HPV REPLICATION REGULATION BY ACETYLATION OF A CONSERVED LYSINE IN THE E2 PROTEIN

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Yanique Serge Gillana Thomas

HPV REPLICATION REGULATION BY ACETYLATION OF A CONSERVED LYSINE IN THE E2 PROTEIN

Papillomaviruses (PVs) are non-enveloped DNA viruses that are the primary etiological agents of cervical and oropharyngeal cancers. Vaccines for H(human)PV have proven to be effective prophylactic treatments; however, there is no treatment available for those currently infected. To develop new therapies, we require a clear understanding of viral pathogenesis and regulation.

The Papillomavirus E2 protein is a sequence specific DNA binding protein that recruits cellular factors to its genome in infected epithelial cells. E2 also binds to and loads the viral E1 DNA helicase at the origin of replication. Post-translational modifications of PV E2 have been identified as potential regulators of E2 functions. We recently reported lysine (K) 111 as a target of p300 acetylation in B(bovine)PV that is involved in the regulation of viral transcription. K111 is conserved in most papillomaviruses, so we pursued a mutational approach to query the functional significance of lysine in HPV E2. Amino acid substitutions that prevent acetylation, including arginine, were unable to stimulate transcription and E1 mediated DNA replication. The arginine K111 mutant retained E2 transcriptional repression, nuclear localization, DNA and chromatin binding, and association with E2 binding partners involved in PV transcription and replication.

When directly investigating origin unwinding, the replication defective E2 K111R mutant recruited E1 to the viral replication origin, but surprisingly, unwinding of the duplex DNA did not occur. In contrast, the glutamine K111 mutant increased origin melting and stimulated replication compared to wild type E2. We have identified Topoisomerase I as a key host factor involved in viral replication whose recruitment is dependent on K111 acetylation, and propose a new model for viral origin dynamics during replication initiation. This work reveals a novel activity of E2 necessary for denaturing the viral origin that likely depends on acetylation of highly conserved lysine 111.

Elliot J. Androphy, MD (Chair)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Alanine
ATP	Adenosine triphosphate
BID	Basic residue-enriched interaction domain
BPV-1	Bovine papillomavirus
BRD4	Bromodomain-containing protein 4
ChIP	Chromatin Immunoprecipitation
Co-IP	Co-immunoprecipitation
CTM	C-terminal motif of Brd4
E2BS	E2 binding site
FAIRE	Formaldehyde-assisted isolation of regulatory elements
GPS2	G-protein pathway suppressor 2
HPV	Human papillomavirus
IP	Immunoprecipitation
K	Lysine
LCR	Long control region
Ori	Origin of replication
PCR	Polymerase chain reaction
PDID	Phosphorylation-dependent interaction domain
PEI	Poly(ethylenimine)
Q	Glutamine
RPA	Replication protein A
SDM	Site directed mutagenesis
ssDNA	Single stranded DNA
TopBP1	Topoisomerase binding protein I
Topo1	Topoisomerase I
URR	Upstream regulatory region
Wt	Wild type

CHAPTER 1

INTRODUCTION

1.1 Papillomaviruses

Papillomaviruses (PVs) are a family of non-enveloped DNA viruses, that are the major etiological agents of cervical and oropharyngeal cancers. The circular, double stranded DNA genome is approximately 8 kb, and is comprised of a long control region (LCR) (Hou, Wu, Zhou, Thomas, & Chiang) and eight open reading frames (Hebner & Laimins, 2006; Kadaja, Silla, Ustav, & Ustav, 2009; Mohr et al., 1990a; Soeda, Ferran, Baker, & McBride, 2006). This viral family is diverse and infects a wide range of species (Campo, 2002), currently over 30 genotypes of PVs and over 200 human papillomaviruses (HPVs) have been identified. PVs are mostly host specific as well as tissue-tropic, and all HPVs demonstrate an affinity for basal epithelial cells (Burd, 2003). These viruses are widespread and cause a wide range of diseases that vary based on the genotype and species in question (Table 1.1).

1.2 Human Papillomaviruses and Disease

There are over 200 HPVs that have been described and they manifest in a variety of ways; they can be asymptomatic, or they can result in benign lesions (papillomas) or progress to malignancy (de Villiers, Fauquet, Broker, Bernard, & zur Hausen, 2004). In the United States, HPV is the most common sexually transmitted infection and nearly all sexually active adults contract it at least once.

Although 90% of infections are asymptomatic and resolve easily (Ho, Bierman, Beardsley, Chang, & Burk, 1998), HPV 16 and 18 alone are responsible for 70% of all cervical cancers, which are almost entirely caused by HPV infection (Bosch et al., 1995; Clifford, Smith, Plummer, Munoz, & Franceschi, 2003). Interestingly, 60-90% of other cancers have also been linked to HPV.

PVs are categorized into high-risk and low-risk according to disease outcome. High risk HPVs including 16, 18, and 31, can lead to the development of intraepithelial neoplasia. HPV16 and 18 are the most prevalent and are responsible for over 70% of all cervical cancers (Munoz et al., 2004). Low-risk HPVs are the major causative factors of benign lesions such as laryngeal papillomas and genital warts.

A large number of HPVs can be classified as mucosal since they infect the mucosal epithelium, particularly the mucosal epithelial lining of the anogenital and oropharyngeal tracts. PVs are classified into genera based sequence variations in the L1 gene. Most of the high risk HPVs are mucosal and belong to the alpha genus. Cutaneous HPVs belong mainly to the beta and gamma genera. Beta HPVs are reported to be co-carcinogens with ultra violet radiation (UVR) to cause various skin cancers (Accardi & Gheit, 2014). Patients who suffer from epidermodysplasia verruciformis (EV) and those who are immune compromised experience frequent occurrences of squamous cell carcinoma

(SCC) when exposed to certain beta HPVs (Patel, Morrison, Rady, & Tyring, 2010).

HPV is a highly prevalent infection in both developed and un-developed countries, but there is currently no treatment for HPV infections. In 2006 the first HPV vaccine was licensed, and there are currently three vaccines available which cover up to nine HPV types ("Human papillomavirus vaccines: WHO position paper, October 2014," 2014). All of the current vaccines cover HPV types 16 and 18, which are responsible for over 70% of all cervical cancer cases. The vaccines are formulated from the major capsid protein L1, and have so far demonstrated a 100% success rate. The multi-dose prophylactic treatment is recommended for use in children prior to sexual activity and potential exposure to the virus. Due to its high cost; however, global accessibility has been a slow process and many parts of the world still have no vaccine program available.

Table 1.1

HPV Strain	Genus	Oncogenic Risk	Disease Outcome
HPV1 *(cutaneous)*	Beta	Low	Plantar warts
HPV5 *(cutaneous)*	Beta	Low	Epidermodysplasia verruciformis
HPV11 *(mucosal)*	Alpha	Low	Benign lesions Respiratory papillomatosis
HPV16 *(mucosal)*	Alpha	High	Cervical carcinoma Cervical adenocarcinoma Oropharyngeal cancer
HPV18 *(mucosal)*	Alpha	High	Cervical carcinoma Head and neck cancers
HPV31 *(mucosal)*	Alpha	High	Cervical carcinoma Anal dysplasia

Table 1.1 HPV can be classified and described by their disease outcomes. Different HPVs depending classification can cause a diverse array of disease states in human hosts.

1.3 Genome Structure and Viral Protein Functions

The circular, double stranded DNA genome is approximately 8 kb, and is comprised of a long control region (LCR) (Hou et al.), an upstream regulatory region (URR), and eight open reading frames (ORF) (Hebner & Laimins, 2006; Kadaja et al., 2009; Mohr et al., 1990b; Soeda et al., 2006). The eight ORFs code for six early genes and two late genes and multiple splice variants. The LCR (Figure 1.1) contains the p97 early promoter, silencer and enhancer sequences, the origin of replication, and binding sites for viral and host factors involved in the regulation of transcription and replication (Desaintes & Demeret, 1996). Though transient activity can be seen in cells of various human origins, p97 is only stably active in human keratinocytes (HFKs) (Cripe et al., 1990) and the late promoter is only active in differentiating keratinocytes (del Mar Pena & Laimins, 2001). Transcriptional regulation of viral genes is dependent on a collaboration of viral proteins and host factors such as activator protein 1 (AP1), transcription factor II domain (TFIID), and bromodomain containing 4 (BRD4) (Chan, Chong, Bernard, & Klock, 1990; Chong, Apt, Gloss, Isa, & Bernard, 1991; McPhillips, Oliveira, Spindler, Mitra, & McBride, 2006). The viral genome contains binding sites for some of these factors within the LCR.

The proteins expressed by the virus are conserved in both structure and function across the various PVs (Conway & Meyers, 2009). These multifunctional proteins and splice variants regulate viral mechanisms and manipulate infected cells directly and through interactions with host proteins (D'Abramo & Archambault, 2011). The ORFs (Figure 1.2) can be divided into those that code for Early or Late viral proteins. The early proteins E1, E2, E4, E5, E6, and E7 are expressed throughout the life of the virus and are responsible for transcription, replication, immune evasion, and oncogenesis (Graham, 2010). The Late proteins L1 and L2 are involved in the production of infectious particles and form the viral capsid. Splice variants are also expressed in certain HPVs including a transcriptional repressor E8^E2, pro- genome amplification E1^E4, and E6* (Lace, Anson, Thomas, Turek, & Haugen, 2008; Wilson, Fehrmann, & Laimins, 2005).

Figure 1.1

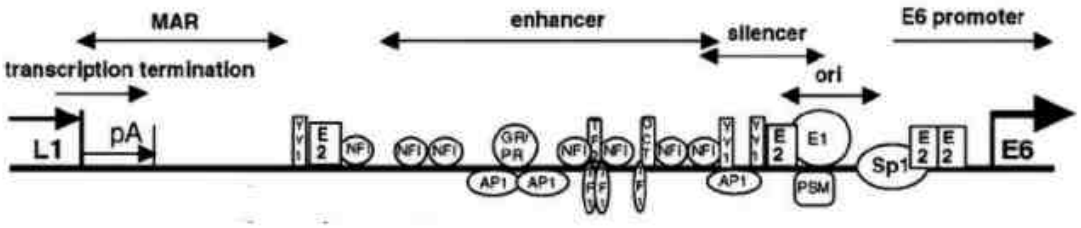


Figure 1.1 Conserved LCR structure of HPV. The LCR of HPV16 highlighting the early promoter p97, replication origin, E2 binding sites, and enhancer and silencer regions. The diagram also features the location of binding sequences for various host factors involved in HPV regulation. This figure was modified from (Tan, Baker, Stunkel, & Bernard, 2003).

Figure 1.2

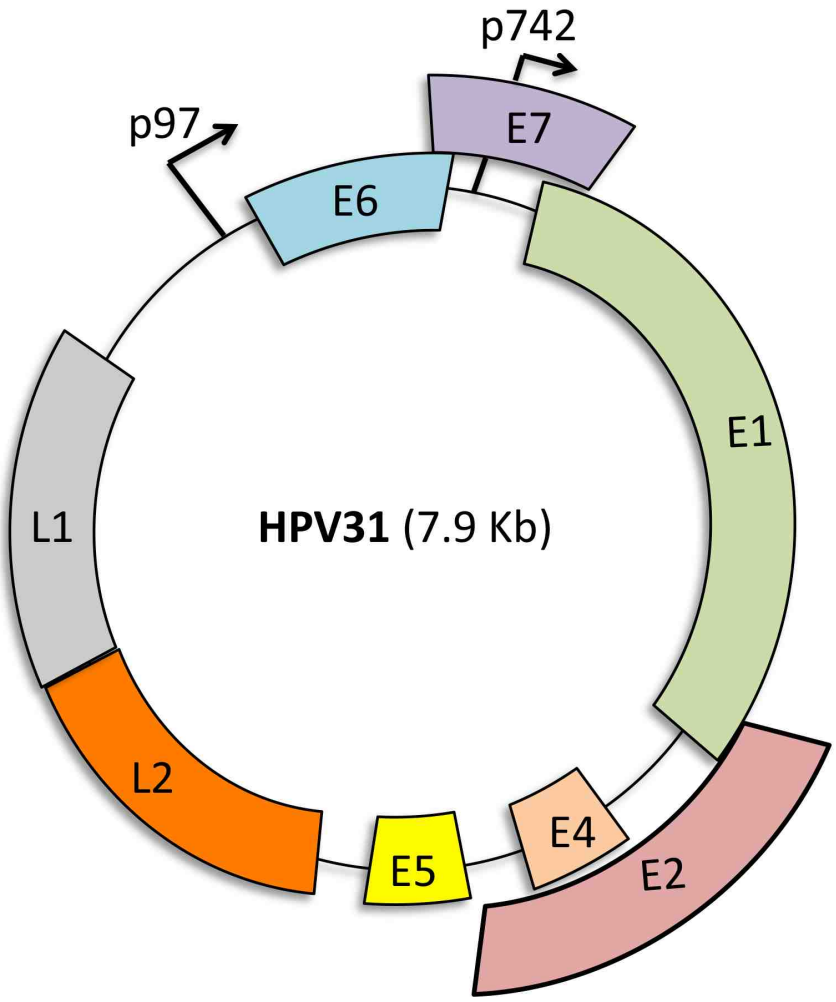


Figure 1.2 HPV31 genome structure and function. This diagram shows the HPV31 whole genome containing the 8 ORFs and the two major (early and late) promoters.

1.3.1 E1

The PV E1 protein is an essential viral replication factor (Lusky & Botchan, 1985; Ustav & Stenlund, 1991; Ustav, Ustav, Szymanski, & Stenlund, 1991). E1 is the viral helicase and initiates replication by binding to specific sequences on the viral genome with the assistance of the E2 protein (Frattini & Laimins, 1994). There is sequence and functional homology between PV E1 and SV (simian virus) 40 Large T Antigen which allowed for many of the predictions concerning E1 in foundational studies (Mansky, Batiza, & Lambert, 1997). The c-terminus of E1 contains the helicase and oligomerization domains, as well as the E2 and DNA polymerase α interacting regions (Chen & Stenlund, 1998; Hughes & Romanos, 1993; Masterson, Stanley, Lewis, & Romanos, 1998) (Figure 1.3). The DNA binding domain (DBD) is located upstream and the localization regulatory region (LRR) is in the N-terminal region (Abbate, Berger, & Botchan, 2004; Amin et al., 2000; Sun, Han, & McCance, 1998). The LRR contains a nuclear export signal (NES) and a nuclear localization signal (NLS), which are regulated by phosphorylation.

During viral replication initiation, E1 functions not only as a helicase, but is also reported to recruit host factors essential to viral replication including replication factors and chromatin remodeling complexes. E1 is reported to interact with replication protein A (RPA), and recruit it to sites of replication in order to stabilize single stranded (ss) DNA resulting from E1 helicase activity (Loo & Melendy,

2004).The N terminus of E1 is reported to interact with histone 1 (H1) and displace it from DNA, facilitating the removal histones and the melting of viral chromatin during replication (Swindle & Engler, 1998). H1 binds to DNA between nucleosomes and serves as a linker that enhances the condensing of chromatin (Thoma, Koller, & Klug, 1979). E1 displacement of H1 may be an essential step to ensure the binding of E1-E2 at the origin. E1 has also been reported to bind the switching deficient/sucrose non-fermentable (SWI/SNF) complex (D. Lee, Sohn, Kalpana, & Choe, 1999). SWI/SNF functions complex that disrupts chromatin to allow factors to access DNA (Travers, 1992). Other host factors E1 is reported to functionally interact with include topoisomerase 1 (Topo1), DNA polymerase α primase (pol α-primase) complex, and the polymerase δ (pol δ) (King et al., 2010; Kuo, Liu, Broker, & Chow, 1994; P. Park et al., 1994).

The E1 protein assembles as a double hexamer at the replication origin and unwinds viral DNA. The helicase function of E1 is driven by adenosine triphosphate (ATP) hydrolysis (Enemark & Joshua-Tor, 2006). The structure of the E1 helicase is conserved in SV40 LT helicase complexes, where each subunit of the helicase possesses a β-hairpin structure that is oriented to the center of the structure and DNA (Figure 1.3) (Enemark, Chen, Vaughn, Stenlund, & Joshua-Tor, 2000; Enemark & Joshua-Tor, 2006; Hickman & Dyda, 2005). The β-hairpins pull DNA apart while the hexamers translocate bi-directionally using ATP hydrolysis for energy, resulting in unwound ssDNA.

Figure 1.3

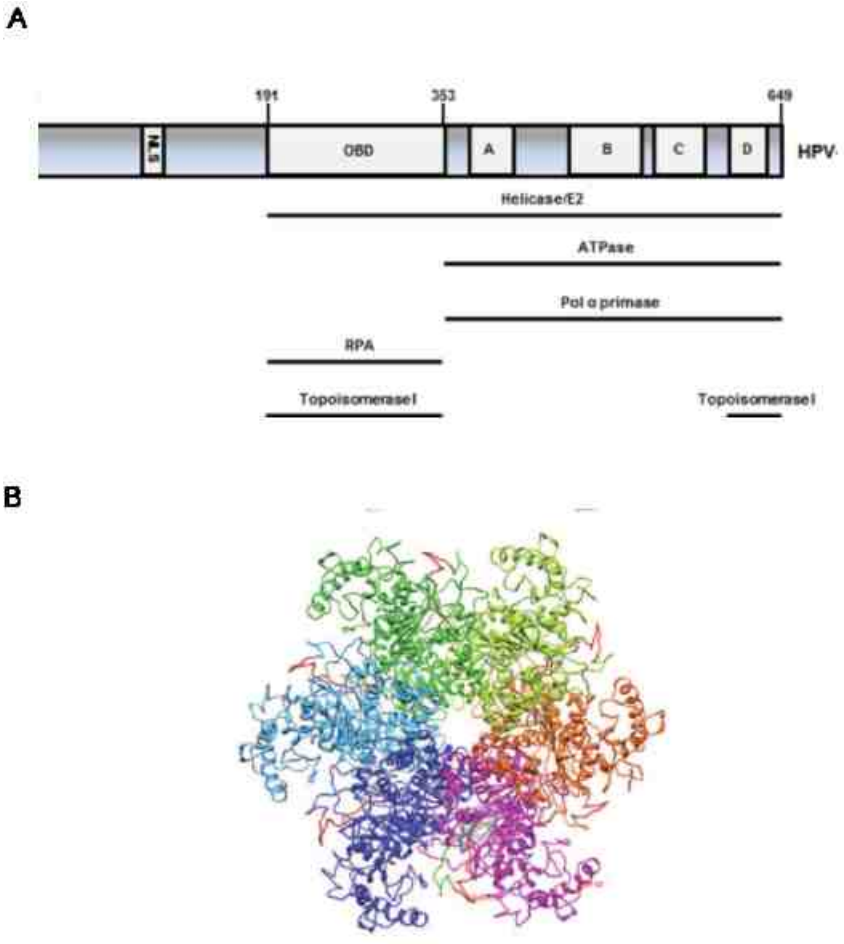


Figure 1.3 Structure of PV E1 helicase protein. A shows the structure of the E1 protein, identifying the functional regions for E1 localization, helicase activity, and interaction with host factors essential to its function in DNA unwinding. B illustrates a forward view of one E1 hexamer. Each monomer is indicated by a different color and the center of this structure is where DNA is pulled apart by β-hairpin processes. This figure is adapted from (Sabol, Matovina, Si-Mohamed, & Grce, 2012) and https://www.intechopen.com/books/the-mechanisms-of-dna-replication/replicative-helicases-as-the-central-organizing-motor-proteins-in-the-molecular-machines-of-the-elon

1.3.2 E2

The PV E2 protein is the major transcriptional regulator of viral gene expression and a viral replication initiation protein (Androphy, Lowy, & Schiller, 1987; Melendy, Sedman, & Stenlund, 1995; Sedman & Stenlund, 1995; Stenlund, 2003). The structure of E2 consists of a N-terminal trans-activation domain (TAD) and a C-terminal DNA binding domain, held together by an unstructured linker region (Figure 1.4). E2 regulates transcription by recruiting factors to the viral genome that result in either the activation of transcription, or repression of transcription (McBride, 2013), through steric hindrance or by blocking the binding of host transcription factors such as Sp1, TFIIB, and TBP (Hou et al., 2000; Stubenrauch, Leigh, & Pfister, 1996; Stubenrauch & Pfister, 1994; Yao, Breiding, & Androphy, 1998). Transcriptional activation involves the recruitment of certain host factors that are involved in chromatin processing such as chromatin adaptor Brd4, AMF1/GPS2, Tip60 HAT complex subunit EP400, Brm, and the demethylase JARDIC/SMCX (Breiding et al., 1997; Kumar, Naidu, Wang, Imbalzano, & Androphy, 2007; A. Y. Lee & Chiang, 2009; Peng, Breiding, Sverdrup, Richard, & Androphy, 2000; J. A. Smith et al., 2010; S. Y. Wu et al., 2006). Similar to AMF1/GPS2, ubiquitin-binding adaptor Tax1BP1 has been shown to stabilize E2 protein levels in certain PVs (Verstrepen, Verhelst, Carpentier, & Beyaert, 2011). A proposed mechanism for the repression of transcription by E2 due to steric hindrance is based on the location of E2BS adjacent the TATA box, effectively blocking the binding of the TATA-binding protein (TBP) (Steger & Corbach, 1997).

Transcriptional repression of E6 and E7 by E2 is important for the stable maintenance of the virus, and loss of this function and subsequent increases in the levels of E6 and E7 may lead to the progression of malignancy with high-risk PVs. Studies have demonstrated that the levels of the E2 protein expressed in cells directly determine the levels of transcription from the viral promoter. Similar to E1, E2 interactions include various members of the DDR pathways that are proposed to be essential for replication during vegetative amplification (Hong & Laimins, 2013). Topoisomerase II binding protein (TopBP1) and Breast Cancer 1 (BRCA1) were both found to interact with E2 and directly influence transcription/replication (Boner & Morgan, 2002; Boner et al., 2002; Kim, Lee, Gwan Hwang, Hwang, & Choe, 2003).

E2 shows differential affinity for the E2BS and it is proposed that at low levels, E2 binds to the high affinity sites that favor transcription, and at higher levels of E2 transcriptional repression and replication are favored due to binding at the low affinity sequences. Past in-vitro studies have shown that E1 can initiate replication in the absence of E2 (Bonne-Andrea, Tillier, McShan, Wilson, & Clertant, 1997; Grossel, Sverdrup, Breiding, & Androphy, 1996; Sedman & Stenlund, 1995); however, E2 is essential for viral replication in-vivo due to the low sequence specificity of E1 (Ustav & Stenlund, 1991). E2 utilizes its high sequence specificity to recruit E1 to the origin in order to initiate viral replication.

As stated previously, E1 *in-vivo* is not efficient in origin binding due to poor sequence specificity. The recruitment of E1 to the origin by E2 is therefore essential to replication initiation (Stenlund, 2003). A BPV E2 mutant; E39A (also E39G), prevents the E2E1 interaction and maintains transcriptional function but inhibits replication (Sakai, Yasugi, Benson, Dowhanick, & Howley, 1996). Another function of E2 is the equal partitioning of viral episomes during mitosis. E2 is loaded onto mitotic chromosomes with the assistance of host factor ChlR1, where the C-terminus of E2 adheres DNA and to the mitotic spindle (Parish, Bean, Park, & Androphy, 2006; Parish, Rosa, et al., 2006; Skiadopoulos & McBride, 1998; Van Tine et al., 2004). This allows for maintenance of a constant copy number and even division of viral episomes between daughter cells, which is important in a persistent infection.

One marker of HPV in cervical cancer is the integration of viral episomes into host chromatin and the subsequent loss of the E2 gene (Cooper, Herrington, Stickland, Evans, & McGee, 1991; Pett et al., 2006; Romanczuk, Thierry, & Howley, 1990). E2 repression of the p97 promoter regulates the expression of oncoproteins E6 and E7, therefore a loss of E2 results in the de-repression of those genes and the eventual immortalization of the infected cells (Jeon, Allen-Hoffmann, & Lambert, 1995; Jeon & Lambert, 1995; Romanczuk & Howley, 1992). This principle was used to develop the HeLa repression assay used in this study (Demeret, Desaintes, Yaniv, & Thierry, 1997). HPV infected cells with no

E2 expression demonstrate a proliferative advantage that is linked to E6 and E7, but E2 has also been reported to induce apoptosis in a transcription independent manner (Demeret, Garcia-Carranca, & Thierry, 2003).

Figure 1.4

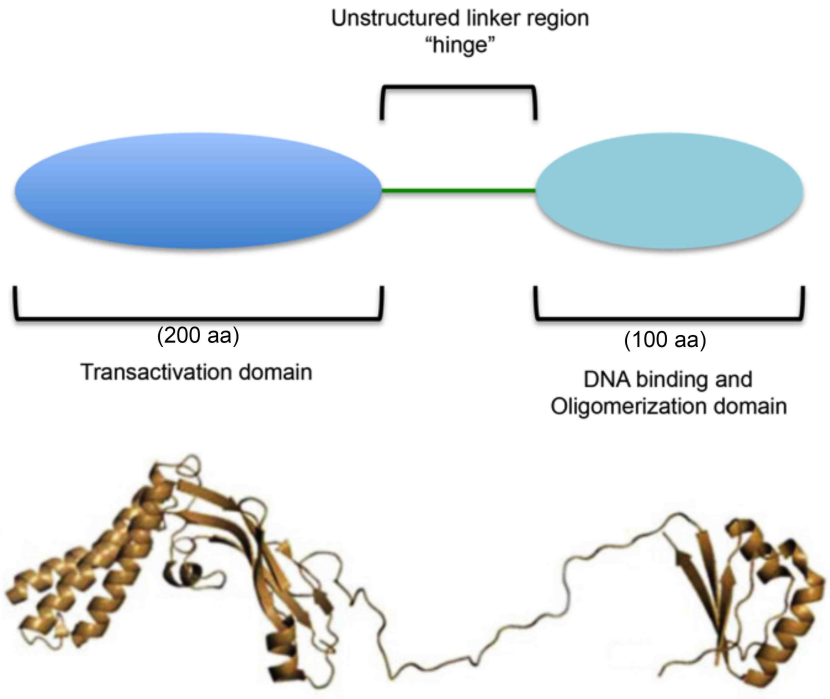


Figure 1.4 Structure of PV E2. E2 has two distinct functional regions, the N-terminal transactivation domain and the C-terminal DNA binding and oligomerization domain. This figure illustrates the monomeric DBD. A flexible and unstructured linker region connects the functional domains of the protein. A portion of this figure was adapted from (M. Muller & Demeret, 2012).

1.4 Papillomavirus Replicative Program

The viral replicative program can be separated into three defined stages; establishment, maintenance, and vegetative amplification. The initiation of a PV infection results in the establishment and maintenance of a low copy-number of double stranded viral episomes within infected basal keratinocytes. The PV replicative program is closely linked to the life cycle of the keratinocyte host cells and is moderated by a combination of viral and host cell factors. During establishment, there is an initial amplification of replication to establish a low copy number of viral genomes in basal cells. The maintenance stage is where the low copy numbers (1-10) of viral episomes are maintained in basal keratinocytes. Maintenance is followed by the vegetative amplification stage in differentiating cells of the upper epithelial strata, where the viral copy number is increased, late genes are expressed, and viral genomes are packaged into new infectious particles (Hebner & Laimins, 2006). There is a distinct correlation between the differentiation of keratinocytes and the switch from the maintenance to the amplification stage; however, the details of how the virus senses and progresses through these stages and how replication is regulated remain unknown.

PVs enter the epithelium through micro-abrasions and infect basal keratinocytes (Schmitt et al., 1996). To facilitate viral entry, viral particles bind to the primary receptor hepran sulfate proteoglycans (HSPGs) on the surface of the basement

membrane that causes a conformational change in the L2 capsid protein (Giroglou, Florin, Schafer, Streeck, & Sapp, 2001; Joyce et al., 1999; Shafti-Keramat et al., 2003). Studies have also indicated the involvement of secondary receptors such as α6 integrin (Evander et al., 1997; McMillan, Payne, Frazer, & Evander, 1999; Yoon, Kim, Park, & Cheong, 2001), CD16 (Da Silva et al., 2001) and laminin-332 (Cerqueira et al., 2013) in membrane attachment. After attachment, viral entry occurs either through caveolar or clathrin mediated endocytosis depending on the PV type (Hindmarsh & Laimins, 2007; J. L. Smith, Campos, & Ozbun, 2007). The L1 protein dissociates and L2 transports the viral DNA to the nucleus where it complexes with promyelocytic leukemia protein (PML) bodies (Day, Roden, Lowy, & Schiller, 1998).

Directly following internalization of the virus and the initial amplified replication to establish copy number, viral replication occurs once per cell cycle (Hoffmann, Hirt, Bechtold, Beard, & Raj, 2006). As the infected basal cells divide, the E2 protein assists in tethering the viral episomes to chromatin, allowing the equal partitioning of genomes, and maintenance of the established copy number in the resulting daughter cells (Skiadopoulos & McBride, 1998). One of the infected daughter cells then enters into the keratinocyte differentiation program. As the cells are actively dividing the virus adheres to the rule of replicating once per cell cycle; however, this changes as the cell travels up through the suprabasal epithelial layers and begins progressing towards terminal differentiation (Figure 1.5) (Kajitani, Satsuka, Kawate, & Sakai, 2012). During the stage where normal

uninfected keratinocytes begin exiting the cell cycle, PV infected cells remain proliferative even though they show some signs of differentiation morphologically.

Differentiation of the infected keratinocytes causes the virus to enter the vegetative amplification stage of its replicative program. During the amplification stage, the virus is able to replicate without the restraint of the cell cycle and to produce high copy numbers of viral genomes (Peh et al., 2002). It has been reported that the virus utilizes the DDR pathway to facilitate this amplified replication (Gillespie, Mehta, Laimins, & Moody, 2012; Moody & Laimins, 2009).

Figure 1.5

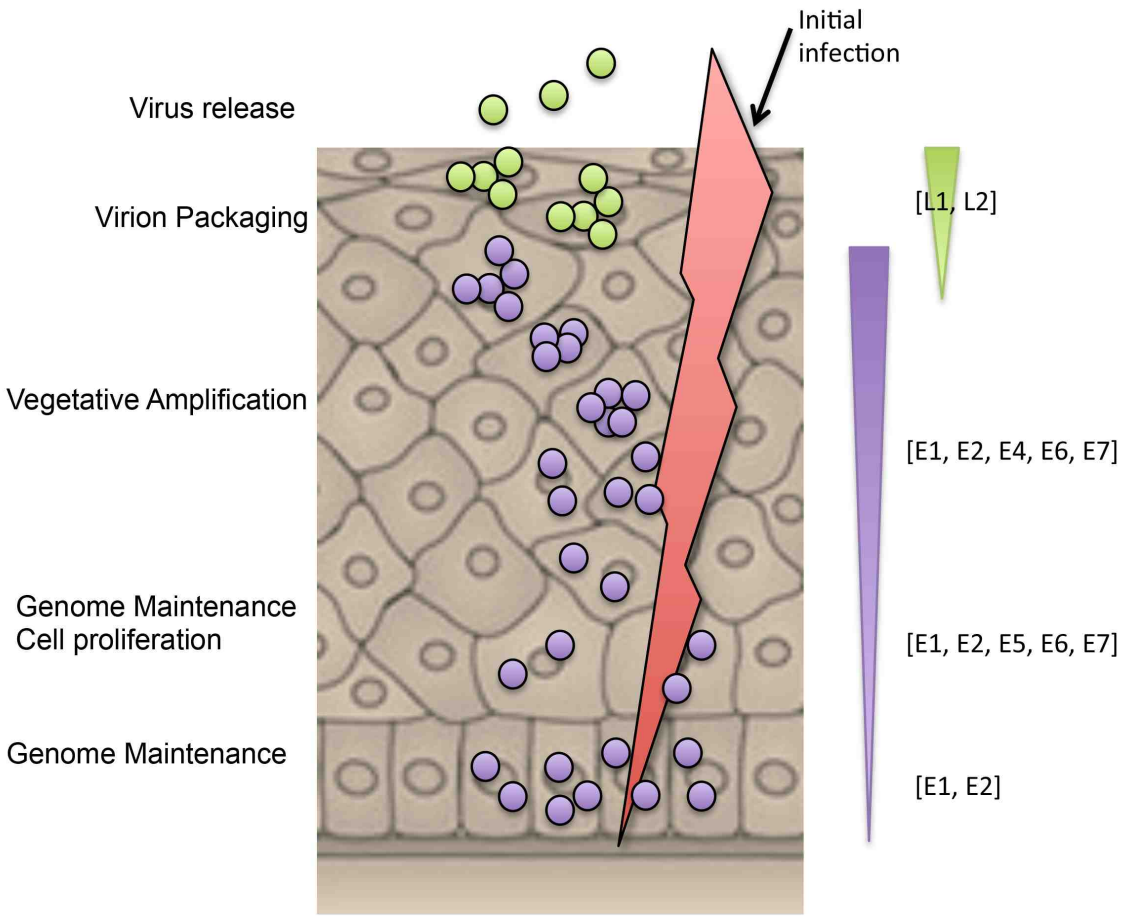


Figure 1.5 The replicative cycle of Papillomaviruses. The life cycle of PVs are closely regulated by their host keratinocytes. This figure shows the stages of the viral replicative cycle, the viral genes expressed at each stage and where these different stages fall in reference to the keratinocyte life cycle. This representation is hypothetical due to the difficulty in identifying the low levels of HPV proteins in epithelial cells. The late proteins E4, L1 and L2 are readily detectable.

The ataxia-telangiectasia mutated (ATM) branch of the DNA damage response pathways is activated by PVs and these factors are recruited to the viral genome. Both the E1 and E7 proteins have been linked to the activation of ATM. E7 is proposed to activate STAT5 phosphorylation which causes the activation of the ATM pathway (Hong & Laimins, 2013). E6 and E7 were found to independently activate caspases in differentiating cells (Moody, Fradet-Turcotte, Archambault, & Laimins, 2007). The activation of caspases was found to be essential for genome amplification, and a conserved caspase 3/7 cleavage site has been identified in E1 (Morin et al., 2011). It is proposed that this cleavage of E1 allows the protein to bind more efficiently and specifically to the replication and utilize DDR factors during amplification. At this stage, the virus shifts from the bi-directional theta mode of replication to the bi-directional Rolling Circle mode (Flores & Lambert, 1997) this is controversial. At this stage, the late genes L1 and L2 that comprise the viral capsid are expressed and viral genomes are packaged into infectious particles.

1.5 Papillomavirus E2 Transcriptional Regulation

In the previous chapter, we established that E2 is responsible for tightly regulating the expression of viral genes throughout the PV life cycle (Chin, Hirochika, Hirochika, Broker, & Chow, 1988; Phelps & Howley, 1987; Spalholz, Lambert, Yee, & Howley, 1987). E2 functions in transcription by binding at E2BS, and depending on the context of this binding and the factors recruited, either activating or repressing transcription. BPV E2 is a strong transcriptional activator. Unlike HPV, the BPV genome has 12 E2BS within the LCR in a region called the minichromosome maintenance element (MME) (Piirsoo, Ustav, Mandel, Stenlund, & Ustav, 1996). This region is involved in the tethering of host chromosomes during mitosis, and the abundance of E2BS allows E2 to recruit host transcription factors to activate transcription. The transactivation domain of E2 interacts with cellular transcription factors AP1 and Sp1, bromodomain binding protein (Brd4), and histone acetyl transferases (HATs) CBP/p300 and pCAF (Ilves, Maemets, Silla, Janikson, & Ustav, 2006; D. Lee, Hwang, Kim, & Choe, 2002; D. Lee, Lee, Kim, Kim, & Choe, 2000; A. Muller, Ritzkowsky, & Steger, 2002).

BPV also encodes for a number of truncated E2 proteins that do not contain a transactivation domain and serve as repressors (Doorbar et al., 1990; Hubbert, Schiller, Lowy, & Androphy, 1988). The truncated E2s such as E2C repress transcription by competing with full-length for the E2 binding sites, or by forming

heterodimers with full-length E2 (McBride, Bolen, & Howley, 1989). HPV E2 has been reported to be a repressor of early genes in HPV (Spalholz et al., 1987; Spalholz, Yang, & Howley, 1985; Thierry & Yaniv, 1987). There are only 4 E2BS in the HPV LCR and E2 represses transcription through steric hindrance, by limiting the binding of host transcription factors and through the recruitment of repressor complexes (Ammermann, Bruckner, Matthes, Iftner, & Stubenrauch, 2008; Fertey et al., 2010). The early promoter of most HPVs is also regulated by the E2BS that coincide with the TATA box and prevent the assembly of the transcription initiation complex when E2 is present (Demeret et al., 1997).

The E2 gene undergoes alternative splicing; splice variant E8^E2 represses both transcription and replication (Stubenrauch, Hummel, Iftner, & Laimins, 2000). Brd4 is an integral binding partner of E2 and is essential for the stability of the E2 protein, tethering of viral genomes to host chromatin, transcription, and replication (McBride & Jang, 2013; McBride, McPhillips, & Oliveira, 2004; McPhillips et al., 2006; Sakakibara et al., 2013; Schweiger, Ottinger, You, & Howley, 2007; Zheng et al., 2009). E2 reacts with Brd4 through the transactivation domain, and splice variants as well as truncations that are unable to form this interaction also regulate viral processes (Abbate, Voitenleitner, & Botchan, 2006).

During productive infection, E2 regulates the expression of the early genes and maintains low levels of oncoproteins E6 and E7. The E6 and E7 proteins are best

known for their oncogenic properties. E6 in high-risk HPV strains binds with the E3-ubiquitin ligase E6 associated protein (E6AP) to ubiquitylate p53 and cause its proteosomal degradation (Lechner & Laimins, 1994; Scheffner, Huibregtse, Vierstra, & Howley, 1993). High-risk HPV E7 has a similar effect on the cells by degrading the retinoblastoma tumor suppressor protein (RB) (Munger et al., 1989). The E6 and E7 proteins have other functions apart from bypassing tumor suppressors; both E6 and E7 are required to maintain the viral genome as episomes, and E6 is necessary for stable genome replication (R. B. Park & Androphy, 2002). Our lab previously reported the inability of E6 mutants that are p53 degradation defective to support stable genome replication in primary keratinocytes. Mutation of E2 or integration of the viral genome can lead to the loss of E2 function, and the loss of E2-mediated repression of the E6 and E7 genes causes the cells to progress towards malignancy (Bernard et al., 1989; Schwarz et al., 1985; Thierry & Howley, 1991).

1.6 Papillomavirus Replication

Normal mammalian replication is initiated by the recognition of an origin by the origin recognition complex (ORC). ORC is a complex of seven subunits and serves as the foundation for the assembly of pre-replication complex (pre-RC). ORC 2-6 are found associated with DNA throughout the cell cycle; however, ORC1 is only found on DNA during G1 and is released in S-phase (DePamphilis, 2003; K. Y. Lee et al., 2012; Papior, Arteaga-Salas, Gunther, Grundhoff, &

Schepers, 2012). Mammalian replication is tightly regulated by a process called replication licensing that is modulated by the cell cycle and ensures that replication occurs once per cycle (Sclafani & Holzen, 2007; Truong & Wu, 2011). The pre-RC is composed of ORC, Cdt1 and Cdc6. Cdt1 and Cdc6 load the mini-chromosome maintenance proteins (Mcm2-7) onto the origin, which is followed by the activation of the Mcms due to the loading of the pre-initiation complex (pre-IC). The pre-IC consists of Cdc45, the GINS complex, Mcm10, and DNA polymerase. Replication is then initiated by the unwinding of the origin by the Mcm helicase.

In Eukaryotic replication the sequential loading of replication factors is closely linked to cell cycle progression. The formation of the pre-RC and loading of the pre-IC occur in late mitosis and early G1, and during S phase the Mcm complex is activated (Sclafani & Holzen, 2007). In the maintenance stage of the viral life cycle, viral replication is licensed in a similar manner and only occurs once per cell cycle (Hoffmann et al., 2006). During the amplification stage of the lifecycle; however, PVs are able to overcome the licensing restrictions and replicate several times per cell cycle. The mode of replication may change from what is known as a theta model to rolling circle model of replication (Kusumoto-Matsuo, Kanda, & Kukimoto, 2011). Others have proposed alternative models including homologous recombination. The specific factors involved PV replication licensing are not fully understood.

Figure 1.6

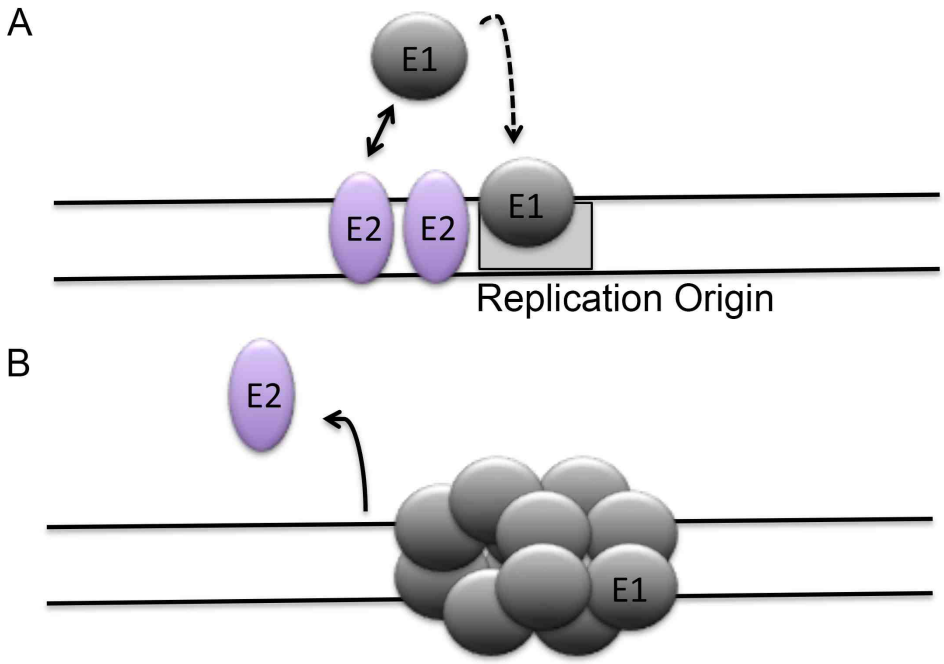


Figure 1.6 Papillomavirus replication initiation. Viral replication begins with the recognition of E2BS flanking the origin by E2 and the recruitment of E1 (A) and the formation of a double-hexamer helicase structure of E1 following the removal of E2 (B).

Viral early proteins E1 and E2 are the only replication proteins since the viruses do not code for a polymerase and previous replication studies have identified the early proteins E1 and E2 as the only viral proteins necessary for transient replication of the viral genome (Sanders & Stenlund, 2001; Ustav & Stenlund, 1991; Ustav et al., 1991). The E1 protein is the major viral replication protein, and it has been reported that E1 alone is sufficient to initiate viral replication in vitro; however, the binding of E1 to DNA is non-specific and E2 is required in-vivo (Ustav & Stenlund, 1991; Ustav et al., 1991).

The PV replication origin contains E1 binding sites, which are flanked by high affinity E2 binding sites. To initiate viral replication, E2 binds to DNA in a sequence specific manner at the E2BS and recruits E1 to bind at the origin (Figure 1.6). When the first pair of E1 molecules binds to the replication origin, E2 is released from its binding sites through a process of ATP hydrolysis (Sanders & Stenlund, 1998). E2 leaving allows the ‘space’ necessary for the bound E1 proteins to recruit more E1 molecules and form a double-hexameric complex at the origin that is similar to the eukaryotic Orc-Mcm complex (Ustav & Stenlund, 1991). This E1 helicase complex unwinds DNA and allows host replication factors to be recruited to the viral genome (Sanders & Stenlund, 1998). The loading of E2 and its regulation of E1 binding, serves as a quasi replication licensing system for viral replication. Without this regulation, the inability of E1 to bind specifically to the replication origin would cause inefficient and inconsistent firing of the viral origin. Though the functions of E1 and E2

during replication initiation have been established (Berg & Stenlund, 1997; Yang et al., 1993), the exact dynamics at the replication origins and the host replication proteins involved in this event are still being investigated.

1.7 E2 Post-translational Modifications

It is proposed that PV E2 undergoes certain post-translational modifications (PTMs) that regulate its function (McBride et al., 1989; Penrose, Garcia-Alai, de Prat-Gay, & McBride, 2004); however, these PTMs have been inadequately characterized. Phosphorylation of E2 has been studied in human and non-human PVs (McBride et al., 1989; Sekhar & McBride, 2012). BPV E2 has phosphorylation sites in the hinge region at serines 290, 298, and 301, which were the first to be identified in BPV (McBride et al., 1989). These phosphorylations were found to regulate E2 activity; consequently, affecting copy number and genome maintenance in infected cells (Lehman & Botchan, 1998). Casein kinase II (CK2) phosphorylation at these highly conserved serine residues signals E2 proteosomal degradation, hereby negatively regulating viral replication, transcription, and copy number maintenance (McBride, Romanczuk, & Howley, 1991; Penrose et al., 2004). Threonine 286 in HPV16 is reported to be a putative phosphorylation site for Cdk2 (Johansson, Graham, Dornan, & Morgan, 2009). This threonine is highly conserved among alpha PVs and is structurally located in a similar position as the BPV serines mentioned previously. Another BPV phosphorylation site was described at serine 235 that enhances E2

activity (Lehman, King, & Botchan, 1997), demonstrating that phosphorylation both positively and negatively regulates E2 functions.

In-vitro studies of E2 have also indicated potential sites of sumoylation. BPV and HPV16 E2 are both bound by ubiquitin carrier protein 9 (Ubc9) (Y. C. Wu, Roark, Bian, & Wilson, 2008). The site of sumoylation is K292; which is conserved in the majority of alpha PVs, is important for E2 transcriptional regulation. Sumoylation has been shown to increase the stability of E2 from HPV11, 16, and 18 (Y. C. Wu, Bian, Heaton, Deyrieux, & Wilson, 2009), and the coincidental increase of sumoylation in differentiating keratinocytes indicates that this PTM may be involved in the switch to amplification mode (Deyrieux, Rosas-Acosta, Ozbun, & Wilson, 2007).

Higher levels of E2 protein are expressed in the upper layers of the epithelium where the virus enters the amplification stage of its replicative program. Recent reports demonstrated that keratinocyte differentiation coincides with and is induced by an increase in expression of the acetyltransferase p300 (EP300) (Wong, Pickard, & McCance, 2010). Our lab has previously identified conserved lysine residues in Bovine PV (BPV1) E2 that were acetylated by p300 *in vitro* and regulate its transcriptional activation capability (Quinlan, Culleton, Wu, Chiang, & Androphy, 2012). Of the several conserved lysines that were identified, a di-lysine motif located in the transactivation domain was of particular interest. These lysines at positions 111 and 112 are conserved in more than 90% of PVs (Figure

1.7), including BPV1 and cancer associated ‘high risk’ HPV types 16, 18, and 31. Studies have shown that mutating these lysines to alanines reduced their ability to activate transcription and alter their nuclear localization (Quinlan, Culleton, Wu, Chiang, & Androphy, 2013).

In this study we examined the effect of mutating these lysines on BPV replication, and the effect of corresponding mutants in HPV31 E2 on the initiation of DNA replication. We discovered that viral replication did not tolerate the conservative mutation of K111 to arginine. Our data reveal that DNA unwinding by the E1 helicase is dependent on the presence of a lysine at position 111 in E2.

Figure 1.7

```
BPV1    CRVPHSVVCQERAKQAIEMQLSLQELSKTEFGDEPWSLLDTSWDRYM-SEPKRCFKKGAF
HPV1    QAVPSLASSQEKAKTAIEMVLHLESLKDSPYGTEDWSLQDTSRELFL-APPAGTFKKSGS
HPV5    QPVPVKAVSETKAKEAIAMVLQLESLQTSDFAHEPWTLVDTSIETFR-SAPEGHFKKGPI
HPV6    QVVPPLKVSEAKGHNAIEMQMHLESLLRTEYSMEPWTLQETSYEMWQ-TPPKRCFKKRGK
HPV8    QPVPPLAVSEAKAKQAIGIMLQLQSLQKSEFADEPWTLVDTSIETYK-NAPENHFKKGAI
HPV11   QVVPPLTVSETKGHNAIEMQMHLESLAKTQYGVEPWTLQDTSYEMWL-TPPKRCFKKQGN
HPV16   QVVPTLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVYL-TAPTGCIKKHGY
HPV18   QVVPAYNISKSKAHKAIELQMALQGLAQSAYKTEDWTLQDTCEELWN-TEPTHCFKKGGC
HPV31   QVVPALSVSKAKALQAIELQMMLETLNNTEYKNEDWTMQQTSLELYL-TAPTGCLKKHGY
MmuPV1  QPLPSTIGAENKAKRAIQMQLVLTSLNESPFGSEEWTMAETSREMYDSTEPYGTFKKSGF
SfPV1   TPVPSLLTSQECAKQAIEMVLYIESLLRSPYSDEPWTLQDTSRERFE-SPPQKTFKKNPA
```

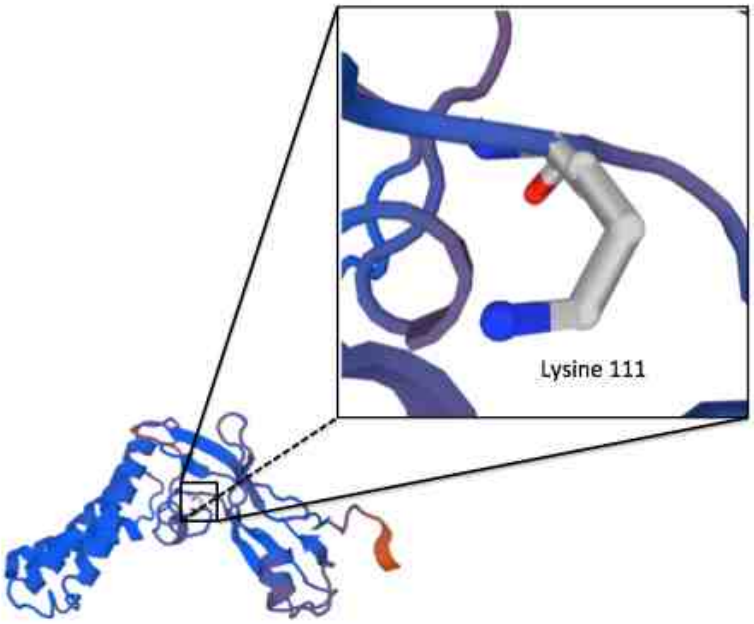


Figure 1.7 Sequence alignment of PV E2 and location of K111. Lysine 111 is a highly conserved amino acid residue is most PVs, including high and low risk HPVs and non-human PVs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell culture, Plasmids, Reagents

C33A and HeLa (ATCC), HEK293TT (J. Schiller, C. Buck), H1299 (Giaccone et al., 1992), CV-1 Douglas Lowy), and J23T3 (H. Green) were maintained in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% FBS (Atlas Biologicals) and 100 U/ml penicillin/streptomycin (Life Technologies). Specific information about all cell lines used in this study can be found in Table A-5.

Spontaneously immortalized keratinocyte cell line (NIKS) were co-cultured in F-Media (Allen-Hoffmann et al., 2000) with mitomycin-C treated J23T3.

Primary HFKs were harvested from donated human neonatal foreskins, and were maintained in KSFM (Life Technologies) supplemented with 100 U/ml penicillin/streptomycin. After transfection they were cultured on feeder cells in E-media.

F-Media

3 parts Ham’s F12 nutrient mix (Life Technologies)

1 part Dulbecco’s Modified Eagle Medium (Life Technologies)

5% FBS (Atlas Biologicals)

400 ng/ml hydrocortisone (Sigma)

5μg/ml insulin (Sigma)

0.18 mM adenine (Sigma)

0.1 nM cholera toxin (Sigma)

100 ng/mi epidermal growth factor (EGF) (Life Technologies)

100 U/ml penicillin/streptomycin (Life Technologies)

E-Media

1 parts Ham’s F12 nutrient mix (Life Technologies)

3 part Dulbecco’s Modified Eagle Medium (Life Technologies)

5% FBS (Atlas Biologicals)

400 ng/ml hydrocortisone (Sigma)

5μM transferrin (sigma)

5 μg/ml insulin (Sigma)

0.18 mM adenine (Sigma)

0.1nM cholera toxin (Sigma)

100ng/mi epidermal growth factor (EGF) (Life Technologies)

100 U/ml penicillin/streptomycin (Life Technologies)

Southern blot analyses were conducted using the Brightstar BioDetect Kit (Ambion) and the Random Primed DNA Labeling Kit (Roche).

HPV31 E2 mutant plasmids were constructed by site-directed mutagenesis (Aligent; Quick-Change II).

HA tagged HPV31 E2 was obtained from P. Howley (Powell et al., 2010), codon optimized flag tagged HPV31 E2 was supplied by A. McBride (Oliveira, Colf, & McBride, 2006), and the HPV31 E1, pCI-RLuc, and pFLOri31 constructs were obtained from J. Archambault (Fradet-Turcotte, Morin, Lehoux, Bullock, & Archambault, 2010) (Table A-4).

Antibodies used include anti-Flag M2 (Sigma Aldrich), anti-HA (HA7; Sigma Aldrich), TopBP1 (Bethyl), mouse anti-FLAG M2 and mouse anti-HA (Sigma Aldrich). Anti-Brd4 antibodies were obtained from C. Chiang, and goat anti-mouse 594 and goat anti-rabbit 488 HRP were from the Jackson ImmunoResearch. Alexa Fluor antibodies were purchased from Thermo Fisher.

All primer sequences for PCR. Mutagenesis, and sequencing are located in Tables A-1,2,and 3.

2.2 Methods

2.2.1 Transfections

Various transfection methods were utilized in this study. C33A and HeLa were transfected with Lipofectamine2000 at a ratio of 1:1, 293TT were transfected with PEI at a ratio of 1:2, and NIKs were transfected using X-tremeGene HP DNA Transfection Reagent (Roche) at a ratio of 1:2 DNA to transfection reagent. C33A cells for BPV replication assays were transfected by $CaPO_4$ transfection.

Lipofectamine2000™ transfection.

Cells were transfected at 50% confluence unless otherwise specified. Plasmids were prepared for transfection with equivalent DNA loads for each sample. In two separate tubes containing 100 µl of Opti-MEM® (Life-Technologies) each, the plasmid mixes were added to one tube and the corresponding amount of Lipofectamine to the other. The tubes were incubated at room temperature for 5 minutes, and then the contents mixed by pipetting. After 10 minutes the transfection mix was added to the cells. The cells were incubated at 37°C and 5% CO_2 over night, and then the transfection media was replaced with fresh media.

PEI transfection

Plasmid mixes were prepared for transfection with equivalent DNA loads for each sample. In a tube containing 200 µl of Opti-MEM® (Life-Technologies) and PEI, the plasmid mixes were added and mixed by pipetting. Plasmid mixes were incubated at room temperature for 10 minutes, and then the transfection mix was added to the cells. The cells were incubated at 37°C and 5% CO_2.

Calcium Phosphate Transfection

Cells were seeded for experiments overnight and were transfected at ~50% confluence. The cells were given fresh growth media 3 hours prior to transfection and the reagents were thawed to room temperature. Two tubes were prepared for each transfection; one containing HEBS and the second DNA and $CaCl_2$ diluted in H_2O. The DNA tube contents were thoroughly mixed, then the HEBS was added to the DNA tube drop-wise while air is bubbled through the sample for mixing. The tubes were then incubated at room temperature for 30 minute before adding to the cells.

X-tremeGENE HP transfection

The transfection reagent was allowed to thaw at room temperature prior to transfection. The reagent was diluted in Opti-MEM® and the DNA was added to a

ratio of 1 µg plasmid DNA to 100 µl medium. Samples were incubated at room temperature for 15 minutes and then added directly to cells in growth medium.

Amaxa®Human Keratinocyte Nucleofector® Transfection

Keratinocytes were passaged at 70% confluence 4 days prior to transfection. Plates of culture (E media) with J2 feeders were prepared for each transfection. The cells were trypsinized and the trypsin was neutralized with trypsin inhibitor. The cells were collected and centrifuged at ~200g at room temperature. The supernatant was discarded and the pellet was resuspended in 100 µl of Nucleofector® solution, then combined with 100 µl DNA (in water). The mixture was transferred to a cuvette and electroporation was conducted with a Nucleofector® device under the high efficiency setting for keratinocytes. The cells were immediately transferred to E media.

Nucleofector® Solution

82 µl Nucleofector® Solution

18 µl supplement

2.2.2 Keratinocytes

2.2.2.1 Harvesting Keratinocytes

Foreskins were collected in antibiotic solution, and stored at 4°C until harvesting. The tissue was cut into strips, and the subcutaneous layer of fat and hypodermis were removed with tweezers and a scalpel. The strips were placed epidermis side down into a sterile dish with collagenase dispase solution. 5 ml of Keratinocyte Serum Free Media (KSFM) were added to the dish and the entire dish was wrapped in plastic and stored at 4°C overnight. After incubation the epidermis was peeled off using tweezers and transferred into a dish of KSFM. The tissue was minced using a scalpel, added to a tube containing Trypsin/EDTA (Life Technologies), and incubated at 37°C for 45 minutes with frequent agitation. The trypsin was neutralized using trypsin inhibitor (Life Technologies) and the tube was centrifuged at ~200g for 5 minutes. The supernatant was discarded and the cells were resuspended in KSFM. The cells were then plated and incubated at 37°C.

Antibiotic Solution (store at 4°C)

1% penicillin/streptomycin

250 ng/ml amphotericin B

Antibiotic Dispase Solution

1% penicillin/streptomycin

250 ng/ml amphotericin B

1% dispase solution

2.2.2.2 Keratinocyte culture

Primary cells (HFKs) are maintained in KSFM and passaged at ~70% confluence. Primary HFKs are passaged lightly, always maintaining a confluence between 30 and 70%. Cells are trypsinized with 0.05% trypsin/EDTA and this is neutralized with trypsin inhibitor (never a full serum growth medium), and cells are frozen in KSFM supplemented with 1% DMSO. HFK cell lines containing HPV31 genomes are maintained in either E or F-media and co-cultured with feeder cells. Feeder cells are replenished every 72 hours by trypsinizing the plate at room temperature for 1 minute, then aspirating the cells off and replacing them with new feeders and fresh media. Feeder cells are J23T3 (mouse epithelial fibroblasts) that are treated with mitomycin C (2 µg/ml for 6 hours).

Transfections of Keratinocytes are conducted by electroporation or XtremeGene-HP in the case of the NIKS. Primary keratinocytes are maintained on feeders in E-media following transfection and are allowed to proliferate for 72 hours prior to any selecting.

2.2.3 DNA Extraction and Purification

2.2.3.1 Hirt Extraction

Low molecular weight DNA is extracted from mammalian cells by Hirt extraction (Hirt, 1967). Cells on plates were rinsed with 1x PBS and harvested by scraping into a 1.5 ml tube. The cells were centrifuged at 4000 rpm for 5 minutes and the supernatant discarded. The cell pellet was resuspended in Hirt extraction buffer; SDS and EDTA were added first and incubated at room temperature for 5 minutes before adding the NaCl. The samples were incubated at 4°C overnight. The following day, the high molecular weight DNA was removed by centrifugation at maximum speed for 30 minutes at 4°C. DNA was purified from the supernatant by phenol:chloroform extraction.

Hirt extraction Buffer:

0.6% SDS

10 mM EDTA

1 M NaCl

2.2.3.2 Genomic DNA Extraction

Cells on plates were rinsed with 1x PBS and harvested by scraping into a 1.5 ml tube. The cells were centrifuged at 4000 rpm for 5 minutes and the supernatant discarded. The cell pellet was then resuspended in Extraction buffer and incubated in a 50°C water bath overnight. DNA was extracted from the samples by phenol:chloroform extraction.

Extraction buffer:

100 mM NaCl

10 mM TrisCl, pH 8

25 mM EDTA, pH 8

0.5% SDS

0.1 mg/ml proteinase K

2.2.3.3 Phenol:Choloroform DNA Extraction

Samples were brought to a volume of 500 µl and added to an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sambrook & Russell, 2006). The tubes were vortexed for 1 minute and centrifuged at 14000 g for 15 minutes at room temperature. The aqueous (top) layer was transferred by pipette to a fresh

microfuge tube and the process was repeated. After the second centrifugation, the top layer was transferred to a tube containing 500 µl of chloroform, vortexed and separated by centrifugation.

2.2.3.4 Ethanol Precipitation

This process usually follows the phenol:chloroform extraction procedure. The sample was added to 100% ethanol and 3 M sodium acetate at a ratio of 1:2:0.1. The samples were vortexed and incubated at -20°C for 1-2 hours. DNA was pelleted by centrifugation at 14000 g and 4°C for 30 minutes. The supernatant was discarded, and incubating the open tubes at room temperature for 10 minutes evaporated any residual ethanol. The DNA was then resuspended in TE or water.

2.2.4 Viral Replication Assays

2.2.4.1 Luciferase

C33A cells were seeded at a density of 25000cells/well 24 hours prior to transfection into 96-well clear bottom plates (Corning). Luciferase based replication assay as previously reported (Fradet-Turcotte et al., 2010). 10 ng each of HPV31 E1, E2 and 2.5 ng pFLORI31/pFLORIBPV-1 constructs were co-transfected into C33As in a 96 well plate, 0.5 ng of pCI-RLuc construct was

transfected per well to normalize for transcriptional activation of the firefly luciferase gene, and the total DNA quantity was adjusted to 100 ng with pCI. Cells were incubated in the transfection media overnight then media was replaced with normal growth media (DMEM). Cells were lysed in Dual-Glo luciferase reagent (Promega) 72 hours post transfection and both firefly and renilla luciferase activities were determined using the PHERAStar FS (BMG Labtech). Firefly luciferase activity was normalized to renilla luciferase activity and the E2 control for each sample.

2.2.4.2 Southern Blot

Southern Blot analysis was conducted by a protocol derived from (Brown, 2001). C33A cells were transfected at 50% confluence with BPV-LCR, E1, and E2 plasmids; 0.5, 3, and 1µg respectively. 72 hours after transfection the cells were harvested by scraping. Episomal DNA was obtained by Hirt extraction at 4°C overnight. Samples were centrifuged at maximum speed in a microcentrifuge for 30 minutes at 4°C. The Supernatants were collected, and DNA was collected by phenol chloroform extraction (phnenol:chloroform:isoamylalcohol 25:24:1). Replicates of the samples were digested with BamH1 and Dpn1, and run on a 0.8% argarose gel. The DNA gel was rinsed in 2x SSC and transferred overnight to BrightStar®-plus membrane in alkaline transfer buffer. The DNA was UV crosslinked to the membrane, then rinsed in 2x SSC. The membrane was blocked in hybridization buffer with heat denatured salmon sperm DNA at 65°C

for ~4 hours. Non-radioactive southern probes were made as instructed with the BrightStar® BioDetect™ Kit using the linearized BPV1-LCR plasmid. After blocking the membrane was placed in fresh hybridization buffer and heat denatured probe, and incubated overnight at 68°C. The membrane was prepared for imaging using the commercial buffers from the kit and accompanying protocols.

In the case of HPV31 stable cell lines, DNA was harvested by scraping, low molecular weight DNA was obtained by Hirt extraction, and the same steps were followed to prepare the membrane. Radioactive (α-^{32}P) probes were prepared with the Random Primed DNA Labeling Kit (Roche) according to the instructions. 25 ng linear HPV31 genome in 9 µl H_2O were denatured at 95°C for 10 minutes then immediately moved to an ice bath. dNTPs (dATP, dGTP, dTTP; 1 µl each), commercial reaction mixture, 50 µCi [α-^{32}P]dCTP (5 µl) and Klenow enzyme (1 µl) were added to the DNA solution and mixed thoroughly. The mix was incubated for 1 hour in a 37°C water bath. The reaction was stopped by heating to 65°C for 10 minutes. The probe was then purified using a DNA purification column. The probe mixed with fresh APH, added to the membrane, and was incubated for 48 hours at 65°C. The membrane was washed several times in SSC and the exposed to the film for 48 hours before analysis on a phosphor-imager.

Alkaline transfer Buffer:

0.4 M NaOH

1 M NaCl

Hybridization Buffer:

5x Denhart's solution

5x SSC

1% SDS

100 µg/ml salmon sperm DNA

2.2.5 Flow Cytometry

C33a cells at 50% confluence were transfected with 2 µg of HPV31 E2 in 60cm dishes. 48 hours after transfection, the cells were harvested with 0.1% Trypsin EDTA, spun down and fixed in 90% ethanol. The samples were washed in PBS and treated with 50 µg/µl each of propidium iodide (PI) and RNase A for 20 minutes. The samples were analyzed using a BD FACS Caliber APS flow cytometer (10,000 events were counted; FL2A for PI: excitation at 488 nm) and FlowJo software.

2.2.6 Immunofluorescence

C33A and NIKs cells on coverslips were transfected with 2.5 ng of each plasmid. Cells were fixed 48 hours post transfection for 15 minutes in 4% formaldehyde solution in PBS, washed three times with PBS, and permeabilized in PBT (0.2% tritonX/PBS) for 10 minutes. The cells were blocked for 1 hour in 10% normal goat serum/PBT, and incubated with primary antibodies (1:5000 in 10% normal goat serum/PBT) overnight. The cells were washed three times and incubated with secondary antibodies (1:5000 in 10% normal goat serum/PBT) for one hour. Cover slips were washed and then mounted with VECTASHIELD antifade mounting medium with DAPI (Vector Labs).

2.2.7 Transcriptional Repression

2.2.7.1 Luciferase Assay

25 ng of HPV31 E2 (wt or mutant) or empty vector control (pCI) and 100 ng HPV31-luc constructs were co-transfected into C33As in each well of a 12-well plate. 48 hours post transfection; cells were lysed and processed with the Dual-Glo luciferase kit (Promega). Luminescence was measured using the PHERAStar FS (BMG Labtech).

2.2.7.2 HeLa repression assay

HeLa cells in 6 well dishes were transfected at 50% confluence with 1 ug HPV31 E2 (WT or mutants). 48 hours post transfection the cells were rinsed with cold PBS and lysed directly in plates using 1 ml TRIZOL. The cells were collected by scraping, and homogenized by several passes through an 18-gauge syringe. RNA was isolated by phase separation and resuspended in DEPC-treated water. cDNA synthesis was conducted with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and RNA was analyzed by quantitative PCR using HPV18 E6 and actin primers. Figure 2.1 illustrates the mechanism of E2 repression of the E6 gene. The oligos were obtained from Integrated DNA Technologies®.

HPV18 LCR F04: 5' – TGG TGT TTG CTG GCA TAA TC – 3'

HPV18 LCR R04: 5' – TCC GTG CAC AGA TCA GGT AG – 3'

HeLabActin F01: 5' – GGA CAT CCG CAA AGA CCT GT – 3'

HeLabActin R01: 5' – CCA GGG CAG TGA TCT CCT TC – 3'

Figure 2.1

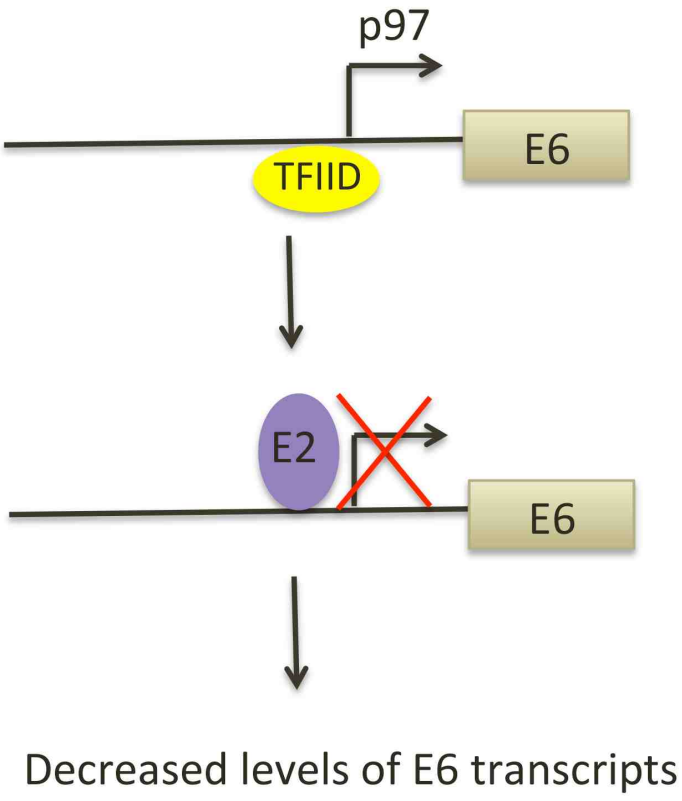


Figure 2.1 HeLa repression assay. Illustration of the concept behind the HeLa repression assay as used for measuring E2 transcriptional repression. Re-introduction of E2 into HeLa cells leads to reduction in E6 transcripts.

2.2.8 Co-Immunoprecipitation

293TT cells were co-transfected with plasmids expressing a codon optimized and flag tagged HPV31 E2 and the protein of interest (Sakakibara, Mitra, & McBride, 2011). The cells were harvested 48 hours post transfection and lysed in 1 ml NP40 buffer (0.5% NP40, 50mM Tris, 150 mM NaCl) for 10 minutes on ice. 100 units of Benzonase endonuclease were added to each sample, and they were rotated at 4°C for 30 minutes. Samples were centrifuged, and supernatants were collected in fresh tubes for immunoprecipitation and inputs. For protein binding the samples were rotated overnight at 4°C with either antibody conjugated beads, or Sepharose A/G beads and ~1μg of antibody. The beads were washed five times with NP40 lysis buffer and resuspended in SDS sample buffer. The samples and their respective inputs were then analyzed by western blot.

2.2.9 Chromatin Immunoprecipitation

C33A cells were transfected with plasmids expressing HPV31 E1, E2 and the HPV31 origin of replication (PFLOri31). 48 hours post transfection, the cells were crosslinked in 1% para-formaldehyde for 10 minutes at room temperature. The cells were rinsed with cold PBS and the crosslinking stopped with 0.125 M

glycine. The plates were rinsed with cold PBS 3 times, and the samples were collected by scraping. These samples were processed using the ChIP-It Enzymatic Shearing kit (Active Motif). The DNA from the samples and their inputs (Figure 2.2) were analyzed by quantitative PCR using primer pairs designed against the HPV 18 or 31 LCR.

Figure 2.2

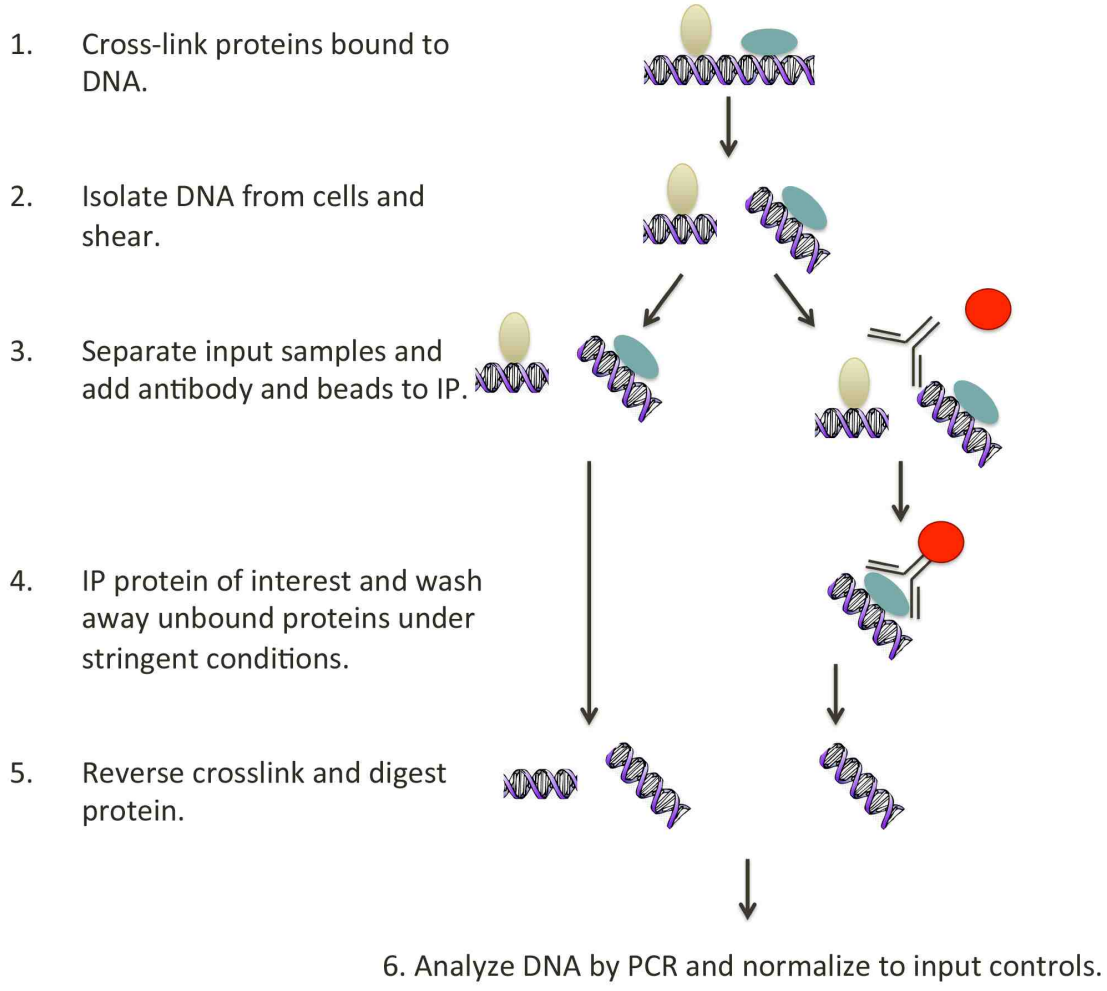


Figure 2.2 Chromatin Immunoprecipitation. Illustration of the ChIP assay showing both IP and input controls.

2.2.10 Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE)

Plasmids expressing HPV31 E2 and E1, along with a plasmid containing the LCR of HPV31 (PFLOri31), were transfected into C33A cells (Figure 2.3). Cells were harvested 48 hours post transfection. One replicate of each sample was harvested immediately by scraping in to cold PBS, and the other was formaldehyde crosslinked with 1% para-formaldehyde for 10 minutes at room temperature. The crosslinking was stopped with 0.125 M glycine for 1 minute, and after washing three times with cold PBS, the samples were collected by scraping. After shearing with the ChIP-It Enzymatic Shearing kit (Active Motif), the chromatin was extracted by phenol-chloroform extraction followed by ethanol precipitation, and resuspended in TE buffer. The crosslinked and respective uncrosslinked control samples were analyzed by quantitative PCR with primer sets designed for the HPV31 replication origin (DeSmet et al., 2016).

LCR primers used for FAIRE:

LCR3 F: 5’ – GTT CTG CGG TTT TTG GTT TC - 3’

LCR3 R: 5’ – TGT TGG CAA GGT GTG TTA GG – 3’

LCR4 F: 5’ – AAA GTG GTG AAC CGA AAA CG – 3’

LCR4 R: 5’ –CAT GCA ATT TCC GAG GTC TT – 3’

Figure 2.3

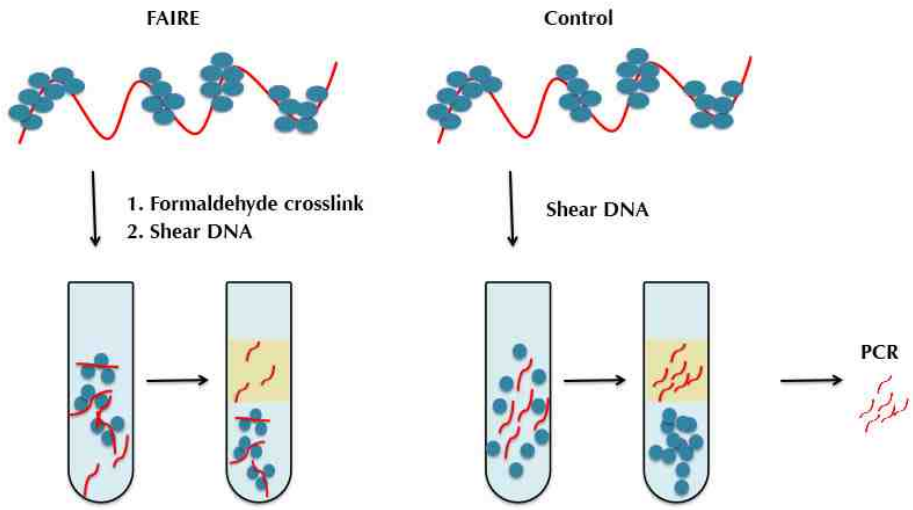


Figure 2.3 FAIRE Assay. This diagram illustrates the concept and protocol for FAIRE assay used to analyze nucleosome structure at the replication origin.

2.2.11 Formation of E1 double hexamer in vivo

C33A cells were transfected with different combinations of pFLORI31, HPV31 E1, and E2. 48 hours post transfection, the media was aspirated off of the cells, and they were rinsed with PBS and fixed with 4% para-formaldehyde for 5 minutes at room temperature. The formaldehyde was removed and the plates were rinsed twice with cold PBS. The cells were collected into 1.5 ml tubes by scraping and lysed with NP40 lysis buffer for 10 minutes on ice prior to adding 100 units of Benzonase to each sample and rotating at 4°C for 1 hour. The samples where then immunoprecipitated (see 2.2.8) with an anti-HPV16 E1 antibody that recognizes HPV31 E1. Protein samples were run on a 4-15% gradient gel (BioRad), and analyzed by western blot analysis with anti-E1 and flag antibodies.

2.2.12 HPV31 Stable Cell Lines

2.2.12.1 HPV31 Whole Genome Cloning

E2 K111 mutations were inserted into the 10.3 Kb genome by a method that required moving an E2 fragment into a smaller temporary plasmid, site directed mutagenesis, and then shuttling the fragment back into the larger plasmid (Figure 2.4). The pBR322 HPV31 plasmid was digested with XbaI:HindIII, HindIII:PvuI, and XbaI:PvuI. pUC18 was digested with HindII:XbaI at the multiple cloning site

(MCS). The digests were all run on a 1% agarose gel and bands were excised based on expected sizes of the resulting fragments. DNA was extracted from the gel slices with a Gel Extraction Kit (Qiagen). The DNA fragment resulting from the digestion of pUC18 and the XbaI:PvuI fragment from the HPV31 plasmid were treated with Antarctic phosphatase prior ligation procedures. The HPV31 HindII:XbaI fragment was inserted into pUC18 using T4 ligase overnight at 16°C. The DNA was transformed into DH5α cells the following day, streaked onto ampicillin plates, and incubated at 37°C overnight. Colonies were picked the following day and grown in 4 ml cultures. DNA was extracted and purified from bacterial cultures with a [plasmid mini-prep kit (Life Technologies) according to manufacturer's instructions. The DNA was run of an agarose to confirm insertion by size.

Mutations at K111 were produced by site directed mutagenesis, and mutants were confirmed by sequencing. The HPV31 mutant fragment and pUC18 are equal in size, so the plasmids were digested out of the pUC18 plasmid with HindIII:XbaI:PvuI to produce only one fragment at the desired size. The DNA was run on a gel and the band excised followed by a DNA extraction with the gel purification kit. To reconstitute the HPV31 plasmid, the three DNA fragments were ligated using T4 ligase overnight. DNA was transformed into DH5 α cells the following day, streaked onto ampicillin plates, and incubated at 37°C overnight. Colonies were screened for presence of mutations at K111.

Figure 2.4

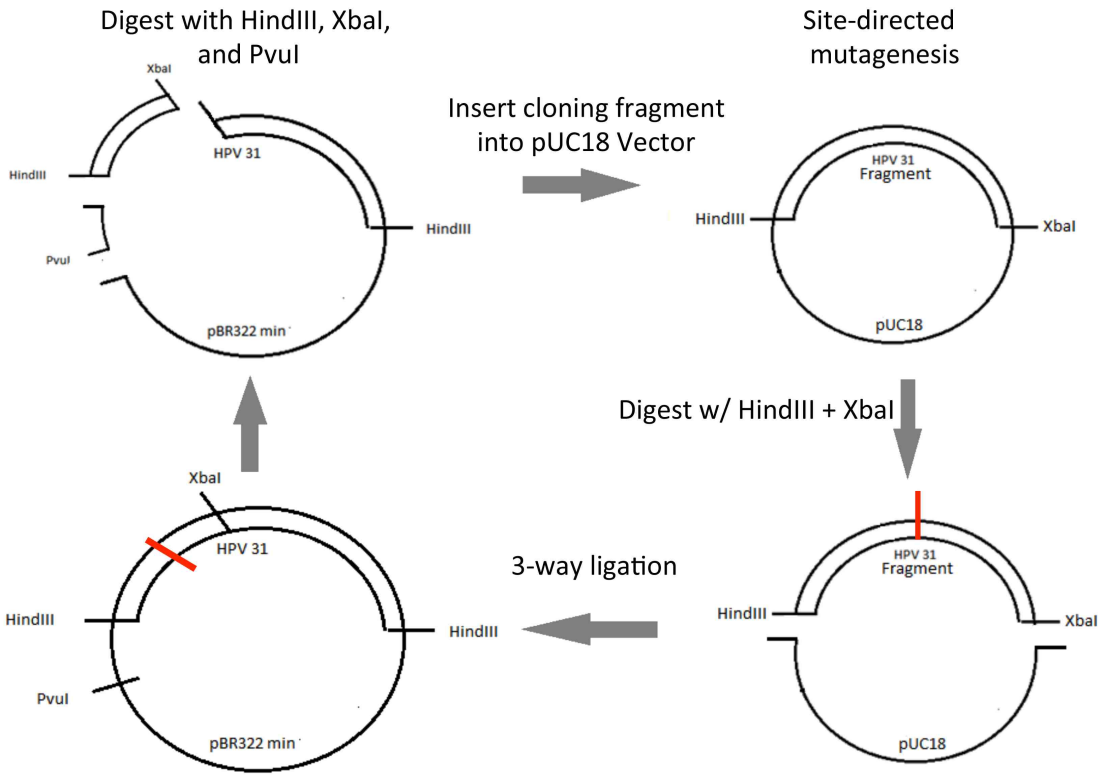


Figure 2.4 Cloning strategy for the HPV31 whole genome mutants. Cloning the K111 mutants into the whole genome plasmid was a multistep process. It required moving a portion of E2 into a temporary vector, site-directed mutagenesis, and a 3-way ligation to re-constitute the large plasmid.

2.2.12.2 Site Directed Mutagenesis

Site directed mutagenesis was conducted using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instructions. The PCR reaction was set up as below and the parameters in Table 2.1:

Strand Synthesis Reaction

5 μl 10x reaction buffer

5 ng/μl plasmid DNA

100 ng/μl of each primer

1 μl dNTP mix

H_2O to bring the total volume to 50 μl.

1 μl of *PfuUltra* HF DNA polymerase (added last)

PCR parameters:

Table 2.1

# of Cycles	Temperature (°C)	Time (sec)
1	95	30
16	95	30
	55	60
	68	300

The PCR products were cooled to 37°C, and then digested with DpnI (1 μl was added directly to the PCR product at 37°C). After digestion the products were transformed into bacteria. Following extraction and purification the K111 mutations in the resulting DNA were confirmed by sequencing.

CHAPTER 3

RESULTS

3.1 K111 is necessary for transient viral replication

Two lysine residues, K111 and K112, are highly conserved among human and animal PVs (Figure 1.7). We previously demonstrated the requirement of K111 for BPV1 E2 transcriptional activation (Quinlan et al., 2012). While E2 is known to bind and localize the E1 helicase to the replication origin, which is flanked by high affinity E2 binding sites, additional role(s) for E2 in DNA replication have not been elucidated. We began testing both BPV and HPV-31 E2 K111 and K112 substitution mutants for stimulation of E1 dependent viral replication following co-transfection with E1 and the PV origin containing luciferase reporter construct. This di-lysine (K111 & K112) motif has been previously studied in BPV1, and mutation at both lysines resulted in a decreased viral replication, whereas a mutation at K111 alone (K111R) caused a more severe reduction in viral replication (Abroi, Kurg, & Ustav, 1996; Brokaw, Blanco, & McBride, 1996).

To further explore the role(s) of E2 K111/K112 in viral replication, we used the luciferase system and southern blots to measure transient replication of K111 and K112 mutants as well as a number of other conserved lysines that we have previously reported as targets for p300 acetylation (Figure 3.1). The southern blot results initially showed that K111R mutant was able to support viral replication,

however, a further analysis of pre-digested samples indicated variation of E1 and E2 levels among samples. Due to these inconsistencies with the southern blot analysis, we used a luciferase based replication assay to quantifiably measure transient replication (Figure 3.2). Compared to wild type BPV E2, mutations at both lysines had deleterious effects on replication, while K112 resulted in a reduction and K111R completely abrogated transient DNA replication (Figure 3.2). The W145R mutant was included in the luciferase assay as a negative control, since this g-protein pathway supressor 2 (GPS2) binding deficient mutation has been described as replication defective (Breiding, Grossel, & Androphy, 1996).

The conservative arginine mutant as well as alanine mutation were tested in HPV31 E2. The results indicate that mutations that prevent acetylation (alanine) at K111 or mimic a deacetylated lysine (arginine) do not induce replication (Figure 3.3). However, the HPV31 K111 glutamine (Q) mutation, which resembles an acetyl lysine, stimulated replication to levels that surpassed wild type E2. We ensured that the abrogation of replication from the K111R mutant HPV31 E2 protein was not due to reduced cell viability (Figure 3.4). These experiments also demonstrated that while replication is affected by K112 in the context of BPV1 (Quinlan et al., 2012), mutations at this position in HPV31 E2 did not exhibit a similar dependence (Figure 3.3).

Figure 3.1

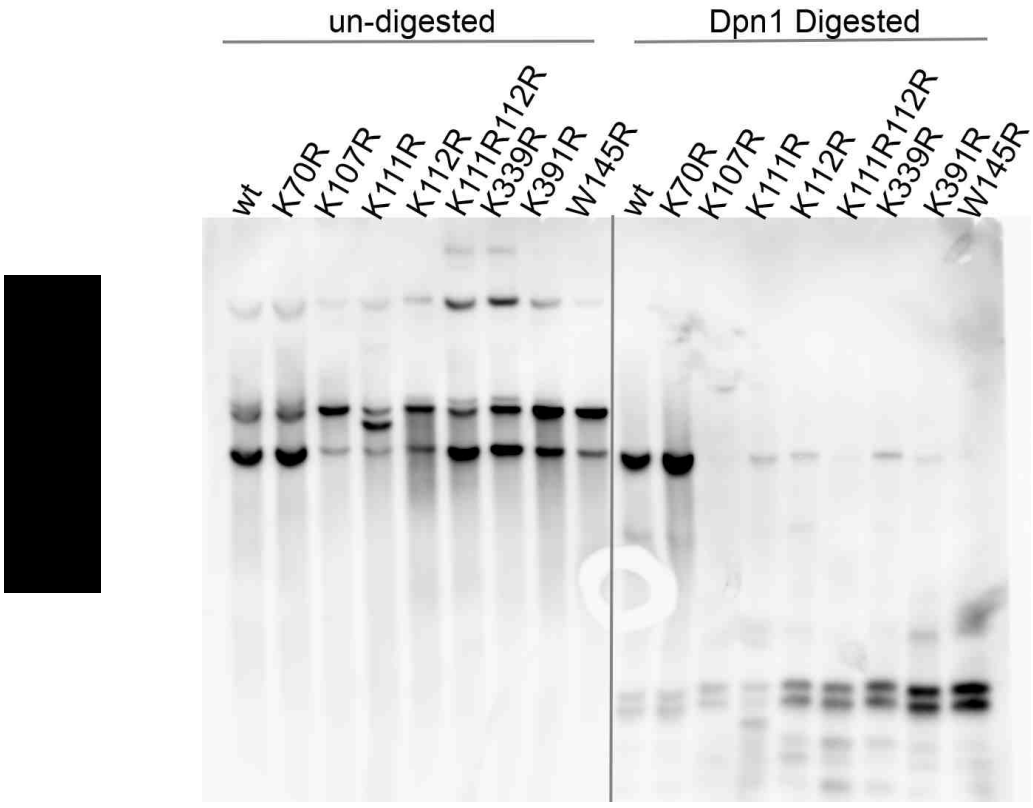


Figure 3.1 Replication of BPV-1 E2 lysine mutants. Southern blot analysis of lysine to arginine mutants of BPV-1 E2, indicating the expression of E1, E2 and the quantity of replicated LCR plasmid. Inconsistent levels of E1 and E2 plasmid were evident in the undigested lanes.

Figure 3.2

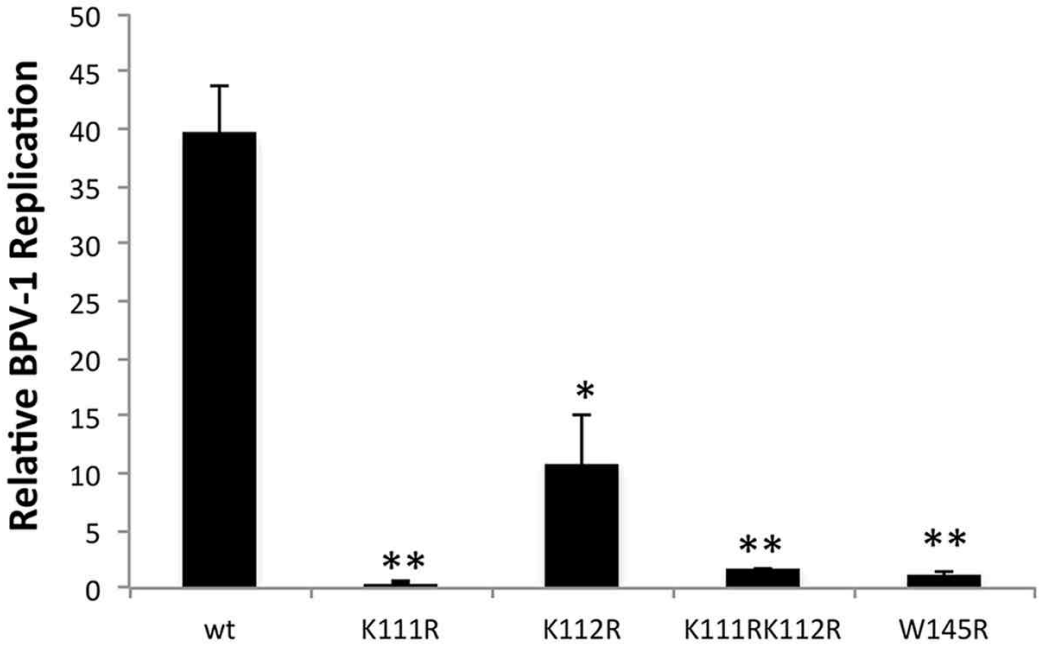


Figure 3.2 BPV-1 E2 K111 mutants affect transient replication. C33A cells were co-transfected with BPV-1 E2 K111 mutants, pFLORI-BPV-1, and pRRL. Results were normalized to renilla luciferase and E2 transcription and presented as fold over E1 only control. *p <0.05, ** p <0.005

Figure 3.3

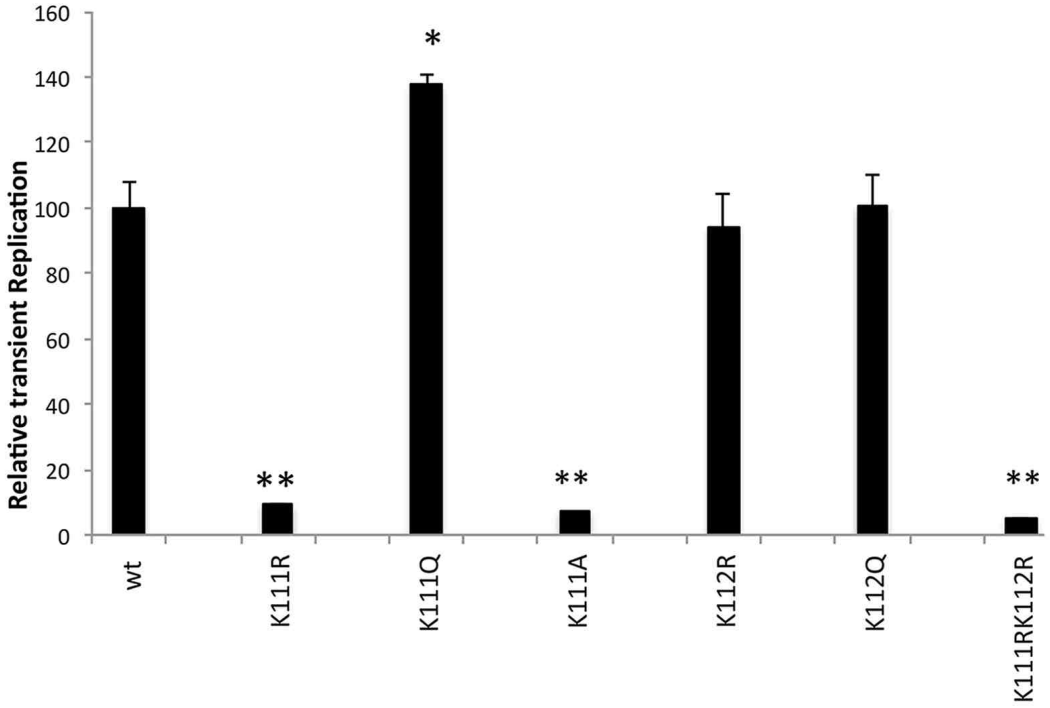


Figure 3.3 Transient replication activities of HPV31 E2 K111 mutants. C33A cells were co-transfected with HPV31 E2 K111 mutants. E2 mutants were co-transfected with pFLOri31. The luminescence was normalized to renilla luciferase and the respective background E2 luminescence. Each of the samples was normalized to their respective control without E1 to cancel out any background effect. * $p < 0.05$, ** $p < 0.005$

Figure 3.4

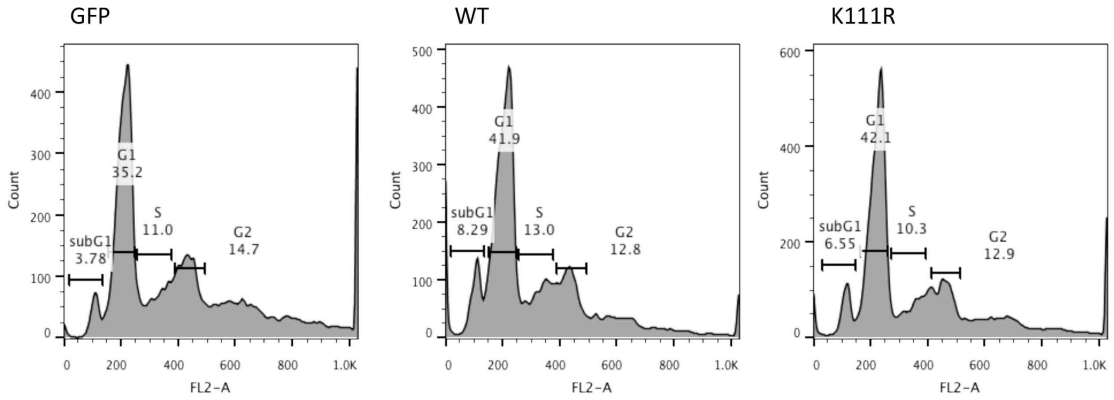


Figure 3.4 Effects of K111 mutations on cell cycle. The effect of K111R mutant on cell viability and cell cycle was determined by flow cytometry.

3.2 Nuclear Localization of E2 occurs Independently of K111

Since K111 of E2 is crucial for viral replication, we further investigated whether mutation(s) at K111 also affects nuclear localization. Though unlikely due to arginine preserving the charge at 111, a possible explanation for this loss of activity is that K111 is located near the putative nuclear localization sequence (NLS) within the trans-activation domain of E2 (Figure 1.4). Deletion mutants in this region of BPV1 are reported to cause E2 to be diffused throughout the cell rather than nuclear (Skiadopoulos & McBride, 1996).

To determine if the mutants demonstrate the same effect in HPV31, we used immunofluorescence technique and observed nuclear localization of K111R mutant compared to wt-E2. A common cell line used in immunofluorescence experiments was a monkey kidney cell line CV-1. E2 K111R had an adverse effect on the nuclei of transfected CV-1 cells. This effect was not observed with wt or K111Q. The nuclei of K111R cell developed a fragmented appearance (Figure 3.5). To study this phenotype we repeated the experiment with other cell lines (C33A, NIKS, and H1299s), and also observed the nuclei of HFKs transfected with the HPV31 genome. Our results indicated that this phenotype is cell line specific.

In transfected C33A, NIKS, and H1299 cells, E2 nuclear localization was unaffected by the K111 mutations (Figure 3.4). These results clearly indicate that

mutations at K111 do not inactivate the putative NLS and E2 remains nuclear. This differs from what was observed with BPV-1 E2 where mutations at K111 resulted in a sub-population of E2 that was localized to the cytoplasm (Quinlan et al., 2013).

Figure 3.5

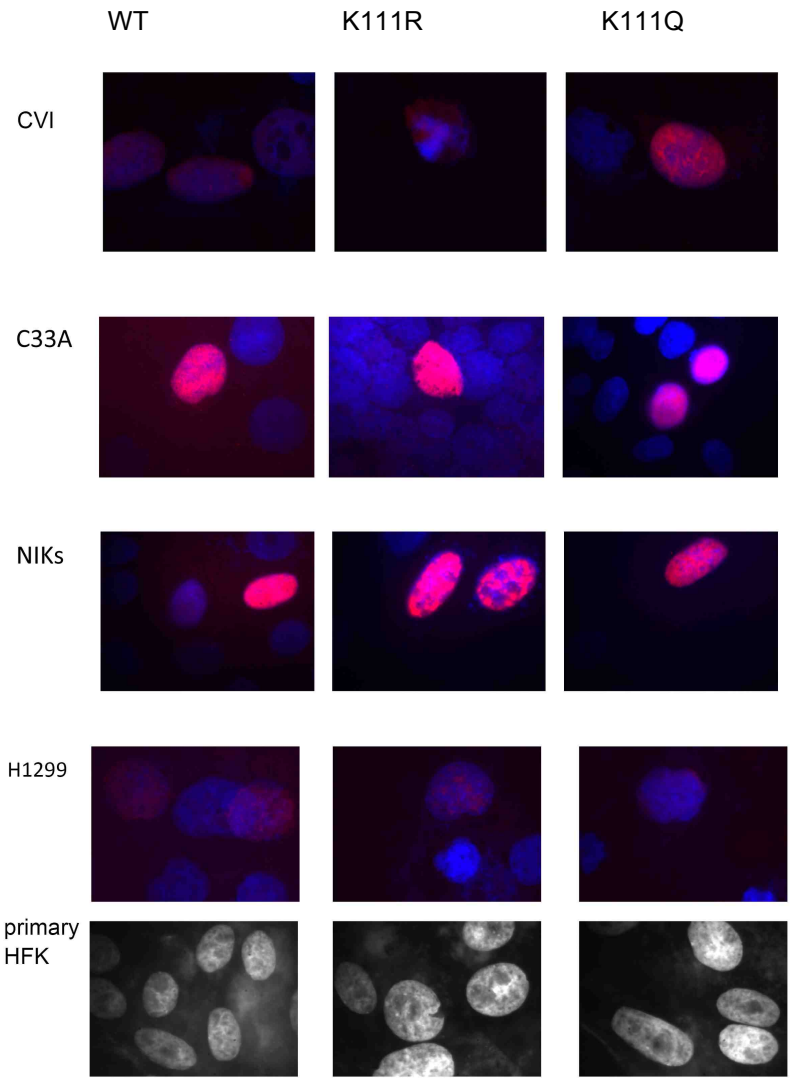


Figure 3.5 K111 mutants do not affect E2 localization. CV1, C33A, NIKS, and H1299 cells were transfected with HPV31 E2wt and the K111 mutants. The cells were incubated with HA7 antibody 48 hours post transfection and analyzed by immunofluorescence for E2 (red) and DAPI (blue). Nuclei of primary HFKs stably transfected with HPV31 wt and mutant genomes are observed (bottom row).

3.3 Mutations of HPV31 E2 K111 facilitate transcriptional repression.

Although K111 is located in the transactivation domain of E2 and not the C-terminal DNA binding region, it may still affect E2 binding to chromatin DNA. Chromatin immunoprecipitation was used to test whether K111 is involved in DNA binding activity of E2 (Figure 3.6). Our results showed that both HPV31 E2 K111R and K111Q were able to bind near the origin with no significant differences compared to wild type E2.

Due to similar results on the effect of the K111 mutants on replication in BPV1 and HPV31, we investigated the transcriptional activities of the K111 mutants. Unlike BPV E2 protein's strong activation, the HPV31 E2 protein acts as an efficient repressor of viral gene transcription (Bernard et al., 1989; Romanczuk et al., 1990; Thierry & Howley, 1991). To examine the effects of mutating K111 on E2 transcriptional repression, we used an HPV31-luciferase assay.

C33A cells were transfected with plasmids expressing HPV31 E2, and a plasmid containing a portion of the HPV31 LCR containing the E2 binding sites and p97 promoter upstream of a firefly luciferase gene. Expression of wild type E2 levels was measured to validate the concentrations of E2 used in the assay (Figure 3.7). I found that the most robust repression effect occurs with low levels of E2. As the quantity of E2 transfected into the cells was increased we observed a dose-dependent increase in transcription due to a squelching effect. Squelching

is a process where a transcription factor inhibits the expression of another gene by directing critical factors away from their targets. E2 in excess may lose its ability to repress transcription due to E2 protein sequestering other transcriptional co-factors away from the E2 DNA binding sequences. BRD4 is a potential suspect in this phenomenon. Since BRD4 is integral to HPV E2 transcriptional repression (McPhillips et al., 2006), free E2 sequestering BRD4 away from E2 bound near the p97 promoter may cause de-repression (Figure 3.7). We selected a low quantity of E2 for the repression luciferase assay. K111R, K111Q, and K111A mutants repressed transcription at levels similar to those of wild type E2 (Figure 3.8). This indicates that K111 is not necessary for HPV31 transcriptional repression.

The luciferase results with C33A cells were also confirmed by a transcriptional repression assay with HeLa cells in order to determine if the K111 mutants were able to access chromatin. In the integrated HPV18 genome of HeLa cells, E2 represses transcription of the early promoter resulting in a reduction of E6 transcripts. HeLa cells were transfected with either a vector control or HPV31 E2 plasmid. When compared to the normal HeLa negative control, cells transfected with wild type E2 as well as the E2 mutants all repressed transcription of the E6 gene (Figure 3.9). Together with the ChIP, our data suggest that the K111 mutants are able to access E2 binding sites in the chromatin context (Table 3.1).

Figure 3.6

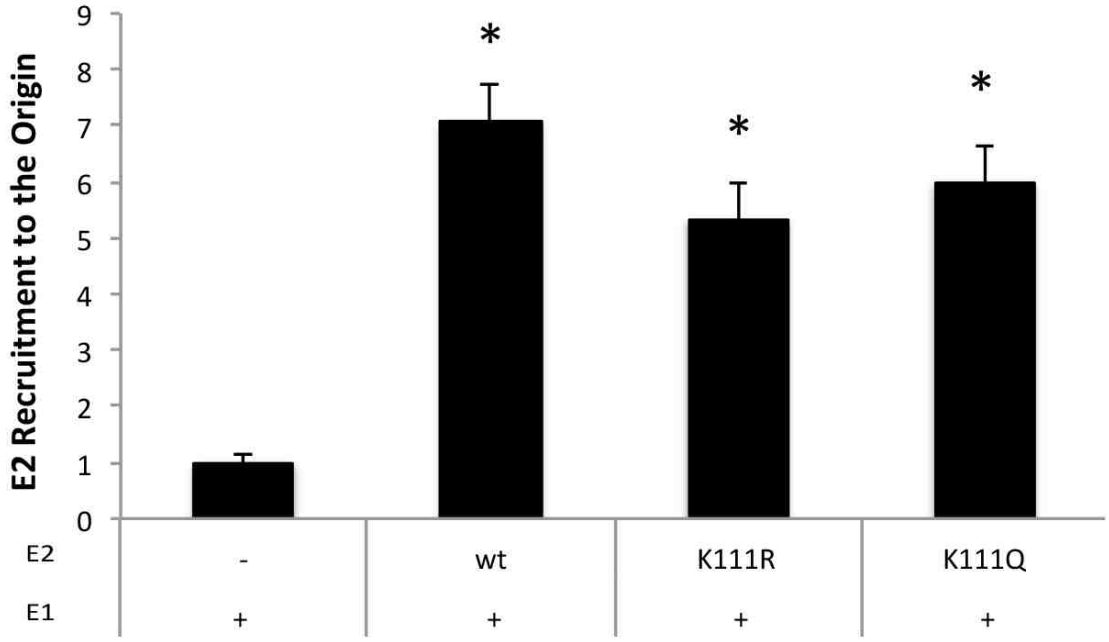


Figure 3.6 HPV31 E2 K111 mutants (K111R & K111Q) bind to the origin DNA. C33A cells were co-transfected with 31-Fluc and HPV31 FLAG-E2 wt or K111 mutants. The amount of E2 at the origin was determined by ChIP. * $p < 0.05$ compared to E1 in the absence of E2.

Figure 3.7

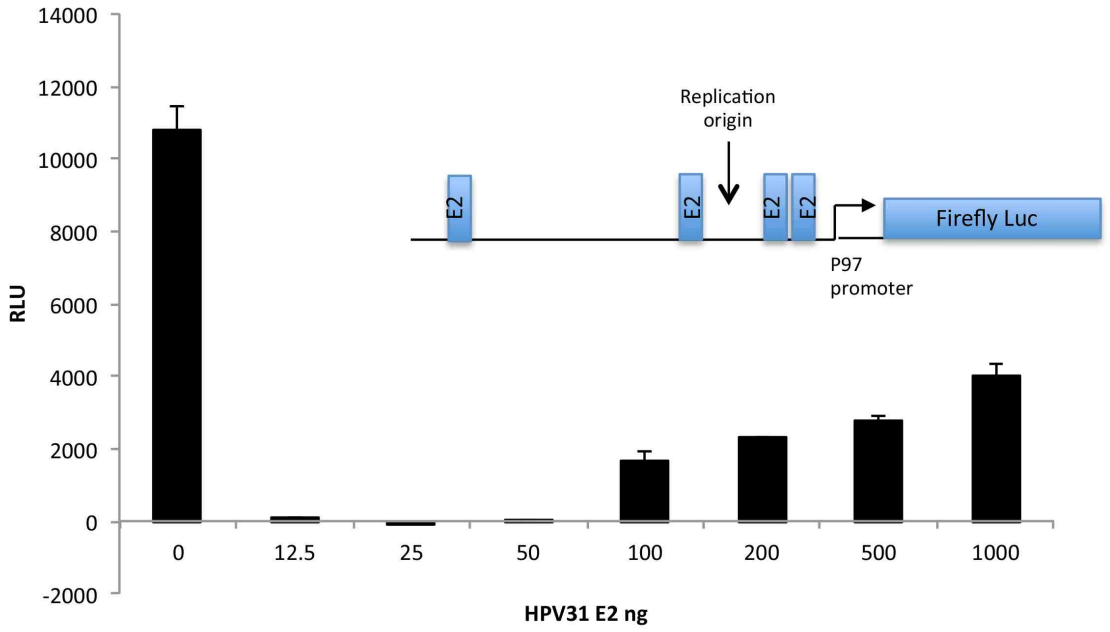


Figure 3.7 E2 most efficiently represses transcription at low concentrations. C33A cells were transfected with 31-FLuc and HPV31 wt FLAG-E2 at increasing amounts of transfected E2. Luciferase was measured by luminometry and repression was presented relative to baseline transcription.

Figure 3.8

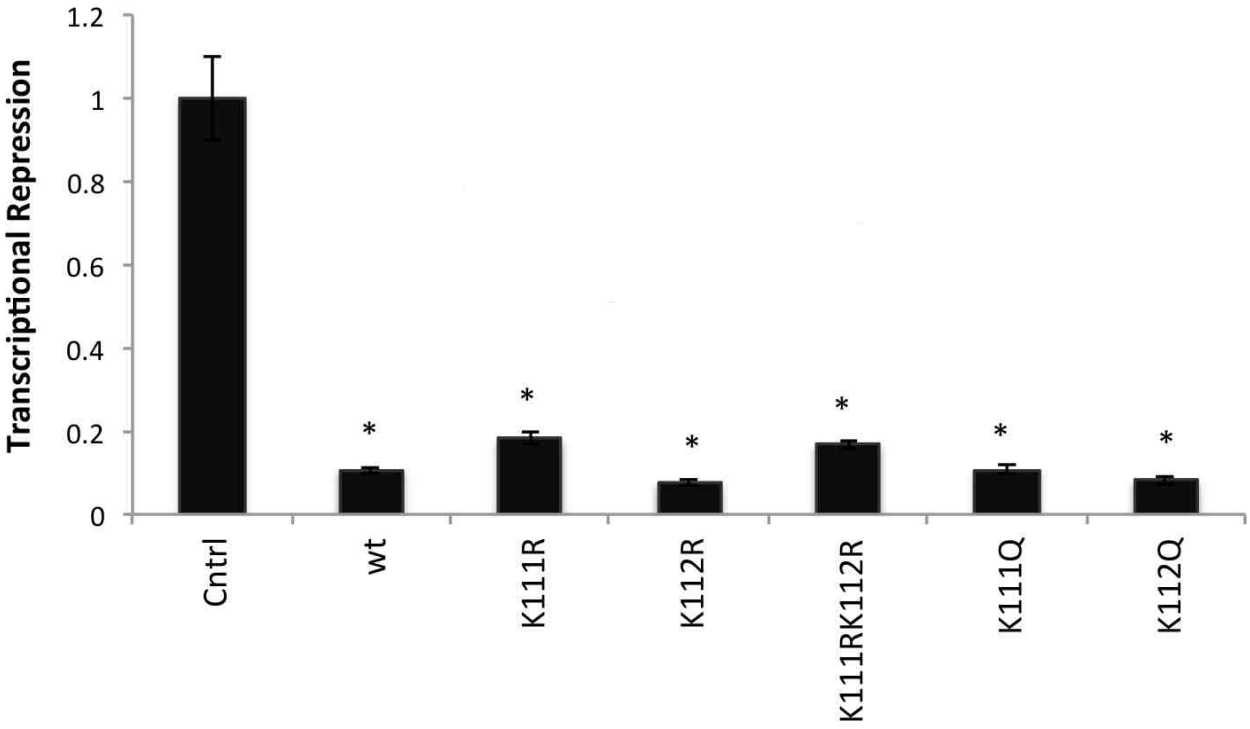


Figure 3.8 HPV31 E2 lysine 111 mutants repress transcription. C33A cells were co-transfected with 31-FLuc and HPV31 FLAG-E2 wt or the K111 mutants. Luciferase was measured by luminometry and repression was presented as a fraction of baseline transcription. * $p < 0.05$

Figure 3.9

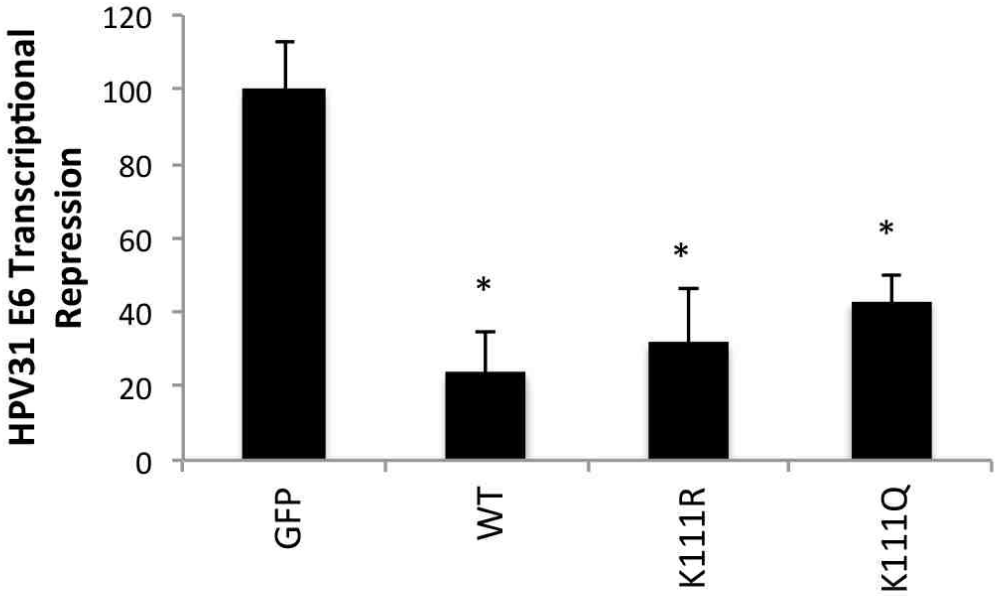


Figure 3.9 HPV31 E2 lysine 111 mutants repress transcription at the HPV18 early promoter. HeLa cells were transfected with HPV31 wt HA-E2 or the mutant (K111R or K111Q), and harvested after 48 hours. E6 transcripts quantified by q-PCR and presented as a percentage transcription in the absence of E2. * $p < 0.05$

Table 3.1

HPV31 E2	Transcription Repression	Nuclear Localization	Viral Replication
wt	**+**	**+**	**+**
K111R	**+**	**+**	**-**
K111Q	**+**	**+**	**++**

Table 3.1 Summary of HPV31 E2 functional assays. HPV31 E2 is localized to the nucleus and represses transcription regardless of the status of K111. K111R is replication defective and the K111Q acetylation mimic shows enhanced replication.

3.4 HPV31 E2 interactionS with host factors are not perturbed by mutations at K111

E2 interacts with a large number of host factors, some of which have adverse effects on replication when binding is abrogated. Potentially p300 acetylation at K111 may be required for recruiting a necessary host factor. We investigated a selection of these co-factors to determine whether E2 K111R impeded binding and resulted in a loss of replication function (Figure 3.10), and a summary of the results can be found in Table 3.2.

In the BPV1 system, interaction between E2 and Brd4 has been shown to be critical for E2 transcriptional activation and repression functions. The HPV16 E2 Brd4 binding deficient mutant R37A/I73A is also defective in viral replication (Wang, Helfer, Pancholi, Bradner, & You, 2013). Brd4 is known to bind to the E2 N-terminal transcriptional activation domain, and localizes to replication foci during the initiation of viral replication before being displaced from actively replicating origins (Sakakibara et al., 2013; Wang et al., 2013). There are three specific regions of Brd4 reported to potentially interact with E2 (McBride & Jang, 2013); CTM, BID, and PDID (S. Y. Wu et al., 2016). The interaction of HPV31 E2 was investigated by co-immunoprecipitation experiments with each of the Brd4 binding regions (Figure 3.11). A mutation at K111 of E2 had no effect on Brd4 CTM binding. We observed differences in binding to BID and PDID according to our co-immunoprecipitation experiments; however, these differences do not

correlate with the loss of activity with K111R and the enhanced activity of K111Q. The variation of protein levels may be resultant from the expression levels of the protein.

Figure 3.10

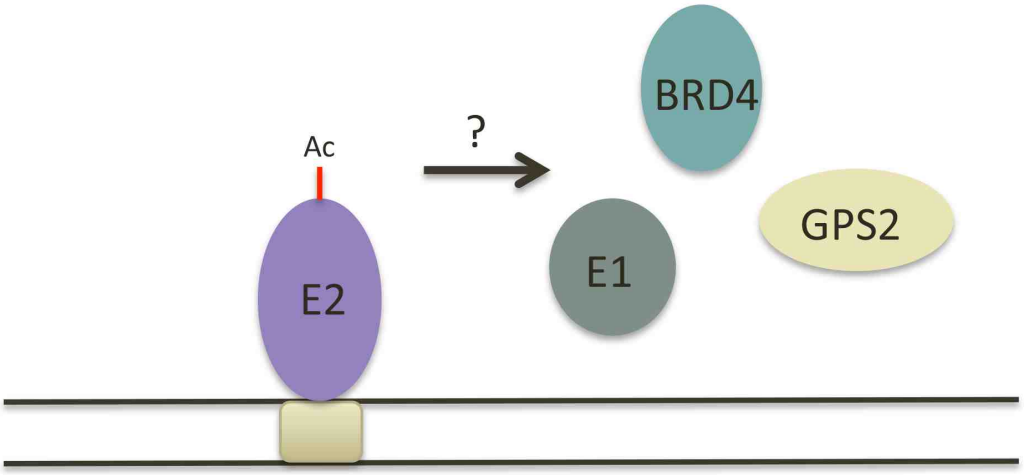


Figure 3.10 Acetylation of E2 K111 on its interaction with co-factors. A proposed mechanism shows that p300 acetylation of E2 is involved in interaction with key E2 binding partners.

Figure 3.11

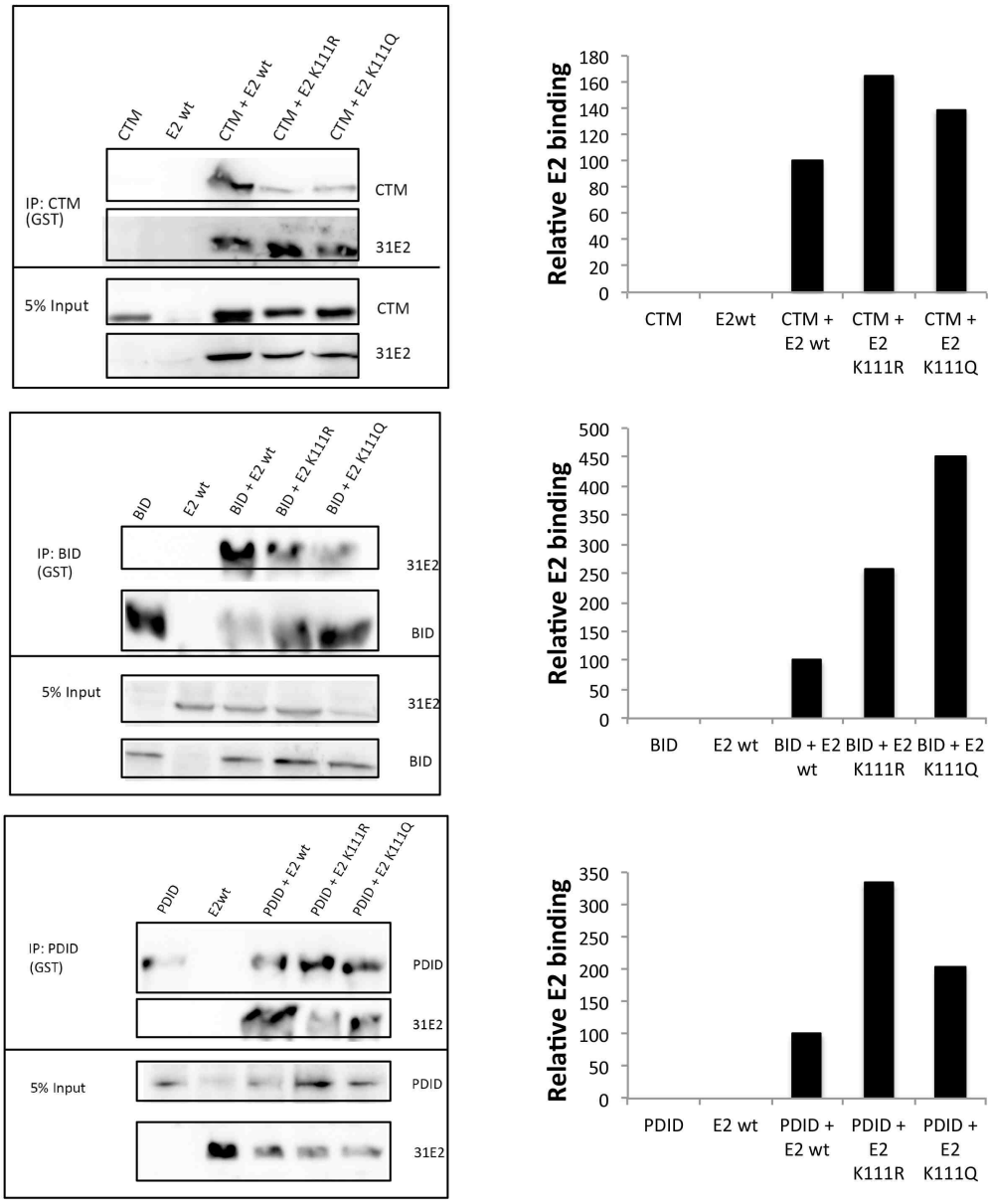


Figure 3.11 HPV31 E2 K111 mutants maintain interactions with Brd4. Human 293TT cells were co-transfected with HPV31 FLAG-E2 and plasmids expressing BRD4 fragments CTM, BID, and PDID. BRD4 fragments and E2 proteins were immunoprecipitated using GST antibody and detected by western blotting. BRD4 (fragments) co-IP relative to E2 immunoprecipitated was calculated by densitometry and presented graphically.

The AMF1/GPS2 protein is another factor known to interact with E2 proteins. A binding deficient mutant of BPV1 E2, W145R, is also transcription and replication defective (Breiding et al., 1997; Peng et al., 2000). We investigated the interaction of the HPV31 E2 mutants with AMF1/GPS2 by co-immunoprecipitation and found that the K111 mutants interacted with GPS2 (Figure 3.12). Interestingly, we also found that the E2 mutants differentially affected the expression of GPS2. K111Q transfected cells showed higher levels of GPS2 while K111R cells showed reduced levels when compared to cells transfected with wild type E2. The reason for the fluctuations of GPS2 levels is unknown.

Topoisomerase II-binding protein 1 (TopBP1) is involved in DNA damage response and DNA replication (Makiniemi et al., 2001). TopBP1 influences viral replication (Kanginakudru, DeSmet, Thomas, Morgan, & Androphy, 2015), and co-localizes with PV E2 at viral replication origins as part of a DNA damage response (Boner et al., 2002; Reinson et al., 2013). TopBP1 complexes with HPV E2 and is important for establishing the copy number of viral genomes (Donaldson et al., 2012), and it also increases replication and transcriptional activities of E2. We investigated the TopBP1 interaction with HPV31 E2 and the effects of the K111 mutants by co-immunoprecipitation (Figure 3.13). Both K111R and K111Q bound TopBP1 in a similar manner to wild type HPV31 E2.

Figure 3.12

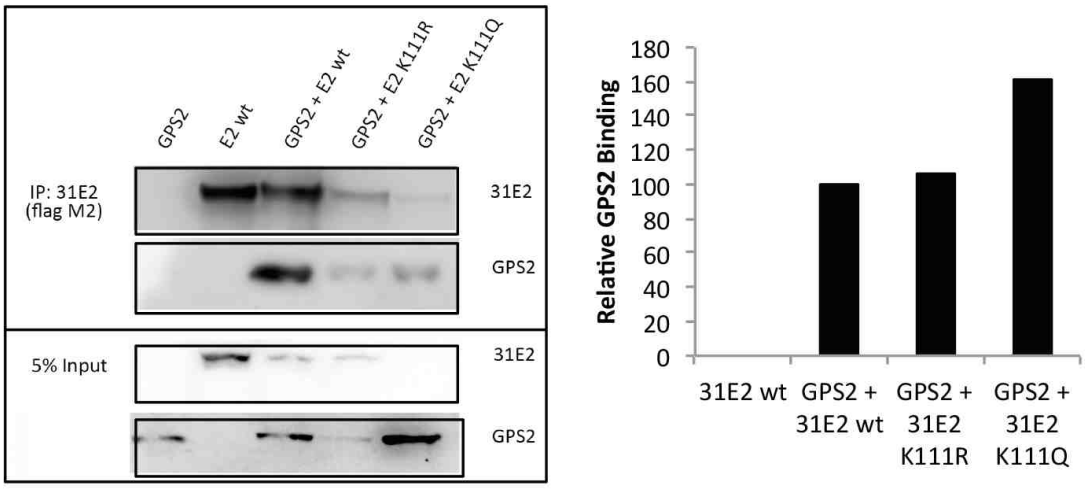


Figure 3.12 HPV31 E2 K111 mutants maintain interactions with GPS2. Human 293TT cells were co-transfected with HPV31 FLAG-E2 and HA-GPS2. HPV31 E2 protein IP was executed with M2 antibody, and blotted with HA7 and M2 antibodies. GPS2 co-IP relative to E2 immunoprecipitated was calculated by densitometry and presented as a western blot and graphically.

Figure 3.13

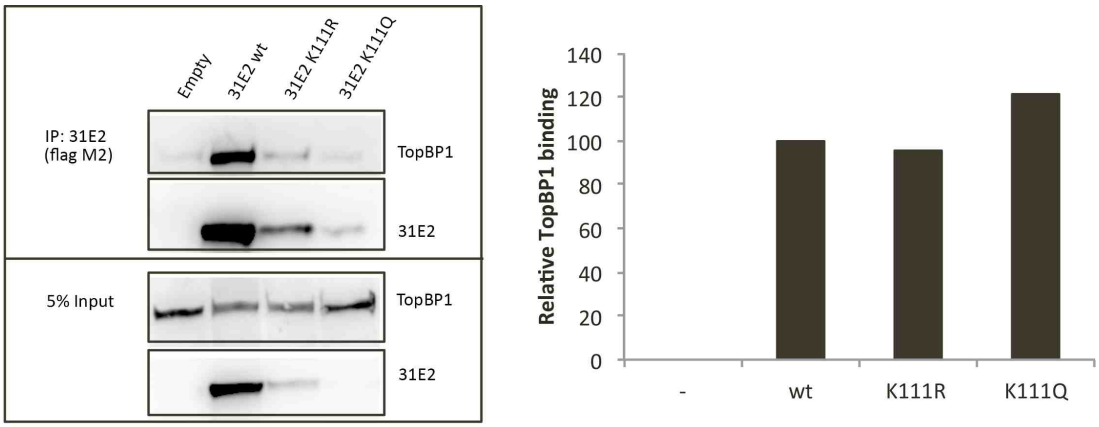


Figure 3.13 HPV31 E2 K111 mutants maintain interactions with TopBP1. 293TT cells were transfected with HPV31 FLAG-E2 followed by E2 protein immunoprecipitation with M2 antibody. Amounts of endogenous TopBP1 co-immunoprecipitated were determined by blotting with anti-TopBP1 and M2 antibodies, and TopBP1 co-IP relative to E2 immunoprecipitated was calculated by densitometry and represented by western blot and graphically.

3.5 E1 protein complexes with and is recruited to the replication origin by the K111 mutants.

An E2 mutation that reduces the interaction between E2 and E1 would likely have an effect on viral replication, so we investigated the E1-E2 interaction to determine if that was affected by the K111 mutations. Co-immunoprecipitation studies were conducted in 293TT cells that were co-transfected with HA-E1 and FLAG-E2. E1 was immunoprecipitated western blot analysis revealed that E1 interacted with both of the E2 mutants (Figure 3.14).

During initiation E2 interacts with and recruits E1 to the replication origin (Stenlund, 2003). Although we already established that the E2 mutants bind both DNA and E1 (Figure 3.6 & 3.14), we investigated the efficiency at which the K111 mutants were able to recruit E1 to the origin. We used ChIP assays compare levels of E1 bound at the origin in the presence of the different E2 proteins. C33A cells were co-transfected with E1, E2 and pFLOri (Figure 3.15), and HeLa cells were co-transfected with HPV31 E1 and E2 (Figure 3.16). We found that the K111R and K111Q mutants both effectively recruited E1 to the viral replication origin in both models. In the C33A experiment we observed a high amount of E1 at the origin in the absence of E2 (Figure 3.15). This result is likely due to the ability of E1 to bind plasmid DNA at a low efficiency, in the absence of E2 despite the lack of sequence specificity. In the HeLa experiment, E1 needs to access

chromatin in order to bind at the origin, and in the absence of E2 we see negligible amounts of E1 at the HPV18 origin (Figure 3.16).

Figure 3.14

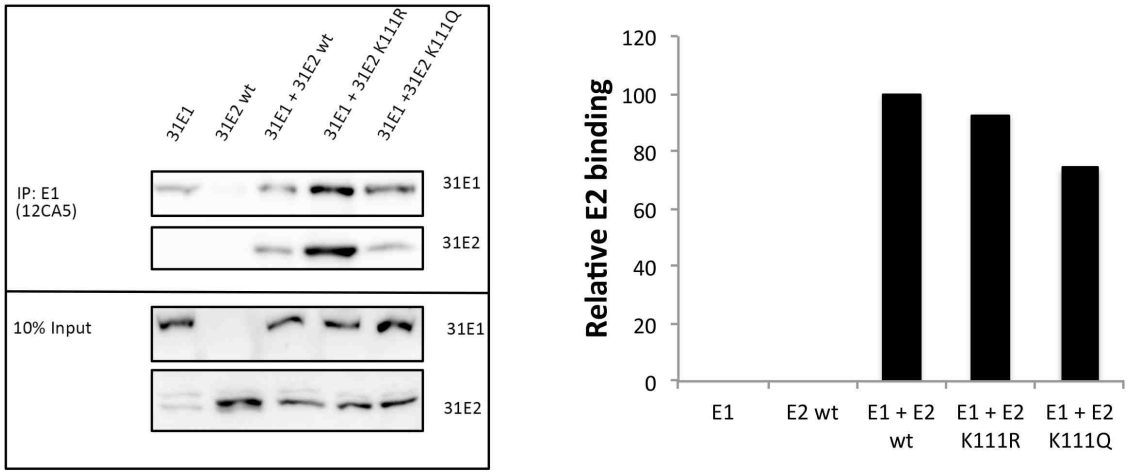


Figure 3.14 HPV31 E1 binds to the mutants and stabilizes E2 protein levels. 293TT cells were co-transfected with HPV31 FLAG-E2 and HA-E1. E1 protein pull down was conducted using 12CA5 antibody and analyzed by western blot. E2 co-IP relative to E1 immunoprecipitated was calculated by densitometry and represented by western blot and graphically.

Figure 3.15

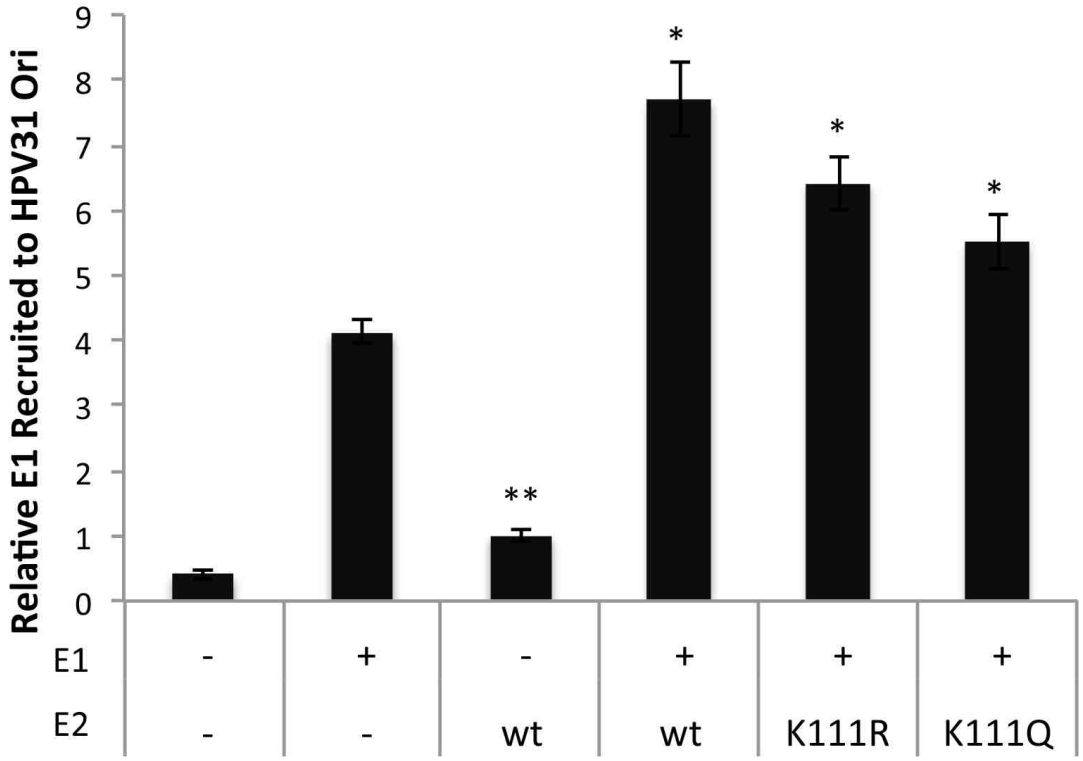


Figure 3.15 Both K111R and K111Q mutants can recruit E1 to the HPV31 replication origin. C33A cells were cotransfected with PFLORI31, HPV31 HA-E2 and FLAG-E1. ChIP assay to detect E1 at the HPV31 LCR was conducted with M2 antibody and LCR4 primers. * $p < 0.05$ ** $p < 0.005$

Figure 3.16

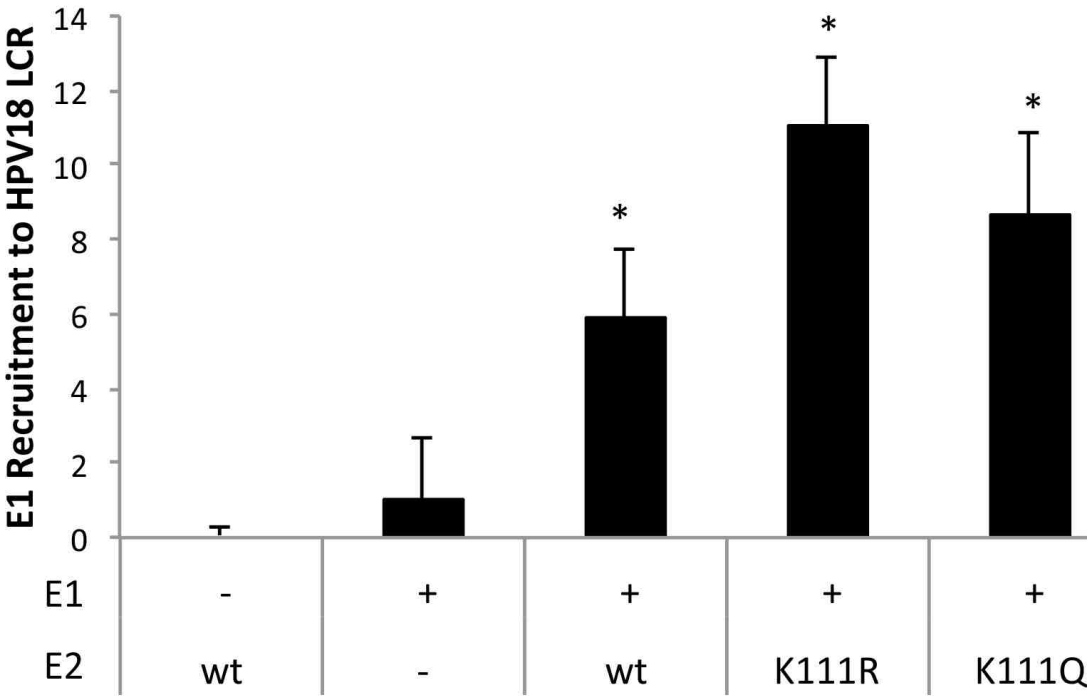


Figure 3.16 K111 mutants recruit E1 to the HPV31 replication origin. HeLa cells were transfected with HPV31 FLAG-E1 and HA-E2, and the presence of E2 at the HPV18 replication origin was determined by ChIP. * $p < 0.05$

Table 3.2

HPV31 E2	TopBP1 Binding	E1 Binding	GPS2 Binding	Brd4 Binding
Wt	**+**	**+**	**+**	**+**
K111R	**+**	**+**	**+**	**+**
K111Q	**+**	**+**	**+**	**+**

Table 3.2 Summary of E2 interaction studies. E2 showed positive K111 independent binding with each of the subset of co-factors investigated.

3.6 The K111R mutant of E2 reduces unwinding of the replication origin.

These results with the K111R E2 protein were intriguing. Our current understanding of HPV replication suggests that since the K111R mutant binds chromatin and recruits E1 to the origin, replication initiation should be unaffected; however, that was not the case. During initiation the assembly of the E1 double hexamer follows the recruitment of E1 to the origin by E2. To determine whether the E1 helicase was active, a FAIRE assay was modified to infer DNA unwinding at the replication origin. Faire assays are typically used to identify active promoters by detecting sites in chromatin that are nucleosome-free (Giresi, Kim, McDaniell, Iyer, & Lieb, 2007; Hogan, Lee, & Lieb, 2006; Tsompana & Buck, 2014). This principle was applied to investigate E1 + E2-mediated unwinding of the viral replication origin by measuring the quantity of nucleosome free origin DNA in the presence of wt E2 or the K111 mutants..

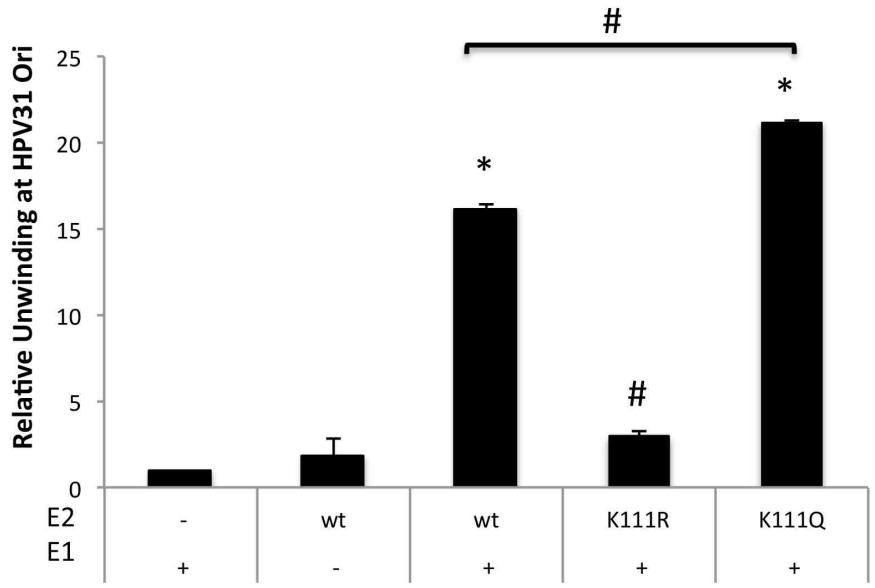
Either E1 or E2 alone poorly supported unwinding of the replication origin-containing DNA, but E1 co-transfected with wild type E2 resulted in 16 fold unwinding at the origin (**Figure** 3.17). The E2 K111R mutant failed to reduce nucleosome occupancy at the viral origin, suggesting that E1 helicase activity is restricted by this mutation (Figure 3.17). Surprisingly, however, the K111Q mutant supported origin unwinding activity 21-fold higher than E1 alone, which is even higher than wild-type E2. Together, these results imply that E2 K111R did

not support origin unwinding, while K111Q enhanced unwinding of the replication origin.

We corroborated the FAIRE results with a second assay. Replication protein A (RPA) is a host replication factor that binds to ssDNA at the origin (Loo & Melendy, 2004). We therefore performed a ChIP assay in the presence of endogenous RPA (Schuck & Stenlund, 2015) at the viral origin in cells co-transfected with E1 and E2 (Figure 3.18). K111R abrogates the helicase activity of E1 resulting in low RPA binding at the origin. Interestingly, K111Q shows an ~3 fold increase in unwinding when compared to wild-type E2. Topoisomerase 1 (Topo1) is a protein that relieves torsional stress in supercoiled DNA during transcription or replication in an ATP-independent manner, and does so by cleaving only one strand of DNA (Champoux, 2001). Cells transfected with E1 and cells with E2 K111R did not show recruitment of Topo1 compared to cells expressing wt E2 protein (Figure 3.19). In the presence of K111Q we observed a 6 fold increase in Topo1 at the origin. These data show that E2 K111R did not allow origin unwinding and K111Q enhanced unwinding of the replication origin, and support the findings of the FAIRE assay to demonstrate that origin melting is the event in replication initiation that is dependent upon acetylation of K111.

Figure 3.17

A



B

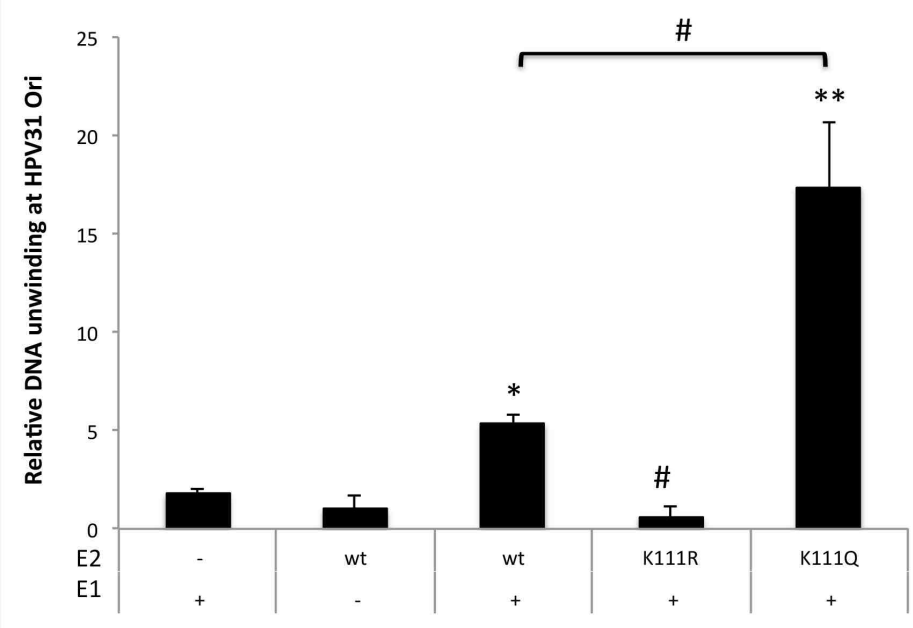


Figure 3.17 Nucleosome free DNA is not found at the replication origin in the presence of K111R. C33A cells were transfected with FLAG-E1, FLAG-E2, PFLORI31. Unwinding at origin DNA was analyzed by FAIRE assay using HPV31 LCR primers (A) LCR3 and (B) LCR4. * $p < 0.05$ by one-way ANOVA comparing to wt in absence of E1. # $p<0.05$ by one-way ANOVA compared to wt E2 + E1.

Figure 3.18

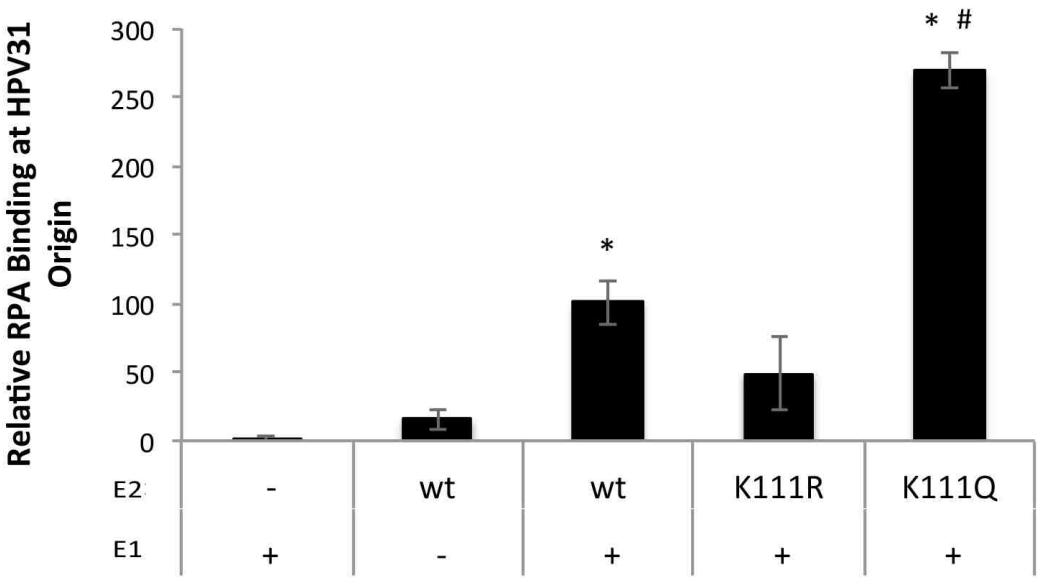


Figure 3.18 The K111R mutant reduces RPA binding at the replication origin. C33A cells were transfected with FLAG-E1, FLAG-E2, PFLORI31. ChIP for endogenous RPA protein at the LCR used anti-RPA (70) antibody and LCR4 primers. * $p < 0.05$ by one-way ANOVA comparing to wt in absence of E1. # $p < 0.05$ by one-way ANOVA compared to wt E2 + E1.

Figure 3.19

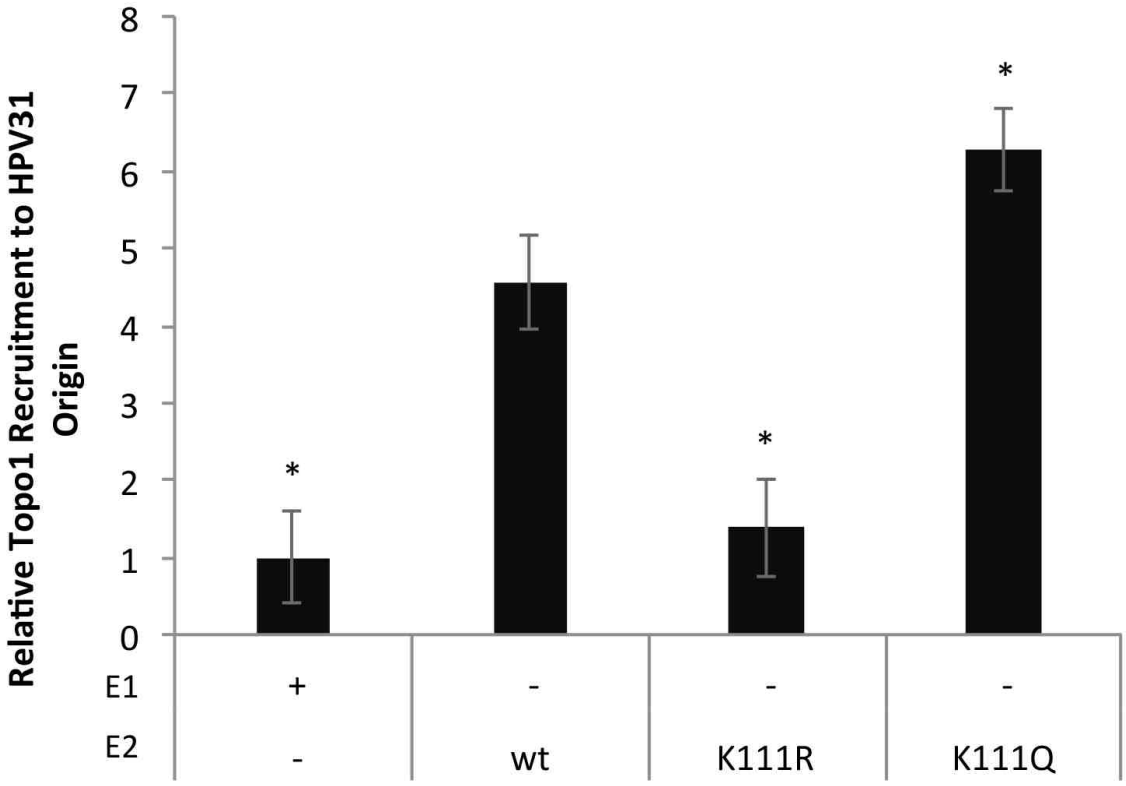


Figure 3.19 E2 K111R failed to support recruitment of Topo1 to the replication origin. C33A cells were transfected with FLAG-E1, FLAG-E2, PFLORI31. Chip for endogenous Topo1 at the HPV31 LCR with anti-Topo1 antibody and LCR3 primers. * $p < 0.05$ by one - way ANOVA comparing to wt in absence of E1. # $p<0.05$ by one-way ANOVA compared to wt E2 + E1.

Table 3.3

HPV31 E2	Viral Replication	Origin Unwinding	RPA Binding	Topoisomerase 1 Binding
wt	**+**	**+**	**++**	**+**
K111R	**-**	**-**	**+/-**	**-**
K111Q	**++**	**++**	**+++**	**++**

Table 3.3 Replication origin unwinding is K111 dependent. Summary of the effects of K111 mutation on the functions of E2 in replication initiation.

3.7 E1 Hexamers formation assay in the presence of the E2 mutants

The lack of unwinding at the replication origin in the presence of K111R (Table 3.3) indicates that the helicase activity of E1 is dependent on K111 acetylation. We previously concluded that the E2 mutants bind to E1 and recruit it to the origin, so we attempted to capture the E1 at the origin to determine the effects of the mutant E2 proteins on E1 double hexamer assembly. We aimed to detect large E1 complexes bound to origin DNA. The results of this experiment were inconclusive. We did not observe clear bands at the appropriate size for E1 hexamers or double hexamers, nor was the western clear enough to attempt quantification or comparisons of the bands (Figure 3.20). We repeated this experiment using a native gel to maintain the complexes; however, under those conditions the antibodies failed to detect any proteins.

Figure 3.20

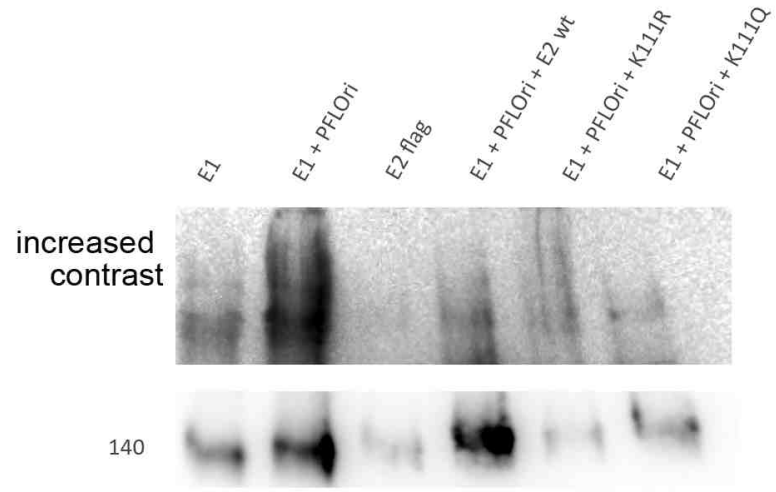


Figure 3.20 Hexamer formation assay. C33A cells were transfected with FLAG-E1, FLAG-E2, PFLORI31. Proteins were chemically crosslinked and ran on a native gel electrophoresis. Immunoprecipitation and western blotting were conducted using HPV16 E1 antibody. The upper portion of the blot was treated with enhanced contrast to better visualize the large bands.

3.8 Episomal E2 mutant Genomes in Stable Cell Lines

Several attempts were made to establish stable keratinocyte cell lines containing the mutant HPV31 whole genomes. Due to the large size of the pBR322 HPV31 (Thomas, Hubert, Ruesch, & Laimins, 1999) plasmid (10.3 kb) we decided to shuttle a fragment containing part of the E2 gene into a smaller plasmid (pUC18) before performing site directed mutagenesis. Following mutagenesis the colonies were screened for the lysine mutants by DNA sequencing, and fragments were shuttled from successful clones back into the large plasmid. Since the HPV31 insert is the same size as linearized pUC18, the plasmids were also digested with PvuI, which cuts at multiple sites in pUC18.

The HPV31 genomes were transfected into primary HFKs and NIKS by electroporation and XtremeGene HP respectively, and either selected with puromycin or; in the case of the primary cells, passaged until the normal cells senesced. The HFKs produced many clones that became immortalized following transfection; however, these cells either did not maintain the genomes long term or the genome could not be detected in them. The NIKS were more successful, resulting in cells that maintained the HPV31 genome which we detected by both PCR analysis (Figure 3.21 A) and by southern blot (Figure 3.21 B). Repeated rounds of transfections were needed to successfully obtain an HPV31 wt cell line, but we were able to develop one that demonstrated copy numbers higher than that of the CIN612 (Figure 3.21 C).

Figure 3.21

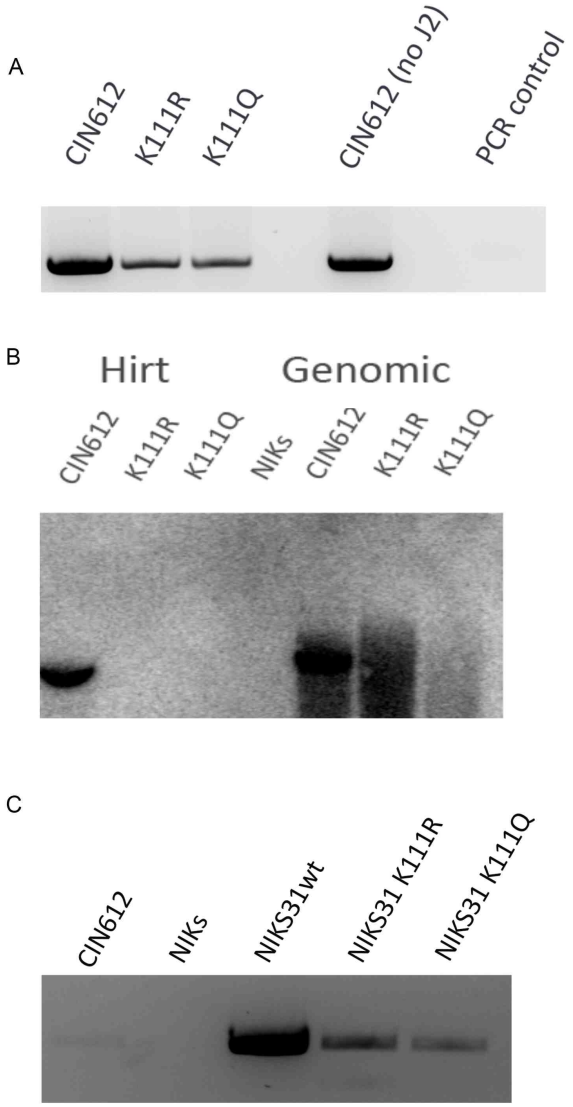


Figure 3.21 Stable NIKS cell lines with the HPV31 whole genome maintain episomes. (A) PCR analysis of Hirt extracted DNA using HPV31 primers. (B) Southern blot of Hirt and Genomic extracts of HPV31 stable cell lines. (C) PCR of Hirt extracts from HPV31 stable cell lines.

CHAPTER 4

DISCUSSION

The role of PV E2 in replication initiation has been extensively studied; however, the mechanisms by which replication and copy number are regulated, and the specific involvement of E2 beyond E1 recruitment remain undefined. Activation of p97 leads to the expression of proteins E1, E2, E6 and E7 (Abroi et al., 1996; Sedman & Stenlund, 1995; Ustav & Stenlund, 1991), where viral early proteins E1 and E2 are the replication initiation proteins of PVs. The loading of E2 and its regulation of E1 binding, serves as a replication licensing system for viral replication. The E1 protein is the main viral replication protein. It has been reported that *in vitro*, E1 alone is capable of initiating viral replication; however, the binding of E1 to DNA is non-specific and E2 is also required in-vivo (Loo & Melendy, 2004). To initiate viral replication, E2 binds to DNA in a sequence specific manner at sites flanking the origin of replication, and recruits E1 monomers to the origin. When the first pair of E1 molecules binds to the replication origin, E2 is released from its binding sites through a process of ATP hydrolysis (Chen & Stenlund, 2002). More E1 molecules are recruited and form a double-hexameric complex at the origin that is similar to the eukaryotic Orc-Mcm complex. This E1 complex then serves as a helicase and unwinds the DNA to allow replication factors to be recruited. Without E2 serving as a viral origin recognition factor, the inability of E1 to bind specifically to the replication origin would cause inefficient and inconsistent firing of the viral origin.

We first identified this site through a p300 in-vitro acetylation assay. It has been reported that p300 levels in basal keratinocytes are low, while differentiating cells show high levels (Wong et al., 2010). This difference in p300 levels coincides with the switch of viral genomes from maintenance to vegetative amplification in infected cells. To explore the importance of this acetylation, we generated substitution mutants that represent de-acetylated and acetylated K111 (K111R and K111Q respectively). The acetylation deficient mutant of E2 K111R was observed to abrogate replication in both BPV1 and in HPV31 as shown here. The acetylation mimetic E2 K111Q demonstrated significantly higher levels of replication than wild type E2. This supports the hypothesis that acetylation at the 111 residue is a necessary step in the initiation of replication. Furthermore, the K111Q result suggests that acetylation at K111 may be an integral regulatory step in switching the viral replicative program from maintenance to vegetative amplification (Reinson, Henno, Toots, Ustav, & Ustav, 2015).

To determine how K111 affects replication, we analyzed and characterized the K111R and K111Q mutants. Since K111 is located in the putative NLS, we investigated the localization of E2 and found that E2 was nuclear in all experiments. After verifying that the K111R mutant was nuclear, we addressed other functions of E2. Knowing that a DNA binding deficient mutant would be detrimental to viral replication we conducted a ChIP of E2 at the origin that allowed us to directly compare the K111 mutants to wild type E2. We observed

no difference between the E2 proteins, which confirmed that the c-terminal DNA binding domain was not influenced by the K111 mutations. HPV31 E2 serves as a strong repressor of transcription, and according to our results from two corroborating experiments; this function is maintained at levels similar to wild type for both mutants. HPV31 E2 ability to repress transcription results from binding at sites located on the TATA box of the p97 promoter, as well as interaction with host cell transcription factors (Demeret et al., 1997; Dostatni et al., 1991; Hou et al., 2000; McBride, 2013; Schweiger et al., 2007). The K111 mutants allow transcriptional repression, which indirectly indicates that the DNA binding ability is unaffected and is consistent with the ChIP results.

There are known cellular factors that interact with E2 and have been reported to directly affect E2 functions; transcriptional regulation and replication. We investigated the ability of the K111 mutants to interact with a subset of these proteins and found that K111R was not binding deficient for any of them. An interesting observation was that the E2 mutants exhibited differential expression levels. K111R was lower expressing than wild type and K111Q was expressed at extremely low levels when compared to wild type or K111R. The co-immunoprecipitation experiments with the fragments of BRD4 were an exception. The CTM of BRD4 stabilizes E2 (Zheng et al., 2009) and that effect was clearly demonstrated, and the BID and PDID fragments also produced E2 stabilization to a lesser degree. The AMF1/GPS2 co-immunoprecipitation experiment also revealed something interesting. In the presence of the K111R mutant there were

lower levels of AMF1/GPS2 detected in the inputs; however, the results show that the K111R still interacts with AMF1/GPS2. Further experimentation would be needed to fully clarify this observation. While the expression of K111Q was low, it remained highly active in all of the experiments, supporting the hypothesis that an acetylation at K111 would result in enhanced viral replication.

K111 is located in the TAD of E2, which is responsible for E1 binding (Abbate et al., 2004). The interaction between E1 and E2 during the initiation of replication is dynamic, and there is a direct interaction between E1 and the TAD of E2. We found that E1 binds to both K111 mutants and the E2 mutants bound DNA and subsequently recruited E1 to the replication origin at levels comparable to wild type. This interaction also demonstrates that the TAD of E2 is functional and therefore not mis-folded in the mutants. E2 recruitment of E1 to the replication origin should initiate viral replication, the first stage of which is to form the E1 double hexamer. In order to form the E1 double hexameric helicase complex, E2 cannot remain bound to E1 at the origin. One possible explanation is that the K111R mutant binds strongly to E1 and is not released, thereby hindering formation of the helicase complex and preventing unwinding at the origin. We investigated the formation of the helicase complex by determining if the origin becomes exposed under replication conditions by FAIRE assay. The FAIRE assay was modified to measure the open chromatin at the origin during replication conditions. Our results confirm that K111R is not capable of facilitating the initiation and unwinding of the replication origin.

To corroborate the results from the FAIRE we performed a ChIP for RPA at the replication origin. Our data reveal that there is reduced RPA at the replication origin with K111R and a significant increase with K111Q, suggesting a direct effect of E2 on the E1 recruitment of RPA to the origin. RPA is recruited to ssDNA by E1 and though there is a direct interaction, it is inhibited by ssDNA (Han, Loo, Militello, & Melendy, 1999; Loo & Melendy, 2004). Since RPA is necessary for DNA replication Loo et al. proposed a mechanism by which E1 loads RPA onto ssDNA during unwinding. The role of RPA in viral replication has been more extensively studied in SV40. SV40 large T-antigen (Tag) has been reported to interact with RPA, and during replication the SV40(Tag)-RPA interaction is essential for assembly of the primosome complex (Melendy & Stillman, 1993). This specific function has not been determined for the E1-RPA interaction, but a similar mechanism could explain how a lack of RPA loading at the origin inhibits replication.

Topo I has been reported to directly interact with both PV E1 and E2, both of which are proposed to stimulate Topo1 enzymatic activity (Clower, Fisk, & Melendy, 2006; Clower, Hu, & Melendy, 2006; Hu, Clower, & Melendy, 2006). Furthermore, E2 and Topo1 enhance E1 binding at the replication origin independently (Hu et al., 2006). Our ChIP results show no change in E1 recruitment (Figure 3.15) but there is a no DNA unwinding in the presence of K111R, which suggests that K111R recruits E1 to the origin but does not recruit

or stimulate Topo1 activity. We report that E2 rather than E1 is responsible for recruiting Topo1 to the replication origin, and K111R does not support Topo1 recruitment while K111Q demonstrates enhanced recruitment (Figure 3.19). These data propose that E2 recruitment and stimulation of Topo1 is integral to DNA unwinding (Figure 4.1). This is intriguing as previously the hypothesis was that E2 and Topo1 recruitment and stimulation were parallel and redundant mechanisms (Archambault & Melendy, 2013).

This study characterized an acetylation site on HPV E2 that directly impacts viral replication. Manipulation of K111 left the E2 localization, transcription function, DNA binding, and E1 recruitment intact, and only affected viral replication. K111R was replication defective while the acetylation-mimetic K111Q enhanced viral replication, which lends credence to the idea that it is an acetylation at this site that regulates viral replication. We found that K111R abrogates replication by preventing the helicase activity of E1. It is possible that E2 blocks the formation of the E1 helicase complex, or that another factor like RPA that is involved in DNA unwinding is not recruited by the K111R mutant.

We attempted to investigate the formation of the E1 double hexamer on the replication origin but the results were inconclusive. E2 recruits E1 to the origin, but E2 can also be inhibitory if it is not subsequently removed from DNA. Reports have demonstrated that chaperone proteins such as Hsp70 are required for the release of E2 from the E2BS (Lin, Makhov, Griffith, Broker, & Chow, 2002).

Experiments investigating the recruitment of these factors to replication origins in the presence of the E2 mutants may help to further elucidate mechanistically how K111 regulates replication.

Further investigation of the interactions of HPV E2 K111 with factors directly involved in replication initiation and DNA unwinding will be necessary to further our understanding of how the K111 acetylation regulates replication mechanistically. Studying the effects of these mutants in stable keratinocyte cell lines during the different stages of the PV replicative cycle would also be necessary in furthering our understanding of how the virus is regulated. An interesting observation made here is that K111Q not only rescued the deficiencies of K111R, but this mutant outperformed wild type E2. This possible acetylation presents a novel regulatory step in the PV replication program, and could be directly involved in the transition from maintenance stage replication to vegetative amplification.

Figure 4.1

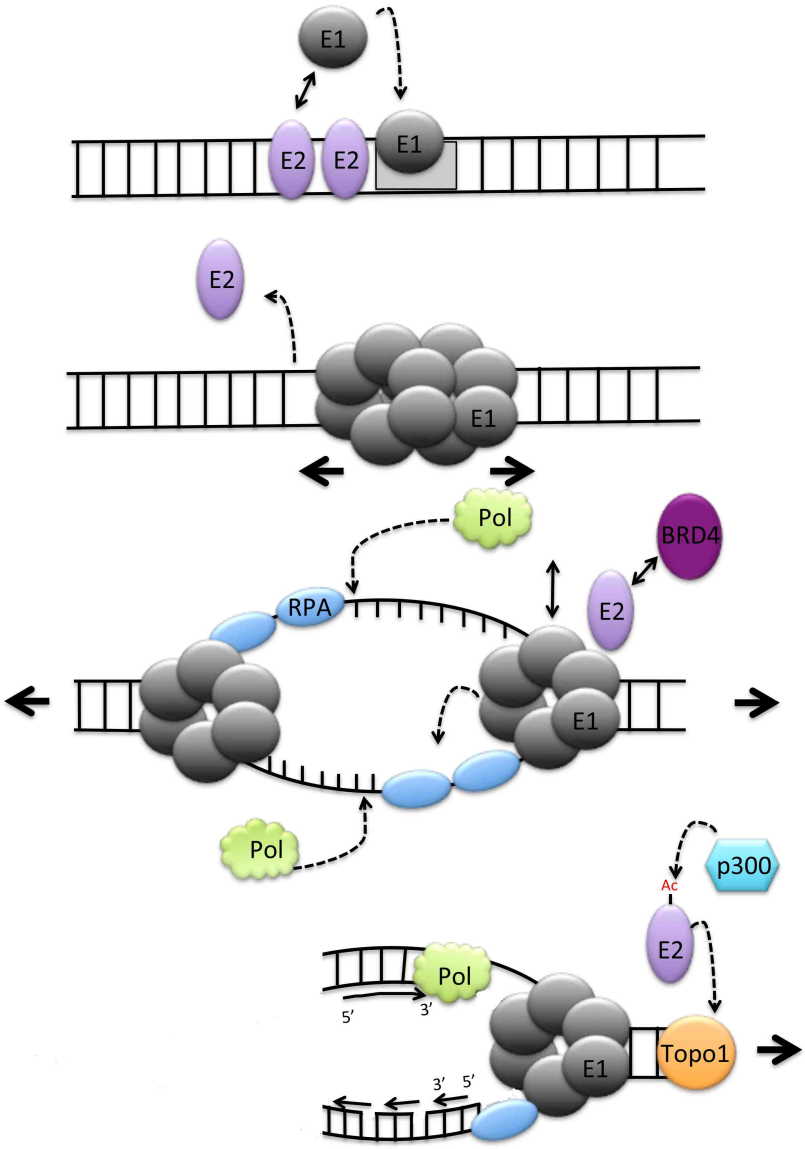


Figure 4.1 A proposed mechanism for the regulation of replication by acetylation of E2 K111. HPV31 E2 regulates replication initiation through K111 acetylation dependent Topo1 recruitment. This illustration depicts the dynamics between E1, E2, RPA, and Topo1 at the replication fork, and the role of K111 acetylation.

APPENDIX

Table A-1

HPV31 HA-E2 Cloning Primers

Primer Name (mutation)	Primer Sequence
HPV31-K111R F	CCTACAGGGTGTTTAAGAAAACATGGATATACTG
HPV31-K111R R	CAGTATATCCATGTTTTCTTAAACACCCTGTAGG
HPV31-K112R F	CCTACAGGGTGTTTAAAAAGACATGGATATACTG
HPV31-K112R R	CAGTATATCCATGTCTTTTTAAACACCCTGTAGG
HPV31-K111R112R F	CCTACAGGGTGTTTAAGAAGACATGGATATACTG
HPV31-K111R112R R	CAGTATATCCATGTCTTCTTAAACACCCTGTAGG
HPV31-K111Q F	CCTACAGGGTGTTTACAAAAACATGGATATACTG
HPV31-K111Q R	CAGTATATCCATGTTTTTGTAAACACCCTGTAGG
HPV31-K112Q F	CCTACAGGGTGTTTAAAACAACATGGATATACTG
HPV31-K112Q R	CAGTATATCCATGTTGTTTTAAACACCCTGTAGG
HPV31-K111Q112Q F	CCTACAGGGTGTTTACAACAACATGGATATACTG
HPV31-K111Q112Q R	CAGTATATCCATGTTGTTGTAAACACCCTGTAGG
HPV31-K111Q112R F	CCTACAGGGTGTTTACAAAGACATGGATATACTG
HPV31-K111Q112R R	CAGTATATCCATGTCTTTGTAAACACCCTGTAGG
HPV31-K111Q112A F	CCTACAGGGTGTTTCAAGCAACATGGATATACTG
HPV31-K111Q112A R	CAGTATATCCATGTTGCTTGAAACACCCTGTAGG
HPV31-K111A F	CCTACAGGGTGTTTAGCAAAACATGGATATACTG
HPV31-K111A R	CAGTATATCCATGTTTTGCTAAACACCCTGTAGG

Table A-2

HPV31 Codon-optimized flag-E2 Cloning Primers

Primer Name (mutation)	Primer Sequence
HPV31-K111R F	ACCGGCTGCCTGAGGAAGCACGGCTAC
HPV31-K111R R	GTAGCCGTGCTTCCTCAGGCAGCCGGT
HPV31-K112R F	CACCGGCTGCCTGAAGAGGCACGGCTA
HPV31-K112R R	TAGCCGTGCCTCTTCAGGCAGCCGGTG
HPV31-K111R112R F	TCCCACCGGCTGCCTGAGGAGGCACGGCTA
HPV31-K111R112R R	TAGCCGTGCCTCCTCAGGCAGCCGGTGGGA
HPV31-K111Q F	CACCGGCTGCCTGCAGAAGCACGGCTA
HPV31-K111Q R	TAGCCGTGCTTCTGCAGGCAGCCGGTG
HPV31-K112Q F	CGGCTGCCTGAAGCAGCACGGCTACAC
HPV31-K112Q R	GTGTAGCCGTGCTGCTTCAGGCAGCCG

Table A-3

HPV31 Sequencing Primers

Primer Name (mutation)	Primer Sequence
HPV31-E2 Seq F	GGAAGGGCAAGTTAATTGTAAGGGC
HPV31-E2 Seq R	CCTGACCACCCGCATGCACTTC

Table A-4

Bacterial (Glycerol) Stocks

Plasmid	Resistance	Vector	Cell	Notes
pCG-BPV E2	AMP	pCG	DH5α	
pCG-BPV E2 K70R	AMP	pCG	DH5α	
pCG-BPV E2 K107R	AMP	pCG	DH5α	
pCG-BPV E2 K111R	AMP	pCG	DH5α	
pCG-BPV E2 K112R	AMP	pCG	DH5α	
pCG-BPV E2K111R112R	AMP	pCG	DH5α	
pCG-BPV E2K339R	AMP	pCG	DH5α	
pCG-BPV E2 K391R	AMP	pCG	DH5α	
pCG-BPV E2 W145R	AMP	pCG	DH5α	
pCG-BPV E1	AMP	pCG	DH5α	
pGL2-E2BS-LUC	AMP	pGL2	DH5α	
HPV31 HA-E2	AMP	pCI	DH5α	
HPV31 HA-E2 K111R	AMP	pCI	DH5α	
HPV31 HA-E2 K111A	AMP	pCI	DH5α	
HPV31 HA-E2 K111Q	AMP	pCI	DH5α	
HPV31 HA-E2 K112R	AMP	pCI	DH5α	
HPV31 HA-E2 K112Q	AMP	pCI	DH5α	
HPV31 HA-E2 K111R112R	AMP	pCI	DH5α	
HPV31 HA-E2 K111R112Q	AMP	pCI	DH5α	
HPV31 HA-E2	AMP	pCI	GM119	Dam-/Dcm-
HPV31 HA-E2 K111R	AMP	pCI	GM119	Dam-/Dcm-
HPV31 HA-E2 K111A	AMP	pCI	GM119	Dam-/Dcm-
HPV31 HA-E2 K111Q	AMP	pCI	GM119	Dam-/Dcm-
HPV31 HA-E2 K112R	AMP	pCI	GM119	Dam-/Dcm-
HPV31 HA-E2 K112Q	AMP	pCI	GM119	Dam-/Dcm-
HPV31 HA-E2 K111R112R	AMP	pCI	GM119	Dam-/Dcm-

Plasmid	Resistance	Vector	Cell	Notes
HPV31 Flag-E2	AMP	pCI	DH5α	Codon optimized
HPV31 Flag-E2 K111R	AMP	pCI	DH5α	Codon optimized
HPV31 Flag-E2 K111A	AMP	pCI	DH5α	Codon optimized
HPV31 Flag-E2 K111Q	AMP	pCI	DH5α	Codon optimized
HPV31 Flag-E2 K112R	AMP	pCI	DH5α	Codon optimized
HPV31 Flag-E2 K112Q	AMP	pCI	DH5α	Codon optimized
HPV31 Flag-E2 K111R112R	AMP	pCI	DH5α	Codon optimized
HPV31 3x-Flag-E1	KAN		DH5α	Codon optimized
Flag-Brd4	AMP	pcDNA3	DH5α	
hBRD4: BID	AMP	pGEX	DH5α	
hBRD4: PDID	AMP	pGEX	DH5α	
hBRD4: CTM	AMP	pGEX	DH5α	
HPV31 Genome	AMP	pBR322	DH5α	
pCI-Rluc	AMP	pCI	DH5α	
pFLORI31	AMP	pCI	DH5α	
pFLORI-BPV	AMP	pCI	DH5α	
			DH5α	
HA-AMF1	AMP	pCDNA	DH5α	
pUC18	AMP		DH5α	
pUC19	AMP		DH5α	
pCI	AMP		DH5α	
pBabe-puro	AMP		DH5α	

Table A-5

Cell Lines

Cell Line	Media	Notes
C33A	DMEM	
293TT	DMEM	
J23T3	DMEM	
H1299	DMEM	
CV-1	DMEM	
NIKS	F-MEDIA	on feeders
CIN612	E-MEDIA	on feeders
HPVFP	KSFM	
HK1	KSFM	primary HFK
HK2	KSFM	primary HFK
HK3	KSFM	primary HFK
HK4	KSFM	primary HFK
NIKS31 wt	F-MEDIA	on feeders
NIKS31 K111R	F-MEDIA	on feeders
NIKS31 K111Q	F-MEDIA	on feeders

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CURRICULUM VITAE

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EDUCATION

Indiana University, *Indianapolis, IN.* (2010-2017)

Ph.D. in Microbiology and Immunology

Minor in Cancer Biology.

Morgan State University, *Baltimore, MD.* (2008-2010)

Master's degree Candidate in Chemistry

Concentration in Analytical Chemistry**.**

Graduate Fellowship recipient.

Morgan State University, *Baltimore, MD.* (2004-2008)

B.S. in Chemistry with High Honors

Minor in French. Golden Key International Honors Society. Reagents Award recipient.

RESEARCH EXPERIENCE

Indiana University School of Medicine, *Indianapolis IN*

Graduate Research Assistant (2011-2017)

Mentor: Elliot J. Androphy, MD

HPV replication regulation by acetylation of a conserved lysine in the E2 protein

- Conducted independent research and performed data analyses
- Presented findings at annual departmental student seminar
- Presented at the DNA Tumor Virus meeting (2014)

Morgan State University Chemistry Department, *Baltimore MD*

Graduate Research Assistant (2007– 2010)

Mentor: Yongchao Zhang, PhD

An Amperometric Biosensor for Phenols Based on Immobilized Tyrosinase.

- Conducted research independently, documented findings, and reported results.
- Trained undergraduate research assistants on experimental design and methodology.
- Supervised undergraduate researchers and students visiting from summer high school programs.
- Presented annually American Chemical Society conference. Presented annually National Organization for the Professional advancement of Black Chemists and Chemical Engineers (NOBCChE) meeting.

Morgan State University Chemistry Department, *Baltimore MD*

Undergraduate Research Assistant (2006-2007)

Mentor: Maurice Iwunze, PhD

The effectiveness of electrochemical processes on the destruction of polychlorophenols using cyclic wave voltametry.

- Conducted research independently, documented findings, and reported results.
- Presented annualy National Organization for the Professional advancement of Black Chemists and Chemical Engineers (NOBCChE) meeting.

TEACHING EXPERIENCE

Indiana University School of Nursing, *Indianapolis IN*

Graduate Teaching Assistant (2013)

- Facilitated the microbiology laboratory class.
- Lectured and led discussions about the course material.
- Administered exams.

Morgan State University Chemistry Department, *Baltimore MD*

Graduate Teaching Assistant (2008- 2009).

- Taught and facilitated Chemistry 101and 105 Laboratory classes.
- Advised students on course material during regularly scheduled office hours.
- Trained undergraduate research assistants in research design and presentation.
- Guest lectured classes on Analytical methods and instrumentation.

AWARDS

American Chemical Society Award: Most Outstanding Chemistry Student (2008)

The George H. Spaulding Science Award (2008)

Honorable Mention at NOBCChE annual national research symposium. (2008)

Morgan State University Deans List: University Honors program. (2004 - 2008)

Honors Reagent's Award: Honors program, Morgan State University. (2004 - 2007)

University Dean's List. (2004–2008)

PROFESSIONAL AFFILIATIONS

Golden Key International Honors Society. (2007 – present)

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PUBLICATIONS AND PRESENTATIONS

Thomas Y, Androphy, EJ. 2017. HPV Replication Regulation by Acetylation of a Conserved Lysine in the E2 Protein. Submitted to J Virol.

Kanginakudru S, DeSmet M, Thomas Y, Morgan I, Androphy, EJ. 2015. Levels of the E2 interacting protein TopBP1 modulate papillomavirus maintenance stage replication. Virology 478:135-142. PMID: 25666521

Zhang, Y., Thomas, Y., Kim, E., Payne, G. F. 2012. pH- and voltage-responsive chitosan hydrogel through covalent cross-linking with catechol. J Phys Chem B 116(5):1579-1585. PMID: 22229705

Thomas Y, Acetylation of Papillomavirus E2 Regulates Viral Replication. Molecular Biology of DNA Tumor Viruses Conference, 2014.