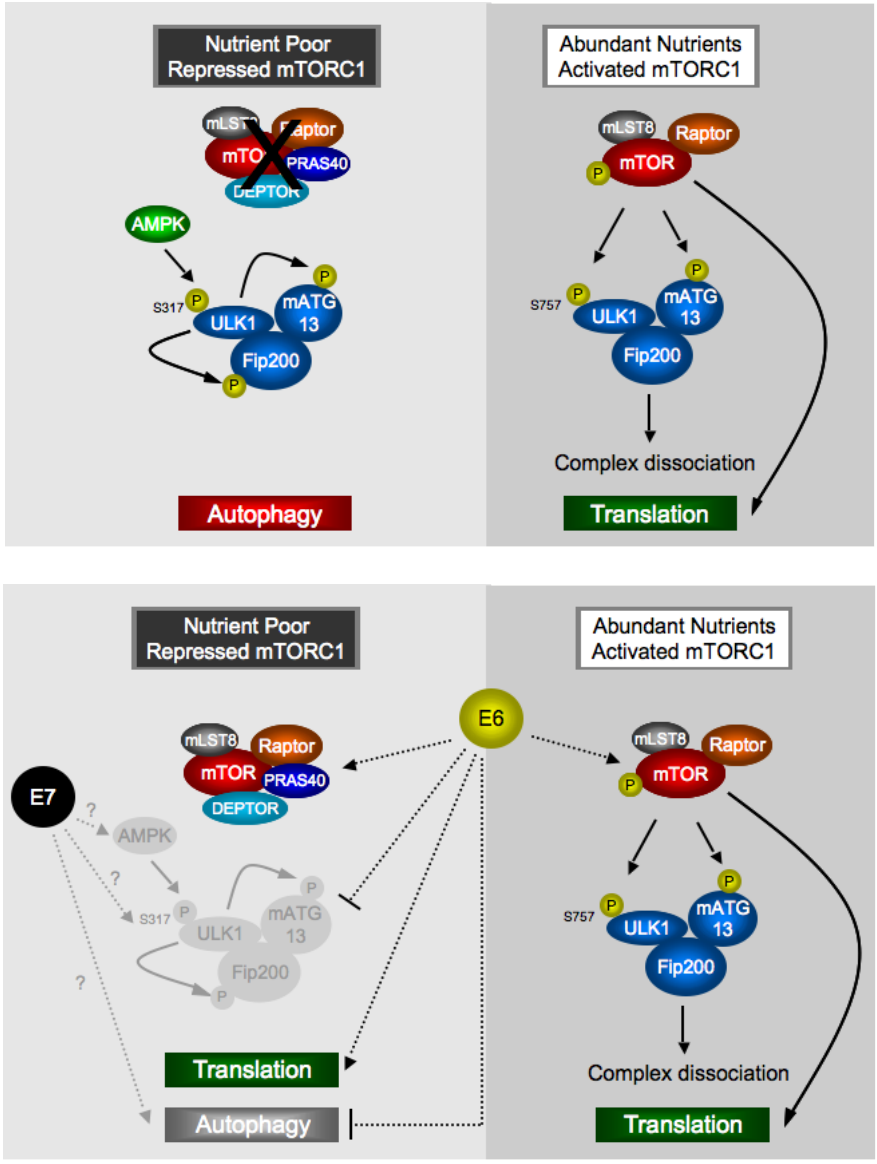


**Figure 5.3. The regulation of mTORC1 and autophagy in the presence and absence of the HPV16 E6 and E7 oncoproteins.** (Top) In the absence of HPV infection, abundant growth factors, energy and nutrients cause the activation of mTORC1. mTORC1 phosphorylates the autophagy regulated kinase ULK1, inactivating ULK1 and causing the dissociation of the ULK1/mAtg13/Fip200 complex (right). Under conditions of restricted growth factors, energy, and nutrients, mTORC1 is inactive. The AMP activated kinase (AMPK1) then phosphorylates ULK1, activating the ULK1/mAtg13/Fip200 complex and promotes autophagy (left). (Bottom) However, upon HPV16 infection, the HPV16 E6 and E7 oncoproteins are expressed. Conditions of limited nutrients such as serum deprivation causes an E7 induced trophic sentinel response and autophagy, through an as-yet identified mechanism that is dependent on the ability of E7 to target pRb for degradation. However, co-expression of HPV16 E6 counterbalances the effects of E7. HPV16 E6 causes ligand independent activation of RPTKs, in the absence of growth factors. This activates downstream cascades including mTORC1, which dampens the E7 mediated increase in autophagy by phosphorylating and inactivating the ULK1/mAtg13/Fip200 complex.

genes with long and highly structured 5'UTRs, typically greater than several hundred base pairs, including HIF1α, CyclinD1, and Myc. We have shown that the protein levels of the mTORC1 target genes CyclinD1 and HIF1α are increased in HPV16 E6 expressing primary cells (data not shown). The post transcriptional increase of CyclinD1 level is promote completion of the viral lifecycle and tumorigenesis by promoting DNA synthesis at the G1 to S phase transition by activating the CyclinD1/CDK4/CDK6 complexes. HPV16 E6 mediated increase in HIF1α may increase its activity as a transcription factor, inducing the expression of target genes including glucose transporters that promote the uptake and utilization of energy. We have also detected an increase in the protein level of the HIF1α target gene and glucose transporter GLUT 1 under normal growth conditions and conditions of nutrient deprivation (data not shown). Collectively these data support HPV16 E6 mediated activation of mTORC1. Given that HPV transcripts contain a relatively short 5' UTR, it is possible that one or several may contain a 5' TOP that is regulated by mTORC1. In fact, nuclease protection assays demonstrated that a subset of the HPV31b E6/E7 transcripts include a short 5'UTR of 33 nucleotides and contains a four nucleotide polypyrimidine tract (5' TOP) (Ozbun and Meyers, 1998). It is unknown if this short 5' UTR is unstructured; if so, these data would suggest that the translation of some HPV transcripts may be regulated by mTORC1. There is evidence HPV16 E7 expression is increased upon induction of cellular differentiation, and mTORC1 inhibition abrogates increased HPV16 E7 expression. This further supports the hypothesis that the translation of some HPV transcripts is regulated by mTORC1 (Oh et al., 2006). I also evaluated the ability of HPV16 E6 to increase general protein synthesis with a pulse chase experiment using $^{35}$S-labelled Methionine incorporation. Unfortunately I was unable to detect an E6-mediated increase in general protein synthesis.

Interestingly, all HPVs, including those that infect the cutaneous epithelia, require adequate production of viral and cellular proteins necessary for viral genome replication and progeny virion production. It is thus surprising that cutaneous HPV E6 proteins do not detectably stimulate cap-dependent translation (Fig. 3.1) or activate mTORC1 signaling (preliminary data not shown). Tissue tropism may have driven mucosal HPV E6 proteins to evolve a distinct repertoire of biological properties and could explain the specificity of mucosal HPV E6 mediated activation of mTORC1 and cap dependent translation. Infection of the mucosal epithelium and successful viral genome replication and progeny virion production involves unique requirements. This may be the result of different gene expression profiles between cutaneous and mucosal epithelium. Transcriptional regulation of viral genes is also different between mucosal and cutaneous HPVs. Introduction of HPV16 and HPV5 long control region (LCR) reporter constructs into cutaneous and mucosal epithelial cells demonstrated that appropriate cellular tropism is important for robust transcriptional activation of the LCR (Mistry et al., 2007). This cell type dependent promoter activation may be caused by the differential expression and participation of transcription factors or transcriptional coactivators. Given these apparent differences in the cellular environment of cutaneous and mucosal epithelia, it is tempting to speculate that corresponding HPV types may have evolved distinct molecular strategies to exploit the available host cellular environment.

HPVs infect the basal epithelium, which is nutrient rich. Basal cells then divide asymmetrically, giving rise to one daughter cell that remains in the basal epithelia and maintains stem cell like properties, and one daughter cell that begins the process of differentiation. Differentiated cells are non-dividing and, therefore DNA replication does not occur. However, expression of high-risk HPV E6 and E7 proteins maintain S phase competence and promote

DNA replication through the targeted degradation of p53 and pRb, respectively. The maintenance of RPTKs and mTORC1 activity in an environment that is presumably limited in energy, nutrients, and growth factors is likely important to the viral life cycle. IR/IGFR and ErbB RPTK ligand binding stimulates the activation of multiple downstream signaling cascades including mTORC1 that are implicated in cell migration, proliferation and growth (Fig 5.4). The stimulation of RPTK associated pathways may serve to indirectly promote genome replication and/or package progeny virions by increasing protein synthesis. Since HPV genome replication is dependent on the expression of host replication factors, one could envision that the ability of HPV16 E6 to increase the translation of capped mRNAs ensures adequate expression of cellular proteins that are necessary for viral genome replication. Increased mTORC1 activation may also directly increase the translation of viral proteins. This is possible considering the viral mRNAs are capped and polyadenylated by cellular machinery and are therefore suitable substrates for canonical cap dependent translation (Stacey et al., 2000; Zhao et al., 2005). As previously described, several HPV31b transcripts may contain a 5' TOP and are therefore suitable substrates for mTORC1 mediated activation of cap dependent translation. There is evidence for translational regulation of early protein synthesis during epithelial cell differentiation in HPV-positive cells. When HPV16-positive CaSki cervical carcinoma cells were cultured in methylcellulose- or $CaCl_2$-containing medium to induce differentiation, increased expression of the E7 oncoprotein was observed. This increase was not at the level of transcription or protein stability, but rather an increase in the association of HPV E7-encoding mRNAs to polysomes. Sustained phosphorylation of 4E-BP1 upon differentiation of CaSki cells but not with HPV-negative HaCaT cells or primary HFKs was also observed. Moreover, mTORC1 inhibition by rapamycin treatment reduced 4E-BP1 phosphorylation and HPV16 E7 oncoprotein expression in

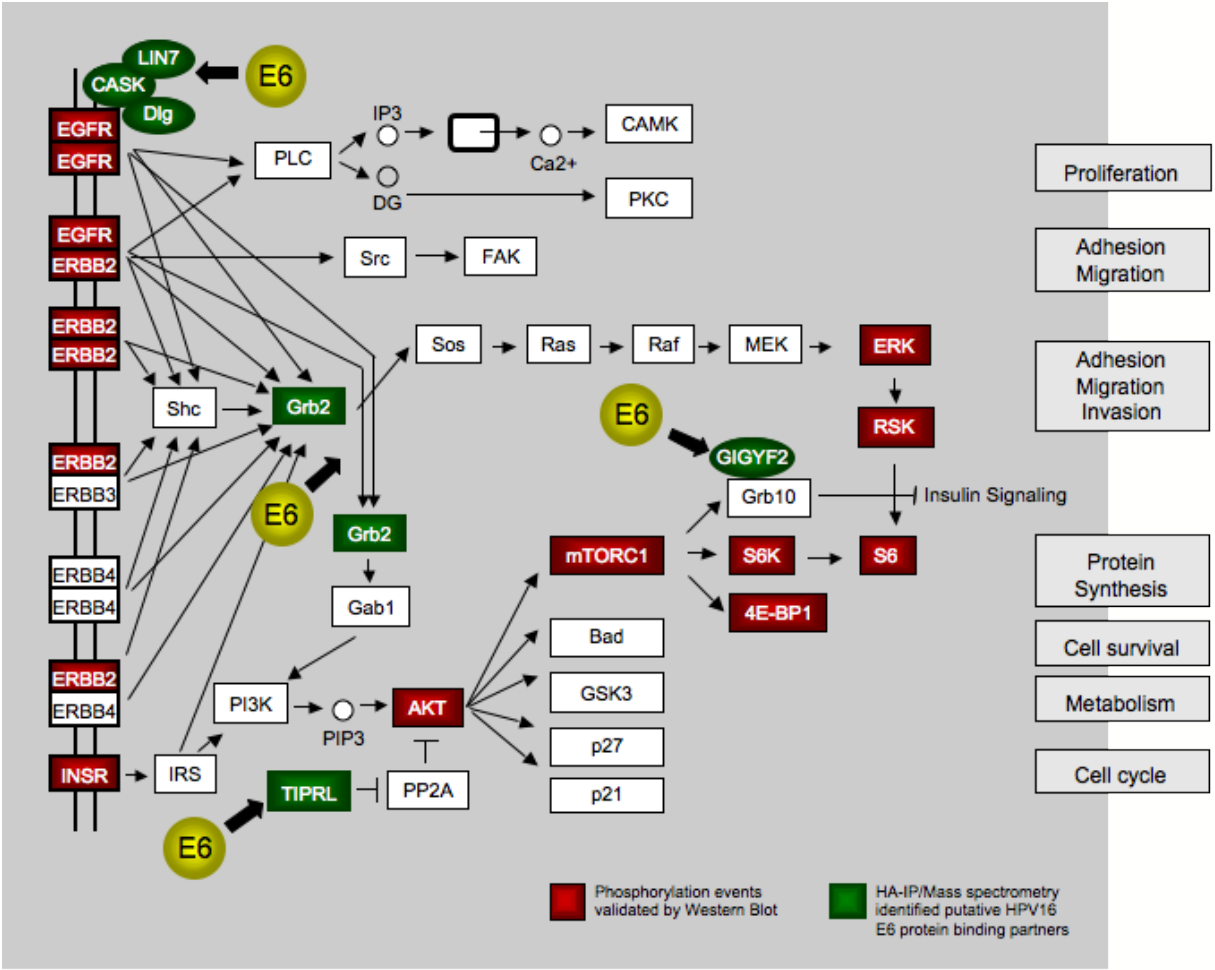


**Figure 5.4. HPV16 E6 mediated activation of RPTK/mTORC1 signaling networks regulates cellular processes that may be important in the viral lifecycle and transformation.** As shown in Figure 5.2, HPV16 E6 activates the RPTK and mTORC1 signaling networks through multiple proposed mechanisms including the association with Grb2. Ultimately this activation may maintain or further activate the cellular processes shown on the right (light grey boxes). Diagram modified from KEGG pathways.

these cells (Oh et al., 2006). Additionally, high levels of viral proteins, especially the L1 and L2 capsid proteins, need to be abundantly expressed during the late stage of productive viral replication. Given the virus replicates in the differentiated epithelium that is presumably nutrient deprived, we hypothesize that RPTK and mTORC1 activation are important to the late events of the viral life cycle, such as late gene expression or production of progeny virus. To delineate whether RPTK and mTORC1 activation is important for late events in the viral lifecycle, viral titer and infectivity could be assayed under conditions of RPTK and mTORC1 inhibition in the organotypic raft culture system. This system is a well established tissue culture model that mimics the differentiation state of natural stratified epithelia and is amenable to productive HPV infection (Meyers et al., 1992). Primary HFKs that stably express a control vector or the HPV16 genome can be grown in the presence of feeder cells at the media-air interface, promoting the formation of the stratified epithelia. The resulting raft cultures could be treated with RPTK or mTORC1 inhibitors and quantitative PCR would allow for monitoring viral titer and infectivity.

The mutational analysis of high-risk and low-risk HPV E6 proteins clarify that although the LXXLL binding motif is important for high- and-low-risk mucosal HPV E6 mediated activation of mTORC1 and cap dependent translation. Moreover, the association with p53 and PDZ proteins additionally contributes to the activation of mTORC1 and cap dependent translation by high-risk HPV E6 proteins. We favor a model in which mTORC1 activation that is mediated by low-risk mucosal HPV E6 proteins through the LXXLL binding motif does not support transformation. However, the association of high-risk HPV E6 proteins with p53, PDZ proteins, and proteins through the LXXLL binding motif leads to a more pronounced activation of mTORC1 that may promote a transformed phenotype. It remains to be seen whether HPV 16 E6 mediated RPTK or mTORC1 activation promote transformation. Interestingly, an EGFR

mutant (ΔEGFR) that lacks the extracellular domain and thus activates downstream signaling cascades independent of ligand binding has been reported. ΔEGFR supports enhanced tumorigenicity and is associated with glioblastomas (Nishikawa et al., 1994). HPV16 E6 activates RPTKs including EGFR, also in the absence of ligand (EGF withdrawal or PBS starvation), which would suggest that E6 may similarly contribute to transformation and tumorigenesis. In the future we would like to evaluate the effects of RPTK and mTORC1 inhibition on cell migration and anchorage independent growth in HPV16 E6 expressing HFKs. The use of dominant negative p53 in place of HPV16 E6 in these assays may address if the additional HPV E6 mediated effects are due to the targeted degradation of p53. It is also possible that other functions of the high-risk HPV E6 protein may synergize with mTORC1 activation or even contribute to mTORC1 activation, and promote transformation.

mTORC1 integrates environmental cues of energy status, growth factor and nutrient availability and couples them with the activation of downstream signaling pathways. This allows for the coordinate regulation of multiple cellular processes including growth and proliferation based on the availability of nutrients. mTORC1 regulation is critical for normal cellular processes. The uncoupling of the upstream energy and nutrient supply with downstream signaling events is commonly observed in tumor cells. There is direct evidence that mTORC1 activation is associated with cancers. Several heritable genetic disorders in which mTORC1 negative regulators are mutated are associated with tumorigenesis. These include germline mutations in the TSC1/TSC2 and PTEN genes. Mutations of either the TSC1 or TSC2 gene products are associated with benign tumors or hamartomas in multiple organ systems (reviewed in (Tomasoni and Mondino, 2011)). Cowden's syndrome, caused by germline mutations in the gene that encodes the dual specificity phosphatase PTEN, is also associated with hamartomas

and an increased risk in the development of thyroid, breast, and endometrial cancers (Eng, 1998; Rustad et al., 2006). The importance of mTORC1 signaling is further highlighted by the observation that multiple proteins in the mTORC1 signaling cascade are tumor suppressors or oncogenes, and are frequently amplified or mutated in human cancers. Gene amplification of the receptor protein tyrosine kinase ErbB2 is a common event in breast cancers, which can lead to the activation of many downstream signaling pathways (Kallioniemi et al., 1992). The class $I_A$ PI3K catalytic subunit p110$\alpha$ (PIK3CA) is frequently amplified in cervical and ovarian cancers (Ma et al., 2000; Shayesteh et al., 1999). Alternatively, somatic PTEN mutations are amongst the most common mutations in multiple cancers, including glioblastoma, endometrial, and prostate cancers (Trotman et al., 2003).

The high-risk HPV E6 and HPV E7 oncoproteins share biological functions with proteins encoded by other DNA tumor viruses, including polyomaviruses SV40, Merkel cell polyomavirus (MCPyV), and murine polyomavirus, and adenoviruses. The PI3K/AKT/mTORC1 signaling axis is targeted for activation by many DNA tumor viruses. The ability of plasma membrane bound mouse polyoma Middle T antigen to activate AKT and other downstream mitogenic pathways through association and subsequent recruitment of the Class I PI3K p85 regulatory subunit has been well documented (Ichaso and Dilworth, 2001; Kaplan et al., 1987; Summers et al., 1998; Whitman et al., 1985). It was recently reported that the MCPyV Small T antigen causes aberrant hyperphosphorylation and activation of eukaryotic translation initiation factor 4E binding protein (4E-BP1). This study suggested that 4E-BP1 activation occurred independent of mTORC1 or mTORC2, but no actual mechanism was proposed (Shuda et al., 2011). DNA tumor viruses may activate the PI3K/AKT/mTORC1 axis through multiple distinct mechanisms, which are not mutually exclusive. The activation of RPTKs by DNA tumor viruses

may account for the activation of multiple downstream signaling pathways including MAPK, mTORC1 and FAK, as we described for HPV16 E6 in this thesis. Divergent types of DNA tumor viruses have possibly evolved unique mechanisms to perturb the same signaling pathways, as these are limiting for tumor viruses in general. This appears to be the case for AKT signaling, and the activation of RPTKs may also be shared, be it through similar or divergent mechanism(s). It is therefore tempting to speculate that oncoprotein mediated RPTK activation through engaging Grb2 and promoting the internalization of activated receptors is conserved amongst multiple DNA tumor viruses. We are currently addressing this question by evaluating MCPyV small T antigen for its effects on RPTK signaling.

The results of this thesis clearly indicate that HPV16 E6 activates RPTKs and mTORC1 signaling. The activation of RPTKs or mTORC1 may promote transformation together with other functions of HPV16 E6, but either function alone may not be sufficient. Nonetheless, high-risk HPV infection is the leading cause of cervical cancers. Therefore the ability of high-risk HPV E6 proteins to activate growth factor associated signaling cascades that are aberrantly activated in human cancers cannot be ignored. Inhibition of these signaling cascades appears to be a potential therapeutic target for HPV associated malignancies. Currently mTORC1 inhibition is in Phase I-III clinical trials as a viable treatment for a variety of cancer types. According to the National Institutes of Health, there are currently a number of clinical trials in which the mTORC1 inhibitors Rapamycin and Rapamycin analogues, or 'rapalogues,' Temsirolimus®, Sirolimus®, Everolimus® are being tested for the treatment of cervical cancers (clinicaltrials.gov). These drugs are also in various stages of clinical trials for treatment of head and neck cancers, which are approximately 25% HPV positive (Kreimer et al., 2005). Moreover, Rapamycin has been used successfully to treat HPV associated anal cancers in two preclinical

mouse models (Stelzer et al., 2010). EGFR inhibition is also under evaluation as a suitable treatment for cervical cancers. Moreover, mTORC1 inhibition with Rapamycin analogues is especially promising in cervical cancers as Rapamycin treatment has been shown to sensitize HPV positive CaSki cells to the apoptotic inducing agent paclitaxel/Taxol® (Faried et al., 2006). The EGFR monoclonal antibody inhibitor Cetuximab® is being tested for treatment of advanced cervical cancer (clinicaltrials.gov). Interestingly, combinatorial EGFR and mTORC1 inhibition is being tested in the treatment of multiple types of cancers including those of the head and neck. We could not identify any existing trials that aim to use combined EGFR and mTORC1 inhibition in the treatment of HPV associated cervical cancers (clinicaltrials.gov). This combined approach might be more promising in order to eliminate aberrant activation of EGFR and mTORC1 in HPV associated cervical cancer. It should be noted, however, that inhibition of EGFR can enhance the activation of signaling events downstream of IGFR (Knowlden et al., 2008).

To date, research with HPV16 E6 has identified many novel functions of this small protein, both in the viral life cycle and mechanisms that contribute to transformation. Together with HPV16 E7, these proteins have demonstrated their potent oncogenicity through targeting the tumor suppressor p53 and pRb, respectively, for degradation This dissertation identified novel functions of the HPV16 E6 oncoprotein that potentially contribute to unrestricted cell growth and proliferation during productive HPV infection. Therefore, these studies provide a basis for the continued exploration of using mTORC1 and RPTK inhibitors in the treatment of HPV positive human cancers. Furthermore, these studies provide insights into similarities and differences between high- and low-risk mucosal HPV E6 proteins and how they may respectively contribute to the viral life cycle. Therefore, studies regarding the perturbation of

growth factor associated signaling cascades by HPV E6 proteins will broaden our knowledge of the mechanisms by which HPV replicates in nutrient deprived tissues and potentially aid in the development of new therapies and treatments for high-and low-risk HPV associated lesions.

# REFERENCES

Akerman, G.S., Tolleson, W.H., Brown, K.L., Zyzak, L.L., Mourateva, E., Engin, T.S., Basaraba, A., Coker, A.L., Creek, K.E., and Pirisi, L. (2001). Human papillomavirus type 16 E6 and E7 cooperate to increase epidermal growth factor receptor (EGFR) mRNA levels, overcoming mechanisms by which excessive EGFR signaling shortens the life span of normal human keratinocytes. Cancer Res *61*, 3837-3843.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol *7*, 261-269.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K., and Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*, 912-915.

Barbosa, M.S., Lowy, D.R., and Schiller, J.T. (1989). Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. J Virol *63*, 1404-1407.

Batzer, A.G., Rotin, D., Urena, J.M., Skolnik, E.Y., and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol Cell Biol *14*, 5192-5201.

Bazenet, C.E., Gelderloos, J.A., and Kazlauskas, A. (1996). Phosphorylation of tyrosine 720 in the platelet-derived growth factor alpha receptor is required for binding of Grb2 and SHP-2 but not for activation of Ras or cell proliferation. Mol Cell Biol *16*, 6926-6936.

Belfiore, A., Frasca, F., Pandini, G., Sciacca, L., and Vigneri, R. (2009). Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev *30*, 586-623.

Bernard, H.U., Burk, R.D., Chen, Z., van Doorslaer, K., Hausen, H., and de Villiers, E.M. (2010). Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology *401*, 70-79.

Brimer, N., Lyons, C., and Vande Pol, S.B. (2007). Association of E6AP (UBE3A) with human papillomavirus type 11 E6 protein. Virology *358*, 303-310.

Budanov, A.V., and Karin, M. (2008). p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell *134*, 451-460.

Camus, S., Menendez, S., Cheok, C.F., Stevenson, L.F., Lain, S., and Lane, D.P. (2007). Ubiquitin-independent degradation of p53 mediated by high-risk human papillomavirus protein E6. Oncogene *26*, 4059-4070.

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Charette, S.T., and McCance, D.J. (2007). The E7 protein from human papillomavirus type 16 enhances keratinocyte migration in an Akt-dependent manner. Oncogene *26*, 7386-7390.

Chen, C.C., Jeon, S.M., Bhaskar, P.T., Nogueira, V., Sundararajan, D., Tonic, I., Park, Y., and Hay, N. (2010). FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor. Dev Cell *18*, 592-604.

Chen, J.J., Reid, C.E., Band, V., and Androphy, E.J. (1995). Interaction of papillomavirus E6 oncoproteins with a putative calcium-binding protein. Science *269*, 529-531.

Cheng, J., DeCaprio, J.A., Fluck, M.M., and Schaffhausen, B.S. (2009). Cellular transformation by Simian Virus 40 and Murine Polyoma Virus T antigens. Semin Cancer Biol *19*, 218-228.

Cole, S.T., and Danos, O. (1987). Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. J Mol Biol *193*, 599-608.

Day, P.M., Kines, R.C., Thompson, C.D., Jagu, S., Roden, R.B., Lowy, D.R., and Schiller, J.T. (2010). In vivo mechanisms of vaccine-induced protection against HPV infection. Cell Host Microbe *8*, 260-270.

Degenhardt, Y.Y., and Silverstein, S.J. (2001). Gps2, a protein partner for human papillomavirus E6 proteins. J Virol *75*, 151-160.

Dibble, C.C., Asara, J.M., and Manning, B.D. (2009). Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1. Mol Cell Biol *29*, 5657-5670.

Dong, G., Broker, T.R., and Chow, L.T. (1994). Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. J Virol *68*, 1115-1127.

Dong, L.Q., Landa, L.R., Wick, M.J., Zhu, L., Mukai, H., Ono, Y., and Liu, F. (2000). Phosphorylation of protein kinase N by phosphoinositide-dependent protein kinase-1 mediates insulin signals to the actin cytoskeleton. Proc Natl Acad Sci U S A *97*, 5089-5094.

Doorbar, J. (2006). Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) *110*, 525-541.

Doorbar, J., Ely, S., Sterling, J., McLean, C., and Crawford, L. (1991). Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature *352*, 824-827.

Dow, L.E., Elsum, I.A., King, C.L., Kinross, K.M., Richardson, H.E., and Humbert, P.O. (2008). Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling. Oncogene *27*, 5988-6001.

Dowling, R.J., Topisirovic, I., Fonseca, B.D., and Sonenberg, N. (2010). Dissecting the role of mTOR: lessons from mTOR inhibitors. Biochim Biophys Acta *1804*, 433-439.

Eichten, A., Rud, D.S., Grace, M., Piboonniyom, S.O., Zacny, V., and Munger, K. (2004). Molecular pathways executing the "trophic sentinel" response in HPV-16 E7-expressing normal human diploid fibroblasts upon growth factor deprivation. Virology *319*, 81-93.

Elston, R.C., Napthine, S., and Doorbar, J. (1998). The identification of a conserved binding motif within human papillomavirus type 16 E6 binding peptides, E6AP and E6BP. J Gen Virol *79 ( Pt 2)*, 371-374.

Emlet, D.R., Moscatello, D.K., Ludlow, L.B., and Wong, A.J. (1997). Subsets of epidermal growth factor receptors during activation and endocytosis. J Biol Chem *272*, 4079-4086.

Eng, C. (1998). Genetics of Cowden syndrome: through the looking glass of oncology. Int J Oncol *12*, 701-710.

Evander, M., Frazer, I.H., Payne, E., Qi, Y.M., Hengst, K., and McMillan, N.A. (1997). Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. J Virol *71*, 2449-2456.

Faried, L.S., Faried, A., Kanuma, T., Nakazato, T., Tamura, T., Kuwano, H., and Minegishi, T. (2006). Inhibition of the mammalian target of rapamycin (mTOR) by rapamycin increases chemosensitivity of CaSki cells to paclitaxel. Eur J Cancer *42*, 934-947.

Feng, W., Duan, X., Liu, J., Xiao, J., and Brown, R.E. (2009). Morphoproteomic evidence of constitutively activated and overexpressed mTOR pathway in cervical squamous carcinoma and high grade squamous intraepithelial lesions. Int J Clin Exp Pathol *2*, 249-260.

Fernandes, H., Cohen, S., and Bishayee, S. (2001). Glycosylation-induced conformational modification positively regulates receptor-receptor association: a study with an aberrant epidermal growth factor receptor (EGFRvIII/DeltaEGFR) expressed in cancer cells. J Biol Chem *276*, 5375-5383.

Ferrari, S., Bandi, H.R., Hofsteenge, J., Bussian, B.M., and Thomas, G. (1991). Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. J Biol Chem *266*, 22770-22775.

Flores, E.R., Allen-Hoffmann, B.L., Lee, D., and Lambert, P.F. (2000). The human papillomavirus type 16 E7 oncogene is required for the productive stage of the viral life cycle. J Virol *74*, 6622-6631.

Foster, K.G., Acosta-Jaquez, H.A., Romeo, Y., Ekim, B., Soliman, G.A., Carriere, A., Roux, P.P., Ballif, B.A., and Fingar, D.C. (2010). Regulation of mTOR complex 1 (mTORC1) by raptor Ser863 and multisite phosphorylation. J Biol Chem *285*, 80-94.

Fukuda, M., and Longnecker, R. (2007). Epstein-Barr virus latent membrane protein 2A mediates transformation through constitutive activation of the Ras/PI3-K/Akt Pathway. J Virol *81*, 9299-9306.

Gao, D., Inuzuka, H., Tan, M.K., Fukushima, H., Locasale, J.W., Liu, P., Wan, L., Zhai, B., Chin, Y.R., Shaik, S.*, et al.* (2011). mTOR Drives Its Own Activation via SCF(betaTrCP)-Dependent Degradation of the mTOR Inhibitor DEPTOR. Mol Cell *44*, 290-303.

Gao, Q., Kumar, A., Srinivasan, S., Singh, L., Mukai, H., Ono, Y., Wazer, D.E., and Band, V. (2000). PKN binds and phosphorylates human papillomavirus E6 oncoprotein. J Biol Chem *275*, 14824-14830.

Gao, Q., Srinivasan, S., Boyer, S.N., Wazer, D.E., and Band, V. (1999). The E6 oncoproteins of high-risk papillomaviruses bind to a novel putative GAP protein, E6TP1, and target it for degradation. Mol Cell Biol *19*, 733-744.

Garcia-Martinez, J.M., and Alessi, D.R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J *416*, 375-385.

Gardiol, D., Kuhne, C., Glaunsinger, B., Lee, S.S., Javier, R., and Banks, L. (1999). Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. Oncogene *18*, 5487-5496.

Gewin, L., Myers, H., Kiyono, T., and Galloway, D.A. (2004). Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. Genes Dev *18*, 2269-2282.

Gingras, A.C., Gygi, S.P., Raught, B., Polakiewicz, R.D., Abraham, R.T., Hoekstra, M.F., Aebersold, R., and Sonenberg, N. (1999). Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev *13*, 1422-1437.

Gingras, A.C., Raught, B., Gygi, S.P., Niedzwiecka, A., Miron, M., Burley, S.K., Polakiewicz, R.D., Wyslouch-Cieszynska, A., Aebersold, R., and Sonenberg, N. (2001). Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev *15*, 2852-2864.

Gingras, A.C., and Sonenberg, N. (1997). Adenovirus infection inactivates the translational inhibitors 4E-BP1 and 4E-BP2. Virology *237*, 182-186.

Giroglou, T., Florin, L., Schafer, F., Streeck, R.E., and Sapp, M. (2001). Human papillomavirus infection requires cell surface heparan sulfate. J Virol *75*, 1565-1570.

Glaunsinger, B.A., Lee, S.S., Thomas, M., Banks, L., and Javier, R. (2000). Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. Oncogene *19*, 5270-5280.

Glondu-Lassis, M., Dromard, M., Lacroix-Triki, M., Nirde, P., Puech, C., Knani, D., Chalbos, D., and Freiss, G. (2010). PTPL1/PTPN13 regulates breast cancer cell aggressiveness through direct inactivation of Src kinase. Cancer Res *70*, 5116-5126.

Gloss, B., Bernard, H.U., Seedorf, K., and Klock, G. (1987). The upstream regulatory region of the human papilloma virus-16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. EMBO J *6*, 3735-3743.

Goh, L.K., Huang, F., Kim, W., Gygi, S., and Sorkin, A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol *189*, 871-883.

Goh, L.K., Huang, F., Kim, W., Gygi, S., and Sorkin, A. (2010). Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol *189*, 871-883.

Goldstein, B.J., Bittner-Kowalczyk, A., White, M.F., and Harbeck, M. (2000). Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein-tyrosine phosphatase 1B. Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein. J Biol Chem *275*, 4283-4289.

Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A., and Carraway, K.L., 3rd (1994). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci U S A *91*, 8132-8136.

Haj, F.G., Markova, B., Klaman, L.D., Bohmer, F.D., and Neel, B.G. (2003). Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. J Biol Chem *278*, 739-744.

Haj, F.G., Verveer, P.J., Squire, A., Neel, B.G., and Bastiaens, P.I. (2002). Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. Science *295*, 1708-1711.

Halbert, C.L., Demers, G.W., and Galloway, D.A. (1992). The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells. J Virol *66*, 2125-2134.

Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R.*, et al.* (2004). The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol *166*, 213-223.

Havre, P.A., Yuan, J., Hedrick, L., Cho, K.R., and Glazer, P.M. (1995). p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res *55*, 4420-4424.

Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*, 905-909.

Hellner, K., and Munger, K. (2011). Human papillomaviruses as therapeutic targets in human cancer. J Clin Oncol *29*, 1785-1794.

Hernandez-Sanchez, C., Blakesley, V., Kalebic, T., Helman, L., and LeRoith, D. (1995). The role of the tyrosine kinase domain of the insulin-like growth factor-I receptor in intracellular signaling, cellular proliferation, and tumorigenesis. J Biol Chem *270*, 29176-29181.

Holz, M.K., and Blenis, J. (2005). Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. J Biol Chem *280*, 26089-26093.

Howie, H.L., Katzenellenbogen, R.A., and Galloway, D.A. (2009). Papillomavirus E6 proteins. Virology *384*, 324-334.

Hsu, P.P., Kang, S.A., Rameseder, J., Zhang, Y., Ottina, K.A., Lim, D., Peterson, T.R., Choi, Y., Gray, N.S., Yaffe, M.B*., et al.* (2011). The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. Science *332*, 1317-1322.

Huh, K., Zhou, X., Hayakawa, H., Cho, J.Y., Libermann, T.A., Jin, J., Harper, J.W., and Munger, K. (2007). Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor. J Virol *81*, 9737-9747.

Humbert, P.O., Grzeschik, N.A., Brumby, A.M., Galea, R., Elsum, I., and Richardson, H.E. (2008). Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module. Oncogene *27*, 6888-6907.

Hwang, E.S., Nottoli, T., and Dimaio, D. (1995). The HPV16 E5 protein: expression, detection, and stable complex formation with transmembrane proteins in COS cells. Virology *211*, 227-233.

Ichaso, N., and Dilworth, S.M. (2001). Cell transformation by the middle T-antigen of polyoma virus. Oncogene *20*, 7908-7916.

Ilves, I., Kivi, S., and Ustav, M. (1999). Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites. J Virol *73*, 4404-4412.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.

Inoki, K., Zhu, T., and Guan, K.L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol *11*, 113-127.

Jastrzebski, K., Hannan, K.M., Tchoubrieva, E.B., Hannan, R.D., and Pearson, R.B. (2007). Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. Growth Factors *25*, 209-226.

Jing, M., Bohl, J., Brimer, N., Kinter, M., and Vande Pol, S.B. (2007). Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. J Virol *81*, 2231-2239.

Johnston, D., Hall, H., DiLorenzo, T.P., and Steinberg, B.M. (1999). Elevation of the epidermal growth factor receptor and dependent signaling in human papillomavirus-infected laryngeal papillomas. Cancer Res *59*, 968-974.

Julien, L.A., Carriere, A., Moreau, J., and Roux, P.P. (2010). mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol Cell Biol *30*, 908-921.

Kallioniemi, O.P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L.C., Smith, H.S., Waldman, F.M., Pinkel, D., and Gray, J.W. (1992). ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. Proc Natl Acad Sci U S A *89*, 5321-5325.

Kaplan, D.R., Whitman, M., Schaffhausen, B., Pallas, D.C., White, M., Cantley, L., and Roberts, T.M. (1987). Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell *50*, 1021-1029.

Keshwani, M.M., von Daake, S., Newton, A.C., Harris, T.K., and Taylor, S.S. (2011). Hydrophobic motif phosphorylation is not required for activation loop phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1). J Biol Chem *286*, 23552-23558.

Kessis, T.D., Slebos, R.J., Nelson, W.G., Kastan, M.B., Plunkett, B.S., Han, S.M., Lorincz, A.T., Hedrick, L., and Cho, K.R. (1993). Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc Natl Acad Sci U S A *90*, 3988-3992.

Khalifa, Y.B., Teissier, S., Tan, M.K., Phan, Q.T., Daynac, M., Wong, W.Q., and Thierry, F. (2011). The human papillomavirus E6 oncogene represses a cell adhesion pathway and

disrupts focal adhesion through degradation of TAp63beta upon transformation. PLoS Pathog *7*, e1002256.

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol *13*, 132-141.

Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T., and Ishibashi, M. (1997). Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A *94*, 11612-11616.

Klingelhutz, A.J., Foster, S.A., and McDougall, J.K. (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature *380*, 79-82.

Knipe, D.M., Howley, P.M. (2006). Fields Virology. *5*.

Knowlden, J.M., Jones, H.E., Barrow, D., Gee, J.M., Nicholson, R.I., and Hutcheson, I.R. (2008). Insulin receptor substrate-1 involvement in epidermal growth factor receptor and insulin-like growth factor receptor signalling: implication for Gefitinib ('Iressa') response and resistance. Breast Cancer Res Treat *111*, 79-91.

Kranjec, C., and Banks, L. A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. J Virol *85*, 1757-1764.

Kranjec, C., and Banks, L. (2011). A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. J Virol *85*, 1757-1764.

Kreimer, A.R., Clifford, G.M., Boyle, P., and Franceschi, S. (2005). Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev *14*, 467-475.

Kumar, V., Sabatini, D., Pandey, P., Gingras, A.C., Majumder, P.K., Kumar, M., Yuan, Z.M., Carmichael, G., Weichselbaum, R., Sonenberg, N.*, et al.* (2000). Regulation of the rapamycin and FKBP-target 1/mammalian target of rapamycin and cap-dependent initiation of translation by the c-Abl protein-tyrosine kinase. J Biol Chem *275*, 10779-10787.

Kung, C.P., and Raab-Traub, N. (2008). Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor through effects on Bcl-3 and STAT3. J Virol *82*, 5486-5493.

Lane, M.D., Ronnett, G.V., Kohanski, R.A., and Simpson, T.L. (1985). Posttranslational processing of the insulin proreceptor. Curr Top Cell Regul *27*, 279-292.

Laprise, P., Viel, A., and Rivard, N. (2004). Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. J Biol Chem *279*, 10157-10166.

Lazarczyk, M., Cassonnet, P., Pons, C., Jacob, Y., and Favre, M. (2009). The EVER proteins as a natural barrier against papillomaviruses: a new insight into the pathogenesis of human papillomavirus infections. Microbiol Mol Biol Rev *73*, 348-370.

Lechner, M.S., and Laimins, L.A. (1994). Inhibition of p53 DNA binding by human papillomavirus E6 proteins. J Virol *68*, 4262-4273.

Lee, S.S., Glaunsinger, B., Mantovani, F., Banks, L., and Javier, R.T. (2000). Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. J Virol *74*, 9680-9693.

Lee, S.S., Weiss, R.S., and Javier, R.T. (1997). Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A *94*, 6670-6675.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27-42.

Levkowitz, G., Waterman, H., Ettenberg, S.A., Katz, M., Tsygankov, A.Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A.*, et al.* (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell *4*, 1029-1040.

Lin, C.Y., Strom, A., Vega, V.B., Kong, S.L., Yeo, A.L., Thomsen, J.S., Chan, W.C., Doray, B., Bangarusamy, D.K., Ramasamy, A.*, et al.* (2004). Discovery of estrogen receptor alpha target genes and response elements in breast tumor cells. Genome Biol *5*, R66.

Liu, X., Dakic, A., Zhang, Y., Dai, Y., Chen, R., and Schlegel, R. (2009). HPV E6 protein interacts physically and functionally with the cellular telomerase complex. Proc Natl Acad Sci U S A *106*, 18780-18785.

Liu, X., Yuan, H., Fu, B., Disbrow, G.L., Apolinario, T., Tomaic, V., Kelley, M.L., Baker, C.C., Huibregtse, J., and Schlegel, R. (2005). The E6AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein. J Biol Chem *280*, 10807-10816.

Liu, Y., Chen, J.J., Gao, Q., Dalal, S., Hong, Y., Mansur, C.P., Band, V., and Androphy, E.J. (1999). Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. J Virol *73*, 7297-7307.

Lobo, G.P., Waite, K.A., Planchon, S.M., Romigh, T., Houghton, J.A., and Eng, C. (2008). ATP modulates PTEN subcellular localization in multiple cancer cell lines. Hum Mol Genet *17*, 2877-2885.

Longworth, M.S., and Laimins, L.A. (2004). Pathogenesis of human papillomaviruses in differentiating epithelia. Microbiol Mol Biol Rev *68*, 362-372.

Lu, Z., Hu, X., Li, Y., Zheng, L., Zhou, Y., Jiang, H., Ning, T., Basang, Z., Zhang, C., and Ke, Y. (2004). Human papillomavirus 16 E6 oncoprotein interferences with insulin signaling pathway by binding to tuberin. J Biol Chem *279*, 35664-35670.

Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol *10*, 307-318.

Ma, Y.Y., Wei, S.J., Lin, Y.C., Lung, J.C., Chang, T.C., Whang-Peng, J., Liu, J.M., Yang, D.M., Yang, W.K., and Shen, C.Y. (2000). PIK3CA as an oncogene in cervical cancer. Oncogene *19*, 2739-2744.

Mamane, Y., Petroulakis, E., LeBacquer, O., and Sonenberg, N. (2006). mTOR, translation initiation and cancer. Oncogene *25*, 6416-6422.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.

Masri, J., Bernath, A., Martin, J., Jo, O.D., Vartanian, R., Funk, A., and Gera, J. (2007). mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor. Cancer Res *67*, 11712-11720.

Massimi, P., Gammoh, N., Thomas, M., and Banks, L. (2004). HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. Oncogene *23*, 8033-8039.

Mazurek, S., Zwerschke, W., Jansen-Durr, P., and Eigenbrodt, E. (2001a). Effects of the human papilloma virus HPV-16 E7 oncoprotein on glycolysis and glutaminolysis: role of pyruvate kinase type M2 and the glycolytic-enzyme complex. Biochem J *356*, 247-256.

Mazurek, S., Zwerschke, W., Jansen-Durr, P., and Eigenbrodt, E. (2001b). Metabolic cooperation between different oncogenes during cell transformation: interaction between activated ras and HPV-16 E7. Oncogene *20*, 6891-6898.

McCormack, S.J., Brazinski, S.E., Moore, J.L., Jr., Werness, B.A., and Goldstein, D.J. (1997). Activation of the focal adhesion kinase signal transduction pathway in cervical carcinoma cell lines and human genital epithelial cells immortalized with human papillomavirus type 18. Oncogene *15*, 265-274.

McLaughlin-Drubin, M.E., Huh, K.W., and Munger, K. (2008). Human papillomavirus type 16 E7 oncoprotein associates with E2F6. J Virol *82*, 8695-8705.

McLaughlin-Drubin, M.E., and Munger, K. (2009a). Oncogenic activities of human papillomaviruses. Virus Res *143*, 195-208.

McLaughlin-Drubin, M.E., and Munger, K. (2009b). The human papillomavirus E7 oncoprotein. Virology *384*, 335-344.

Menges, C.W., Baglia, L.A., Lapoint, R., and McCance, D.J. (2006). Human papillomavirus type 16 E7 up-regulates AKT activity through the retinoblastoma protein. Cancer Res *66*, 5555-5559.

Menon, S., and Manning, B.D. (2008). Common corruption of the mTOR signaling network in human tumors. Oncogene *27 Suppl 2*, S43-51.

Meyers, C., Frattini, M.G., Hudson, J.B., and Laimins, L.A. (1992). Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science *257*, 971-973.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. Eur J Biochem *267*, 6321-6330.

Mistry, N., Simonsson, M., and Evander, M. (2007). Transcriptional activation of the human papillomavirus type 5 and 16 long control region in cells from cutaneous and mucosal origin. Virol J *4*, 27.

Moody, C.A., and Laimins, L.A. (2010). Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer *10*, 550-560.

Moody, C.A., Scott, R.S., Amirghahari, N., Nathan, C.O., Young, L.S., Dawson, C.W., and Sixbey, J.W. (2005). Modulation of the cell growth regulator mTOR by Epstein-Barr virus-encoded LMP2A. J Virol *79*, 5499-5506.

Moore, C.E., Wiatrak, B.J., McClatchey, K.D., Koopmann, C.F., Thomas, G.R., Bradford, C.R., and Carey, T.E. (1999). High-risk human papillomavirus types and squamous cell carcinoma in patients with respiratory papillomas. Otolaryngol Head Neck Surg *120*, 698-705.

Mothe-Satney, I., Brunn, G.J., McMahon, L.P., Capaldo, C.T., Abraham, R.T., and Lawrence, J.C., Jr. (2000a). Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. J Biol Chem *275*, 33836-33843.

Mothe-Satney, I., Yang, D., Fadden, P., Haystead, T.A., and Lawrence, J.C., Jr. (2000b). Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. Mol Cell Biol *20*, 3558-3567.

Munger, K., Phelps, W.C., Bubb, V., Howley, P.M., and Schlegel, R. (1989). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol *63*, 4417-4421.

Nakagawa, S., and Huibregtse, J.M. (2000). Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. Mol Cell Biol *20*, 8244-8253.

Nakamura, M., Bodily, J.M., Beglin, M., Kyo, S., Inoue, M., and Laimins, L.A. (2009). Hypoxia-specific stabilization of HIF-1alpha by human papillomaviruses. Virology *387*, 442-448.

Nave, B.T., Ouwens, M., Withers, D.J., Alessi, D.R., and Shepherd, P.R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J *344 Pt 2*, 427-431.

Nawaz, Z., Lonard, D.M., Smith, C.L., Lev-Lehman, E., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1999). The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. Mol Cell Biol *19*, 1182-1189.

Neary, K., and DiMaio, D. (1989). Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells. J Virol *63*, 259-266.

Nguyen, M.L., Nguyen, M.M., Lee, D., Griep, A.E., and Lambert, P.F. (2003). The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia in vivo. J Virol *77*, 6957-6964.

Nishikawa, R., Ji, X.D., Harmon, R.C., Lazar, C.S., Gill, G.N., Cavenee, W.K., and Huang, H.J. (1994). A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc Natl Acad Sci U S A *91*, 7727-7731.

Nourry, C., Grant, S.G., and Borg, J.P. (2003). PDZ domain proteins: plug and play! Sci STKE *2003*, RE7.

Novosyadlyy, R., Vijayakumar, A., Lann, D., Fierz, Y., Kurshan, N., and LeRoith, D. (2009). Physical and functional interaction between polyoma virus middle T antigen and insulin and IGF-I receptors is required for oncogene activation and tumour initiation. Oncogene *28*, 3477-3486.

O'Shea, C., Klupsch, K., Choi, S., Bagus, B., Soria, C., Shen, J., McCormick, F., and Stokoe, D. (2005). Adenoviral proteins mimic nutrient/growth signals to activate the mTOR pathway for viral replication. EMBO J *24*, 1211-1221.

Oh, K.J., Kalinina, A., Park, N.H., and Bagchi, S. (2006). Deregulation of eIF4E: 4E-BP1 in differentiated human papillomavirus-containing cells leads to high levels of expression of the E7 oncoprotein. J Virol *80*, 7079-7088.

Oh, W.J., Wu, C.C., Kim, S.J., Facchinetti, V., Julien, L.A., Finlan, M., Roux, P.P., Su, B., and Jacinto, E. (2010). mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. EMBO J *29*, 3939-3951.

Oliveira, J.G., Colf, L.A., and McBride, A.A. (2006). Variations in the association of papillomavirus E2 proteins with mitotic chromosomes. Proc Natl Acad Sci U S A *103*, 1047-1052.

Ou, Y.H., Torres, M., Ram, R., Formstecher, E., Roland, C., Cheng, T., Brekken, R., Wurz, R., Tasker, A., Polverino, T.*, et al.* (2011). TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. Mol Cell *41*, 458-470.

Ozbun, M.A., and Meyers, C. (1998). Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. J Virol *72*, 2715-2722.

Pearce, L.R., Komander, D., and Alessi, D.R. (2010). The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol *11*, 9-22.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C., and Thomas, G. (2004). S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol *24*, 3112-3124.

Petersen, C.P., Bordeleau, M.E., Pelletier, J., and Sharp, P.A. (2006). Short RNAs repress translation after initiation in mammalian cells. Mol Cell *21*, 533-542.

Peterson, R.T., Desai, B.N., Hardwick, J.S., and Schreiber, S.L. (1999). Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc Natl Acad Sci U S A *96*, 4438-4442.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., and Sabatini, D.M. (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell *137*, 873-886.

Pim, D., and Banks, L. (1999). HPV-18 E6*I protein modulates the E6-directed degradation of p53 by binding to full-length HPV-18 E6. Oncogene *18*, 7403-7408.

Pim, D., Collins, M., and Banks, L. (1992). Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. Oncogene *7*, 27-32.

Pim, D., Massimi, P., and Banks, L. (1997). Alternatively spliced HPV-18 E6* protein inhibits E6 mediated degradation of p53 and suppresses transformed cell growth. Oncogene *15*, 257-264.

Pim, D., Massimi, P., Dilworth, S.M., and Banks, L. (2005). Activation of the protein kinase B pathway by the HPV-16 E7 oncoprotein occurs through a mechanism involving interaction with PP2A. Oncogene *24*, 7830-7838.

Pratt, R.L., and Kinch, M.S. (2002). Activation of the EphA2 tyrosine kinase stimulates the MAP/ERK kinase signaling cascade. Oncogene *21*, 7690-7699.

Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A., and Thomas, G. (1998). Phosphorylation and activation of p70s6k by PDK1. Science *279*, 707-710.

Raught, B., Peiretti, F., Gingras, A.C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., and Hershey, J.W. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. EMBO J *23*, 1761-1769.

Richards, R.M., Lowy, D.R., Schiller, J.T., and Day, P.M. (2006). Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc Natl Acad Sci U S A *103*, 1522-1527.

Rodriguez-Viciana, P., Collins, C., and Fried, M. (2006). Polyoma and SV40 proteins differentially regulate PP2A to activate distinct cellular signaling pathways involved in growth control. Proc Natl Acad Sci U S A *103*, 19290-19295.

Rojas, M., Yao, S., and Lin, Y.Z. (1996). Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. J Biol Chem *271*, 27456-27461.

Romanowski, B. (2011). Long term protection against cervical infection with the human papillomavirus: review of currently available vaccines. Hum Vaccin *7*, 161-169.

Roulin, D., Cerantola, Y., Dormond-Meuwly, A., Demartines, N., and Dormond, O. (2010). Targeting mTORC2 inhibits colon cancer cell proliferation in vitro and tumor formation in vivo. Mol Cancer *9*, 57.

Ruesch, M.N., Stubenrauch, F., and Laimins, L.A. (1998). Activation of papillomavirus late gene transcription and genome amplification upon differentiation in semisolid medium is coincident with expression of involucrin and transglutaminase but not keratin-10. J Virol *72*, 5016-5024.

Rustad, C.F., Bjornslett, M., Heimdal, K.R., Maehle, L., Apold, J., and Moller, P. (2006). Germline PTEN mutations are rare and highly penetrant. Hered Cancer Clin Pract *4*, 177-185.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35-43.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell *25*, 903-915.

Sapp, M., and Bienkowska-Haba, M. (2009). Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. FEBS J *276*, 7206-7216.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell *22*, 159-168.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Schalm, S.S., Tee, A.R., and Blenis, J. (2005). Characterization of a conserved C-terminal motif (RSPRR) in ribosomal protein S6 kinase 1 required for its mammalian target of rapamycin-dependent regulation. J Biol Chem *280*, 11101-11106.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and Howley, P.M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495-505.

Schiffman, M., Castle, P.E., Jeronimo, J., Rodriguez, A.C., and Wacholder, S. (2007). Human papillomavirus and cervical cancer. Lancet *370*, 890-907.

Schiffman, M., Rodriguez, A.C., Chen, Z., Wacholder, S., Herrero, R., Hildesheim, A., Desalle, R., Befano, B., Yu, K., Safaeian, M.*, et al.* (2010). A population-based prospective study of carcinogenic human papillomavirus variant lineages, viral persistence, and cervical neoplasia. Cancer Res *70*, 3159-3169.

Scholle, F., Bendt, K.M., and Raab-Traub, N. (2000). Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J Virol *74*, 10681-10689.

Sedman, S.A., Barbosa, M.S., Vass, W.C., Hubbert, N.L., Haas, J.A., Lowy, D.R., and Schiller, J.T. (1991). The full-length E6 protein of human papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. J Virol *65*, 4860-4866.

Shahbazian, D., Roux, P.P., Mieulet, V., Cohen, M.S., Raught, B., Taunton, J., Hershey, J.W., Blenis, J., Pende, M., and Sonenberg, N. (2006). The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO J *25*, 2781-2791.

Shair, K.H., Bendt, K.M., Edwards, R.H., Bedford, E.C., Nielsen, J.N., and Raab-Traub, N. (2007). EBV latent membrane protein 1 activates Akt, NFkappaB, and Stat3 in B cell lymphomas. PLoS Pathog *3*, e166.

Shayesteh, L., Lu, Y., Kuo, W.L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G.B., and Gray, J.W. (1999). PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet *21*, 99-102.

Shelly, M., Mosesson, Y., Citri, A., Lavi, S., Zwang, Y., Melamed-Book, N., Aroeti, B., and Yarden, Y. (2003). Polar expression of ErbB-2/HER2 in epithelia. Bimodal regulation by Lin-7. Dev Cell *5*, 475-486.

Shin, S., Wolgamott, L., Yu, Y., Blenis, J., and Yoon, S.O. (2011). Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation. Proc Natl Acad Sci U S A *108*, E1204-1213.

Shor, B., Gibbons, J.J., Abraham, R.T., and Yu, K. (2009). Targeting mTOR globally in cancer: thinking beyond rapamycin. Cell Cycle *8*, 3831-3837.

Shuda, M., Kwun, H.J., Feng, H., Chang, Y., and Moore, P.S. (2011). Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator. J Clin Invest.

Sibbet, G., Romero-Graillet, C., Meneguzzi, G., and Campo, M.S. (2000). alpha6 integrin is not the obligatory cell receptor for bovine papillomavirus type 4. J Gen Virol *81*, 327-334.

Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., and Di Fiore, P.P. (2008). Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell *15*, 209-219.

Sizemore, N., Choo, C.K., Eckert, R.L., and Rorke, E.A. (1998). Transcriptional regulation of the EGF receptor promoter by HPV16 and retinoic acid in human ectocervical epithelial cells. Exp Cell Res *244*, 349-356.

Skolnik, E.Y., Batzer, A., Li, N., Lee, C.H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993). The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science *260*, 1953-1955.

Slieker, L.J., Martensen, T.M., and Lane, M.D. (1986). Synthesis of epidermal growth factor receptor in human A431 cells. Glycosylation-dependent acquisition of ligand binding activity occurs post-translationally in the endoplasmic reticulum. J Biol Chem *261*, 15233-15241.

Spangle, J.M., and Munger, K. (2010). The human papillomavirus type 16 E6 oncoprotein activates mTORC1 signaling and increases protein synthesis. J Virol *84*, 9398-9407.

Spanos, W.C., Hoover, A., Harris, G.F., Wu, S., Strand, G.L., Anderson, M.E., Klingelhutz, A.J., Hendriks, W., Bossler, A.D., and Lee, J.H. (2008). The PDZ binding motif of human papillomavirus type 16 E6 induces PTPN13 loss, which allows anchorage-independent growth and synergizes with ras for invasive growth. J Virol *82*, 2493-2500.

Stacey, S.N., Jordan, D., Williamson, A.J., Brown, M., Coote, J.H., and Arrand, J.R. (2000). Leaky scanning is the predominant mechanism for translation of human papillomavirus type 16 E7 oncoprotein from E6/E7 bicistronic mRNA. J Virol *74*, 7284-7297.

Stauffer, Y., Raj, K., Masternak, K., and Beard, P. (1998). Infectious human papillomavirus type 18 pseudovirions. J Mol Biol *283*, 529-536.

Stelzer, M.K., Pitot, H.C., Liem, A., Lee, D., Kennedy, G.D., and Lambert, P.F. (2010). Rapamycin inhibits anal carcinogenesis in two preclinical animal models. Cancer Prev Res (Phila) *3*, 1542-1551.

Straight, S.W., Herman, B., and McCance, D.J. (1995). The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. J Virol *69*, 3185-3192.

Summers, S.A., Lipfert, L., and Birnbaum, M.J. (1998). Polyoma middle T antigen activates the Ser/Thr kinase Akt in a PI3-kinase-dependent manner. Biochem Biophys Res Commun *246*, 76-81.

Swart, R., Ruf, I.K., Sample, J., and Longnecker, R. (2000). Latent membrane protein 2A-mediated effects on the phosphatidylinositol 3-Kinase/Akt pathway. J Virol *74*, 10838-10845.

Thomas, J.T., Hubert, W.G., Ruesch, M.N., and Laimins, L.A. (1999). Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. Proc Natl Acad Sci U S A *96*, 8449-8454.

Thomas, M., and Banks, L. (1999). Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. J Gen Virol *80 ( Pt 6)*, 1513-1517.

Thomas, M., Massimi, P., Navarro, C., Borg, J.P., and Banks, L. (2005). The hScrib/Dlg apico-basal control complex is differentially targeted by HPV-16 and HPV-18 E6 proteins. Oncogene *24*, 6222-6230.

Titolo, S., Pelletier, A., Sauve, F., Brault, K., Wardrop, E., White, P.W., Amin, A., Cordingley, M.G., and Archambault, J. (1999). Role of the ATP-binding domain of the human

papillomavirus type 11 E1 helicase in E2-dependent binding to the origin. J Virol *73*, 5282-5293.

Tomasoni, R., and Mondino, A. (2011). The tuberous sclerosis complex: balancing proliferation and survival. Biochem Soc Trans *39*, 466-471.

Tong, X., and Howley, P.M. (1997). The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. Proc Natl Acad Sci U S A *94*, 4412-4417.

Tonikian, R., Zhang, Y., Sazinsky, S.L., Currell, B., Yeh, J.H., Reva, B., Held, H.A., Appleton, B.A., Evangelista, M., Wu, Y.*, et al.* (2008). A specificity map for the PDZ domain family. PLoS Biol *6*, e239.

Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T.*, et al.* (2003). Pten dose dictates cancer progression in the prostate. PLoS Biol *1*, E59.

Tungteakkhun, S.S., Filippova, M., Fodor, N., and Duerksen-Hughes, P.J. (2010). The full-length isoform of human papillomavirus 16 E6 and its splice variant E6* bind to different sites on the procaspase 8 death effector domain. J Virol *84*, 1453-1463.

Ugi, S., Imamura, T., Maegawa, H., Egawa, K., Yoshizaki, T., Shi, K., Obata, T., Ebina, Y., Kashiwagi, A., and Olefsky, J.M. (2004). Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes. Mol Cell Biol *24*, 8778-8789.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J.*, et al.* (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature *431*, 200-205.

Underbrink, M.P., Howie, H.L., Bedard, K.M., Koop, J.I., and Galloway, D.A. (2008). E6 proteins from multiple human betapapillomavirus types degrade Bak and protect keratinocytes from apoptosis after UVB irradiation. J Virol *82*, 10408-10417.

Vande Pol, S.B., Brown, M.C., and Turner, C.E. (1998). Association of Bovine Papillomavirus Type 1 E6 oncoprotein with the focal adhesion protein paxillin through a conserved protein interaction motif. Oncogene *16*, 43-52.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science *324*, 1029-1033.

Vecchione, A., Marchese, A., Henry, P., Rotin, D., and Morrione, A. (2003). The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor. Mol Cell Biol *23*, 3363-3372.

Veldman, T., Liu, X., Yuan, H., and Schlegel, R. (2003). Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter. Proc Natl Acad Sci U S A *100*, 8211-8216.

Venuti, A., Paolini, F., Nasir, L., Corteggio, A., Roperto, S., Campo, M.S., and Borzacchiello, G. (2011). Papillomavirus E5: the smallest oncoprotein with many functions. Mol Cancer *10*, 140.

Wang, Q., Villeneuve, G., and Wang, Z. (2005). Control of epidermal growth factor receptor endocytosis by receptor dimerization, rather than receptor kinase activation. EMBO Rep *6*, 942-948.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J *20*, 4370-4379.

Wang, X., Trotman, L.C., Koppie, T., Alimonti, A., Chen, Z., Gao, Z., Wang, J., Erdjument-Bromage, H., Tempst, P., Cordon-Cardo, C.*, et al.* (2007). NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. Cell *128*, 129-139.

Wang, Z., and Moran, M.F. (1996). Requirement for the adapter protein GRB2 in EGF receptor endocytosis. Science *272*, 1935-1939.

White, M.F., Shoelson, S.E., Keutmann, H., and Kahn, C.R. (1988). A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. J Biol Chem *263*, 2969-2980.

Whitman, M., Kaplan, D.R., Schaffhausen, B., Cantley, L., and Roberts, T.M. (1985). Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature *315*, 239-242.

Xie, X., Zhang, D., Zhao, B., Lu, M.K., You, M., Condorelli, G., Wang, C.Y., and Guan, K.L. (2011). IkappaB kinase epsilon and TANK-binding kinase 1 activate AKT by direct phosphorylation. Proc Natl Acad Sci U S A *108*, 6474-6479.

Yang, L., and Botchan, M. (1990). Replication of bovine papillomavirus type 1 DNA initiates within an E2-responsive enhancer element. J Virol *64*, 5903-5911.

Yarden, Y., and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. Nat Rev Mol Cell Biol *2*, 127-137.

Yu, C.F., Liu, Z.X., and Cantley, L.G. (2002). ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. J Biol Chem *277*, 19382-19388.

Yu, Y., and Alwine, J.C. (2002). Human cytomegalovirus major immediate-early proteins and simian virus 40 large T antigen can inhibit apoptosis through activation of the

phosphatidylinositide 3'-OH kinase pathway and the cellular kinase Akt. J Virol *76*, 3731-3738.

Yu, Y., Yoon, S.O., Poulogiannis, G., Yang, Q., Ma, X.M., Villen, J., Kubica, N., Hoffman, G.R., Cantley, L.C., Gygi, S.P.*, et al.* (2011). Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. Science *332*, 1322-1326.

Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A., and Pan, D. (2003). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol *5*, 578-581.

Zhao, X., Oberg, D., Rush, M., Fay, J., Lambkin, H., and Schwartz, S. (2005). A 57-nucleotide upstream early polyadenylation element in human papillomavirus type 16 interacts with hFip1, CstF-64, hnRNP C1/C2, and polypyrimidine tract binding protein. J Virol *79*, 4270-4288.

Zheng, L., Ding, H., Lu, Z., Li, Y., Pan, Y., Ning, T., and Ke, Y. (2008). E3 ubiquitin ligase E6AP-mediated TSC2 turnover in the presence and absence of HPV16 E6. Genes Cells *13*, 285-294.

Zhou, X., and Munger, K. (2009). Expression of the human papillomavirus type 16 E7 oncoprotein induces an autophagy-related process and sensitizes normal human keratinocytes to cell death in response to growth factor deprivation. Virology *385*, 192-197.

Zhou, X., Spangle, J.M., and Munger, K. (2009). Expression of a viral oncoprotein in normal human epithelial cells triggers an autophagy-related process: is autophagy an "Achilles' heel" of human cancers? Autophagy *5*, 578-579.

Zhu, J.H., Chen, R., Yi, W., Cantin, G.T., Fearns, C., Yang, Y., Yates, J.R., 3rd, and Lee, J.D. (2008). Protein tyrosine phosphatase PTPN13 negatively regulates Her2/ErbB2 malignant signaling. Oncogene *27*, 2525-2531.

Zito, C.I., Qin, H., Blenis, J., and Bennett, A.M. (2007). SHP-2 regulates cell growth by controlling the mTOR/S6 kinase 1 pathway. J Biol Chem *282*, 6946-6953.

Zoncu, R., Efeyan, A., and Sabatini, D.M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol *12*, 21-35.

Zwerschke, W., Mazurek, S., Massimi, P., Banks, L., Eigenbrodt, E., and Jansen-Durr, P. (1999). Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. Proc Natl Acad Sci U S A *96*, 1291-1296.

Zwick, E., Hackel, P.O., Prenzel, N., and Ullrich, A. (1999). The EGF receptor as central transducer of heterologous signalling systems. Trends Pharmacol Sci *20*, 408-412.

## APPENDIX 1

HPV16 E6 associated proteins as determined by HA affinity purification followed by mass spectrometry

| Gene Symbol | #Peptides | # Unique Peptides | Shared w/ HPV16 E6no*I128T | Shared w/ HPV16 E6no*PDZ |
|---|---|---|---|---|
| SCRIB | 54 | 54 | + | - |
| UTRN | 27 | 27 | + | - |
| UBE3A | 23 | 23 | + | + |
| GOPC | 21 | 21 | + | + |
| HUWE1 | 21 | 21 | + | + |
| CASK | 18 | 18 | + | - |
| DLG1 | 17 | 17 | + | - |
| SNX27 | 17 | 17 | + | - |
| IMPDH2 | 15 | 15 | + | + |
| MPP6 | 12 | 12 | + | - |
| AIFM1 | 11 | 11 | + | + |
| USP9X | 11 | 11 | + | + |
| ILK-2 | 11 | 11 | + | + |
| SNTB2 | 10 | 10 | + | - |
| PSMD3 | 10 | 10 | + | + |
| KPNB1 | 9 | 9 | + | + |
| PSMC1 | 8 | 8 | + | + |
| PSMC3 | 8 | 8 | + | + |
| TP53 | 8 | 8 | + | + |
| YWHAE | 8 | 8 | + | + |
| PSMD2 | 8 | 8 | - | + |
| CAPZA1 | 7 | 7 | + | + |
| DTNA | 7 | 7 | + | - |
| PSMD1 | 7 | 7 | + | + |
| MAGI3 | 7 | 7 | + | - |
| SAPS3 | 7 | 7 | + | + |
| PPP2R2A | 6 | 6 | + | + |
| PSMC5 | 6 | 6 | + | + |
| STRAP | 6 | 6 | + | + |
| CAPN2 | 6 | 6 | + | + |
| PPP2R1A | 6 | 6 | + | + |
| PSMC2 | 6 | 6 | + | + |
| YWHAQ | 5 | 5 | + | + |
| CAPZB | 5 | 5 | + | + |
| PSMD11 | 5 | 5 | - | + |
| DDB1 | 5 | 5 | + | + |
| RFC4 | 5 | 5 | + | + |
| ERP44 | 5 | 5 | + | + |
| GIGYF2 | 4 | 4 | + | + |
| PSMC6 | 4 | 4 | + | + |
| CAPNS1 | 4 | 4 | - | + |
| RPL36AP37 | 4 | 4 | + | + |

| | | | | |
|---|---|---|---|---|
| KPNA2 | 4 | 4 | + | + |
| RNH1 | 4 | 4 | + | + |
| SNX27 | 4 | 4 | + | - |
| PSMD4 | 4 | 4 | + | + |
| CPVL | 4 | 4 | + | - |
| UBC | 4 | 4 | + | + |
| PPP2CA | 4 | 4 | + | + |
| PSME3 | 3 | 3 | + | + |
| UBE2L3 | 3 | 3 | + | + |
| MAPK1 | 3 | 3 | + | + |
| PTPN3 | 3 | 3 | + | - |
| LIN7C | 3 | 3 | + | - |
| PDZRN3 | 3 | 3 | + | - |
| CSDE1 | 3 | 3 | + | + |
| USP7 | 3 | 3 | + | + |
| DLD | 3 | 3 | - | + |
| PPP6C | 3 | 3 | + | + |
| EIF4E2 | 3 | 3 | + | + |
| GPS1 | 3 | 3 | - | - |
| TJP2 | 3 | 3 | - | - |
| SNTB1 | 3 | 3 | - | - |
| AMOT | 3 | 3 | + | + |
| PSMC4 | 3 | 3 | - | + |
| CTNNAL1 | 3 | 3 | + | - |
| DLAT | 2 | 2 | + | + |
| MPP7 | 2 | 2 | - | - |
| PSMD12 | 2 | 2 | + | + |
| UBA1 | 2 | 2 | - | - |
| LIN7A | 2 | 2 | - | - |
| TPM3 | 2 | 2 | - | + |
| PSMD13 | 2 | 2 | - | + |
| PPP2R1A | 2 | 2 | + | + |
| PSMD6 | 2 | 2 | + | - |
| LOC646057 | 2 | 2 | + | + |
| PSMD7 | 2 | 2 | - | + |
| EFTUD2 | 2 | 2 | + | + |
| COPS5 | 2 | 2 | + | + |
| TPM1 | 2 | 2 | - | + |
| CBX3 | 2 | 2 | - | + |
| MPP2 | 2 | 2 | + | - |
| GAPVD1 | 2 | 2 | - | + |
| LIN7B | 2 | 2 | - | - |
| TXNDC5 | 2 | 2 | + | + |
| LOC344382 | 2 | 2 | - | - |
| TP53 | 1 | 1 | + | + |
| TPM4 | 1 | 1 | + | - |
| SCRIB | 1 | 1 | + | - |

| | | | | |
|---|---|---|---|---|
| LRRC1 | 1 | 1 | - | + |
| LGMN | 1 | 1 | + | + |
| HDAC2 | 1 | 1 | - | - |
| GIGYF2 | 1 | 1 | + | + |
| RPS26P54 | 1 | 1 | + | + |
| BOLA2 | 1 | 1 | + | + |
| ALDH2 | 1 | 1 | + | + |
| TIPRL | 1 | 1 | + | + |
| SKP1 | 1 | 1 | - | + |
| DTNB | 1 | 1 | + | - |
| IMPDH1 | 1 | 1 | - | - |
| HAT1 | 1 | 1 | + | - |
| WDR68 | 1 | 1 | - | + |
| PSMD8 | 1 | 1 | - | + |
| PPP2R2C | 1 | 1 | + | + |
| SORT1 | 1 | 1 | + | - |
| AKAP8L | 1 | 1 | + | + |
| DICER1 | 1 | 1 | - | + |
| PPM1G | 1 | 1 | + | - |
| SMN2 | 1 | 1 | + | - |
| DLG4 | 1 | 1 | + | - |
| SAPS3 | 1 | 1 | + | + |
| COPS4 | 1 | 1 | - | + |
| UGCGL1 | 1 | 1 | + | + |
| AKAP8 | 1 | 1 | + | - |
| CAPZA2 | 1 | 1 | + | - |
| DCTPP1 | 1 | 1 | - | - |
| CACYBP | 1 | 1 | - | - |
| PSMD14 | 1 | 1 | - | + |
| ECD | 1 | 1 | - | + |
| CALM1 | 1 | 1 | - | - |
| NUP37 | 1 | 1 | - | - |
| PPP1CB | 1 | 1 | + | - |
| SEH1L | 1 | 1 | - | + |
| SNORA7A | 1 | 1 | - | - |
| USP47 | 1 | 1 | - | - |
| SIPA1L1 | 1 | 1 | - | - |
| PGAM5 | 1 | 1 | - | - |
| TPM3 | 1 | 1 | - | + |
| PPP1CA | 1 | 1 | + | - |
| RPA1 | 1 | 1 | - | - |
| HUWE1 | 1 | 1 | - | - |
| GTF3C5 | 1 | 1 | + | - |
| PTGES3 | 1 | 1 | + | - |
| PKLR | 1 | 1 | - | + |
| DCTN2 | 1 | 1 | - | - |
| GRB2 | 1 | 1 | - | + |

## APPENDIX 2

HA-affinity purification/mass spectrometry identified associated proteins with E6 proteins from HPV types 5, 8, 6b, 11, and 18

HPV5 E6 interacting proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| EP300 | 34 | 34 |
| CREBBP | 26 | 26 |
| IDE | 22 | 22 |
| RBL1 | 9 | 9 |
| HUWE1 | 9 | 9 |
| ILK-2 | 7 | 7 |
| RB1 | 7 | 7 |
| ATAD3B | 7 | 7 |
| CTBP2 | 6 | 6 |
| SFPQ | 5 | 5 |
| SMAD3 | 4 | 4 |
| UBC | 4 | 4 |
| MMS19 | 3 | 3 |
| SMAD9 | 1 | 1 |
| HIST1H4J | 1 | 1 |
| NUFIP2 | 1 | 1 |
| INPP5D | 1 | 1 |
| ATAD3A | 1 | 1 |
| PTPLAD1 | 1 | 1 |
| MYO1C | 1 | 1 |
| CDK3 | 1 | 1 |

HPV8 E6 interacting proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| CREBBP | 35 | 35 |
| HUWE1 | 33 | 33 |
| AMOT | 32 | 32 |
| EP300 | 31 | 31 |
| MCM3 | 29 | 29 |
| MCM5 | 20 | 20 |
| ATAD3B | 16 | 16 |
| LRPPRC | 13 | 13 |
| UBAP2L | 12 | 12 |
| PLEKHA5 | 12 | 12 |
| RB1 | 11 | 11 |
| PFKM | 10 | 10 |
| RBL1 | 9 | 9 |
| SFPQ | 8 | 8 |
| PEF1 | 7 | 7 |
| NSUN2 | 7 | 7 |
| ATAD3A | 7 | 7 |
| IWS1 | 7 | 7 |
| ILK-2 | 7 | 7 |
| ZNHIT2 | 6 | 6 |

| | | |
|---|---|---|
| NDUFS2 | 6 | 6 |
| YWHAQ | 5 | 5 |
| AMBRA1 | 5 | 5 |
| UBR5 | 5 | 5 |
| CTBP2 | 4 | 4 |
| AKAP8 | 4 | 4 |
| ATAD3B | 4 | 4 |
| AKAP8L | 3 | 3 |
| MCM7 | 3 | 3 |
| HOOK1 | 3 | 3 |
| ARNT | 3 | 3 |
| UBC | 3 | 3 |
| WDR68 | 3 | 3 |
| NDUFS3 | 3 | 3 |
| FAM115A | 3 | 3 |
| LONP2 | 3 | 3 |
| KIF5B | 3 | 3 |
| KIF22 | 2 | 2 |
| PFDN2 | 2 | 2 |
| SMAD3 | 2 | 2 |
| MYO1B | 2 | 2 |
| FAM96B | 2 | 2 |
| CDK2 | 2 | 2 |
| UBAP2L | 2 | 2 |
| RIF1 | 2 | 2 |
| POLR2B | 2 | 2 |
| CHD4 | 2 | 2 |
| SATB2 | 2 | 2 |
| NDUFA5 | 2 | 2 |
| HELLS | 2 | 2 |
| TIMM50 | 2 | 2 |
| YLPM1 | 2 | 2 |
| PGAM5 | 2 | 2 |
| MMS19 | 2 | 2 |
| VBP1 | 2 | 2 |
| PFDN6 | 2 | 2 |
| CACYBP | 2 | 2 |
| ATP2A1 | 2 | 2 |
| LEMD3 | 2 | 2 |
| SPC24 | 1 | 1 |
| CCNA2 | 1 | 1 |
| FOXP4 | 1 | 1 |
| ATAD3B | 1 | 1 |
| NUBP2 | 1 | 1 |
| MTHFD2 | 1 | 1 |
| GNL3 | 1 | 1 |
| SPC25 | 1 | 1 |
| CTTNBP2NL | 1 | 1 |
| USP9X | 1 | 1 |
| TRIM37 | 1 | 1 |
| RABL5 | 1 | 1 |

| | | |
|---|---|---|
| PTCD3 | 1 | 1 |
| SLC39A7 | 1 | 1 |
| PPP2CA | 1 | 1 |
| IQGAP1 | 1 | 1 |
| POLR2A | 1 | 1 |
| KIF11 | 1 | 1 |
| CITED2 | 1 | 1 |
| PMF1 | 1 | 1 |
| SF3B1 | 1 | 1 |
| LZTS2 | 1 | 1 |
| MYO1A | 1 | 1 |
| PRPF8 | 1 | 1 |
| PSMC5 | 1 | 1 |
| WDR77 | 1 | 1 |
| SET | 1 | 1 |
| PTPLAD1 | 1 | 1 |
| HUWE1 | 1 | 1 |
| CDK3 | 1 | 1 |
| SYMPK | 1 | 1 |
| EFTUD2 | 1 | 1 |
| CANX | 1 | 1 |
| HDAC1 | 1 | 1 |
| NUFIP2 | 1 | 1 |
| IGF2BP3 | 1 | 1 |
| LAS1L | 1 | 1 |
| SIPA1L1 | 1 | 1 |
| AAAS | 1 | 1 |
| PDCD11 | 1 | 1 |
| PSMD4 | 1 | 1 |
| SMAD9 | 1 | 1 |
| MRPS31 | 1 | 1 |
| HUWE1 | 1 | 1 |
| FTSJ3 | 1 | 1 |
| DLD | 1 | 1 |
| LUC7L2 | 1 | 1 |
| OCRL | 1 | 1 |
| SF1 | 1 | 1 |
| SLC25A4 | 1 | 1 |
| TRIM33 | 1 | 1 |
| RARS | 1 | 1 |
| USP7 | 1 | 1 |
| MTHFD1L | 1 | 1 |

HPV6b E6 interacting proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| UBE3A | 16 | 16 |
| HUWE1 | 15 | 15 |
| SFPQ | 7 | 7 |
| CANX | 5 | 5 |
| ILK-2 | 5 | 5 |

| | | |
|---|---|---|
| ATAD3B | 5 | 5 |
| UBC | 4 | 4 |
| MTHFD2 | 3 | 3 |
| PSMA2 | 3 | 3 |
| ATP2A2 | 3 | 3 |
| HM13 | 3 | 3 |
| BAT3 | 3 | 3 |
| ATP2A1 | 3 | 3 |
| ATP1A2 | 2 | 2 |
| PTPLAD1 | 2 | 2 |
| HLA-B | 2 | 2 |
| SLC39A7 | 1 | 1 |
| TIMM50 | 1 | 1 |
| HLA-H | 1 | 1 |
| PSMC5 | 1 | 1 |
| U2AF1 | 1 | 1 |
| AKAP8L | 1 | 1 |
| SNRPF | 1 | 1 |
| SLC1A5 | 1 | 1 |
| YWHAE | 1 | 1 |
| PSMD3 | 1 | 1 |
| ANXA2P2 | 1 | 1 |
| CNP | 1 | 1 |
| ATP1B3 | 1 | 1 |
| AMOT | 1 | 1 |
| PSMA7 | 1 | 1 |
| PSMB1 | 1 | 1 |
| PSMC1 | 1 | 1 |

HPV11 E6 interacting proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| UBE3A | 25 | 25 |
| STXBP3 | 16 | 16 |
| HUWE1 | 9 | 9 |
| TRMT61B | 8 | 8 |
| STX4 | 7 | 7 |
| ILK-2 | 7 | 7 |
| ATAD3B | 7 | 7 |
| AIFM1 | 5 | 5 |
| SFPQ | 5 | 5 |
| KIF3A | 4 | 4 |
| UBC | 4 | 4 |
| USP7 | 3 | 3 |
| LRPPRC | 3 | 3 |
| CANX | 2 | 2 |
| PGAM5 | 2 | 2 |
| PYCRL | 2 | 2 |
| UBE2L3 | 2 | 2 |
| YWHAE | 2 | 2 |

| | | |
|---|---|---|
| PHGDH | 2 | 2 |
| PSMC1 | 1 | 1 |
| ATP1A2 | 1 | 1 |
| AKAP8L | 1 | 1 |
| CBLB | 1 | 1 |
| IMPDH2 | 1 | 1 |
| NUP188 | 1 | 1 |
| HERC2 | 1 | 1 |
| UBA1 | 1 | 1 |
| MYBBP1A | 1 | 1 |
| AGK | 1 | 1 |
| SLC25A4 | 1 | 1 |
| PSMD11 | 1 | 1 |
| RANBP2 | 1 | 1 |
| SLC1A5 | 1 | 1 |
| SLC39A7 | 1 | 1 |
| PSMC6 | 1 | 1 |
| ATAD3A | 1 | 1 |
| TARDBP | 1 | 1 |
| PSMA6 | 1 | 1 |

HPV18 E6 interacting proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| UBE3A | 26 | 26 |
| SCRIB | 26 | 26 |
| DLG1 | 21 | 21 |
| UTRN | 20 | 20 |
| CASK | 16 | 16 |
| CLPX | 14 | 14 |
| HUWE1 | 10 | 10 |
| MPP7 | 9 | 9 |
| PSMD1 | 9 | 9 |
| SNTB2 | 9 | 9 |
| CANX | 9 | 9 |
| TP53 | 8 | 8 |
| PSMD4 | 7 | 7 |
| PSMD2 | 7 | 7 |
| PSMC3 | 6 | 6 |
| SFPQ | 6 | 6 |
| MAGI3 | 6 | 6 |
| PSMD3 | 5 | 5 |
| PSMC2 | 4 | 4 |
| PSMC1 | 4 | 4 |
| PSMC6 | 4 | 4 |
| DMD | 4 | 4 |
| PSMC5 | 4 | 4 |
| UBC | 4 | 4 |
| DLG4 | 4 | 4 |
| PSME3 | 3 | 3 |
| PSMB1 | 3 | 3 |

| | | |
|---|---|---|
| AMOT | 3 | 3 |
| DTNA | 3 | 3 |
| LIN7C | 3 | 3 |
| MPP2 | 3 | 3 |
| SNX27 | 3 | 3 |
| PSMD11 | 3 | 3 |
| PSMD12 | 3 | 3 |
| IRS4 | 3 | 3 |
| MPP6 | 3 | 3 |
| ATAD3B | 3 | 3 |
| DLG1 | 2 | 2 |
| KPNB1 | 2 | 2 |
| PSMB6 | 2 | 2 |
| PSMD6 | 2 | 2 |
| PSMB5 | 2 | 2 |
| PSMD14 | 2 | 2 |
| YWHAE | 2 | 2 |
| PGAM5 | 2 | 2 |
| CTNNAL1 | 2 | 2 |
| AIFM1 | 2 | 2 |
| LIN7B | 2 | 2 |
| PSMC4 | 2 | 2 |
| ADRM1 | 2 | 2 |
| TP53 | 1 | 1 |
| YWHAQ | 1 | 1 |
| MTHFD2 | 1 | 1 |
| SLC39A7 | 1 | 1 |
| ATP1A2 | 1 | 1 |
| UCHL5 | 1 | 1 |
| USP4 | 1 | 1 |
| U2AF1 | 1 | 1 |
| SNX27 | 1 | 1 |
| PSMB2 | 1 | 1 |
| MYO6 | 1 | 1 |
| PSMD13 | 1 | 1 |

## APPENDIX 3

HPV16 E6 * associated proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| TGM3 | 3 | 3 |
| CALML5 | 2 | 2 |
| LGALS7 | 2 | 2 |
| SFPQ | 2 | 2 |
| ANXA2P2 | 2 | 2 |
| NCL | 1 | 1 |
| CALML3 | 1 | 1 |
| ASPRV1 | 1 | 1 |
| EPPK1 | 1 | 1 |

HPV16 E6 ** associated proteins

| Gene Symbol | #Peptides | # Unique Peptides |
|---|---|---|
| XRCC6 | 8 | 8 |
| WDHD1 | 7 | 7 |
| MTHFD2 | 4 | 4 |
| EPRS | 4 | 4 |
| SFPQ | 4 | 4 |
| ACLY | 3 | 3 |
| IMPDH2 | 3 | 3 |
| PPP2R1A | 3 | 3 |
| DARS | 3 | 3 |
| ASNA1 | 2 | 2 |
| LUC7L | 1 | 1 |
| BOLA2 | 1 | 1 |
| KPNB1 | 1 | 1 |
| PPP2CA | 1 | 1 |
| UPF1 | 1 | 1 |
| RCC2 | 1 | 1 |
| HAX1 | 1 | 1 |
| MCM7 | 1 | 1 |
| PKLR | 1 | 1 |
| KPNA2 | 1 | 1 |