

REGULATION OF PAPILLOMAVIRUS E2 PROTEIN BY POST-
TRANSLATIONAL MODIFICATION

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Sara Poirier Culleton

Regulation of Papillomavirus E2 Protein by Post-translational Modification

Papillomaviruses (PVs) are small, double-stranded DNA viruses. Hundreds of species have evolved to replicate in mammals, birds, and reptiles. Approximately two hundred species are estimated to infect humans alone, and these human papillomaviruses (HPVs) cause diseases ranging from benign warts to anogenital and oropharyngeal cancers. While vaccination is effective at preventing the majority of these infections and their disease outcomes, there are no successful treatments for existing infections; thus, exploration of novel therapeutic targets is warranted.

PVs control expression and function of their gene products through alternative splicing, alternate start codons, and post-translational modification (PTM). The viral E2 protein regulates transcription, replication, and genome maintenance in infected cells, and PTMs have been demonstrated for E2 proteins from multiple papillomavirus types. Serine phosphorylation events were reported to influence E2 stability, and our laboratory was the first to describe in vitro acetylation events with implications for E2 transcription function. Here we report confirmation of these acetylation events in vivo and additional data elucidating the role of these PTMs in viral transcription. Moreover, we present a novel phosphorylation site for bovine papillomavirus type 1 (BPV-1) E2 at tyrosine 102 (Y102). Using phospho-deficient and phospho-mimetic point mutants, we found that this site influences E2-mediated transcription and replication, and we hypothesize that phosphorylation at Y102 regulates these activities by interrupting the association of E2

with its binding partners. We also report interaction of BPV-1 E2 and HPV-31 E2 with different receptor tyrosine kinases (TKs), most notably members of the fibroblast growth factor receptor family. We hypothesize that Y102 phosphorylation by these receptors occurs early in infection to limit viral replication and gene expression. Further studies will cement the role of RTKs in PV biology and could reveal novel therapeutic strategies.

Elliot Androphy, M.D., Chair

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CHAPTER 1
INTRODUCTION

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Human Papillomaviruses and Disease

HPV is the most common sexually transmitted infection in the United States. According to the Centers for Disease Control and Prevention, 79 million Americans are currently infected with at least one type of HPV, and most sexually active Americans will have at least one HPV infection during their lifetime (cdc.gov/std/hpv). There are at least two hundred types of human papillomaviruses (HPVs). Different HPV types are associated with different disease outcomes, ranging from genital warts (*condyloma acuminata*) caused by HPV-6 and HPV-11 to the cancers caused by HPV-16 and HPV-18. These two types, classified as “high-risk” due to their oncogenic potential, are the two most common HPVs implicated in cervical cancer, causing an estimated 70% of all cases (Guilfoile and Babcock, 2012). These and other high-risk types such as HPV-31 have also been detected in oropharyngeal cancers (Michaud et al., 2014). Persistent infection with a high-risk HPV is a causative factor in almost all cases of cervical cancer as well as other anogenital cancers.

Although cervical cancer was once one of the top causes of cancer-related death in America, the addition of the Papanicolaou (Pap) smear for cervical dysplasia screening to routine physical exams reduced incidence and deaths from this disease by 60% in less than 40 years (report.nih.gov/nihfactsheets). Even more promising for eliminating cervical cancer is the introduction of the HPV vaccines Gardasil and Cervarix in the early 2000s. These vaccines, which consist of virus-like particles (VLPs) made from L1 protein of specific HPV types (6, 11, 16, and 18 for Gardasil, 16 and 18 for Cervarix), are nearly 100% effective at preventing HPV infection. This means that Gardasil will theoretically eliminate the 70% of cervical cancer

cases caused by HPV-16 and 18 as well as the 90% of genital warts cases caused by HPV-6 and 11 (report.nih.gov/nihfactsheets).

Despite the significant benefits provided by vaccination, there is currently no treatment for the tens of millions of existing HPV infections. About 90% of HPV infections are estimated to clear spontaneously within two years, but the few that persist beyond this point can cause cellular anomalies that lead to cancer (who.int/mediacentre/factsheets). Currently, because it can take decades to progress from the initial infection to cancer, regular Pap smears and prompt removal of pre-cancerous cervical dysplasias are the best option for avoiding complications of HPV infection. Even more preferable would be treatment to eradicate HPV infection in its early stages, before signs of cellular abnormalities occur. Further study into the processes that control infection is therefore necessary to identify potential therapeutic targets.

Papillomavirus Structure and Taxonomy

Papillomaviruses (PVs) comprise a family of hundreds of non-enveloped DNA viruses which infect mammals, birds, or reptiles. All PVs are divided into genera (designated with Greek letters) based on sequence variations in the L1 gene. For example, the α -PVs include HPVs that infect either mucosal or cutaneous keratinocytes and can be further divided into high- or low-risk types based on their ability to immortalize infected cells. In contrast, β -PVs preferentially infect cutaneous epithelium (de Villiers et al., 2004) and cause cutaneous malignancies in immunosuppressed individuals (Doorbar et al., 2015), including patients with the rare skin disorder epidermodysplasia verruciformis (EV).

PVs contain a double-stranded, circular DNA genome about 8 kilobases (kb) in length. The bovine papillomavirus type 1 (BPV-1) genome shown in Figure 1.1 contains coding regions for six early genes (E1-E8) and two late genes (L1 and L2), as well as a non-coding upstream regulatory region (URR, or long control region, LCR) that includes the viral origin of replication (*ori*) and the minichromosome maintenance element (MME), which facilitates efficient viral genome partitioning in dividing host cells. Certain PVs also produce splice variants of the major genes, including E8^{E2}, E1^{E4}, and a group of splice products from within the E6 gene collectively designated E6*.

In BPV-1, a widely used model for papillomavirus research, there are two major classes of promoter that drive expression from the genome. Early promoters (major promoter: P₈₉; see Figure 1.1) regulate transcription of the early gene products with the involvement of several host transcription factors such as nuclear factor I (NF-I) (Gloss et al., 1989), activator protein 1 (AP-1) (Offord and Beard, 1990), specificity protein 1 (SP1) (Gloss and Bernard, 1990), and the transcription factor II-D (TFIID) complex (Carrillo et al., 2004). Regulation of the early promoter has been found at least in part to be differentiation-dependent; in infected cells, early gene transcripts are more abundant in differentiated cells than in undifferentiated cells (Yukawa et al., 1996, Durst et al., 1992, O'Connor et al., 2000). Differential regulation of other promoters (i.e. P₂₄₄₃ and P₃₀₈₀; see Figure 1.1) also modulates early promoter function (Linz and Baker, 1988). The late promoter P_L is active only in differentiating cells such as those found in fibropapillomas (warts) (Linz and Baker, 1988), and its regulation is not as well understood. It controls expression of the capsid proteins in preparation for virion assembly and exit from host cells.

FIGURE 1.1

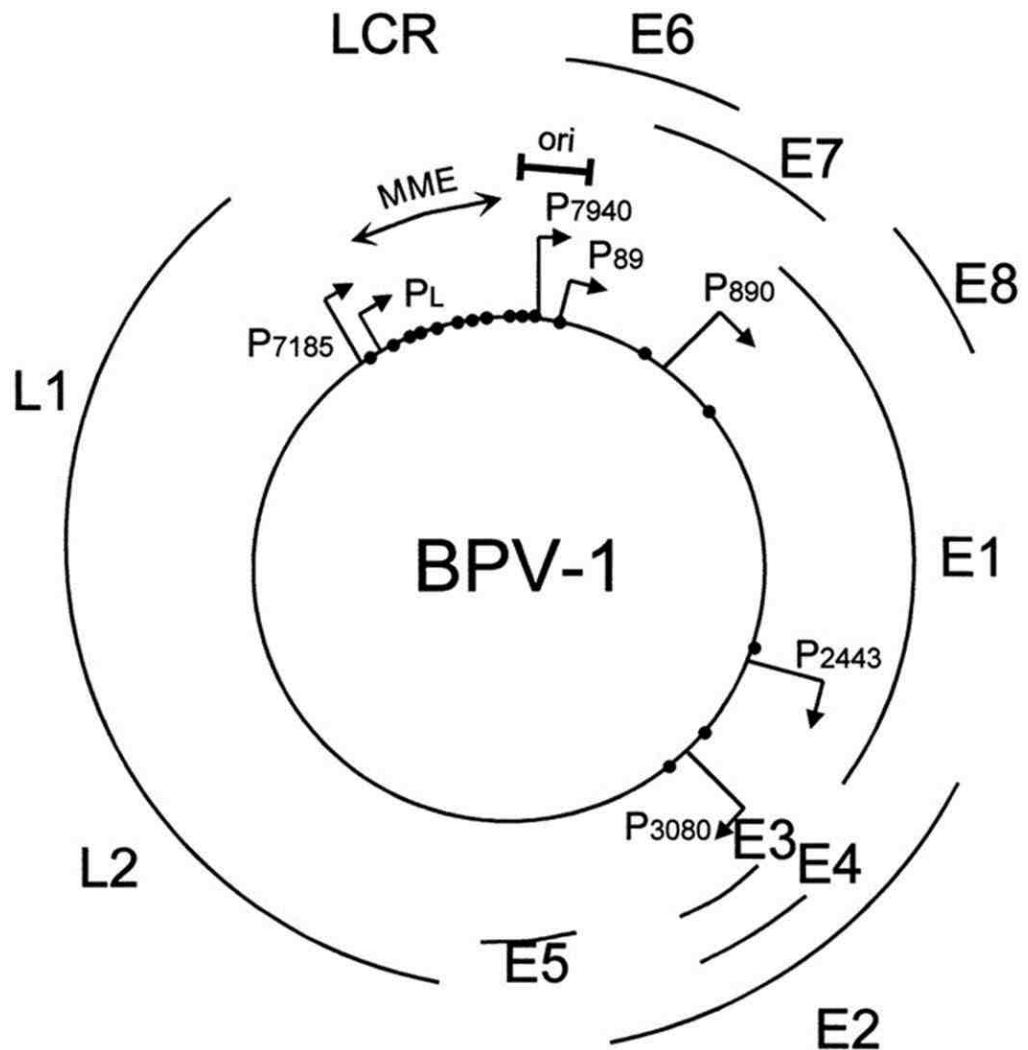


Figure 1.1. The bovine papillomavirus type 1 (BPV-1) genome. The papillomavirus genome is comprised of circular, double-stranded DNA approximately 8 kilobases (kb) in length. BPV-1 encodes several early genes (E1-E8; curved lines are scaled to length of open reading frames (ORFs)) and two late genes (L1 and L2). The transcription and replication factor E2 has several binding sites in the genome with the consensus sequence ACC(N₆)GGT (black dots). The long control region (LCR), also called the upstream regulatory region (URR), is a large region of noncoding DNA containing the origin of replication (*ori*, bar) and the minichromosome maintenance element (MME, two-headed arrow). Transcription is initiated from several promoters (bent arrows), including the major early promoter P₈₉, the late promoter P_L, and the regulatory promoters P₂₄₄₃ and P₃₀₈₀ (Linz and Baker, 1988). Reprinted with permission from (Skiadopoulos and McBride, 1998).

Infection and Entry

PVs preferentially infect keratinocytes in the basal layer of the epithelium. They gain access to these cells by means of microscopic abrasions or lesions which expose the basement membrane (Figure 1.2). PVs are thought to bind the basement membrane rather than basal keratinocytes themselves (Roberts et al., 2007). Viral particles primarily bind to heparan sulfate proteoglycans (HSPGs) (Shafti-Keramat et al., 2003), though other proteins such as laminin-332 may also aid in viral attachment (Cerqueira et al., 2013). Studies using pseudovirions (comprised of L1, L2, and reporter DNA such as the GFP gene) have shown that HSPG binding causes a conformational change in L2 which exposes a furin protease cleavage site. Furin cleavage is necessary for subsequent conformational changes which allow binding to an as yet unidentified cell surface receptor (Kines et al., 2009). Viral entry occurs 12-24 hours after binding (Culp and Christensen, 2004).

PVs are internalized by different mechanisms depending on viral type. HPV-31 and bovine papillomavirus type 1 (BPV-1) seem to depend on clathrin and caveolin for their entry into cells (Raff et al., 2013, Horvath et al., 2010). HPV-16, however, employs actin-dependent endocytosis initiated by the phosphoinositide 3-kinase (PI3K) pathway (Surviladze et al., 2013). Interestingly, PI3K is emerging as a potential therapeutic target for head and neck squamous cell carcinoma (HNSCC) (Yap et al., 2008). The PI3K encoding gene *PIK3CA* acquires an activating mutation in HPV-positive head and neck cancers (Lui et al., 2013). A study using HPV-16 pseudovirus (PsV) to study viral entry mechanisms revealed that the small-molecule PI3K inhibitor LY294002 prevented the majority of PsV infection of HaCaT cells (immortalized

keratinocytes) (Surviladze et al., 2013), and another study reported that siRNA against PI3K prevents E6 and E7 mediated Akt signaling downstream of the kinase (Zhang et al., 2014a).

Once the virus is internalized, it enters the endosomal pathway, where the major capsid protein L1 dissociates from the L2-DNA complex. Retrograde transport of this complex from the Golgi to the ER by a γ -secretase-dependent mechanism has been suggested as a means of getting viral DNA close to the nucleus (Zhang et al., 2014b). Actin filaments may also assist in L2-DNA nuclear transport (Schelhaas et al., 2012), though it is thought that PV DNA enters the nucleus after cell division as the nuclear envelope re-forms (McBride, 2008). Once viral DNA arrives in the nucleus, it associates with promyelocytic leukemia protein (PML) complexes, called PML bodies, to initiate transcription and replication (Day et al., 2004). The factors required to first express and maintain the early proteins in the host are unknown.

FIGURE 1.2

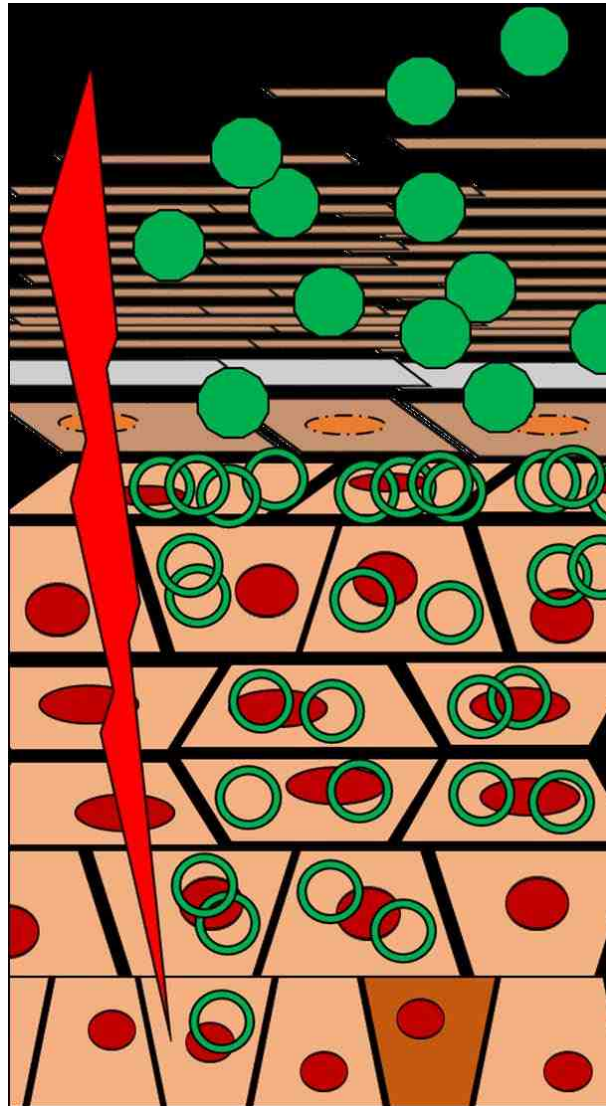


Figure 1.2. Viral infection and replicative cycle in the context of the keratinized epithelium. Papillomavirus gains access to the basal layer of the keratinized epithelium (pale cells, keratinocytes; brown cell, melanocyte) via micro-abrasions (red bolt). The virus establishes its copy number in the basal cells (green rings, bottom cell layers). As host cells migrate upward and differentiate, viral genome copy number is amplified (green rings, top cell layers). Amplification prepares cells to produce new viral particles (green polygons), which are released into the environment as dead cells slough off the epithelial surface.

Replicative Cycle

PV replication requires complex crosstalk between viral and cellular proteins across the different life phases of the host cell, from proliferation and maturation to differentiation and death. There are three major stages in the viral replicative cycle: initiation, maintenance, and amplification. Each one requires PVs to exploit cellular resources available at different times in keratinocyte development.

In the initial stage of PV replication, the genome is copied at a low level, usually between 1 and 10 copies per cell depending on the system used for study (Chesters and McCance, 1985, Botchan et al., 1986, Geimanen et al., 2011). The viral helicase E1 and the replication and transcription regulator E2 are required for PV replication. E2 recruits E1, which binds viral DNA with low specificity, to the viral origin of replication (*ori*) (Ustav and Stenlund, 1991) (Figure 1.3). The E2:E1 interaction is transient, however, since E2 must dissociate for E1 to form a double hexamer and initiate replication (Sanders and Stenlund, 1998, Abbate et al., 2004). The cellular factors that contribute to viral replication include: DNA polymerase (Pol) α , Pol δ , RPA, and topoisomerases (Topo) I and II. All of these factors were required for replication in a cell-free system (Kuo et al., 1994), with Topo I acting similarly to E2 in recruiting E1 to the *ori* before dissociating (Hu et al., 2006).

After initial replication, PVs enter their maintenance stage. When an infected cell divides, PV genome copies are passed on to the daughter cells via interaction of E2-DNA complexes with cellular proteins. Some of these proteins, including Brd4 (You et al., 2004) and Chlr1 (Parish et al., 2006), tether the complexes to host chromosomes, while others such as Mklp2 mediate

interaction with the mitotic spindle (Van Tine et al., 2004, Yu et al., 2007). During maintenance replication, the majority of E1 protein is sequestered in the cytoplasm (Fradet-Turcotte et al., 2010a), likely because high amounts of nuclear E1 are toxic to cells (Sakakibara et al., 2013). Expression of E1 and E2 is low at this time, which limits replication activity (Lambert, 1991) and may be controlled by the E6 and E7 proteins, though the exact mechanism is unknown. Stable maintenance of HPV episomes (versus integration) depends on E6 and E7 (Thomas et al., 1999), particularly the p53 degradation function of E6 (Park and Androphy, 2002). While PVs can maintain their genomes strictly in the episomal form, BPV-1 genomes in the ID13 cell line (mouse fibroblasts stably expressing BPV-1) were detected as monomeric episomes as well as tandem repeats integrated within host chromosomes (Schvartzman et al., 1990). Multimeric episomes have also been observed in cell lines and patient samples (Kennedy et al., 1987, Choo et al., 1989, Kristiansen et al., 1994, Orav et al., 2013).

Keratinocytes terminally differentiate as they migrate towards the epithelial surface. In infected cells, in a process that is not fully understood, PVs sense and exploit cellular differentiation to transition to the amplification stage of their replication cycle. In this stage, PV genome copy number increases to thousands of copies per cell. The DNA damage response pathway (DDR) assists in amplification (Gillespie et al., 2012): activation of one branch of the DDR, the ATM pathway, recruits DNA damage proteins to viral replication centers in the nucleus (Moody and Laimins, 2009). Multiple factors that stimulate amplification do so through activating the DDR; these include chemical and biological mutagens (Schmitt et al., 1989) and the viral E7

oncoprotein (Hong and Laimins, 2013).

E7 as well as the E6 oncoprotein promotes amplification by preventing complete cell-cycle arrest, allowing the virus to utilize host replication machinery despite activation of DNA repair pathways (Gillespie et al., 2012). The E5 oncoprotein has also been shown to promote amplification (McBride, 2008). While most PVs manipulate their hosts only to the extent needed for viral propagation, the high-risk HPVs can undergo aberrant replication which has mutagenic effects on the host cell. They integrate their genomes into host cell chromosomes, abrogating virion production while further dysregulating cell cycle arrest and DNA repair. Since integration usually disrupts the E2 gene, which is responsible for limiting expression of E6 and E7, these oncoproteins can act unchecked. Inhibiting the tumor suppressors p53 and Rb, they produce genomic instability leading to neoplastic changes and progression to cancer (Shin et al., 2006a, Shin et al., 2006b). In the context of cervical cancer, both integration and the absence of an intact E2 gene (i.e. from episomal HPV DNA co-existing with integrated DNA in the same cell) are associated with poor prognosis (Shin et al., 2014).

We hypothesize that there is a means to limit E2 function even before loss of the gene through integration. Generally, we assert that post-translational modification of viral proteins contributes to the coordinated timing of the viral replicative cycle with the different stages of the host-cell life cycle. The next section and the following chapters build a case for E2 as a crucial recipient of this form of regulation because it is a highly conserved protein of diverse functions.

FIGURE 1.3

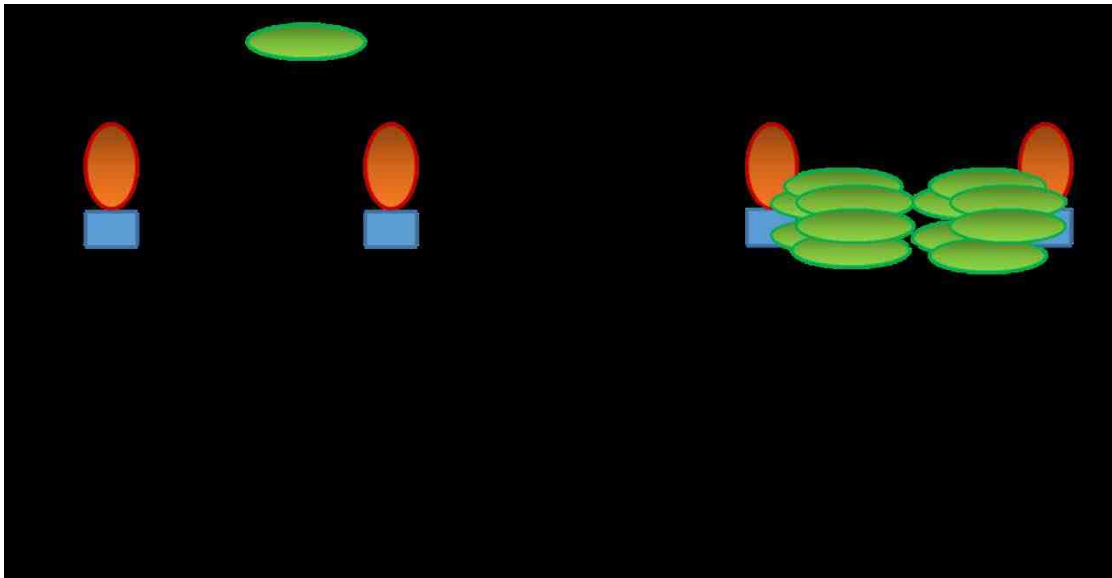


Figure 1.3. Recruitment of the viral helicase E1 to DNA by the E2 protein. E2 (orange oval) binds with high specificity to the consensus sequence ACC(N₆)GGT (E2BS, blue box). When E2 binds these sites in the *ori* (Figure 1.1), it can recruit E1 monomers (green ovals) to efficiently assemble on DNA (E1 binds the *ori* with low specificity in the absence of E2). After E1 forms a double hexamer (hence “12 x” E1) and E2 dissociates, cellular replication factors bind and assist E1 in initiating replication.

E2: Structure and Functions

E2 is a modular protein containing two distinct domains, an amino-terminal transactivating domain (TAD) and a carboxy-terminal DNA-binding domain (DBD) (Figure 1.4), which are highly conserved among PVs (Giri and Yaniv, 1988). The two domains are connected by a less conserved, flexible hinge region. In BPV-1 E2, which is 410 amino acids (aa) in length and 48 kilodaltons (kDa) in mass, aa 1-216 comprise the TAD and aa 286-410 comprise the DBD.

E2 monomers interact at their DBDs to form an 8-stranded antiparallel β barrel which interacts with DNA, and each monomer also contributes α helices outside the barrel to enhance sequence-specific binding interactions (Hegde et al., 1992, Hegde and Androphy, 1998, Hegde, 2002). The β barrel binds the palindromic DNA consensus sequence ACC(N₆)GGT (Androphy et al., 1987, Hegde, 2002). The identity of the variable bases in each E2 binding site (E2BS) determines the affinity of E2 for that site, and both high- and low-affinity E2BS are found in the viral upstream regulatory region (URR) (Figure 1.1). E2 association with high-affinity E2BS results in transcriptional activation, while association with low-affinity E2BS results in transcriptional repression and replication (Hegde, 2002). Thus, viral gene transcription (e.g. of E6 and E7) is favored when E2 levels are low, and replication is favored when E2 levels are high.

The placement of the E2BS within the viral genome also determines the functional outcome of E2 binding. High-affinity sites are distant from the *ori* and presumably activate transcription by promoting E2 multimerization, DNA looping, and recruitment of cellular transcription factors. Low-affinity sites are

close to the *ori* to recruit E1 and cellular replication factors (Ustav et al., 1993). Indeed, E1:E2 complex formation at these sites prevents nucleosome assembly (Li and Botchan, 1994) and binding of transcription factors such as TATA binding protein (TBP) (Hartley and Alexander, 2002). E2 occupies other E2BS in the minichromosome maintenance element (MME) within the URR during S phase and mitosis to mediate genome tethering to host chromosomes and ensure efficient partitioning to dividing host cells (Kadaja et al., 2009, Melanson and Androphy, 2009, Piirsoo et al., 1996).

Mutation studies with different PVs suggest that individual amino acids in the E2 protein sequence contribute to each of its functions, since some mutants which cannot transactivate can still drive replication or vice versa (Grossel et al., 1996, Brokaw et al., 1996, Ferguson and Botchan, 1996, Abroi et al., 1996). Indeed, PVs have evolved to express multiple forms of E2 which separate these functions in a natural infection. Use of alternate start codons or splicing in the BPV-1 E2 open reading frame (ORF) yield gene products with masses of 31 kDa and 28 kDa (Hubbert et al., 1988). The former, referred to as E2C or E2R, spans aa 162-410 (Figure 1.4) and generally acts as a repressor of transcription (Lambert et al., 1990) and replication, though it may have more of a limit-setting function (e.g. establishing a set copy number in viral maintenance) since it still promotes these activities to some extent (Lim et al., 1998, Lace et al., 2012). Such fine tuning of E2 activity occurs in part through dimerization: while heterodimers comprised of full-length E2 and E2R activate replication, E2R homodimers repress it (Lim et al., 1998). The 28 kDa protein, E8^ΔE2, is the result of splicing from the E8 ORF to the C-terminus of the E2 ORF (Figures 1.1 and 1.4), and it represses both replication and transcription

for HPV-16 (Straub et al., 2014). Controlled expression of E2R and E8^{E2} may allow PVs to alternate between different E2 functions.

Another means of E2 regulation, post-translational modification (PTM), may provide a more dynamic means of influencing E2 function since PTMs can be added and removed in less time than it takes to alter E2 gene transcription. Dynamic regulation is important for multifunctional proteins such as E2, especially since the actions of E2 directly affect the progression of the viral replicative cycle. The following chapters will discuss the role of different PTMs in E2 function and their implications for understanding PV biology.

FIGURE 1.4

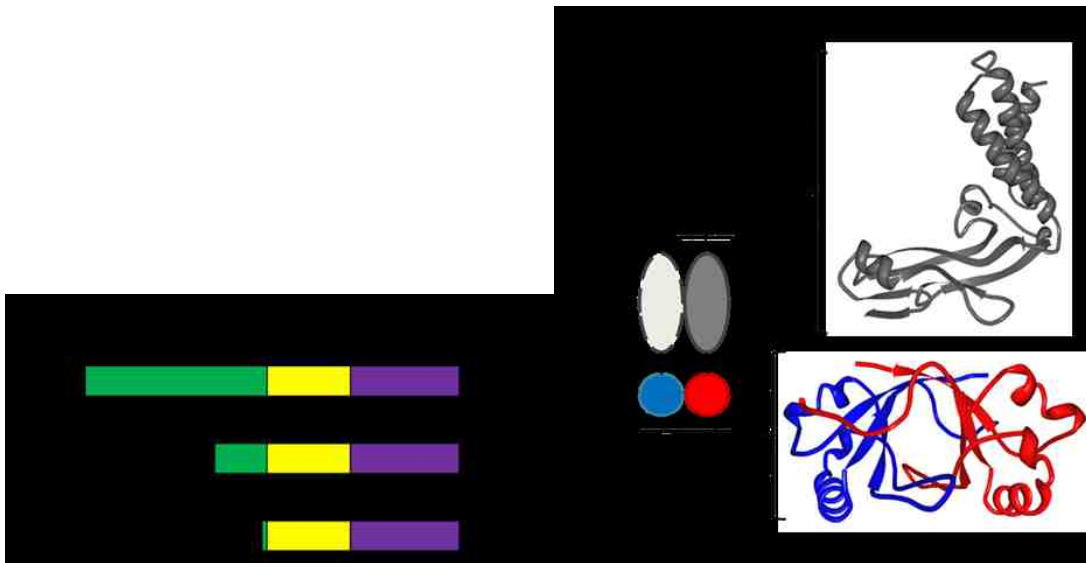


Figure 1.4. Structure of E2. (A) The E2 open reading frame (ORF) encodes multiple gene products through the use of alternate start codons and splicing. In BPV-1, the full-length E2 protein (amino acids/aa 1-410) has an N-terminal transactivating domain (N, green, aa 1-210), a flexible hinge region (yellow), and a C-terminal DNA-binding domain (C, purple, aa 286-410). The truncated gene product E2R (aa 162-410), transcribed from an alternate start codon, lacks the majority of the TAD. E2R generally acts as a repressor, though weak transactivation has been detected in certain systems. The alternate splicing product E8^ΔE2 combines 11 aa of E8 (black) with aa 206-410 of E2; this protein is a transcriptional repressor. Dotted lines, coding sequence not included in protein. (B) Full-length E2 binds E2BS (DNA, black bars) as a dimer. The DNA binding domains (left, blue and red circles; right, blue and red ribbons) interact to form an 8-stranded B barrel (right). Dimerization of the transactivating domains (gray ovals) is not necessary for E2 function. Dotted border, ribbon counterpart not shown (gray ribbon, TAD monomer). The BPV-1 E2 ribbon structure was generated in Protein Workshop (downloaded from rcsb.org/pdb) using the crystal structure 2JEU (Sanders et al., 2007), documented in the Papillomavirus Episteme (PaVE, pave.niaid.nih.gov) and the RCSB Protein Data Bank (PDB).

CHAPTER 2

E2-MEDIATED TRANSCRIPTION AND ACETYLATION

*Note: A portion of this work was published in Quinlan EJ, Culleton SP, Wu SY, Chiang CM, Androphy EJ. 2013. Acetylation of conserved lysines in bovine papillomavirus E2 by p300. *J. Virol.* 87 (3):1497-1507.

Introduction

E2 Transcriptional Regulation

As mentioned in the previous chapter, the ability of papillomavirus E2 protein to activate or repress transcription is linked in part to the location of its binding sites in viral DNA, expression of its truncated forms, and dimerization. BPV-1 E2 is particularly adept at activating transcription compared to other PVs because the BPV-1 URR contains the minichromosome maintenance element (MME), a region upstream of the P₈₉ early promoter rich in E2BS (Figure 1.1). In addition to their roles in genome partitioning mentioned earlier, these E2BS are thought to act as transcriptional enhancers (Spalholz et al., 1987), allowing interaction between E2 binding the MME and E2 binding the early promoter by DNA looping (Knight et al., 1991). Conversely, E2BS within the promoters of HPVs such as HPV-11, 16, and 18 are arranged so that they overlap with the TATA box, preventing binding of cellular factors to viral DNA and formation of the transcription initiation complex (Dostatni et al., 1991, Demeret et al., 1997, Hartley and Alexander, 2002, Dong et al., 1994). These HPVs also lack the MME and only have four E2BS (Kurg, 2011). Indeed, in HPV-transformed cell lines, transfected BPV-1 E2 represses E6 and E7 expression: BPV-1 E2 replaces the missing HPV E2 and binds to the E2BS to block transcription initiation (Goodwin et al., 1998, Wells et al., 2000). Above a certain level of E2 protein, however, E2 dimers form in solution rather than on DNA, reducing transcription activation in a phenomenon known as squelching (Kovelman et al., 1996, Abroi et al., 1996).

Cellular Co-factors for E2 Dependent Transcription

The E2 TAD is essential for E2-dependent transcription (Haugen et al., 1988) partly because it mediates interactions with cellular proteins. The truncated forms of E2 (E2R and E8^ΔE2), which lack the TAD, can therefore act as transcriptional repressors (Lambert et al., 1987, Choe et al., 1989, Vande Pol and Howley, 1990). E2-interacting proteins include factors important for basal transcription, such as Sp1, TFIIB, and TFIID (Sandler et al., 1996, Yao et al., 1998, Rank and Lambert, 1995); factors involved in chromatin remodeling, such as Brd4 and Brm (Ilves et al., 2006, Kumar et al., 2007); and those which regulate cellular transcription through other mechanisms, such as Gps2 and Tax1BP1 (Breiding et al., 1997, Wang et al., 2009). The present work includes interaction studies with BPV-1 E2 and Brd4, Gps2, and Tax1BP1 as a cross-section of E2 binding partners.

Brd4 is a member of the BET family of proteins, which each contain two bromodomains and a conserved extra-terminal (ET) domain (Wu and Chiang, 2007). It is best known in cellular biology for binding the P-TEFb complex, which is required for transcriptional elongation (Vollmuth et al., 2009). Brd4 binds P-TEFb with its extreme C-terminal domain (CTD, or motif, CTM) and interacts with acetylated lysines on histone tails via its bromodomains, thus providing a means for recruiting transcription elongation machinery onto so-called “open” chromatin (Filippakopoulos et al., 2012). In addition to its activities in uninfected cells, Brd4 has been implicated in several processes in PV biology. It was first found to interact with BPV-1 E2 via the CTD and mediate E2 tethering of viral genomes to mitotic chromosomes (You et al., 2004, Abbate et al., 2006) (Figure 2.1). Studies utilizing the isolated CTD, which exerts a dominant negative effect on Brd4 when the two are co-expressed, revealed that PVs also require full-

length Brd4 for transient replication and transcriptional activation (Ilves et al., 2006, Schweiger et al., 2006). For HPVs, Brd4 can act as a chromatin insulator, preventing expression of E6 and E7 (Wu et al., 2006). In multiple PV types, mutation of the conserved residues arginine 37 (R37) and isoleucine 73 (I73) in the E2 TAD not only abrogate Brd4 binding but also inhibit transactivation, further highlighting the importance of Brd4 for PV transcription regulation (Senechal et al., 2007). Brd4 has also been shown to stabilize E2 by an as-yet-unknown mechanism which requires direct interaction between the two proteins and prevents E2 proteasomal degradation (Lee and Chiang, 2009).

Gps2 (AMF-1 in yeast), discovered as an E2 binding protein in a yeast two-hybrid screen (Breiding et al., 1997), directly bound and additively enhanced BPV-1 transactivation with the lysine acetyltransferase (KAT) p300 (Peng et al., 2000). E2 interacts weakly with p300 by itself, but the interaction was strengthened in the presence of Gps2. This finding suggested that Gps2 recruited E2 into a complex with p300, though the function of such a complex was still unknown (Peng et al., 2000).

Tax1BP1 (named for its binding to human T-cell leukemia virus type 1 (HTLV-1) protein Tax1; also abbreviated TXBP), is a partner in the ubiquitin editing complex with the deubiquitinase A20 and has roles in suppressing pro-inflammatory NF- κ B signaling and preventing apoptosis (Verstrepen et al., 2011, Shembade et al., 2011). Tax1BP1 was found to bind BPV-1 E2, HPV-16 E2, and HPV-18 E2; it not only stabilized these proteins in an E2-specific manner but also enhanced BPV-1 transcription activation in a p300-dependent manner (Wang et al., 2009). p300 was also found to bind Tax1BP1 directly.

FIGURE 2.1

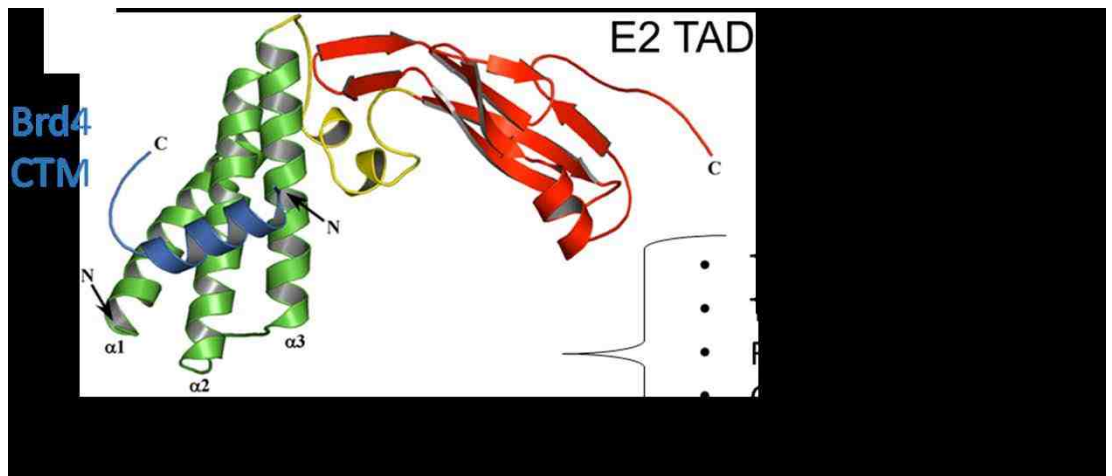


Figure 2.1. The E2:Brd4 interaction. Although Brd4 is over 1300 amino acids in length, only the last 20 have been found necessary to bind E2. The C-terminal domain (CTD, blue helix) binds perpendicular to the N-terminal α helices (green) of the E2 transactivating domain (TAD). This interaction has diverse outcomes depending on PV type and assay conditions, ranging from transcription activation to stabilization of E2 protein. Ribbon structures reprinted with permission from (Abbate et al., 2006).

Lysine Acetyltransferases and E2 Acetylation

Despite the role of p300 in enhancing E2-mediated transcription from two separate protein complexes (E2:Gps2 and E2:Tax1BP1), acetylation of E2 by p300 or any other KAT had not been observed. p300 is one of several KATs, also known as histone acetyltransferases (HATs) due to their initial discovery as post-translational modifiers of lysine residues in histone tails (Allfrey et al., 1964). This function was first thought to loosen chromatin by decreasing the net positive charge on histones, but subsequent studies revealed a complex system with layers of specificity at the level of KATs, lysine residues, chromatin location, and tissue types (Hebbes et al., 1988, Kimura et al., 2005). Like p300 and its close relative CREB-binding protein (CBP), several proteins known for their roles as transcription co-activators were found to possess acetyltransferase activity. These include two major families, GNAT (Gcn5-related N-acetyltransferase) and MYST (MOZ, Ybf2/Sas3, Sas2, Tip60). The targets of these proteins are not limited to histones: the tumor suppressor p53 is a notable target of p300, Tip60, and the GNAT family member PCAF, all of which act at different residues (Sakaguchi et al., 1998, Tang et al., 2006). Specific KAT:p53 interactions allow for fine-tuning of p53 function, such as favoring apoptosis versus cell-cycle arrest as in the case of Tip60 (Tang et al., 2006). We expect that, if E2 were acetylated, it would be for the purpose of similar fine-tuning (e.g. favoring transcription over replication or vice versa).

PVs both act on and depend on pathways in which KATs are involved. HPV-16 E6, for instance, targets the KAT complex component and p53 transcriptional coactivator hAda3 for proteasomal degradation; this process depends on the ubiquitin ligase E6AP and prevents p14ARF-mediated

signaling to p300 for p53 acetylation and subsequent cell senescence (Kumar et al., 2002, Sekaric et al., 2007, Shamanin et al., 2008, Hu et al., 2009). CBP, p300, and PCAF have all been identified as binding partners and transcription co-activators for E2 from various PVs (Peng et al., 2000, Lee et al., 2000, Muller et al., 2002, Lee et al., 2002, Kruppel et al., 2008). Interestingly, both CBP and PCAF lacking intact KAT domains failed to support E2-mediated transactivation (Lee et al., 2002, Lee et al., 2000), suggesting that acetyltransferase activity is important for this function of KATs. However, the target of this activity in these studies was unknown.

These findings led our lab to hypothesize that E2 acetylation is important for its transcription activation function. siRNA knockdown of three different KATs (p300, CBP, and PCAF) could not be functionally complemented by expression of the other two KATs, suggesting an independent mechanism for each in enhancing E2 transactivation (Quinlan et al., 2012). *In vitro* acetylation assays with p300 revealed several lysine acetylation sites on BPV-1 E2. Two of particular interest, K111 and K112 (Figure 2.2), were notable for their extremely high level of conservation among PVs, a previous report of their inclusion in a putative nuclear localization signal (NLS) (Skiadopoulos and McBride, 1996), and differential effects on transactivation of lysine to arginine (K→R) point mutants at these sites (Brokaw et al., 1996). To determine whether acetylation at these sites might influence E2 transactivation, we mutated the residues to arginine. This mutation discourages acetylation while conserving side-chain size and charge. Although K111R and a double mutant, K111R/K112R, had less than 10% wild type transcriptional activity in a luciferase reporter assay, K112R stimulated transcription at about 70% of wild

type levels (Quinlan et al., 2012). These results were not only consistent with an earlier study (Brokaw et al., 1996) but also provided the first indication that E2 acetylation could regulate the protein's activity. However, we sought to determine whether such modifications occurred in E2 expressed in cells. Here we show that both BPV-1 and HPV-31 E2 are acetylated at these sites in a live-cell system. We also confirmed that BPV-1 K111R has a greater transactivation deficiency than K112R using a different cell line from our initial experiments. Furthermore, we initiated co-immunoprecipitation studies with K→R mutants and Tax1BP1. These results support our hypothesis that E2 acetylation at these sites influences its ability to activate transcription, while suggesting future avenues for study to determine the mechanism of action.

FIGURE 2.2

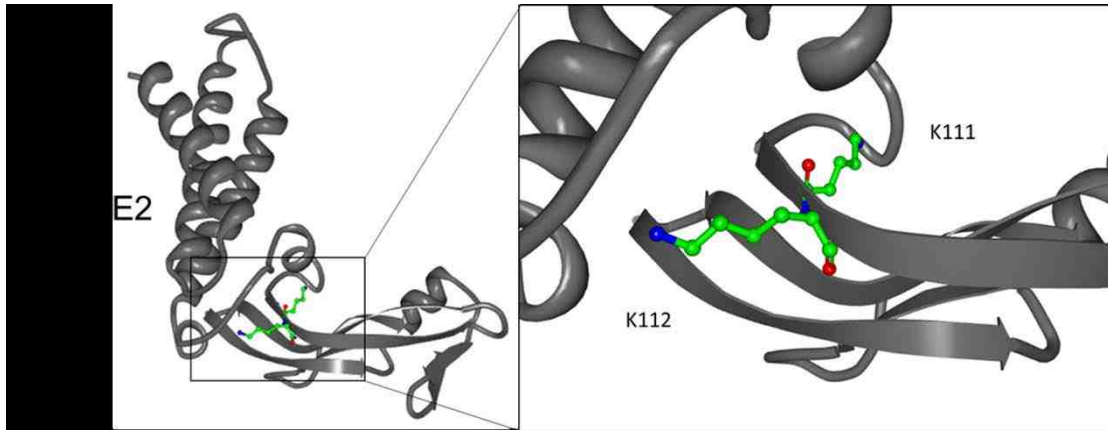


Figure 2.2. Lysines 111 and 112 in the BPV-1 E2 TAD. An *in vitro* acetylation assay with BPV-1 E2 and the acetyltransferase p300, followed by mass spectrometry, identified multiple acetylated lysines (K) including adjacent residues K111 and K112 (ball and stick model, inset: green, carbon; red, oxygen; blue; nitrogen). Gray ribbon, E2 TAD. The BPV-1 E2 ribbon structure was generated as described in Figure 1.4.

Materials and Methods

Cells and Transfections

Cells used include C33a, an HPV-negative human cervical cancer cell line; C33a/E2, C33a cells stably expressing BPV-1 E2; and RPE1, an hTERT-immortalized human retinal epithelial cell line. C33a and C33a/E2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals) and 1% penicillin-streptomycin solution (Pen-Strep, Invitrogen). RPE1 cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (Invitrogen) with 10% FBS and 1% Pen-Strep. C33a/E2 cells were kind gifts of Dr. Peter Howley. Transfection of C33a cells was performed using the calcium phosphate method. C33a/E2 and RPE1 cells were transfected using Lipofectamine 2000 (Invitrogen) at a 2:1 Lipofectamine:DNA ratio, with the DNA and transfection reagent diluted in Opti-MEM serum-free medium (Invitrogen). Media was replaced ~16 hours post-transfection for all experiments.

Mass Spectrometry

Two BPV-1 samples were derived from either C33a/E2 cells or C33a cells transfected with pCG-BPV1 E2 (Table 1, Appendix). For the first sample, C33a/E2 cells were grown on 15-cm dishes and lysed in urea lysis buffer (8M urea, 100 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1% NP-40) for one freeze-thaw cycle. Lysates were diluted in the same lysis buffer minus urea and incubated with Sepharose A and G beads cross-linked with two mouse monoclonal antibodies to BPV-1 E2, B201 and B202. Immunoprecipitants were resolved by SDS-PAGE and stained with InstantBlue Coomassie-based solution

(Expedeon). Two fragments from the 50-65 kDa region of the gel were excised and analysed separately. The second sample was derived from C33a cells grown on 10-cm dishes (Corning) transiently transfected with pCG-E2. Cells were lysed 48 hours post-transfection with NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 2mM Na₃VO₄, 10 mM NaF, protease inhibitor cocktail (Sigma)). Immunoprecipitation conditions were the same as those described above. Immunoprecipitants were resolved, stained, and submitted to the Indiana University Proteomics Core Facility for analysis.

HPV-31 samples were derived from C33a cells transfected with codon-optimized Flag-tagged HPV-31 E2 plasmid (Table 1, Appendix). Cells were grown on 15-cm dishes and lysed in Flag lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100; from the Sigma M2 Affinity Gel Technical Bulletin, Cat. # A2220). Tagged protein was immunoprecipitated with M2 Affinity Gel and resolved, stained, and submitted for analysis as described above.

The gel bands for mass spectrometric analysis were first reduced with 10 mM DTT in 10 mM ammonium bicarbonate and then alkylated with 55 mM iodoacetamide (prepared freshly in 10 mM ammonium bicarbonate). Alkylated samples were digested by trypsin (Promega) overnight at 37°C. Tryptic peptides were injected onto a C18 trapping column (NanoAcquity UPLC® Trap column 180µm x 20mm, 5µm, Symmetry C18) first before an analytical column (NanoAcquity UPLC® column 100µm x 100mm, 1.7µm BEH130 C18). Peptides were eluted with a linear gradient from 3 to 40% acetonitrile in water with 0.1% formic acid developed over 90 min at room temperature at a flow rate of 500 nL/min, and the effluent was electro-sprayed into a Thermo-Fisher Scientific

LTQ Orbitrap Velos Pro mass spectrometer (Thermo-Fisher Scientific) interfaced with a Waters Acquity® UPLC system (Waters). Analysis of phosphopeptides was performed using a data dependent neutral loss scan. Blanks were run prior to the sample run to make sure there were no significant background signals from solvents or the columns. Database search and data analysis were performed using the Thermo-Fisher Scientific Proteome Discoverer™ software (v1.3).

Luciferase Reporter Assays

C33a cells were grown in 12-well dishes (BD Falcon). Transfections were performed in triplicate. 48 hours post-transfection, cells were rinsed with DPBS and lysed with Steady-Glo. Lysates were transferred to 96-well Opti-Plates (PerkinElmer) and luminescence was measured with the Pherastar system. A monomeric RFP-GFP (mRFP-GFP) control was used to measure transfection efficiency.

Western Blot and Co-Immunoprecipitation

For co-immunoprecipitation (co-IP), C33a cells were transfected with BPV-1 E2 wild type or mutant encoding plasmids and 3x-Flag tagged Tax1BP1 (Table 1, Appendix). 48 hours post-transfection, cells were lysed and immunoprecipitated with B201 as described in (Wang et al., 2009). IP samples and inputs were resolved by SDS-PAGE and transferred onto PVDF membranes (Millipore), and proteins were detected by B201, M2 anti-Flag (Sigma), and anti-mouse HRP secondary antibody (Jackson Labs). Blots were developed with SuperSignal West Dura (Thermo Pierce) and were imaged

using ImageQuant LAS 4000 imager and software (GE Healthcare).

Results

E2 lysines are acetylated in cells

After having discovered the *in vitro* acetylation events in BPV-1 E2, we sought to determine whether this modification occurred on the protein in live cells. Two separate BPV-1 E2 protein samples were purified either from transfected C33a cells, an HPV-negative human cervical cancer cell line in which E2 transcription and replication activities are commonly tested, or C33a/E2 cells, a C33a-derived cell line stably expressing E2 at near-physiologic levels (You et al., 2004). Flag-tagged HPV-31 E2 protein was purified from transfected C33a cells. Protein samples were submitted to the Indiana University Proteomics Core for linear ion trap mass spectrometry. Spectra analysis by the Core revealed acetylation on K112 of BPV-1 E2 and K111 of HPV-31 E2 (Tables 2 and 3, Appendix). These results validated the *in vitro* acetyltransferase assay for BPV-1 E2, since acetylation was detected at other residues noted *in vitro* including K25, K70, K107, K226, K339, K346, and K347 (Quinlan et al., 2012).

E2 lysine mutants differentially influence transcription

Since K111 and K112 are conserved across almost all PVs, these residues were selected for functional analysis. We used lysine to arginine (K→R) mutants from our previous work (Quinlan et al., 2012): K111R, K112R, and the double mutant K111R/K112R. Transactivation assays using the pGL2-E2BS-Luc firefly luciferase reporter (Table 1, Appendix) were conducted initially in RPE1 cells, a highly transfectable human retinal epithelial cell line immortalized with hTERT. These assays suggested that K111R and the double

mutant, but not K112R, were deficient for transcription activation (Quinlan et al., 2012). We sought to confirm those findings in a different cell line, choosing C33a cells for their comparable transfectability and closer approximation to natural PV host cells. Again, using the E2BS-Luc reporter, which contains four E2BS upstream of an SV-40 promoter (Kumar et al., 2007), we found that K112R transactivation function was mostly intact, while both K111R and the double mutant diminished transactivation to less than 10% wild type levels (Figure 2.3) (Quinlan et al., 2012). Readouts were normalized to cells transfected with monomeric RFP-GFP (mRFP-GFP) plus empty vector for transfection efficiency detection by fluorescence (Table 1, Appendix).

FIGURE 2.3

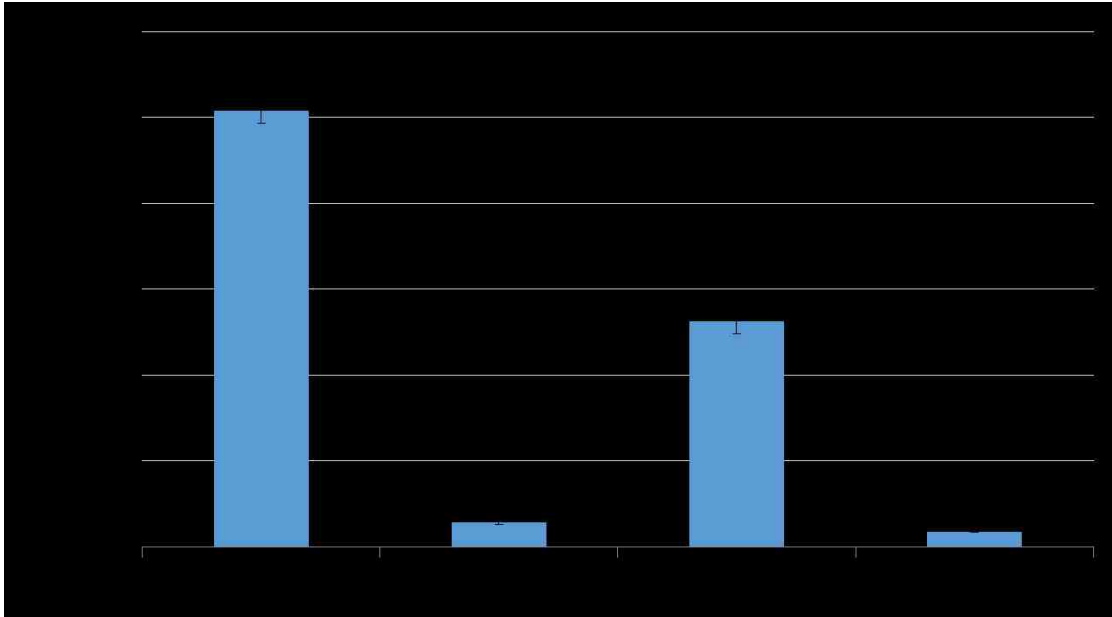


Figure 2.3. Lysine mutants differentially activate transcription. C33a cells were grown in 12-well dishes. Samples were transfected in triplicate wells with 50 ng/well pCG-E2 wild type or mutants (WT, K111R, K112R, K111R/K112R), as well as 75 ng/well pGL2-E2BS-Luc. 48 hours post-transfection, cells were lysed on-plate with 100 μ l Steady Glo reagent. Lysates were mixed with 100 μ l DPBS and transferred to a white 96-well plate for luminescence detection using the Pherastar system. Luminescence for each sample was divided by WT luminescence to get fold WT activation, and Student's t test was used to compare each sample to WT. ****, $p < 0.00001$. Values are expressed as mean \pm SEM.

E2 lysine mutants differentially affect protein expression and interaction with a transcription co-activator

The mutants K111R, K112R, and K111R/K112R were previously demonstrated to be expressed at different levels, with K112R closest to wild type, followed by K111R with less than 50% wild type expression and the double mutant with less than 20% (Quinlan et al., 2012). In addition to protein expression, we wanted to assess the mutants' capacity to bind a known E2 transcriptional co-activator, Tax1BP1. C33a cells were transfected with BPV-1 E2 wild type or mutant and 3x-Flag Tax1BP1, and lysates were immunoprecipitated with B201, a mouse monoclonal antibody against an epitope between aa 160 and 220 of BPV-1 E2 (Wang et al., 2009). Western blotting revealed that, despite comparable levels of E2 wild type and mutant immunoprecipitation, Tax1BP1 co-IP varied drastically. Wild type and K112R pulled down the highest levels of Tax1BP1, while all other mutants showed substantially decreased association with the cellular protein (Figure 2.4).

FIGURE 2.4

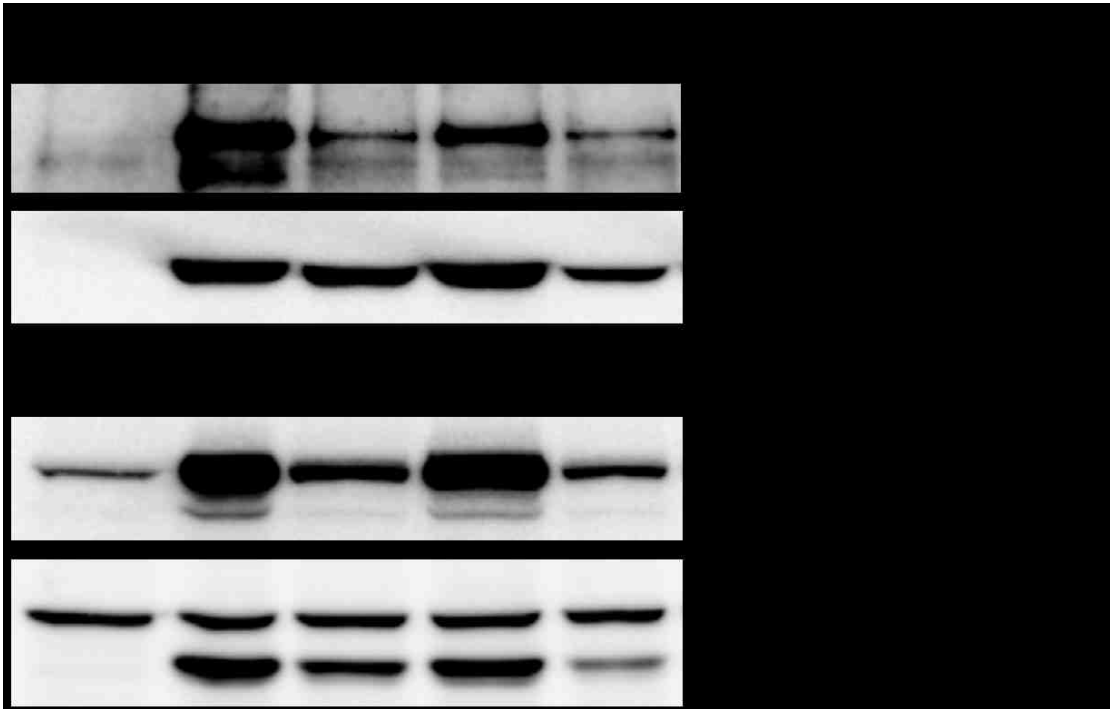


Figure 2.4. Lysine mutants differentially associate with Tax1BP1. C33a cells were grown on 6-cm dishes and transfected with 1 μ g each mRFP-GFP (-), BPV-1 E2 wild type and the K111R, K112R, and double mutants (WT, 1R, 2R, 1R/2R), and 3x-Flag Tax1BP1 (TXBP). Cells were harvested 48 hours post-transfection. 10% of each lysate was set aside for input, while remaining lysate was incubated with protein sepharose A and G beads and the anti-BPV-1 E2 mouse monoclonal antibody B201 at 4°C overnight. Western blots were probed for 3x-Flag TXBP and BPV-1 E2 using the mouse monoclonal anti-Flag antibody M2 and B201, respectively. *, nonspecific band.

Discussion and Future Directions

As small viruses encoding less than a dozen genes, the papillomaviruses are dependent on cellular machinery for assistance in carrying out essential functions. While the E2 protein has been shown to bind numerous cellular proteins, and many of these interactions are important for viral transcription, replication, and other activities, the exact mechanisms remain unclear (Smith et al., 2010, Muller and Demeret, 2012, Muller et al., 2012).

Previous work in our laboratory revealed multiple acetylation sites in BPV-1 E2 *in vitro* and demonstrated the importance of three different KATs – p300, CBP, and PCAF – in supporting E2-mediated transcription activation. This work showed that two K→R mutants, K111R and K112R, differentially affected E2 transactivation and protein expression, as well as DNA binding and subcellular localization (Quinlan et al., 2012). These results confirmed previous reports describing the effects of K111 and K112 on transcription (Brokaw et al., 1996) as well as their inclusion in a putative nuclear localization signal (NLS) in the E2 TAD (Skiadopoulos and McBride, 1996).

In the present study, mass spectrometry analysis of E2 harvested from cells confirmed K112 acetylation for BPV-1 E2 and K111 acetylation for the previously untested HPV-31 E2 protein. The absence of the other PTM (i.e. Ac-K111 for BPV-1 and Ac-K112 for HPV-31) should not be used to rule them out as possibilities; rather, the results may reflect a snapshot of conditions in these cell populations, or may even reflect differences in the cells themselves or the surface exposure of these residues *in vivo*. It is also possible that PTMs besides acetylation occur at these lysines. The use of a single residue for multiple types

of PTM has already been documented for other proteins such as p53, which has several lysines that serve as substrates for acetylation, ubiquitination, sumoylation, and neddylation (Tang et al., 2006, Kim et al., 2011, Liu et al., 2014, Bischof et al., 2006, Guihard et al., 2012).

Luciferase reporter assays in C33a cells confirmed previous findings in RPE1 cells that K111R and K111R/K112R were almost completely defective for transcription activation and K112R was mildly defective (Quinlan et al., 2012). While conformational changes resulting from the mutations themselves cannot be ruled out, transcription impairment may be due in part to reduced protein levels for K111R and the double mutant compared to K112R or wild type (Quinlan et al., 2012). Transiently transfected E2 drives its own transcription, so transactivation-defective mutants are unable to elevate their own protein levels. However, repeating this assay with titrations of the mutants to several-fold higher transfection amounts did not rescue their function (Quinlan et al., 2012). Despite comparable levels of immunoprecipitation for wild type, K111R, and K112R, co-IP of Tax1BP1 varied widely (Figure 2.4). Again, the mutation itself or the lack of acetylation at these sites may induce a conformational change preventing interaction. Interestingly, looking at the Tax1BP1 input levels for this experiment, both wild type and K112R but not K111R seemed to stabilize Tax1BP1. This suggests that intact K111, or acetylation at K111, is important for this ability of E2, or that acetylation at K112 would actually destabilize Tax1BP1. It is difficult to draw conclusions from the double mutant since its expression was low, possibly confounding the co-IP results. Even though diminished interaction with Tax1BP1 may not be the only mechanism responsible for impaired K111R transactivation, the co-IP results

point to altered transcription co-factor binding as a general means of regulation at this site. Thus far, our results suggest that: (1) acetylation at K111 is important for transactivation since the mutant was defective for this function in the luciferase assay; (2) acetylation at K111 may stabilize Tax1BP1, or K112 acetylation may lead to its destabilization (since K111R and K112R were associated with lower and higher levels of TaxBP1 relative to baseline, respectively); and (3) Tax1BP1 can bind all of the mutants, but this interaction is apparently not sufficient for E2 transactivation.

Studies to further elucidate the role of K111 and K112 in PV biology are currently under way in our laboratory. These sites are now being examined in the context of HPV-31 E2, since this PV is more relevant to human disease and was found to be acetylated at K111 in cells. Co-IP studies with Brd4 and Gps2 are also in progress. We expect the results to be similar to those for the BPV-1 E2:Tax1BP1 interaction, supporting the hypothesis that K111 acetylation inhibits co-factor association generally. If just the Gps2 results follow a similar pattern to Tax1BP1, this would suggest the importance of E2 acetylation by p300 in enhancing Gps2- and Tax1BP1-dependent E2 transcription. Brd4 contains bromodomains, which recognize acetylated lysines; although Brd4 has previously been observed to bind E2 through its CTD (Abbate et al., 2006), this does not preclude the use of other interaction sites. Tax1BP1 co-IP should also be repeated in the HPV-31 system to determine whether different viruses use similar PTMs at conserved residues.

We can refine our earlier hypothesis that post-translational modification (PTM) of E2 is one means of controlling the timing and magnitude of various E2 functions: we also assert that this is accomplished by altering the viral

protein's association with cellular factors. We propose that K111 acetylation of E2 occurs late in BPV-1 infection to promote E2 transactivation. This would coincide with elevated p300 expression, which is found in differentiating cells (Wong et al., 2010). Additional experiments of interest include transcription assays with wild type versus KAT-deficient p300 to determine whether acetylation – not just an intact lysine residue – is necessary for transactivation. We would also like to harvest HPV-31 positive cells (such as CIN612 or transfected HaCaT) in different cell-cycle stages or maturation states (i.e. proliferating versus differentiated keratinocytes) for comparison of HPV-31 E2 PTMs by mass spectrometry. Although we reported the first known acetylation events in both BPV-1 E2 and HPV-31 E2, their role in the PV replicative cycle remains to be seen. The suggested experiments would provide further insight into the significance of these PTMs in natural infection.

CHAPTER 3
TYROSINE PHOSPHORYLATION IN BPV-1 E2

*Note: A portion of this work has been submitted for publication and is currently under revision.

Introduction

Papillomavirus Proteins and Phosphorylation

The first PTMs detected on papillomavirus E2 proteins were serine phosphorylation events in the BPV-1 E2 hinge. These included serines 290, 298, and 301 (McBride et al., 1989). An “A3” mutant of the protein, with all three serines changed to alanines, stimulated replication to levels 20-fold higher than wild type in C127 cells, a murine fibroblast cell line (McBride and Howley, 1991). Increased stability of the mutant E2 protein likely contributed to this increase in replication activity; an S301A mutant was found to have a longer half-life and reduced ubiquitination compared to wild type (Penrose and McBride, 2000). Additionally, S301 phosphorylation induced a conformational change that destabilizes E2 (Penrose et al., 2004). Casein kinase II (CKII or CK2) phosphorylates S301 (Penrose et al., 2004). The enzyme recognizes this residue in the context of a PEST sequence, a stretch of amino acids rich in proline (P), glutamate (E), serine (S), and threonine (T) that is common to rapidly degraded proteins (Rogers et al., 1986).

Unlike the original three phospho-serines, an additional phosphorylation event at S235 appeared to enhance E2 activity since it was required for stable replication of viral genomes and tumorigenic transformation in C127 cells (Lehman et al., 1997). Indeed, an “A4” mutant – A3 plus S235A – failed to tether viral DNA to mitotic chromosomes (Lehman and Botchan, 1998). The kinase responsible for this phosphorylation event is unknown. Phosphorylation sites reported in other PVs include S253 in HPV-8 E2 mediated by protein kinase A (PKA) and S243 in HPV-16 E2; the presence of the PTM at both sites allows E2 to bind host chromatin (Sekhar and McBride, 2012, Chang et al., 2014).

Studies in HPV-16 suggest that these sites are dephosphorylated upon differentiation, though this may not apply to all PVs. The phosphatase calcineurin dephosphorylates HPV-16 E2: a scaffold protein, NRIP, brings E2 in contact with calmodulin (CaM), which recruits calcineurin (Chang et al., 2011, Kahl and Means, 2003). This leads to decreased ubiquitination and subsequent stabilization of E2. NRIP is known to bind CaM when calcium levels are high, and incubation in high-calcium media is known to differentiate keratinocytes. Since E2-dependent genome replication is highest in differentiated cells, it is expected that release of E2 from an inhibitory modification would be linked to changes in intracellular calcium by the NRIP/CaM/calcineurin complex.

Upon differentiation, E1 is cleaved by caspases 3 and 7 (Moody et al., 2007, Morin et al., 2011), and its exclusion from the nucleus is relieved by phosphorylation of its nuclear export signal (NES) (Sakakibara et al., 2013); the cleaved E1 is then imported to the nucleus to initiate replication along with E2 in a DDR-dependent manner (McBride et al., 2012).

E2 is not the only viral protein which undergoes serine phosphorylation. CK2 acts on the viral helicase E1 as well as E2, decreasing both proteins' DNA binding specificity and minimizing replication during viral maintenance (Schuck et al., 2013). Upon host-cell differentiation, the phosphorylation on E1 is lost via caspase-dependent cleavage, allowing full activity of the protein. In HPV-31 E1, phosphorylation at S92 and S106 by cyclin-dependent kinase 2 (Cdk2) activates a nuclear export signal (NES) in the protein, relocating it to the cytoplasm (Fradet-Turcotte et al., 2010a). Since E1 export is required for viral genome maintenance and nuclear buildup of the protein is harmful to cells, these two sites are thought to be dephosphorylated upon terminal host-cell

differentiation, when maximal E1 activity is needed and host-cell lethality is no longer an issue.

Tyrosine Kinases and Papillomavirus Infection

Although no tyrosine or threonine phosphorylation events have been reported to date for any PV protein, various tyrosine kinases (TKs) are targets of PV oncoproteins. The E5 protein of BPV-1 is a small (44 aa) oncogene product which is required for cellular transformation (Schlegel et al., 1986, DiMaio et al., 1986, Schiller et al., 1986, Horwitz et al., 1988). Though it carries out its transforming function by activating cellular platelet-derived growth factor β receptor (PDGF-R) (Lai et al., 1998, Klein et al., 1999, DiMaio et al., 2000), E5 can also induce transformation by promoting constitutive activation of c-Src, a non-receptor TK (Supryniewicz et al., 2002). c-Src activation was mediated by E5 mutants defective for PDGF-R binding, indicating that E5 can transform cells by two independent mechanisms. The E6 and E7 oncoproteins also promote increased TK activity. HPV-16 E7 was shown to activate c-Src family members Fyn, Src, and Yes in keratinocytes by promoting increased protein expression and autophosphorylation (Szalmas et al., 2013). E6 from the same virus maintained signaling from the receptor tyrosine kinases (RTKs) epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGF-1R) independent of the receptors' ligands by promoting their internalization (Spangle and Munger, 2013). E6 also assists TKs by inhibiting proteins that counter their activity: both HPV-16 and HPV-18 E6 were found to target protein tyrosine phosphatase H1 for proteasomal degradation (Topffer et al., 2007).

Because PV oncoproteins promote TK activity, we hypothesized that a

TK itself might be involved in a regulatory feedback loop and act on one or more viral proteins. The E2 protein would be a reasonable target since it has the ability to repress viral oncoprotein expression (Wells et al., 2000, Prabhavathy et al., 2015). TK activation may promote transcription repression by E2, or it may be a counter-strategy employed by PV oncoproteins to neutralize E2 and promote conditions conducive to viral DNA integration, loss of E2 expression, and oncogenic transformation.

In our search for E2 acetylation events in cells described in the previous chapter, we found several other PTMs, including tyrosine phosphorylation. Since no tyrosine phosphorylation event had ever been reported for E2, we sought to investigate the potential significance of this PTM. We show that BPV-1 E2 is phosphorylated at tyrosine 102 (Y102), and that point mutants at this site which prevent or mimic phosphorylation have differential effects on E2-mediated transcription, replication, and binding to cellular co-factors. We also demonstrate the first evidence of direct binding of BPV-1 E2 to multiple receptor tyrosine kinases and assert that one or more of these RTKs acts on E2 to modulate its function in the early stages of the viral replicative cycle.

Materials and Methods

Cells and Transfections

C33a, C33a/E2, HEK293TT, CV-1, A3, and ID13 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Atlas Biologicals) and 1% penicillin-streptomycin solution (Pen-Strep, Invitrogen). HaCaT cells were cultured in low-calcium (0.1 mM) DMEM with Pen-Strep. C33a/E2 cells were a kind gift of Drs. Peter Howley and Jianxin You. C33a cells were transfected either by the calcium phosphate method (mass spectrometry and transactivation assays) or by Lipofectamine 2000 (Invitrogen) at a 1:1 Lipofectamine:DNA ratio (replication assays). HEK293TT cells were transfected using the calcium phosphate method. CV-1 cells were transfected using Lipofectamine 2000 at a 2:1 Lipofectamine:DNA ratio. For transfections with Lipofectamine 2000, the DNA and transfection reagent were diluted in Opti-MEM serum-free medium (Invitrogen). Media was replaced ~16 hours post-transfection for all experiments.

Plasmids and Mutagenesis

Expression plasmids used include pCI, pEGFP-C1, mRFP-GFP, pCG-E2 for full-length wild-type (WT) BPV-1 E2, pCG-E2R for the truncated form of BPV-1 E2, pCG-E1 for BPV-1 E1, pHA:AMF1 for HA-tagged Gps2/AMF1, and p3XFLAG-CMV-7.1 Tax1BP1 for Tax1BP1. Tyr-Phe (Y102F) and Tyr-Glu (Y102E) mutations in BPV-1 E2 were generated using the QuikChange II site-directed mutagenesis system according to the manufacturer's instructions (Agilent Technologies). Briefly, pCG-BPV1E2 was used as a template for the

reaction. After the presence of a PCR product was confirmed by DNA gel electrophoresis, DH5 α *E. coli* cells were transformed by heat shock, recovered, and spread onto LB-Ampicillin plates to grow overnight at 37°C. Colonies were selected the following day for DNA isolation, and mutation was confirmed by sequencing. Primers used for the mutagenesis reaction include 5'-CACAAGCTGGGACCGATTCATGTCAGAACCTAAAC-3' (forward) and 5'-GTTTAGGTTCTGACATGAATCGGTCCCAGCTTGTG-3' (reverse) for Y102F and 5'-CACAAGCTGGGACCGAGAGATGTCAGAACCTAAACG-3' (forward) and 5'-CGTTTAGGTTCTGACATCTCTCGGTCCCAGCTTGTG-3' (reverse) for Y102E. pVL-F:hBrd4 for full-length Flag-tagged Brd4, pGEX:hBrd4(1224-1362) for GST-CTM, and pGEX:hBrd4(524-579) for GST-BID were kind gifts of Dr. Cheng-Ming Chiang. Luciferase reporter constructs included pGL2-E2BS-Luc for transactivation assays and pFLORI-BPV1 (firefly luciferase with long BPV-1 *ori*) as well as pRL (*Renilla*) for replication assays. The latter two plasmids were generously provided by Dr. Jacques Archambault, along with pCG-BPV-1 E1 Eag1235 (BPV-1 E1 with enhanced activity) for the E2-FGFR3 replication assays (see below). Dr. Leslie Thompson generously shared plasmids encoding wild-type human FGFR2, Flag-FGFR3, and FGFR4 (pRK7-FGFR2, pRK7-FGFR3-Flag, and pRK7-FGFR4) as well as the constitutively active Flag-tagged FGFR3 mutant K650E (pRK7-FGFR3KE-Flag). The pcDNA3-EphB2 construct encoding chicken EphB2 was a kind gift of Dr. Elena Pasquale. See also Table 1 in the Appendix.

Mass Spectrometry

Two separate BPV-1 E2 samples were analyzed by mass spectrometry.

One sample was derived from C33a/E2 cells, a cell line generated in the Howley lab that stably expresses BPV-1 E2. Cells were grown on 15-cm dishes (Corning), released from plates with 0.05% trypsin solution (Invitrogen), pelleted, and then frozen at -80°C. A total of 15 frozen pellets were thawed, lysed in urea lysis buffer (8M urea, 100 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1% NP-40), pooled, and diluted in buffer minus urea for immunoprecipitation with equal volumes Sepharose A and G beads (Invitrogen) cross-linked with two mouse monoclonal antibodies to BPV-1 E2, B201 and B202 (B201/2 A/G beads). After 4 hours of rotation at 4°C, beads were washed 5 times with TBS, alternating between low and high salt (150 mM and 500 mM). Beads were then suspended in Laemmli SDS-PAGE sample buffer plus 5% β-mercaptoethanol (BME) (Fisher), heated at 95°C, and run on a 12.5% SDS-PAGE mini gel. After staining with InstantBlue Coomassie-based solution (Expedeon), a region spanning 50-65 kDa was excised and divided into 4 fragments to be analyzed separately.

Additional samples were derived from C33a cells transfected with pCG-E2 and cultured on four 10-cm dishes. Cells were lysed 48 hours post-transfection with NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 2mM Na₃VO₄, 10 mM NaF, protease inhibitor cocktail (Sigma)). B201/2 A/G beads were used to immunoprecipitate E2. After low/high salt TBS washes as described above, beads were suspended in Laemmli buffer plus BME, boiled, and loaded on a 10% SDS-PAGE gel. After InstantBlue staining, bands spanning 45-50 kDa were excised.

The gel bands for mass spectrometric analysis were first reduced with 10 mM DTT in 10 mM ammonium bicarbonate and then alkylated with 55 mM

iodoacetamide (prepared freshly in 10 mM ammonium bicarbonate). Alkylated samples were digested by trypsin (Promega) overnight at 37°C. Tryptic peptides were injected onto a C18 trapping column (NanoAcquity UPLC® Trap column 180µm x 20mm, 5µm, Symmetry C18) first before an analytical column (NanoAcquity UPLC® column 100µm x 100mm, 1.7µm BEH130 C18). Peptides were eluted with a linear gradient from 3 to 40% acetonitrile in water with 0.1% formic acid developed over 90 min at room temperature at a flow rate of 500 nL/min, and the effluent was electro-sprayed into a Thermo-Fisher Scientific LTQ Orbitrap Velos Pro mass spectrometer (Thermo-Fisher Scientific) interfaced with a Waters Acquity® UPLC system (Waters). Analysis of phosphopeptides was performed using a data dependent neutral loss scan. Blanks were run prior to the sample run to make sure there were no significant background signals from solvents or the columns. Database search and data analysis were performed using the Thermo-Fisher Scientific Proteome Discoverer™ software (v1.3).

Luciferase Reporter Assays

For transcriptional assays, C33a and CV-1 cells were cultured in 12-well dishes (BD Falcon). Transfections were performed in triplicate. 48 hours post-transfection, cells were rinsed with DPBS (Invitrogen) and lysed with Steady-Glo luciferase substrate (Promega). Lysates were transferred to 96-well Opti-Plates (PerkinElmer) and luminescence was measured with a Pherastar plate reader and software. Transient replication assays were performed using C33a cells on 96-well white-walled, clear-bottom plates (Corning). 72 hours post-transfection, cells were lysed and treated with the Dual-Glo luciferase system

(Promega). Firefly and Renilla luminescence were measured using the Pherastar system.

Statistical Analysis. All experiments were repeated at least 3 times. Student's t test was performed for luciferase assays comparing wild type to mutant function. For all experiments, means are expressed +/- SEM. p-values ≤ 0.05 were considered significant.

Immunoprecipitation and Immunoblotting

Unless otherwise indicated, HEK293TT cells were used for these experiments, were grown on 6-cm dishes (Corning), and were harvested for experiments 48 hours post-transfection. All cells were rinsed with cold DPBS before lysis with buffers described below. Lysis occurred for 30 minutes on ice, followed by centrifugation at 4°C at 12,000 rpm for 10 minutes. For immunoprecipitation (IP) experiments, total supernatant from each sample was used after setting aside 5-10% for input; for non-IP immunoblots, volumes corresponding to equal protein were measured as determined by BCA assay (Pierce Thermo Scientific). Samples were suspended in Laemmli buffer plus BME, boiled, run on SDS-PAGE gels, and transferred onto 0.45 μm PVDF membranes (Millipore) in semi-dry transfer boxes (Bio-Rad). Membranes were blocked in 5% milk, incubated overnight at 4°C with appropriate primary antibodies (below), washed with PBST, and incubated at room temperature with either goat anti-mouse or goat anti-rabbit light chain specific antibodies conjugated to horseradish peroxidase (Jackson Labs). Signals were detected with SuperSignal West Dura ECL solution (Pierce) and Amersham ECL Prime (GE Healthcare) using the ImageQuant LAS 4000 system (GE Healthcare). All

experiments were repeated at least 3 times.

Lysis, wash, and primary antibody specifications are as follows:

BPV-1 E2 wild-type and mutant immunoblot. HEK293TT, C33a, or CV-1 cells were lysed in NP-40 lysis buffer (see Mass Spectrometry, above) and subjected to BCA assay (Pierce Thermo Scientific) after ice incubation and centrifugation. Volumes corresponding to 40 µg were measured, brought to equal volume with lysis buffer, and mixed with Laemmli buffer plus BME prior to western blotting. Blots were probed with B201.

E2:Flag-Brd4 and E2:E1 IPs. Cells were lysed in NP-40 lysis buffer (see Mass Spectrometry, above) and treated with Benzonase nuclease (Millipore) for 1 hour before centrifugation. Protein complexes were immunoprecipitated with M2 anti-Flag conjugated agarose beads (M2 Affinity Gel, Sigma) or B201/2 A/G beads (see Mass Spectrometry, above). Beads were washed 5 times alternating 150 mM and 500 mM TBS. Blots were probed with B201 and either M2 antibody (Sigma) or 502-2 (rabbit polyclonal against BPV-1 E1).

E2:HA-Gps2 IP. Cells were lysed in DPBS (Invitrogen) plus 0.5% NP-40 and protease inhibitors and immunoprecipitated with 1:1 Sepharose A and G bead slurry plus II-I (rabbit polyclonal against BPV1 E2). Beads were washed 3 times with NETN buffer. Blots were probed with B201 and HA-7 (Sigma).

E2:3xFlag-Tax1BP1 IP. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 20 mM NaF, 50 mM KH₂PO₄, 1% Triton X-100, 10% glycerol, 2 mM dithiothreitol (DTT), and protease inhibitor cocktail). After centrifugation, lysates were mixed with equal volumes of binding buffer (50 mM Tris-HCl, pH 8, 100 mM KCl, 0.1 mM EDTA, 0.2% NP-40, 0.1% bovine serum albumin, 2.5% glycerol, 2 mM DTT, and protease inhibitor cocktail) and immunoprecipitated

with 1:1 sepharose A/G bead slurry plus II-I. Beads were washed 5 times with low- or high-salt wash buffer (100 mM Tris-HCl, pH 8, 100 to 300 mM NaCl, 0.5% NP-40, and 2 mM DTT). Blots were probed with M2 and B201.

E2:GST-CTM and E2:GST-BID IPs. Cells were lysed in high-salt lysis buffer (50 mM Tris-HCl, pH 8, 400 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, protease inhibitor cocktail). For IP, equal volume of no-salt lysis buffer was added, as well as glutathione Sepharose beads (GE Healthcare) and ethidium bromide to 0.1 µg/µl. Beads were washed 3 times with binding buffer (50 mM Tris-HCl, pH 8, 100 mM KCl, 0.1 mM EDTA, 0.2% NP-40, 0.1% BSA, 2.5% glycerol). Blots were probed with B201 and SD8 (rabbit polyclonal against GST).

E2:FGFR IPs. Cells were lysed in 1% NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 8, 150 mM NaCl, protease inhibitor cocktail) and immunoprecipitated with 1:1 Sepharose A/G bead slurry plus II-I. Beads were washed 3 times with lysis buffer. Blots were probed with B201 and M2, Bek antibody C-17 (rabbit anti-FGFR2, Santa Cruz Biotechnologies), FGFR4 antibody C-16 (rabbit, Santa Cruz).

Other E2:kinase IPs. Cells were lysed in IGF-1R lysis buffer (300 µM NaCl, 0.5% NP-40, 20 mM Tris-HCl, pH 8, 1 mM EDTA, protease inhibitor cocktail) and immunoprecipitated with 1:1 Sepharose A/G bead slurry plus II-I. Beads were washed 3 times with lysis buffer. Blots were probed with B201 and antibodies against EphB2 (mouse anti-EphB2 AM11064PU-N, Acris Antibodies), Met C-28 (rabbit anti-c-Met sc-161, Santa Cruz), and IGF-1Rβ C-20 (rabbit anti-IGF-1R sc-713, Santa Cruz).

Immunofluorescence

CV-1 cells were grown on 12-well plates (BD Falcon) with 18-mm glass coverslips. 48 hours post-transfection, cells were rinsed with cold DPBS and fixed in 4% paraformaldehyde solution for 15 minutes. After permeabilization in antibody dilution buffer (5% normal goat serum and 0.1% Triton X-100 in DPBS), cells were incubated in dilution buffer plus B201 supernatant at 4°C overnight. Coverslips were incubated in dilution buffer plus AlexaFluor 594 anti-mouse secondary antibody (Invitrogen) and mounted onto slides with ProLong Gold plus DAPI (Invitrogen). Slides were imaged with a Zeiss fluorescent microscope using QCapture Pro 6 software.

Software and Web Resources

The BPV-1 E2 ribbon structure was generated in Protein Workshop (downloaded from rcsb.org/pdb) using the crystal structure 2JEU (Sanders et al., 2007), documented in the Papillomavirus Episteme (PaVE, pave.niaid.nih.gov) and the RCSB Protein Data Bank (PDB). PaVE was also used to obtain information for Tables 4 and 5 in the Appendix. The full-length BPV-1 E2 amino acid sequence was obtained from PaVE for use as input in phosphorylation site predictive software, including GPS 2.1 (downloaded from gps.biocuckoo.org), NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos/), and ScanSite 3 (scansite3.mit.edu). Receptor tyrosine kinase expression information was obtained from GeneCards (www.genecards.org) using the GeneCards ID numbers (GC id) GC10M121401 (FGFR2), GC04P001795 (FGFR3), GC05P177086 (FGFR4), GC01P022710 (EphB2), GC07P116672 (c-Met), and GC15P098648 (IGF-1R).

Results

Identification of phosphorylated tyrosine 102

After having discovered *in vitro* lysine acetylation in BPV-1 E2 (Quinlan et al., 2012), we sought to determine whether other PTMs occurred on this protein in live cells. Two separate samples were prepared for mass spectrometric analysis. The first was obtained from C33a/E2 cells, derived from an HPV-negative human cervical cancer cell line but stably expressing the E2 gene (You et al., 2004). The second was obtained from unaltered C33a cells transiently transfected with full-length BPV-1 E2 (amino acids [aa] 1-410). E2 protein from both samples was purified by immunoprecipitation and analyzed by tandem mass spectrometry using a high-resolution LTQ orbitrap mass spectrometer. Data analysis included PTM identification as well as a confidence score (Table 2, Appendix). This approach detected the previously published phosphorylations at serines 298 and 301 (McBride et al., 1989), as well as acetylation at K112. Phosphorylation at tyrosine (Y) 102 (Figure 3.1) was of particular interest since tyrosine phosphorylation has not been detected for any E2 protein. Furthermore, this PTM was assigned the highest confidence score of any PTM in the report generated by Thermo's Proteome Discoverer™ software, including those at the previously published sites (Table 2, Appendix). Y102 is in the center of the BPV-1 E2 transactivation domain (TAD, aa 1-210) between the three alpha-helices and beta sheet folds (Figure 3.2). While not conserved among all PVs, Y102 (or Y99, or Y103) occurs in 36 HPVs for which complete genomes are available, including the high-risk HPVs HPV-16 and HPV-31 (Table 4, Appendix). Tyrosine residues are also observed at these positions in 55 non-human PVs (Table 5, Appendix).

FIGURE 3.1

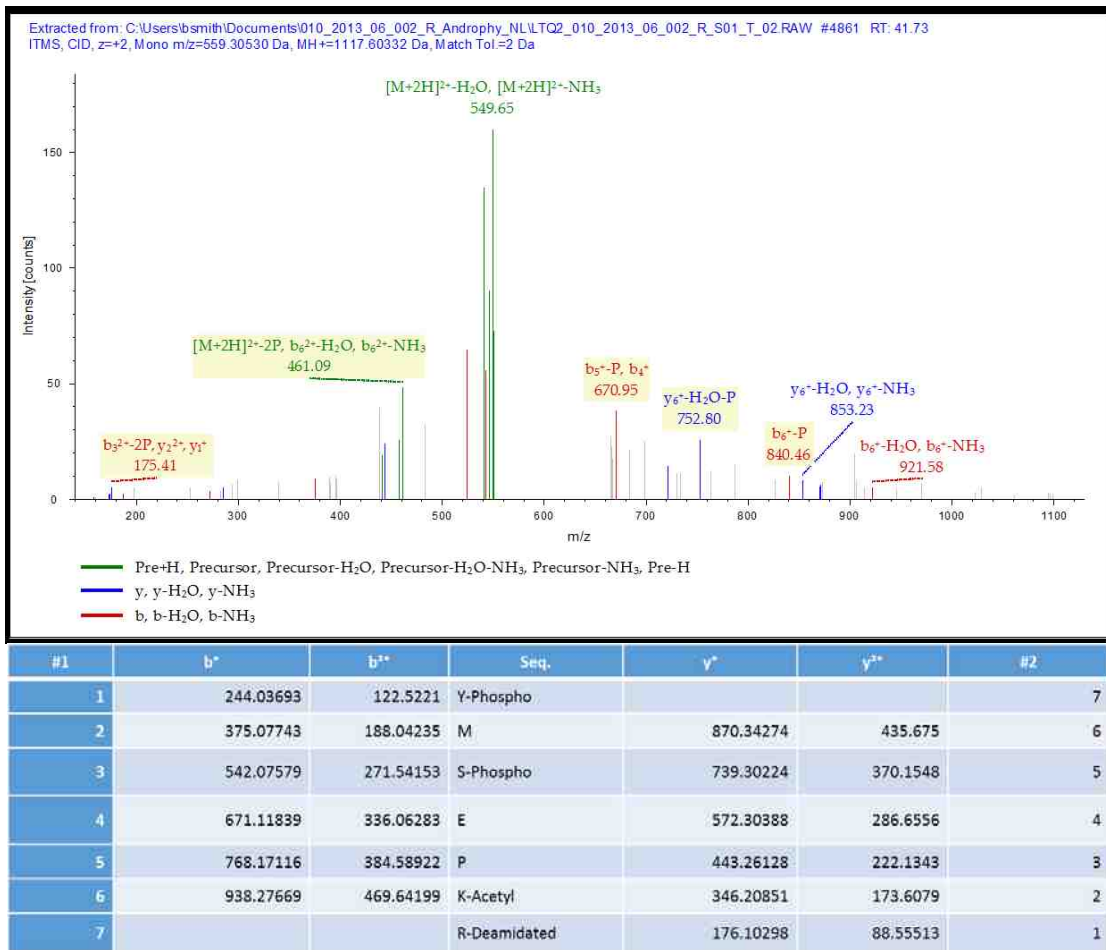


Figure 3.1. Tyrosine 102 of BPV-1 E2 is phosphorylated in cells. Top, tandem mass spectrum of Y-102 phosphorylated peptide of BPV-1 E2. The target protein was immunoprecipitated from cell lysate using mouse monoclonal antibodies against BPV-1 E2. The dominant neutral loss of phosphoric acid, water, and ammonia from the precursor ion $^{102}\text{YMSEPKR}^{108}$ and sequence specific fragment ions were assigned by Proteome Discoverer™. Other modified residues of this particular peptide are indicated in the table below the MS/MS spectrum (bottom).

FIGURE 3.2

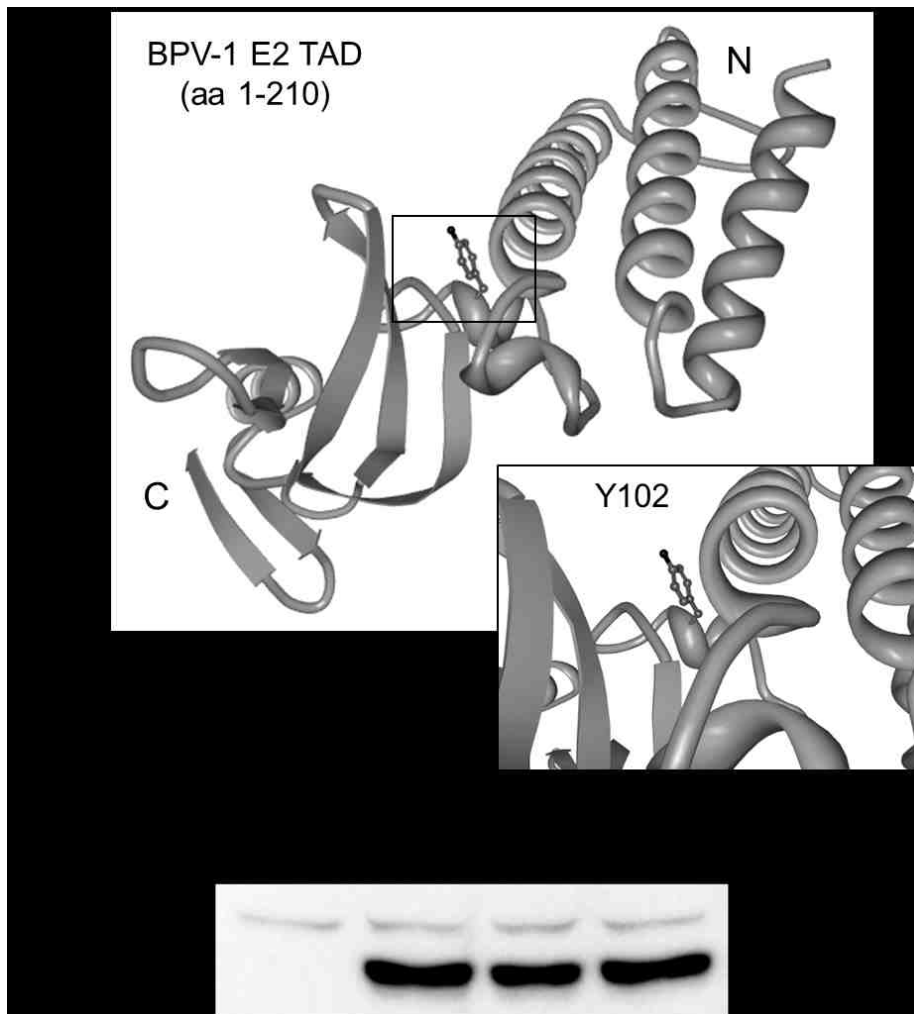


Figure 3.2. Y102 location in the transactivation domain (TAD) and mutagenesis. (A) The BPV-1 E2 TAD crystal structure 2JEU (Sanders et al., 2007) was identified in the Papillomavirus Episteme (PaVE, pave.niaid.nih.gov) and modified in Protein Workshop (rscsb.org/pdb) to highlight Y102 (ball and stick model, inset). N, amino terminus; C, carboxyl terminus. (B) The Agilent QuikChange II site-directed mutagenesis kit was used to generate tyrosine to phenylalanine (Y102F) and tyrosine to glutamate (Y102E) in the pCG-E2 construct. 1 μ g each of wild type and mutant constructs (WT, YF, YE) were expressed in HEK293TT cells grown in 6-cm dishes, and cells were harvested for western blot 48 hours post-transfection. mRFP-GFP was transfected in place of E2 as a negative control ((-)). The blot was probed for BPV-1 E2 with the mouse monoclonal antibody B201 (ascites). E2, full-length BPV-1 E2; *, nonspecific band.

The phospho-mimetic mutant Y102E abrogates BPV-1 E2 mediated transcriptional activation

To investigate the potential functional significance of this PTM, we used site-directed mutagenesis to generate E2 constructs expressing a tyrosine to phenylalanine (Y102F) mutant, which cannot be phosphorylated at this site, and a tyrosine to glutamate (Y102E), which creates a negative charge similar to phosphotyrosine. E2 protein expression in HEK293TT cells was comparable to wild type (WT) for both mutants (Figure 3.2). We began activity studies with the tyrosine mutants by testing how these affected the ability of E2 to stimulate transcription. C33a cells were transfected with plasmids encoding WT BPV-1 E2, Y102F, or Y102E along with a firefly luciferase reporter (pGL2-E2BS-Luc) containing four E2 binding sites upstream of an SV40 promoter (Kumar et al., 2007). While Y102F stimulated luciferase expression at levels comparable to WT (38-fold above negative control), Y102E was inactive (Figure 3.3). However, Y102E protein levels in the C33a cells were lower than WT and Y102F (Figure 3.3). To ensure that these results were not due to lack of expression or cell type dependent differences, the experiments were repeated in CV-1 cells, an African green monkey kidney cell line. As in C33a, Y102F stimulated reporter transcription comparable to WT (35-fold above negative control), while Y102E failed to transactivate (Figure 3.4); its luciferase readout was comparable to that of the truncated BPV-1 E2R protein (aa 162-410), which exhibits low levels of transcription activation (Lace et al., 2012). In these cells, overexpression of Y102E resulted in protein levels comparable to WT and Y102F but did not restore transcriptional activation (Figure 3.4).

To determine whether the transcriptional defect of Y102E might be due

to altered co-factor binding, co-immunoprecipitation (co-IP) experiments were performed with BPV-1 E2 WT and mutants and different binding partners known to enhance E2-mediated transactivation. One of these binding partners, the chromatin modulator Brd4, has been shown in previous studies to interact via its extreme C-terminal domain (CTD, also C-terminal motif, CTM; Figure 3.5) with E2 from multiple PVs (You et al., 2004, You et al., 2005, Zheng et al., 2009, McPhillips et al., 2006). BPV-1 E2 WT and Y102 mutants were expressed in HEK293TT cells along with full-length Flag-tagged Brd4, and protein complexes were immunoprecipitated with mouse monoclonal anti-Flag (M2) conjugated beads. While WT and Y102F E2 co-immunoprecipitated with Brd4, Y102E was not (Figure 3.5). This was surprising since the Brd4 CTM is known to bind to the N-terminal alpha helices of the E2 TAD (Abbate et al., 2006), and contacts to the region of Y102 have not been previously described. To ascertain whether the observed effect was due to lack of Y102E binding to the CTM, GST-tagged CTM was substituted for full-length Brd4 in transfections, and complexes were captured on glutathione beads. Again, the same pattern appeared, with Y102E failing to associate (Figure 3.6). Cheng-Ming Chiang and colleagues reported distinct domains in Brd4 that undergo conformational changes to interact with p53 (Wu et al., 2013). We tested the basic interacting domain (BID) (Figure 3.5) for association with BPV-1 E2. Interestingly, GST-BID pulled down WT, Y102F, and Y102E BPV-1 E2 proteins (Figure 3.6). Thus, Y102E fails to interact with the established E2 binding site of Brd4 but binds a novel site.

To ensure that the inability of Y102E to transactivate was not due to global disruption of the protein's structure, we examined complex formation with

other known E2 binding partners, including Gps2 (also known as AMF1) and Tax1BP1. Gps2 has been found to stimulate transcription activation of E2 and recruit it to form a complex with the cofactor p300 (Breiding et al., 1997, Peng et al., 2000). Tax1BP1 (abbreviated TXBP) stabilizes protein levels of E2 and enhances its transactivation (Wang et al., 2009). BPV-1 E2 WT and mutant constructs were co-expressed with either hemagglutinin (HA)-tagged Gps2 or triple Flag-tagged (3xFlag) Tax1BP1, and complexes were captured with anti-BPV-1 E2 antibody. Both Y102F and Y102E co-precipitated HA-Gps2 and 3xFlag-Tax1BP1 (Figure 3.7). The E2R form was used as a negative control for Tax1BP1 association.

FIGURE 3.3

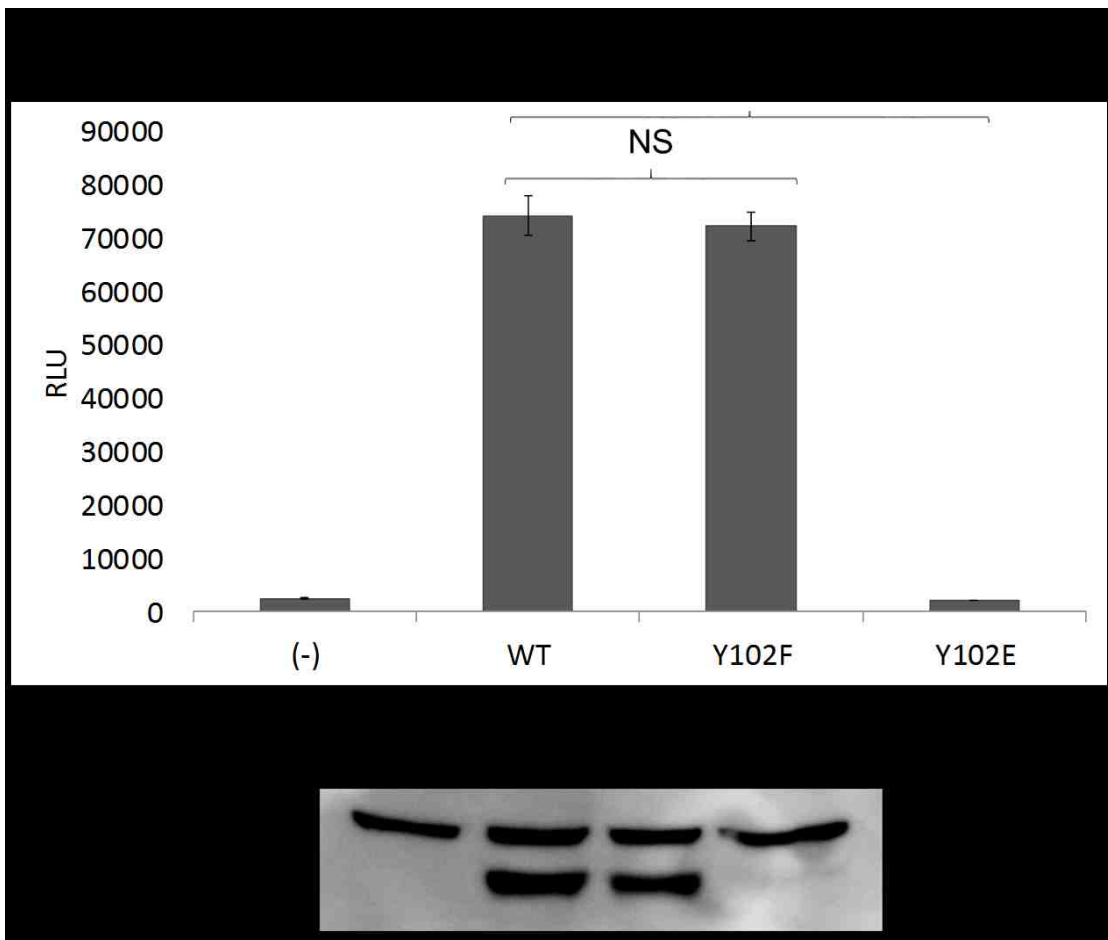


Figure 3.3. The phospho-mimetic Y102E abrogates E2 transcriptional activation. (A) C33a cells were grown in 12-well dishes. Samples were transfected in triplicate wells with 50 ng/well pCG-E2 wild type or mutants (WT, Y102F, Y102E) or mRFP-GFP ((-)), as well as 75 ng/well pGL2-E2BS-Luc. 48 hours post-transfection, cells were lysed on-plate with 100 μ l Steady Glo reagent. Lysates were mixed with 100 μ l DPBS and transferred to a white 96-well plate for luminescence detection using the Pherastar system. Student's t test was used to compare samples to WT. RLU, relative light units; **, $p < 0.01$; NS, not significant. Values are expressed as mean \pm SEM. (B) 1 μ g each of wild type and mutant constructs (WT, YF, YE) or mRFP-GFP ((-)) were expressed in C33a cells grown in 6-cm dishes, and cells were harvested for western blot 48 hours post-transfection. The blot was probed for BPV-1 E2 with B201. E2, full-length BPV-1 E2; *, nonspecific band.

FIGURE 3.4

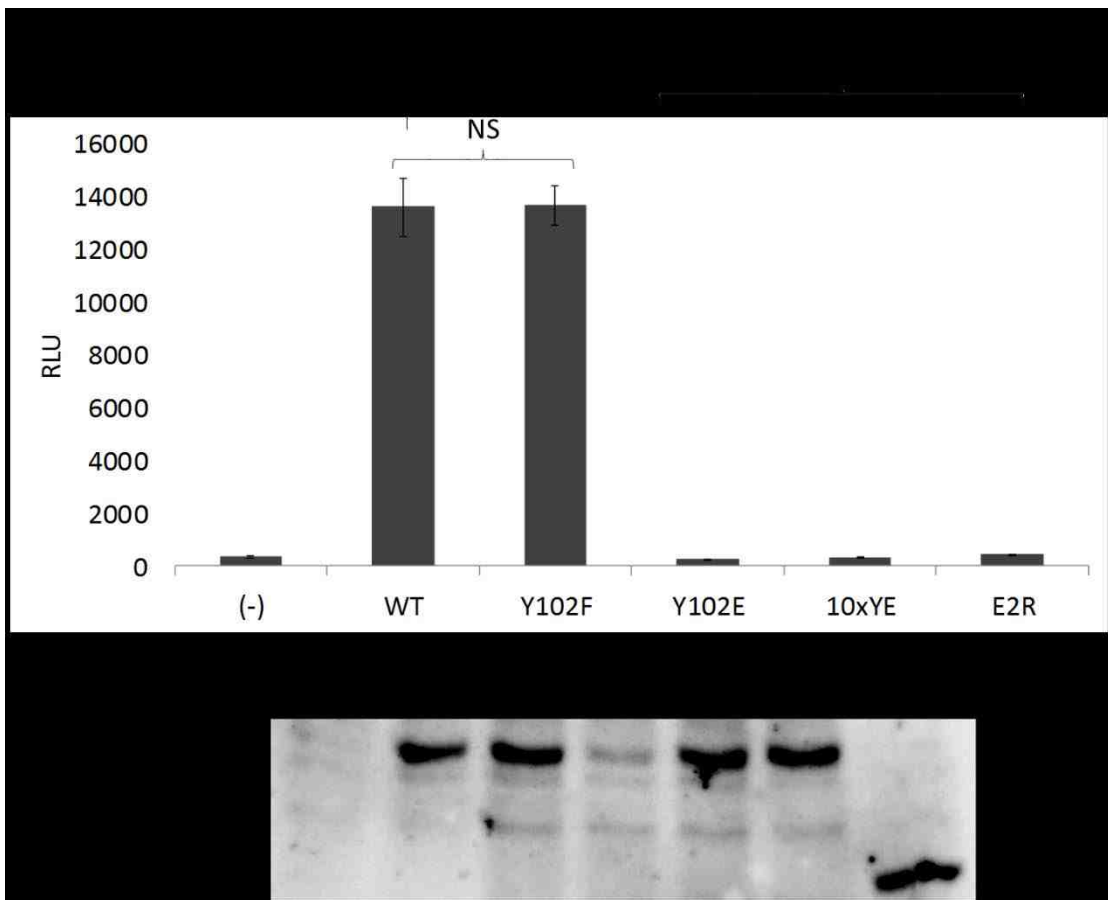


Figure 3.4. Y102E is defective for transactivation in a second cell line. (A) CV-1 cells were cultured, transfected, and lysed as in Figure 3.3, but with 10 ng/well E2, pCG-E2R (E2R, aa 162-410), or mRFP-GFP, plus wells with 10 times the original amount of Y102E (100 ng/well, 10xYE). Student's t test was used to compare samples to WT. RLU, relative light units; **, $p < 0.01$; NS, not significant. Values are expressed as mean \pm SEM. (B) CV-1 cells were transfected and prepared for western blot as in Figure 3.3, with the addition of samples with 5 times Y102E (5xYE), 10 times Y102E (10xYE), or E2R. The blot was probed for BPV-1 E2 with B201. E2, full-length BPV-1 E2; *, nonspecific band.

FIGURE 3.5

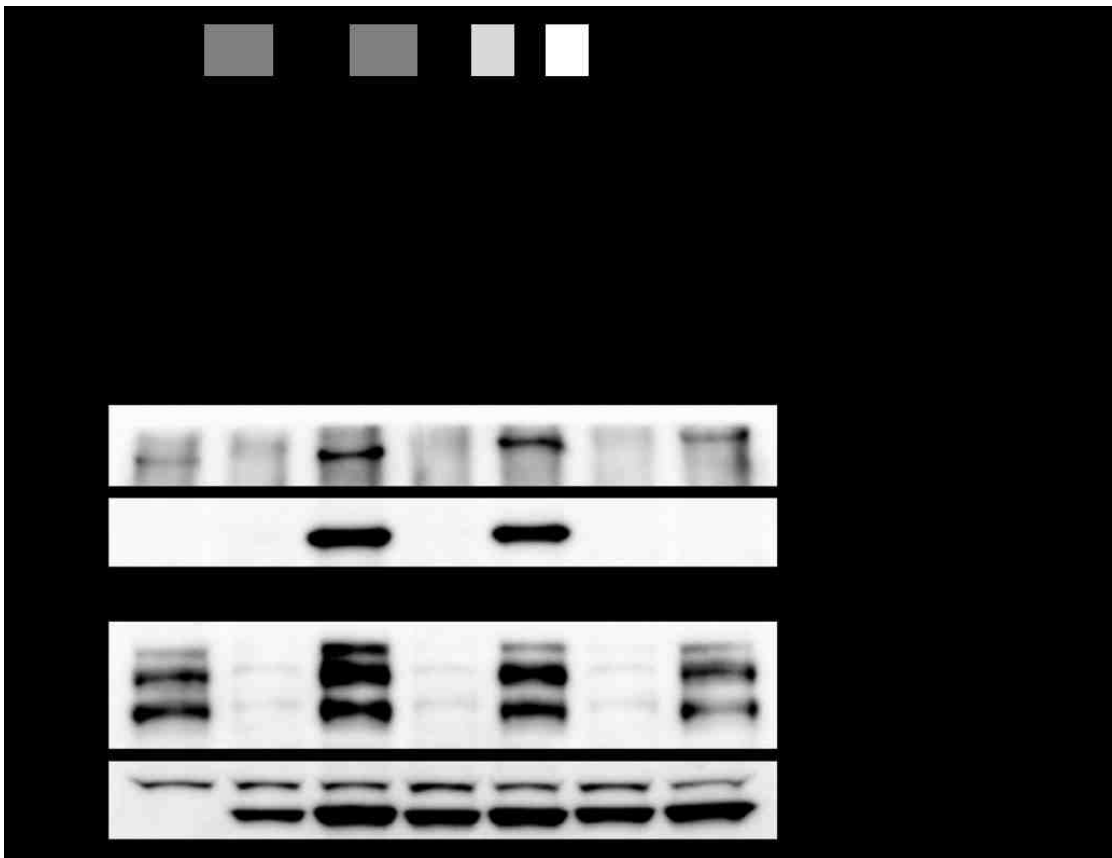


Figure 3.5. Y102E does not bind full-length Brd4. (A) Domains of the Brd4 chromatin modulator protein, modified from (Wu et al., 2013). BD1 and BD2, bromodomains; BID, basic interacting domain; ET, extraterminal domain; CTM, C-terminal motif. (B) HEK293TT cells were grown on 6-cm dishes and transfected with 1 ug each mRFP-GFP (-), BPV-1 E2 wild type and mutants (WT, YF, YE), and Flag-tagged full length Brd4 (pVL-F:hBrd4) or pCI. Cells were harvested 48 hours post-transfection. 10% of each lysate was set aside for input, while remaining lysate was incubated at 4°C overnight with the anti-Flag mouse monoclonal antibody M2 conjugated to agarose beads. Samples were probed by western blot for Flag-Brd4 and BPV-1 E2 using M2 and B201, respectively. E2, full-length BPV-1 E2; *, nonspecific band.

FIGURE 3.6

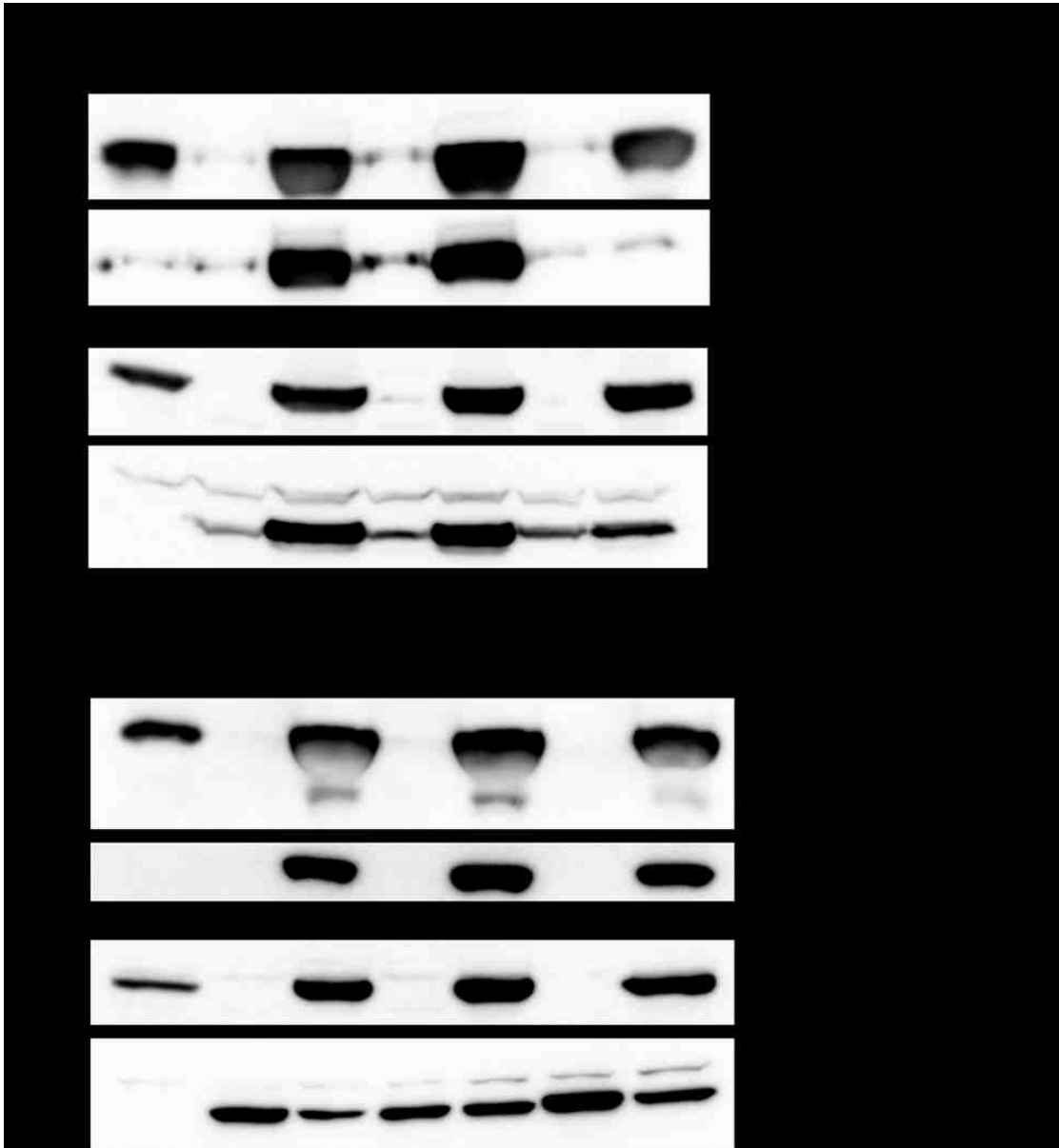


Figure 3.6. Y102E does not bind Brd4 CTM but associates with a novel E2 binding site. Samples were prepared as in Figure 3.5, with GST-tagged CTM substituted for full-length Brd4 in (A) and GST-BID substituted in (B). Complexes were pulled down with glutathione beads. Western blots were probed for GST with rabbit polyclonal antibody SD8 and for E2 with B201. *, nonspecific band.

FIGURE 3.7

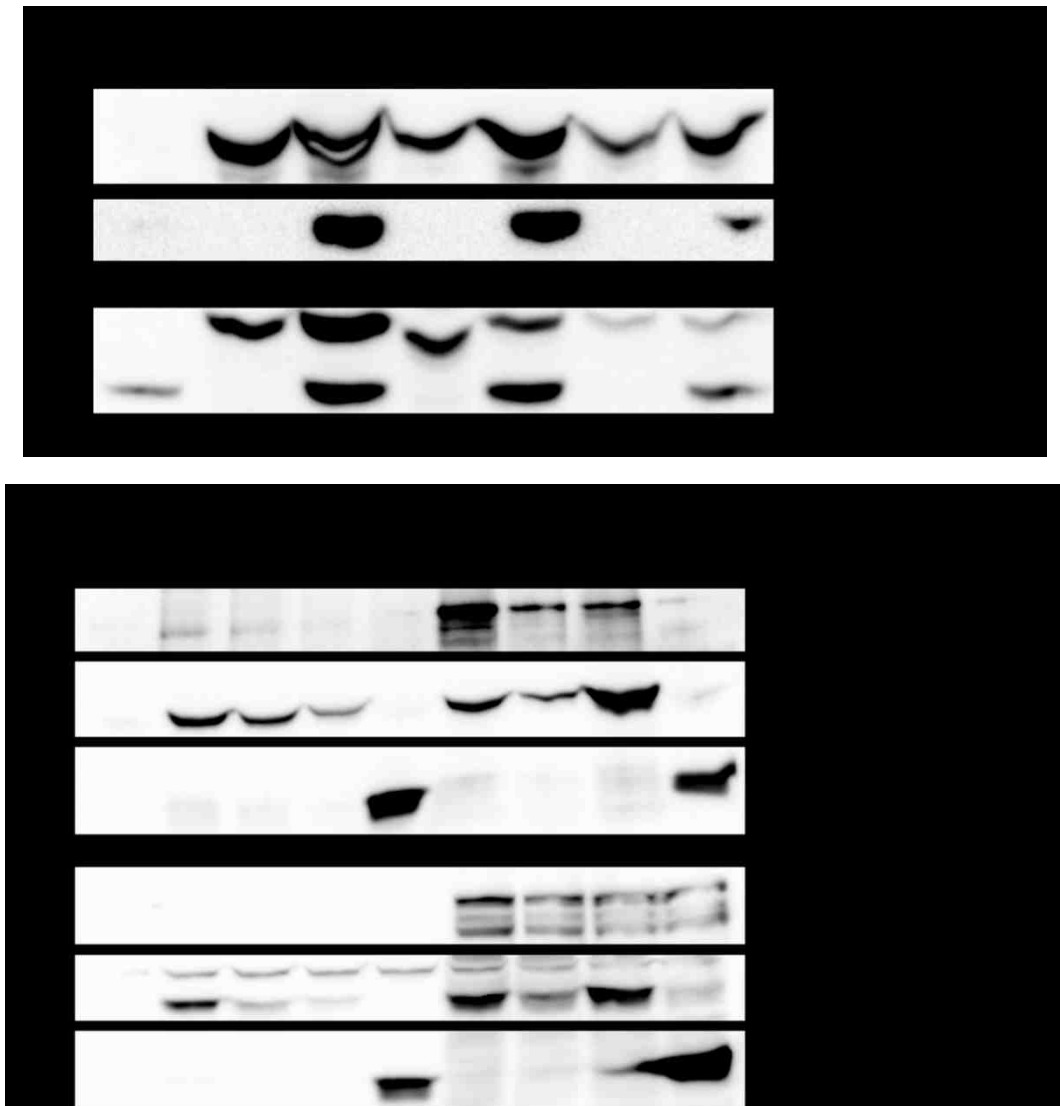


Figure 3.7. Y102 mutants associate with the transcription factors Gps2 and Tax1BP1. (A) HEK293TT cells were grown on 6-cm dishes and transfected with 1 μ g each mRFP-GFP (-), BPV-1 E2 wild type and mutants (WT, YF, YE) plus E2R, and hemagglutinin-tagged Gps2 (HA-Gps2, pHA:AMF1) or pCI. Cells were harvested 48 hours post-transfection. 5% of each lysate was set aside for input, while remaining lysate was incubated with protein sepharose A and G beads and the anti-BPV-1 E2 rabbit polyclonal antibody II-I at 4°C overnight. Western blots were probed for HA-Gps2 and BPV-1 E2 using the mouse monoclonal anti-HA antibody HA-7 and B201, respectively. (B) Samples were prepared as in (A), with p3XFLAG-CMV-7.1 Tax1BP1 substituted for pHA:AMF1. Cells were harvested 48 hours post-transfection. 10% of each lysate was set aside for input, while remaining lysate was used for immunoprecipitation as in (A). Western blots were probed with M2 and B201. Flag-TXBP, 3xFlag-Tax1BP1.

E2 Y102E cannot activate transient BPV-1 replication

E2 stimulates viral replication by recruiting E1 monomers to the viral origin of replication (*ori*). The ability of the Y102 mutants to stimulate transient replication of the viral *ori* was examined in a luciferase-based reporter assay. This method, developed by the Archambault lab for both BPV-1 and HPV-31 in C33a cells, requires co-transfection of plasmids encoding E2, the homologous E1 helicase, *Renilla* luciferase (internal control), and firefly luciferase (target replicon) (Fradet-Turcotte et al., 2010c, Gagnon et al., 2013). The firefly luciferase gene is constitutively expressed due to the presence of a CMV promoter. However, since the construct contains the BPV-1 *ori*, changes in plasmid copy number due to replication affect the amount of luciferase produced. Interestingly, Y102F-dependent luciferase activity was significantly increased above WT (Figure 3.8). In contrast, Y102E did not generate luminescence signal above baseline levels. To examine whether the Y102 mutants are capable of binding E1, BPV-1 E1 and E2 were expressed in HEK293TT cells and immunoprecipitated with anti-E2 antibody. While Y102F co-precipitated E1 at levels comparable to WT, Y102E did not (Figure 3.9).

FIGURE 3.8

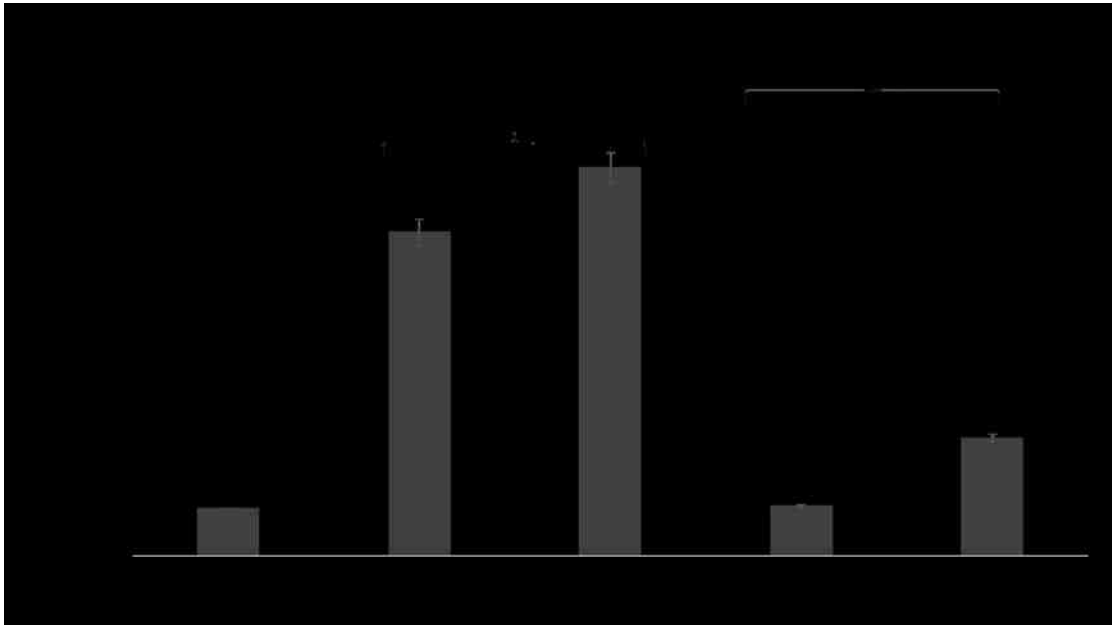


Figure 3.8. Y102E fails to stimulate transient BPV-1 replication. C33a cells were grown in a 96-well clear bottom plate. Samples were transfected in 8 replicate wells with 10 ng/well pCG-E2 wild type or mutants (WT, Y102F, Y102E), E2R, or mRFP-GFP ((-)), as well as 10 ng/well pCG-E1, 2.5 ng/well pFLORI-BPV1 (firefly luciferase reporter), and 0.5 ng/well pRL (*Renilla* luciferase reporter). 72 hours post-transfection, cells were lysed on-plate with 30 μ l Dual Glo reagent. Lysates were mixed with 30 μ l DPBS and luminescence was detected using the Pherastar system. Student's t test was used to compare samples to WT. F/R, firefly output (in relative light units) divided by *Renilla* output; **, $p < 0.01$; ****, $p < 0.00001$. Values are expressed as mean \pm SEM.

FIGURE 3.9

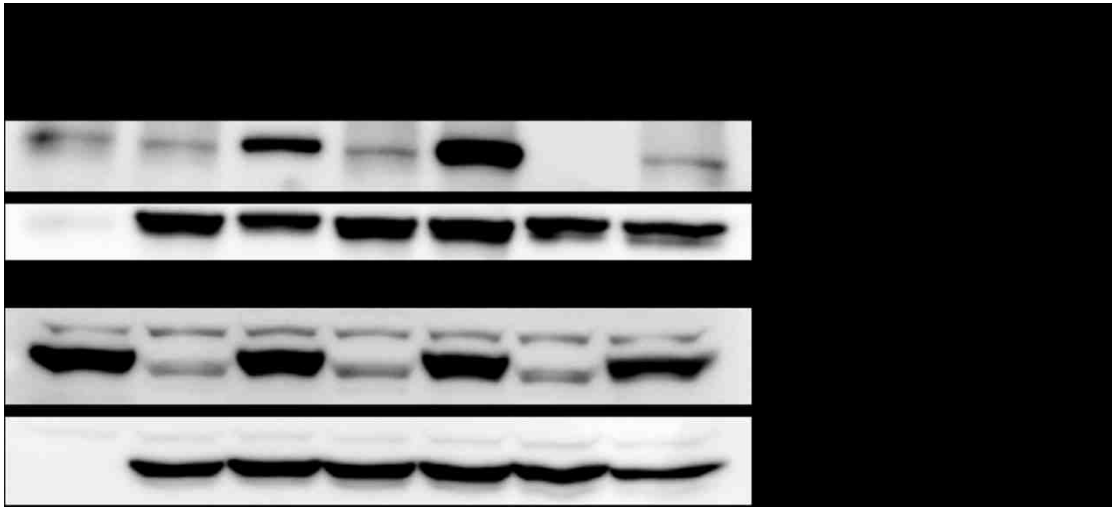


Figure 3.9. Y102E is defective for association with the viral helicase E1. HEK293TT cells were grown on 6-cm dishes and transfected with 1 μ g each mRFP-GFP (-), BPV-1 E2 wild type and mutants (WT, YF, YE), and BPV-1 E1 or pCI. Cells were harvested 48 hours post-transfection. 10% of each lysate was set aside for input, while remaining lysate was incubated with protein sepharose A and G beads and II-I antibody at 4°C overnight. Western blots were probed for BPV-1 E1 and BPV-1 E2 using the rabbit polyclonal anti-E1 antibody 502-2 and B201, respectively. *, nonspecific band.

Y102 mutants localize to nuclei

The full-length E2 protein contains two putative nuclear localization signals, one in a basic region of the C-terminal DBD and another within the TAD (Zou et al., 2000, Skiadopoulos and McBride, 1996). Given the proximity of Y102 just amino terminal of the TAD NLS (aa 111-120), we sought to determine the localization of the Y102F and Y102E proteins. Immunofluorescence experiments were performed in CV-1 cells transfected with WT BPV-1 E2, Y102F, Y102E, or E2R as well as BPV-1 E1. A3 cells, murine fibroblast cells stably carrying BPV-1 genomes at high copy number (McBride and Howley, 1991), were used as a positive control. CV-1 cells transfected with eGFP in place of E2 were used as a negative control. While the E2 distribution pattern in CV-1 cells with WT E2, Y102F, Y102E, and E2R was almost exclusively nuclear (similar to A3 cells), Y102E was mostly nuclear with some cytoplasmic distribution (Figure 3.10).

FIGURE 3.10

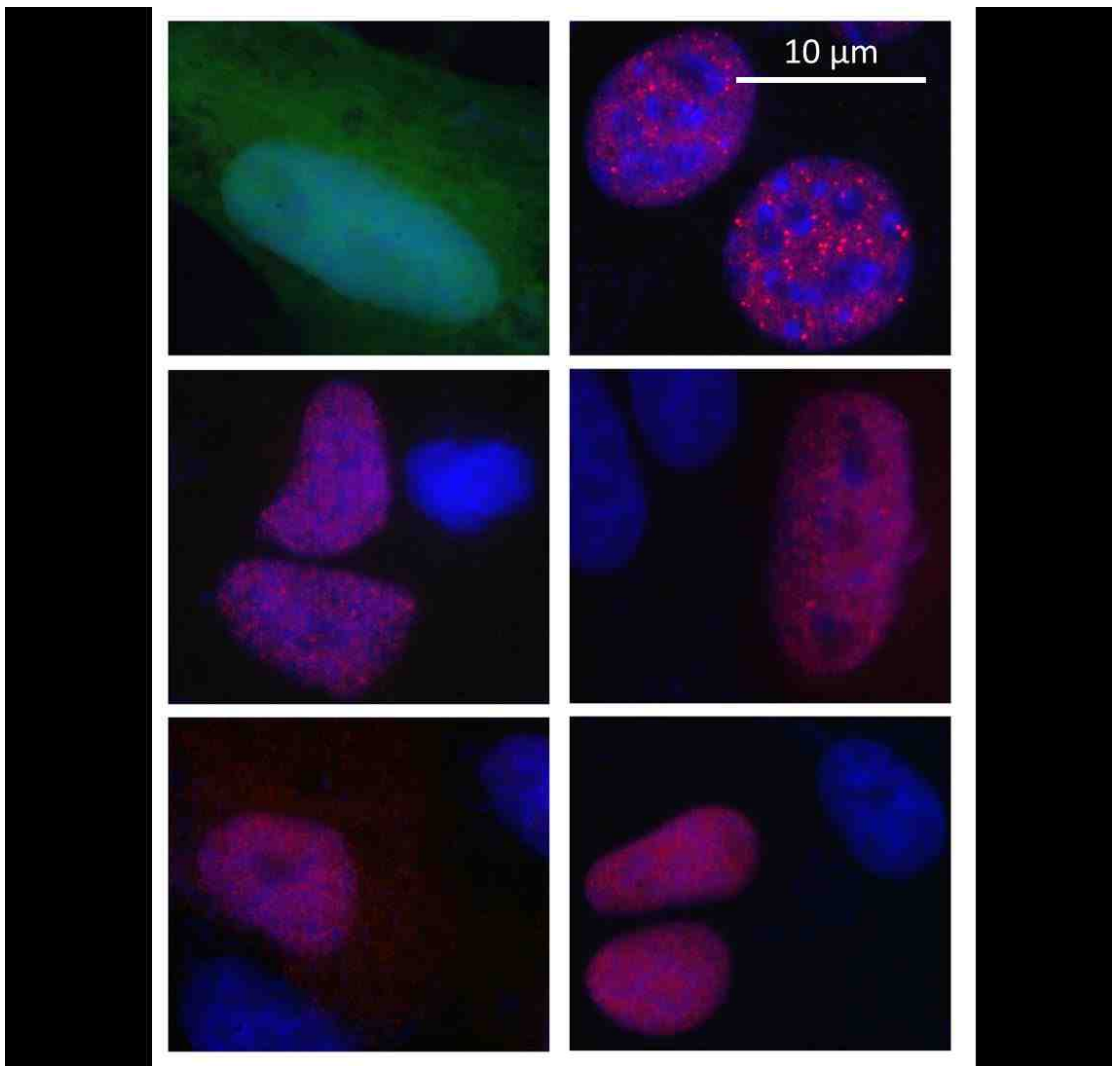


Figure 3.10. Y102 mutants localize to nuclei. CV-1 cells were grown in 12-well plates with 18-mm glass coverslips. A3 cells were grown under the same conditions as a positive control for E2 expression ((+)). CV-1 cells were transfected with 100 ng pCG-E1 and 100 ng pEGFP-C1 (eGFP, (-)), E2 wild type or mutants (WT, Y102F, Y102E), or E2R. 48 hours post CV-1 transfection, cells were fixed and permeabilized, followed by incubation with B201 supernatant at 4°C overnight. Coverslips were washed, stained with Alexa Fluor 594 anti-mouse antibody, washed, and mounted onto glass slides using ProLong Gold with DAPI. Green, eGFP; red, BPV-1 E2; blue, DNA. White bar, 10 μ m. All images taken in all three channels (green, red, blue) at 1000x magnification.

Receptor tyrosine kinases interact with E2

We utilized predictive software systems including NetPhos 2.0, Scansite 3, and GPS 2.1 to inform our search for candidate kinases. NetPhos 2.0 predicts the most likely phosphorylation sites in a protein; Y102 received the highest probability score of 0.968, followed by Y138 with 0.570. The other two algorithms predict kinases that might phosphorylate a given site. While Scansite 3 predicted that the insulin receptor (INS-R) or insulin-like growth factor receptor (IGF-1R) would act on Y102 at its lowest threshold, GPS 2.1 predicted at its highest threshold that the acting kinase would be fibroblast growth factor receptor 3 (FGFR3). The receptor tyrosine kinases EphB2 and c-Met were also suggested to us as candidates (personal communication). mRNA transcripts of all of these tyrosine kinase receptors are expressed in skin and uterus 2- to 10-fold above baseline by microarray analysis (www.genecards.org, biogps.org). Protein expression, however, is not significantly above baseline in skin, uterus, or cervix for any of these receptors (www.genecards.org). After obtaining expression constructs for Flag-tagged FGFR3, the FGFR family members FGFR2 and FGFR4, and EphB2, we conducted co-immunoprecipitation experiments from HEK293TT lysates to determine whether BPV-1 E2 formed a complex with any of the kinases. Interestingly, BPV-1 E2 WT, Y102F, and Y102E immunoprecipitated all of these kinases (Figures 3.11, 3.12, 3.13, and 3.14). We also tested HEK293TT lysates for complex formation between BPV-1 E2 and endogenous c-Met or IGF-1R, but no co-immunoprecipitation was detected (Figures 3.15 and 3.16). All of the kinases probed by co-IP except for c-Met exist in a mostly cytoplasmic distribution in HEK293TT cells (Figure 3.17), though endogenous amounts are not high enough to detect by western blot

(Figures 3.12 and 3.13). Similar distributions were observed in HaCat cells, a spontaneously immortalized human keratinocyte line, and ID13 cells, a BPV-1 transformed murine fibroblast line that maintains BPV-1 genomes at a low copy number (Figure 3.17).

FIGURE 3.11

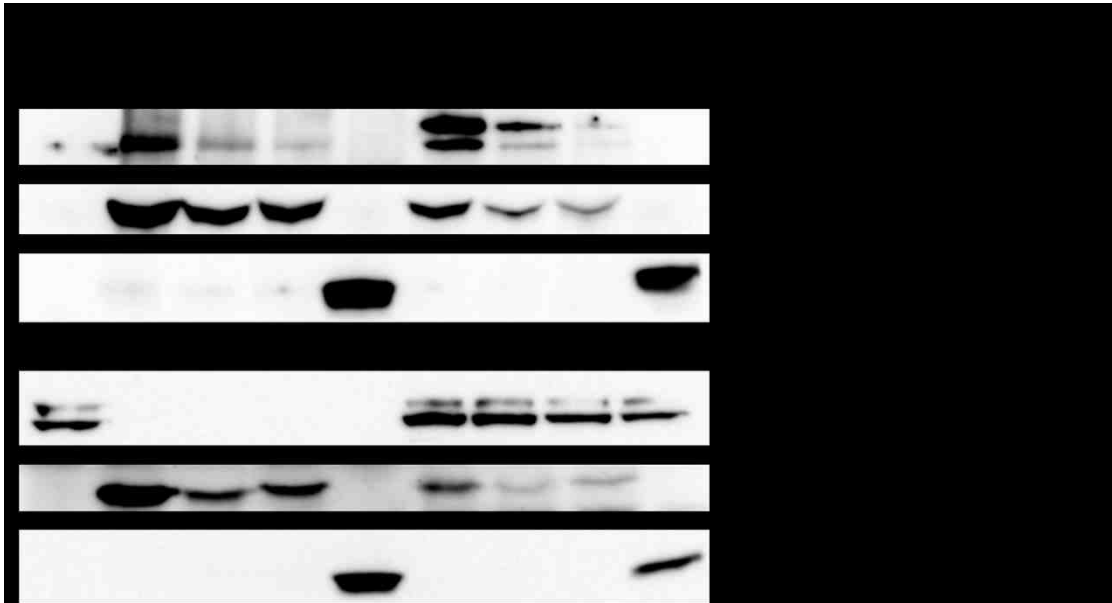


Figure 3.11. BPV-1 E2 associates with FGFR3. HEK293TT cells were grown on 6-cm dishes and transfected with 1 μ g each mRFP-GFP (-), BPV-1 E2 wild type and mutants (WT, YF, YE) plus E2R, and Flag-tagged FGFR3 (pRK7-FGFR3-Flag) or pCI. Cells were harvested 48 hours post-transfection. 5% of each lysate was set aside for input, while remaining lysate was incubated with protein sepharose A and G beads and II-I antibody at 4°C overnight. Western blots were probed for Flag-FGFR3 and BPV-1 E2 using M2 and B201, respectively. *, nonspecific band.

FIGURE 3.12

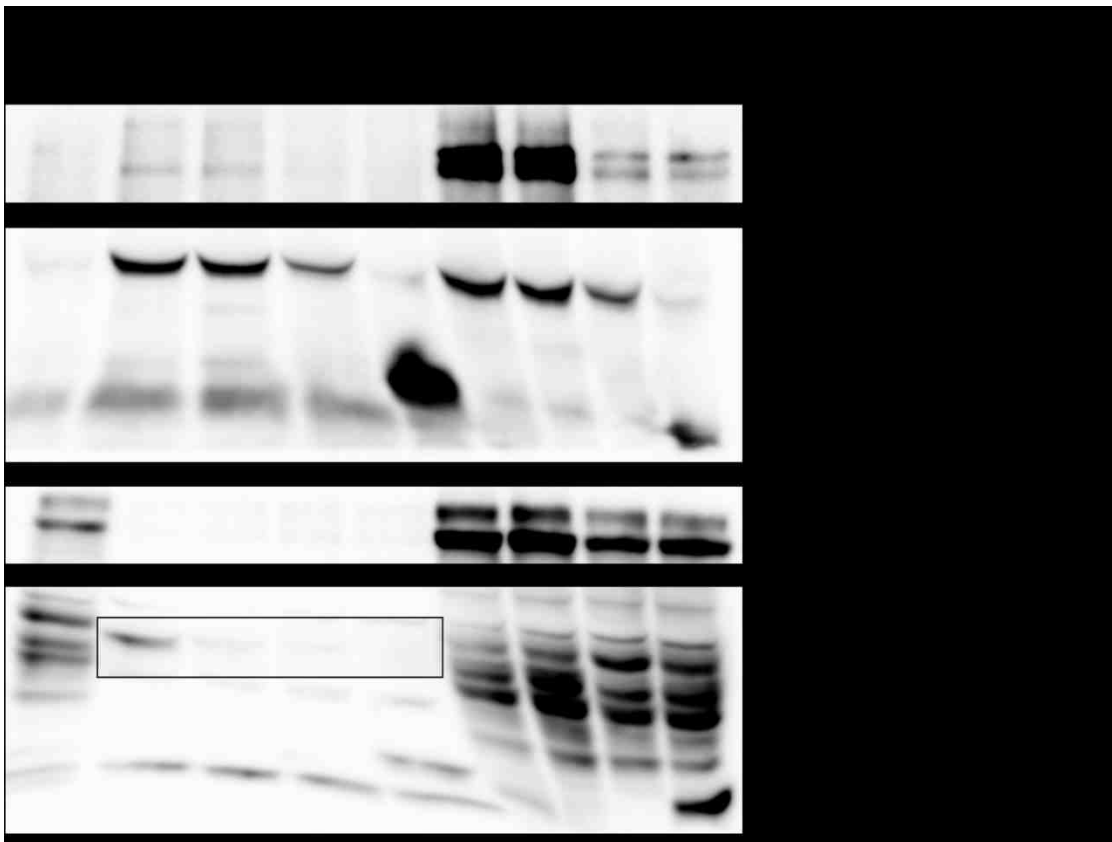


Figure 3.12. BPV-1 E2 associates with FGFR2. Samples were prepared as in Figure 3.11, with FGFR2 substituted for Flag-FGFR3. Western blots were probed with B201 and the rabbit anti-FGFR2/Bek antibody C-17. Inset box in input indicates longer exposure.

FIGURE 3.13

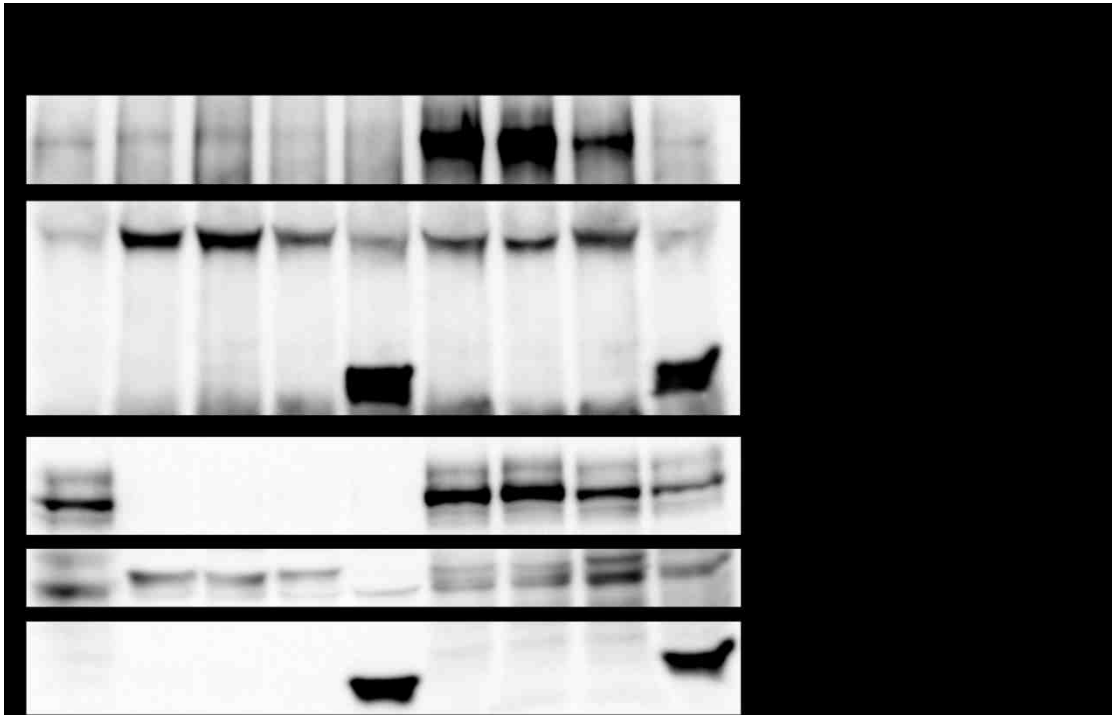


Figure 3.13. BPV-1 E2 associates with FGFR4. Samples were prepared as in Figure 3.11, with FGFR4 substituted for Flag-FGFR3. Western blots were probed with B201 and the rabbit anti-FGFR4 antibody C-16.

FIGURE 3.14

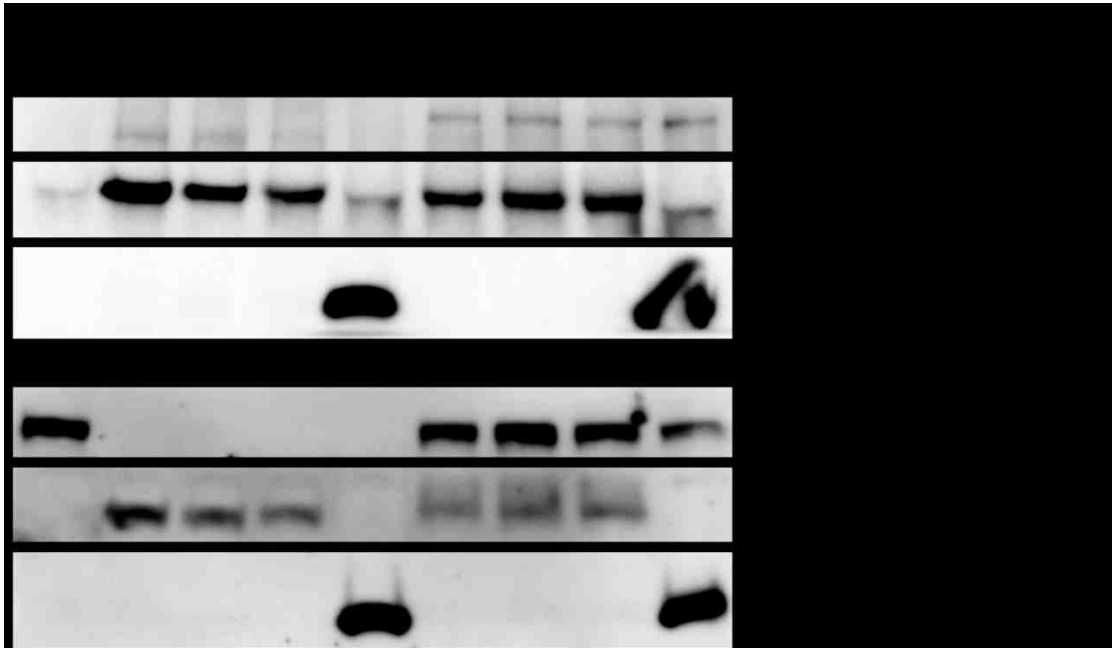


Figure 3.14. BPV-1 E2 associates with EphB2. Samples were prepared as in Figure 3.11, with EphB2 substituted for Flag-FGFR3. Western blots were probed with B201 and a mouse anti-EphB2 antibody.

FIGURE 3.15

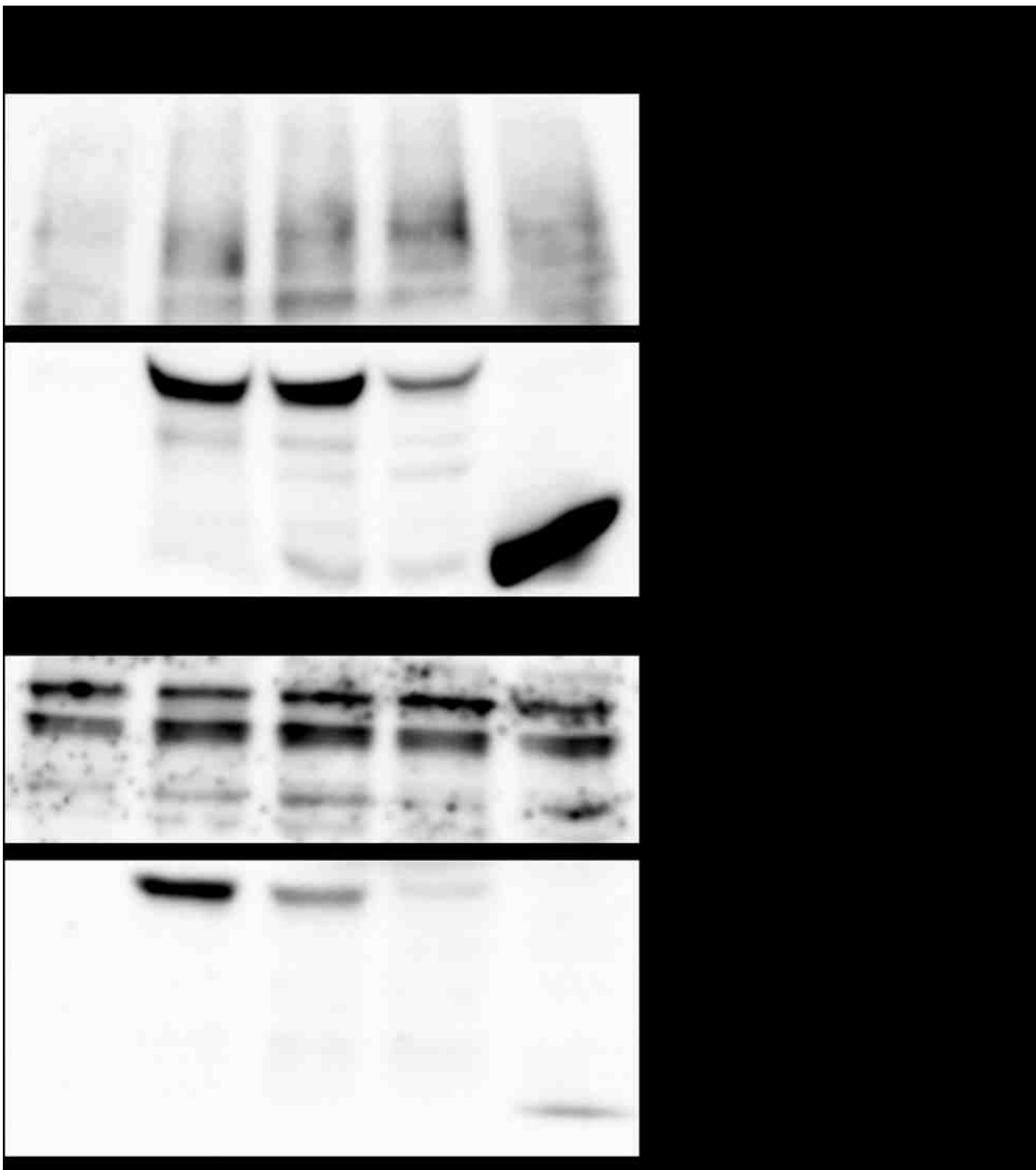


Figure 3.15. BPV-1 E2 does not associate with c-Met. HEK293TT cells were grown on 6-cm dishes and transfected with 1 μ g each mRFP-GFP (-) or BPV-1 E2 wild type and mutants (WT, YF, YE) plus E2R. Cells were harvested 48 hours post-transfection. 5% of each lysate was set aside for input, while remaining lysate was incubated with protein sepharose A and G beads and II-I antibody at 4°C overnight. Western blots were probed for BPV-1 E2 using B201 and for endogenous receptor tyrosine kinases with rabbit anti-Met C-28. Labels in parentheses indicate that the protein was not identified.

FIGURE 3.16

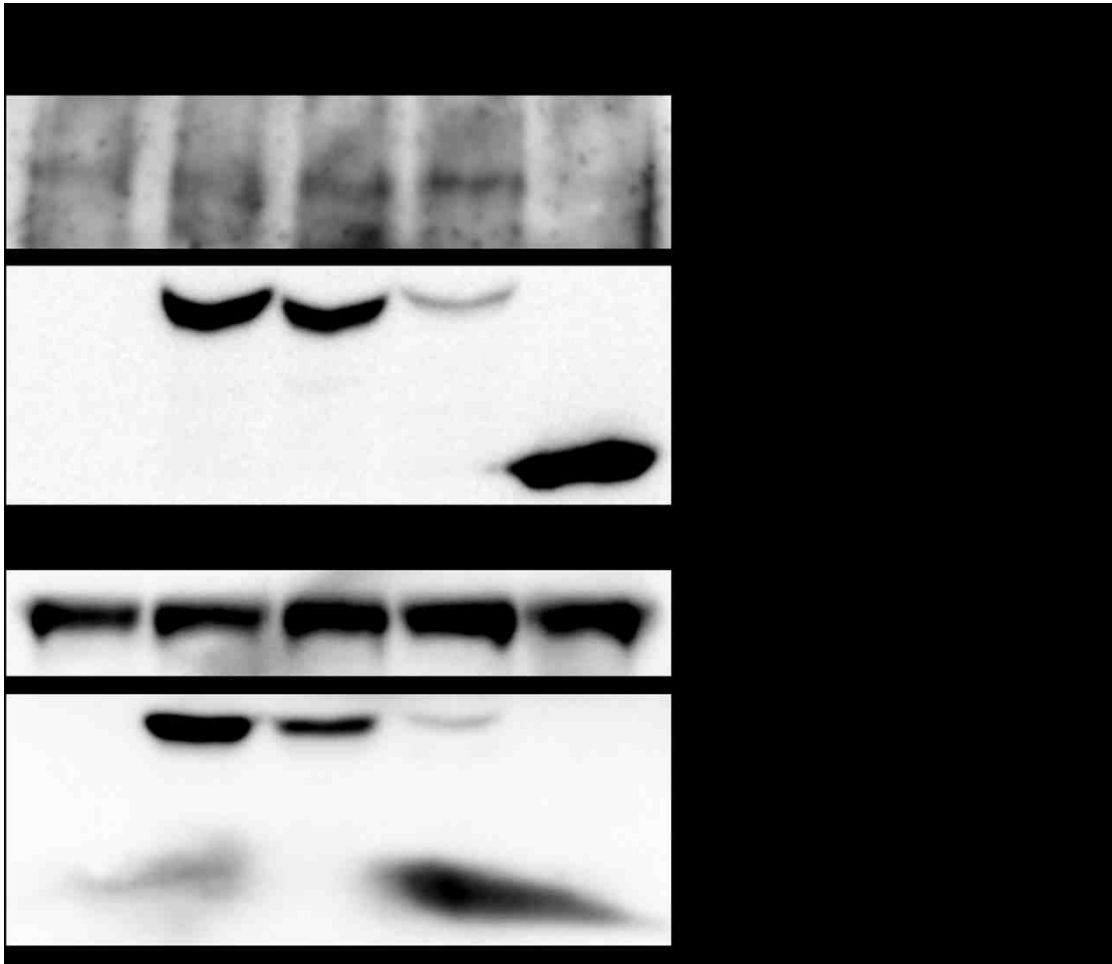


Figure 3.16. BPV-1 E2 does not associate with IGF-1R. Samples were prepared as in Figure 3.15 and probed for BPV-1 E2 using B201 and for endogenous receptor tyrosine kinases with rabbit anti-IGF-1R β C-20. Labels in parentheses indicate that the protein was not identified.

FIGURE 3.17

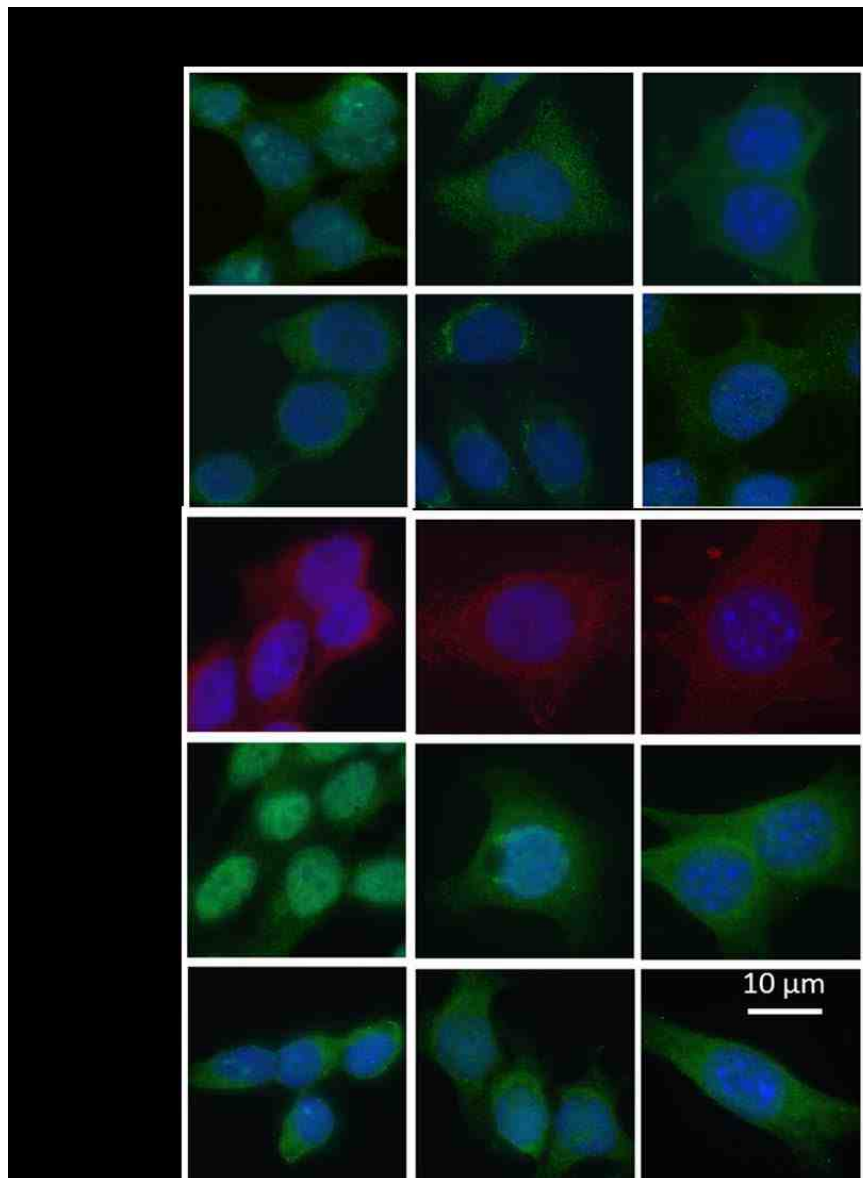


Figure 3.17. Localization of fibroblast growth factor receptors in multiple cell lines. HEK293TT, HaCaT, and ID13 cells were grown in duplicate wells on 12-well plates with 18-mm glass coverslips. Cells were fixed and permeabilized, followed by incubation with anti-FGFR2/Bek C-17, anti-FGFR4 C-16, anti-EphB2, anti-Met C-28, or anti-IGF-1R β C20 at 4°C overnight. Coverslips were washed, stained with either Alexa Fluor 488 anti-rabbit or AF 594 anti-mouse as appropriate, washed, and mounted onto glass slides using ProLong Gold with DAPI. Green, FGFR2, FGFR4, c-Met, or IGF-1R as labeled; red, EphB2; blue, DNA. White bar, 10 μ m. All images taken at 1000x magnification.

Discussion and Future Directions, Part 1: Tyrosine Kinases in Productive Papillomavirus Infection

Here we report the identification and functional characterization of a novel tyrosine phosphorylation in BPV-1 E2. This residue at Y102 is conserved in 91 of the known PV species (Tables 4 and 5, Appendix), including many high-risk HPVs. The detection of phospho-Y102 in two separate samples from live cells, the high confidence score which this PTM received by mass spectrometry analysis (Table 2, Appendix), and the conservation of Y102 among diverse PVs indicate that phosphorylation at this residue is important for one or more functions of the protein *in vivo*.

To begin investigations of its function, we generated at Y102 phospho-defective (Tyr-Phe, Y102F) and phospho-mimetic (Tyr-Glu, Y102E) forms. Although the use of glutamate as a tyrosine phospho-mimetic is variably effective, there is a precedent in tyrosine kinase studies where Tyr-Glu mutants successfully mimic well-known effects of auto-phosphorylation of the enzyme (Zisch et al., 2000, Kassenbrock and Anderson, 2004). The BPV-1 E2 Y102F mutant retains WT function in a transcription assay. Notably, in transient BPV *ori* replication assays, Y102F activity was significantly increased over that of WT. In contrast, the Y102E mutant is completely defective for both transcription and replication (Figures 3.3, 3.4, and 3.8). Future studies to pinpoint the Y102E defect should include time-course assays (in case Y102E function is delayed) and comparison between samples treated with receptor tyrosine kinase ligands or inhibitors versus control. For the latter, however, global alterations in cell signaling may confound the results.

The inability of Y102E to co-immunoprecipitate the BPV-1 E1 viral

helicase (Figure 3.9) likely explains its replication deficit. Y102F binds to E1 and reproducibly demonstrated activity somewhat greater than WT, and it indeed appears to pull down more E1 than WT in Figure 3.9. Our interpretation is that a fraction of WT E2 protein may undergo phosphorylation of Y102, thereby reducing its activity, while Y102F cannot undergo this negatively acting PTM. Indeed, the difference in replication stimulation between WT and Y102F in Figure 3.8 may reflect the size of the WT phospho-Y102 pool; with this pool eliminated by the Y102F mutation, E2 achieves its maximal activation. From these results we conclude that phosphorylation of this tyrosine is not required for the transcription or replication activities of E2. In contrast, the Y102E mutant is defective for the ability to induce transient viral DNA replication, which we attribute to its inability to co-immunoprecipitate the BPV-1 E1 helicase.

The E1 binding surface of BPV-1 E2 spans the majority of the TAD (Baxter and McBride, 2005, Abbate et al., 2004) and includes the small α -helical loop on which Y102 resides (Figure 3.2). The Y102E mutation may prohibit binding of E2 to E1, or may disrupt the conformation of this loop as well as conformation of the N-terminal α -helices in the TAD described above. These α -helices contain multiple residues important for E1 binding (Baxter and McBride, 2005), and binding of transcriptional co-activators including Brd4 (Abbate et al., 2006), Brm (Kumar et al., 2007) and TFIIB (aa 74-134) (Yao et al., 1998). Disruption of these structures would account for our IP and luciferase assay results.

The transcriptional defect of Y102E is more complex to unravel. Both Y102E and Y102F co-immunoprecipitated with the E2 transcription enhancers Gps2/AMF1 and Tax1BP1 (Figure 3.7), which bind to the β -sheet region C-

terminal of Y102 (Breiding et al., 1997, Wang et al., 2009) (see also Figure 3.2). Based on high amounts of Y102E on IP with very little Tax1BP1 co-precipitated (Figure 3.7), this mutant seems like a poor binding partner for this cofactor. Perhaps the mutant allows Tax1BP1 to recruit a stabilizing enzyme (such as the deubiquitinase A20) while minimizing direct interaction with Tax1BP1. The Gps2 and Tax1BP1 co-IP results are consistent with our earlier conclusion that association with these two factors is necessary but not sufficient to stimulate E2 dependent transactivation.

Unlike Y102F, Y102E cannot associate with the full-length chromatin modulator Brd4 or the isolated Brd4 C-terminal motif (CTM) and does not appear to be stabilized (based on input) by the presence of CTM (Figures 3.5 and 3.6). (Gps2 and Tax1BP1, in contrast, do seem to stabilize Y102E; see IP results, Figure 3.7.) In the co-crystal structure, the twenty amino acid Brd4 CTM peptide binds across the α -helices of the E2 TAD (Abbate et al., 2006) (see also Figure 2.1). If mutation of Y102 to glutamate induces a conformational change in the TAD, this could affect the α -helical region. It is also possible that the folding of the full Brd4 protein juxtaposes it in the vicinity of Tyr 102, such that phosphorylation of this residue destabilizes Brd4 interaction with the E2 α -helices. E2 binding to the basic interacting domain (BID) of Brd4 was unexpected as the CTM is the only domain previously reported to interact with E2. The BID domain can bind to p53 (Wu et al., 2013) and to HPV E2 (C-M Chiang, personal communication). In Brd4:p53 interaction studies, BID was found to undergo a conformational change induced by serine phosphorylation within a different Brd4 domain (Wu et al., 2013). It is possible that such conformational changes regulate the association of Brd4 and E2, potentially

favoring E2's interaction with one Brd4 domain over another. Such differential binding could change the functional outcome of E2:Brd4 association.

Because the Y102E mutant failed to activate both transcription and replication and could not associate with key mediators of these processes, we infer that phosphorylation at Y102 is inhibitory. Like the mutation to glutamate, phosphorylation at this site is expected to produce conformational changes that impair binding to co-factors, leading to inhibition of multiple functions. Based on our initial western blot of the Y102 mutants from HEK293TT lysates (Figure 3.2) and several co-IP experiments, we would not expect Y102E or phospho-Y102 to be degraded more quickly than WT due to altered conformation. However, the decrement in Y102E expression in C33a cells (Figure 3.3) and in CV-1 cells without overexpression (Figure 3.4), as well as decreased expression compared to WT in certain co-IPs warrants further examination of the effect of this mutant on E2 stability. As with the CV-1 cells, Y102E overexpression (5 to 10-fold) should be attempted in the C33a system. Co-IP studies assessing Y102E association with Gps2 and Tax1BP1 could also be performed in C33a and CV-1. Decreased interaction with these stabilizing cofactors compared with HEK293TT may explain the low expression of the mutant in these cells. Brd4 interaction with mutants could also be examined in different cell lines, though the lack of association with Y102E even in HEK293TT suggests that Brd4 is not needed for stabilization of this mutant. Interestingly, in Figure 3.4, there is no apparent difference between Y10E levels in CV-1s with 5-fold versus 10-fold overexpression; this may be due to a maximum limit for transcription from this construct reached by the cellular machinery, or Y102E may have some transcription repression activity. HeLa repression assays, in which BPV-1 E2 is

expressed in HeLa cells and evaluated for its ability to decrease E6 and E7 mRNA transcripts, would be helpful in assessing potential Y102E repression activity.

Since dimerization of E2 is important for DNA binding, transcription, and replication (Moskaluk and Bastia, 1989, Prakash et al., 1992, Antson et al., 2000, Cardenas-Mora et al., 2008), it is also possible that Y102 phosphorylation of one of the monomers prevents dimerization or changes conformation of the dimer so that E2 is inhibited in a dominant-negative manner if both phosphorylated and dephosphorylated forms of E2 co-exist within host cells. *Cis* or *trans* effects of Y102 phosphorylation on DNA binding and dimerization are unlikely since the DNA binding domain (DBD) is completely functional in the absence of the TAD, and there is no evidence for their physical association. However, chromatin immunoprecipitation (ChIP) of the Y102 mutants is still warranted to confirm this speculation. Altered subcellular localization preventing function can also be a consequence of PTMs, especially at or near a nuclear localization site (NLS). However, we did not observe phospho-Y102 mis-localization to the extent that it affects E2 function since the Y102E mutant was predominately nuclear (Figure 3.10).

We identified candidate kinases for Y102 phosphorylation and found that FGFR family members 2, 3, and 4 and the kinase EphB2 were co-immunoprecipitated by BPV-1 E2 (Figures 3.11, 3.12, 3.13, and 3.14). This is the first evidence of any receptor tyrosine kinase in complex with E2. The kinase binding site could be in the TAD β -sheet region described above (see also Figure 3.2) since there is little difference between WT and Y102E co-immunoprecipitation of receptors. Docking of a tyrosine kinase typically

involves a peptide region distinct from the targeted tyrosine, so mutating the active site would not necessarily affect binding.

In keratinocytes, the PV replicative cycle is closely linked to the host-cell life cycle, so kinase activity in these cells could be directly correlated with functional outcomes for a particular stage in viral infection. Multiple growth factor receptors including FGFR1 and IGF-1R have been found to translocate and act in the nucleus, where they could encounter E2 (Song et al., 2013). We expect that the kinase responsible for Y102 phosphorylation is most active in undifferentiated, basal keratinocytes, where viral replication and transcription occur at low levels (Figure 3.18). A study comparing the transcriptomes of keratinocytes in monolayer culture, skin, and reconstituted epidermis noted that growth factor receptors (such as the FGFRs, c-Met, and IGF-1R) and ephrin receptors (such as EphB2) were up-regulated in skin and reconstituted epidermis (Gazel et al., 2003). The data indicate that these receptors function in a differentiation-dependent context, though their expression in basal versus suprabasal versus superficial cells of the stratified epithelium remains unclear. We can, however, suggest that future studies of these receptors and E2 should include stratified epithelium, either from tissue samples or raft cultures.

Our data lead us to hypothesize the following scenario. Y102 phosphorylation accumulates in early infection (Figure 3.18) to limit replication and viral gene expression and thereby prevent a lytic infection which would trigger an immune response. As host cells differentiate, we expect that a tyrosine phosphatase restores Y102, thus activating E2 transcriptional and replication functions that result in viral genome amplification.

FIGURE 3.18

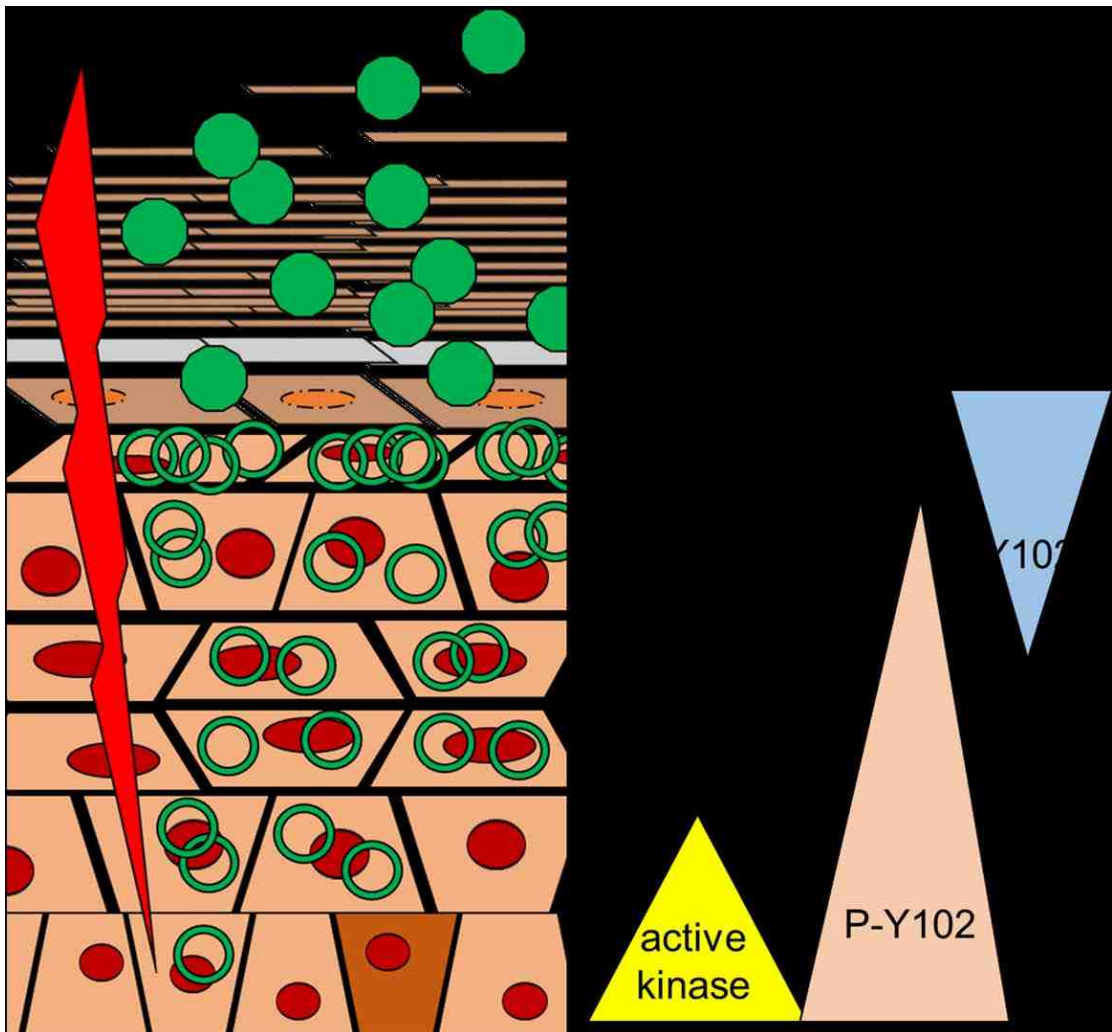


Figure 3.18. Proposed model of E2 tyrosine phosphorylation in the context of the papillomavirus replicative cycle. Refer also to Figure 1.2. The kinase acting on Y102 is expected to be most active in basal keratinocytes (yellow triangle), where viral copy number is kept low in part through inhibition of E2 by phosphorylation (P-Y102, pink triangle). As host cells migrate upward and differentiate, dephosphorylated E2 is expected to predominate (Y102, blue triangle), permitting genome amplification.

Discussion and Future Directions, Part 2: Tyrosine Kinases in Cervical Cancer

Although the majority of our studies focused on Y102 in BPV-1 E2, we have extended our focus to HPV-31. As one of the high-risk HPVs in which Y102 is conserved (Table 4, Appendix), HPV-31 interests us because of the potential for E2 PTMs to affect virally induced tumorigenesis. Although we could not detect P-Y102 in our HPV-31 E2 samples (Table 3, Appendix), we do not rule it out as a possibility since this PTM was predicted to occur by different software systems mentioned previously. Also, the IU Proteomics Core encountered difficulties in obtaining over 50% peptide coverage from their digests due to the unusually hydrophobic nature of the protein, so peptides containing Y102, let alone P-Y102, may have been present below the limits of detection. Nevertheless, the phosphorylation sites detected at serines and threonines in this protein (Table 3, Appendix) have not been reported previously, and may be worth exploring for their functional significance. T216, T220, and S266 are of particular interest since they received the highest confidence scores from the Core's analysis software.

Preliminary studies indicate that Y102E in HPV-31 E2 behaves similar to BPV-1 E2 Y102E in replication assays (Figure 3.19). Construction of cell lines from human foreskin keratinocytes (HFKs) stably expressing HPV-31 wild type or Y102 mutant genomes is currently in progress. Our goals are to not only observe differences in copy number during stable replication but to look for varying effects on copy number upon differentiation through the use of methylcellulose, calcium, or raft culture. Raft culture would be particularly informative since cells could also be used for immunohistochemistry to monitor

kinase distribution and activation in different keratinocyte layers. We expect that, while Y102F copy number would be higher than WT in undifferentiated keratinocytes, WT replication would escalate to Y102F levels upon differentiation. We do not expect Y102E to amplify its genome, though it may maintain a low copy number in undifferentiated cells. We also plan to initiate tyrosine kinase inhibitor studies in CIN612 cells (a human cell line stably expressing HPV-31) to compare viral copy number with and without treatment. It is possible that Y102E genomes could amplify upon differentiation, when E1 is cleaved and activated (Moody et al., 2007, Morin et al., 2011). E1 may be sufficient to drive replication, and it could be recruited to the *ori* by cellular factors such as topoisomerase I (Hu et al., 2006) (see also Chapter 1) in the absence of E2 binding.

Direct visualization of P-Y102 would be invaluable to elucidating the spatial and temporal distribution of this PTM. We have worked with Pacific Immunology (Ramona, California) to develop HPV-31 E2 antibodies specific to P-Y102. These antibodies were raised in New Zealand white rabbits against a peptide sequence of HPV-31 E2 containing phosphorylated or dephosphorylated E2, and the antibodies were affinity purified. Testing of the antibodies for specificity in western blot, co-IP, and immunofluorescence is currently under way.

Findings from several laboratories over the last decade strongly suggest a role for kinases in HPV-associated cancers. HPV-16 E7 promotes overexpression of a cellular oncogene, cancerous inhibitor of protein phosphatase 2A (CIP2A), reducing c-Myc dephosphorylation on serine 62 by PP2A and thereby preventing its degradation (Liu et al., 2011). Conversely,

both low-risk (6 and 11) and high-risk (16 and 18) HPVs drive expression of the SHP-1 and SHP-2 tyrosine phosphatases (Tao et al., 2008), suggesting that these viruses are selective in their regulation of different PTMs in different signaling pathways. Indeed, tyrosine phosphorylation of annexin A1 and DNA-PKcs, as well as other proteins, was found to be increased with advancing disease stage in cervical cancer (Robinson-Bennett et al., 2008).

Receptor tyrosine kinases have been increasingly implicated in cervical and oropharyngeal cancer. Activating mutations in FGFR3 such as S249C were enriched in a subset of cervical cancers, and they were associated with differential gene expression and tissue phenotype compared to cancers with wild type FGFR3. This study indicated that lesions with high FGFR3 activity could be uniquely targeted for treatment in eligible patients (Rosty et al., 2005). Other RTKs, including EphB2 and IGF-1R, have also been found to play roles in HPV-associated cancers. EphB2 is upregulated in proportion to increasing cervical dysplasia (normal epithelium < high-grade squamous lesions < carcinoma), and overexpression of the kinase in cell culture models was found to induce epithelial to mesenchymal transition (Gao et al., 2014). IGF-1R was reported to act in oral cancer cells to down-regulate its own repressor, microRNA-99a, which has been described as a tumor suppressor gene (Yen et al., 2014). Together, these results suggest that RTKs can act as oncogenes in HPV-infected cells, and they have indeed been identified as such in other types of cancer (Chukkapalli et al., 2014, Salazar et al., 2014).

Although E2 gene expression is lost in viral genome integration during tumorigenesis, it is possible that HPV oncoproteins prime cells for this event by inactivating E2 via up-regulation of tyrosine kinase activity (see Introduction to

this chapter). E2 phosphorylation would eliminate E1:E2 complex formation, which may protect the viral genome from nuclease activity and integration. E1 would still be able to conduct replication, albeit less efficiently. E1-dependent replication, combined with unchecked E6 and E7 activity, may lead to increased aberrant replication, subsequent integration and complete loss of the E2 gene, and eventually host chromosomal instability and uncontrolled oncogene expression leading to cancer.

FIGURE 3.19

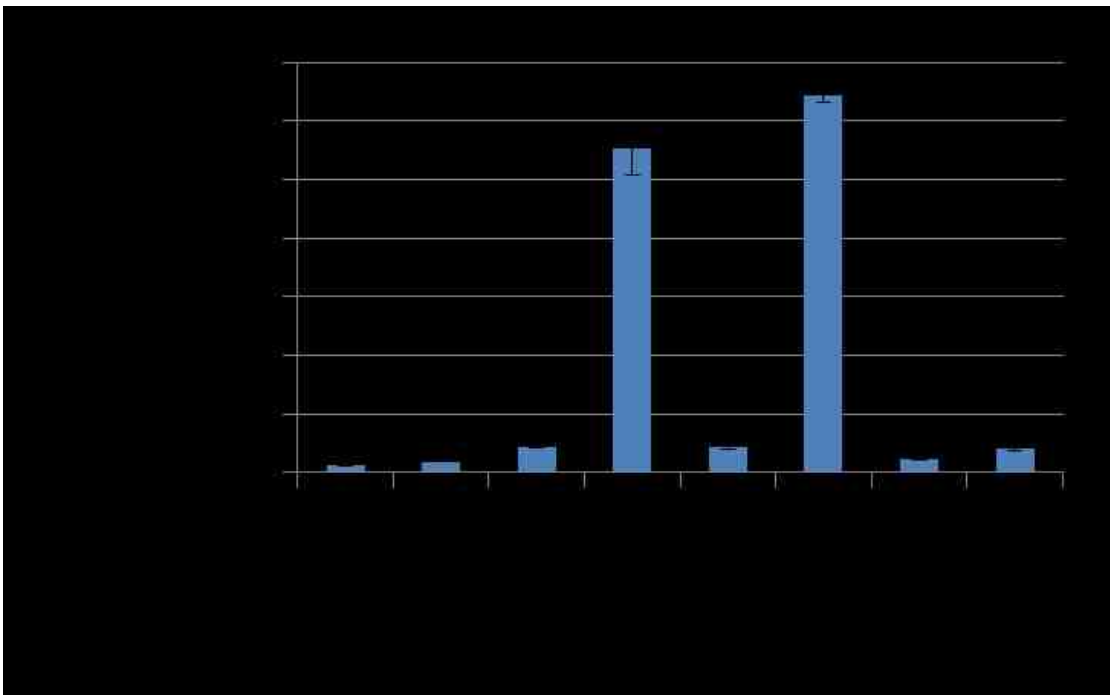


Figure 3.19. Y102E fails to stimulate transient HPV-31 replication. C33a cells were grown in a 96-well clear bottom plate. Samples were transfected in 8 replicate wells with 10 ng/well codon-optimized Flag-HPV-31 E2 wild type or mutants (WT, Y102F, Y102E) or mRFP-GFP ((-)), as well as 10 ng/well 3x-Flag-HPV31-E1 and 2.5 ng/well pFLORI31 (firefly luciferase reporter) (see Table 1, Appendix). 72 hours post-transfection, cells were lysed on-plate with 50 μ l Steady Glo reagent. Lysates were mixed with 50 μ l DPBS and luminescence was detected using the Pherastar system. RLU, relative light units. Values are expressed as mean \pm SEM.

CONCLUSIONS

Post-translational modification represents a dynamic means of controlling the timing and extent of a protein's activity, and a multifunctional protein like papillomavirus E2 may require multiple forms of control. We hypothesized that post-translational modification of E2 controls the staging of the viral replicative cycle in concert with host-cell maturation and differentiation. We refined this hypothesis, clarifying that such control would manifest in differential binding to proteins which normally enhance E2 functions, including transactivation and replication. We demonstrated that BPV-1 E2 and HPV-31 E2 are acetylated in cells, and we affirmed that one residue (K111) of a dilysine motif (K111-K112) is necessary for transcription activation. We propose that acetylation of this residue occurs late in papillomavirus infection to promote viral gene transcription.

We also detected a novel PTM, phosphorylation of tyrosine 102 in BPV-1. An acetyl-mimetic point mutant at this site, Y102E, was defective for transcription activation and replication. This mutant also failed to bind the E2 transcription co-factor Brd4 and the viral helicase E1, though it was able to associate with the transcription co-activators Gps2 and Tax1BP1. We also detected the first known association between BPV-1 E2 and receptor tyrosine kinases. We propose that phosphorylation at Y102 occurs early in infection to limit viral transcription and replication for the purposes of limiting copy number and evading the immune system.

Altogether, our results support our hypothesis since mutations at PTM sites not only had functional effects on E2 which could correspond to differential modification at certain stages of the viral replicative cycle (i.e. late in infection

for acetyl-K111, early in infection for P-Y102), but they also affected association with cellular co-factors such as Brd4, Gps2, and Tax1BP1. Future studies should seek to establish the physiological relevance of these PTMs in the context of infection, including whether these modifications occur simultaneously or are interdependent with one another for effective regulation of E2. Other PTMs detected in our mass spectrometry samples should also be examined for their relevance to papillomavirus biology, including involvement in switch mechanisms (i.e. two or more PTMs at the same site at different stages of the replicative cycle) or as multiple PTMs acting in concert to influence E2 function.

The present work underscores the value of studying post-translational modification in E2 – and potentially other viral proteins – to better understand papillomavirus infection.

TABLES

TABLE 1

Lab Plasmid Stocks	pCG-BPV1-E2 Y102A
HA-HPV31-E2	
HA-p300	Gifts
mRFP-GFP	pCI-Rluc (Archambault)
pBabe-Puro	pFLORI-BPV1 (Archambault)
pcDNA3 Flag-Brd4	pFLORI31 (Archambault)
pcDNA HA-AMF1	3x-Flag-HPV31-E1 (Archambault)
pCI	pGEX (GST) (Chiang)
pCG-BPV1-E1	pGEX:hBrd4(524-579) (BID) (Chiang)
pCG-BPV1-E2	pGEX:hBrd4(1224-1362) (CTM) (Chiang)
pCG-BPV1-E2 K111R	pVL-F:hBrd4 (Chiang)
pCG-BPV1-E2 K112R	pGEM-EGFR (Korc)
pCG-BPV1-E2 K111R/K112R	HPV31 genome in pBR322min (Laimins)
pCG-VP16-E2 125	codon-optimized Flag-HPV31-E2 (McBride)
pCMV 7.1 3x-Flag-Tax1BP1	pcDNA3-EphB2 WT, chicken (Pasquale)
pGL2-E2BS-Luc	pcDNA3-EphB2 KD K662R, chicken (Pasquale)
pUC18	pBabe-Puro IGF-1R (Spandau)
	pRK7-FGFR1 (Thompson)
Constructs Made for this Work	pRK7-FGFR2 (Thompson)
HA-HPV31-E2 Y102F	pRK7-FGFR3-Flag WT (Thompson)
HA-HPV31-E2 Y102E	pRK7-FGFR3-Flag K650E (Thompson)
HPV31 c-o Flag-HPV31-E2 Y102F	pRK7-FGFR4 (Thompson)
HPV31 c-o Flag-HPV31-E2 Y102E	
pBR-HPV31 Y102F-E2	Purchased
pBR-HPV31 Y102E-E2	pBabe-Puro HGF (Addgene)
pCG-BPV1-E2 K111Q	pLenti-Met GFP (Addgene)
pCG-BPV1-E2 K112Q	
pCG-BPV1-E2 Y102F	
pCG-BPV1-E2 Y102E	
pCG-BPV1-E2 WT-A3	
pCG-BPV1-E2 YF-A3	
pCG-BPV1-E2 YE-A3	

Table 1. Plasmid stocks used in experiments. Also included are constructs made for preliminary studies not described in the text, such as a BPV-1 E2 Y102A mutant and Y102 mutants with the three serine→alanine mutations that comprise the “A3” mutation set (YF-A3 and YE-A3). Individuals who generously donated plasmids include Jacques Archambault (McGill University); Cheng-Ming Chiang (UT Southwestern); Murray Korc (Indiana University); Lou Laimins (Northwestern University); Alison McBride (NIAID); Elena Pasquale (Sanford-Burnham Institute); Dan Spandau (Indiana University); and Leslie Thompson (UC Irvine).

TABLE 2

BPV-1 E2 Residue	Modification	Times Observed (out of 3)
S22	Phosphorylation****	1
S23	Phosphorylation****	1
K25	Acetylation	1
Y32	Phosphorylation***	1
K48	Acetylation	2
S62	Phosphorylation****	1
K70	Acetylation	1
T97	Phosphorylation*	1
S98	Phosphorylation*	1
Y102	Phosphorylation****	2
S104	Phosphorylation****	1
K107	Acetylation	1
K112	Acetylation	1
S171	Phosphorylation**	1
S181	Phosphorylation**	1
T182	Phosphorylation**	1
Y186	Phosphorylation**	1
S187	Phosphorylation**	1
S199	Phosphorylation**	1
S200	Phosphorylation**	1
T201	Phosphorylation*	1
S202	Phosphorylation**	1
S203	Phosphorylation**	1
K226	Acetylation	1
S275	Phosphorylation**	1
S276	Phosphorylation**	1
S277	Phosphorylation****	1
T283	Phosphorylation***	1
S298	Phosphorylation***	3
S301	Phosphorylation***	3
T302	Phosphorylation***	1
T313	Phosphorylation****	1
T314	Phosphorylation****	1
S332	Phosphorylation*	1
T334	Phosphorylation***	1
K339	Acetylation	1
Y341	Phosphorylation****	1
K346	Acetylation	1
K347	Acetylation	1

Table 2. Post-translational modifications detected for BPV-1 E2. According to the Thermo-Fisher Scientific Proteome Discoverer™ software (v1.3): *, low-confidence score; **, mid-low confidence score; ***, mid-high confidence score; ****, high confidence score.

TABLE 3

HPV-31 E2 Residue	Modification	Times Observed (out of 3)
K88	Acetylation	1
K111	Acetylation	1
T216	Phosphorylation****	1
T220	Phosphorylation***	1
T222	Phosphorylation**	1
S266	Phosphorylation***	1
S269	Phosphorylation**	1
S276	Phosphorylation*	1

Table 3. Post-translational modifications detected for HPV-31 E2. See Table 2 legend for confidence scoring key.

TABLE 4

HPV Type	Genus	Position of Tyrosine Residue	Surrounding Sequence (-/+ 5 aa)
6	α	99	LQETSYEMWQT
8	β	102	TSIETYKNAPE
11	α	99	LQDTSYEMWLT
12	β	102	TSAETYNNVPE
16	α	102	VSLEVYLTAPT
19	β	102	TSAETYRSAPE
25	β	102	TSTETYKSPPE
26	α	99, 102	MRDTSYEMYMTEPK
31	α	102	TSLELYTLTAPT
32	α	99	LQETSYEMWHA
35	α	103	TSIELYTTVPQ
41	ν	105	TTKERYLAEPS
49	β	102	TSLETYNAPPA
51	α	99	MRETCYELWCV
53	α	103	CESMWYTEPKQ
69	α	99, 102	MRDTCYELYVTEPK
76	β	102	TSLETYPTPI
82	α	99	MRDTCYELWGE
85	α	102	TCQELYQTPPQ
96	β	102	TSLETYRAPPV
98	β	102	TSIETYKNAPE
101	γ	99	LTDTSYELFMT
108	γ	102	TSHELYTTPPE
109	γ	102	TSFELYNAPPQ
115	β	102	TSLETYRTPPS
118	β	102	TSLETYKNAPE
123	γ	102	TSFELYNSSPQ
124	β	102	TSLETYRNQPE
134	γ	102	TSLELYNTEPE
139	γ	102	TSFEVYNAAPF
152	β	102	TSLETYRNRPE
155	γ	99, 102	MQDTSYEIYSAAPK
156	γ	103	SAELTYTAPRN
170	γ	99, 102	LQDTSYEAYTSAPE
174	β	99	LVQTSYETFRS
175	γ	103	STEIYTTPRN

Table 4. Tyrosine 102 conservation among human papillomaviruses. Viruses are listed by type, with genus and presence of Y102 or an analogous site (e.g. Y99, Y103) listed. Amino acid sequence surrounding the tyrosine(s) of interest (bold) is also provided. Genus and sequence information were obtained from the Papillomavirus Episteme (pave.niaid.nih.gov).

TABLE 5

PV Type	Genus	Position of Tyrosine Residue	Context Sequence
AaPV1	δ	102	SWERYQ
AsPV1	π	102	SLELYK
BPV1	δ	102	SWDRYM
BPV2	δ	102	SWDRYM
BPV5	ε	102	SHERYK
BPV8	ε	102	SHERYK
BPV13	δ	102	SWDRYM
BgPV1	δ	102	SWDRYI
BpPV1	Dyo-κ	99, 102	SYETYV
CPV3	χ	99	SYERWT
CPV5	χ	99	SYERWM
CcPV1	Dyo-ζ	99	QYQLVM
CcaPV1	δ	102	SWDRYV
CdPV1	δ	99, 102	SYETYS
CdPV2	δ	99	SYECFS
ChPV1	φ	102	SSMTYL
CmPV1	Dyo-ζ	99	QYSLVM
DdPV1	υ	102	SHEMYT
EaPV1	-	102	SWDRYA
EcPV2	Dyo-ι	102	SREMYA
EcPV3	Dyo-ρ	102	SKETYR
EcPV4	Dyo-ι	99	SYELFK
EdPV1	σ	102	TLERYL
EhelPV1	-	102	SWELYK
EserPV2	-	102	SLEKYS
FIPV1	Dyo-ε	102	SPTVYK
MaPV1	π	102	SLELYE
McPV2	π	102	SLEMYE
MfPV5	α	103	SLEMWY
MmuPV1	π	102	SREMYD
MnPV1	ι	102	SREMYM
MpPV1	τ	102	SLELYL
MscPV1	-	102	SREKYL
OaPV1	δ	99, 102	SYERYL
OaPV2	δ	99, 102	SYERYL
OvPV1	δ	102	SWGRYQ
PmPV1	ι	102	SREMYM
PphPV1	ο	102	SKEMYA
PphPV2	υ	102	TFSMYM
PphPV4	Dyo-π	102	SSEMYM
PsPV1	ο	102	SHDMYM
PsuPV1	π	102	SVEVYE
RaPV1	ψ	102	SRENYM
RnPV1	π	102	SIEMYE

RnPV2	ι	102	SRDMYL
RtPV1	δ	102	SWDRYQ
SsPV1	Dyo-δ	102	TWESYR
TmPV2	ρ	102	SEERYK
TtPV2	υ	102	SKDMYM
TtPV3	υ	99	SYEMFQ
TtPV4	υ	102	SHELYT
TtPV5	ο	102	SIELYD
TtPV7	υ	102	SHELYM
UmPV1	ω	102	SVELYE
ZcPV1	Dyo-ν	102	SWERYT

Table 5. Tyrosine 102 conservation among non-human papillomaviruses. Information is organized as in Table 4 and was also obtained from the Papillomavirus Episteme.

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CURRICULUM VITAE

Sara Poirier Culleton

Education

- October 2015 Ph.D., Microbiology & Immunology, Indiana University – Purdue University Indianapolis
- 2009 – present M.D., Indiana University – Purdue University Indianapolis (expected 2017)
- May 2009 B.S., Molecular & Cell Biology, University of Illinois Urbana-Champaign; Minor in Chemistry; *summa cum laude*

Research Experience

- 2011 – 2015 *Doctoral Research:*
Indiana University – Purdue University Indianapolis, Indianapolis, IN. Effect of lysine acetylation and tyrosine phosphorylation on functions of the papillomavirus E2 protein. Dr. Elliot Androphy, Principal Investigator
- 2007 – 2009 *Undergraduate Research:*
University of Illinois Urbana – Champaign, Champaign, IL. Kinetics of redox reactions at multiple sites within the bc₁ complex of *Rhodobacter sphaeroides*. Dr. Antony Crofts, Principal Investigator
- 2008 *Research:*
University of Chicago, Chicago, IL. Isolation of three domains of hematopoietic cell kinase. Dr. Benoît Roux, Principal Investigator

Honors, Awards, Fellowships

Indiana University – Purdue University Indianapolis

- July 2014 Awarded NIH F30 Grant
- June 2014 Harold Raidt Graduate Student Teaching Award
- June 2012 Appointed to T32 Immunology & Infectious Diseases Training Grant

University of Illinois Urbana-Champaign

- 2009 University Honors (Bronze Tablet)
- 2008 Phi Beta Kappa
- 2008 Mortar Board
- 2006 – 2008 Dean's List
- 2005 Chancellor's Scholar

Poster Presentations

SP Culleton, M DeSmet, EJ Androphy. A novel tyrosine phosphorylation regulates functions of the BPV1 E2 protein. DNA Tumor Virus Meeting, Trieste, Italy. July 21-26, 2015.

SP Culleton, EJ Androphy. A novel phosphorylation site influences multiple regulatory functions of papillomavirus E2 protein. DNA Tumor Virus Meeting, Madison, WI. July 21-26, 2014.

SP Culleton, EJ Androphy. A novel phosphorylation site influences multiple regulatory functions of papillomavirus E2 protein. ASCI/AAP/APSA Joint Meeting, Chicago, IL. Apr. 25-27, 2014.

SP Culleton, EJ Androphy. A novel phosphorylation site influences multiple regulatory functions of papillomavirus E2 protein. American Physician Scientists Association Midwest Regional Meeting, Columbus, OH. Nov. 9, 2013.

Publications

Culleton SP, DeSmet ML, Qi G, Wang M, Androphy EJ. Tyrosine 102 of bovine papillomavirus type 1 E2 is phosphorylated *in vivo* and influences E2 transcription and replication functions. [in revision]

Culleton SP, Androphy EJ, Kanginakudru S. Papillomavirus Replication. HPV-Associated Oropharyngeal Cancer. Ed. MS Stack and DL Miller. Springer, September 2015.

Quinlan EJ, Culleton SP, Wu SY, Chiang CM, Androphy EJ. 2013. Acetylation of conserved lysines in bovine papillomavirus E2 by p300. J. Virol. 87 (3):1497-1507.