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Control of Bovine Papillomavirus E2 Function By Acetylation and the Novel E2 Interacting Protein RINT1: A Dissertation

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CONTROL OF BOVINE PAPILLOMAVIRUS E2 FUNCTION BY ACETYLATION AND THE NOVEL E2 INTERACTING PROTEIN RINT1

A Dissertation Presented

By

Edward James Quinlan

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Virology

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Dedication

I would like to dedicate this work to my family who has stood by me through it all. First, I need to thank my wife Angela. She has been with me through almost my entire graduate career, seen the ups and downs, and supported me and put up with me through it all. She was uniquely able to sympathize with me and say exactly the right thing when needed. She also gave up her time to help proofread this document and a manuscript for publication. We have both been on a long road to this point. She was and is my inspiration and motivation and without her this would not have been possible. It has been an amazing time starting our lives together, and I look forward to turning the page and starting the next chapter.

Next, I would like to thank my parents Stephen and Laureen Quinlan. They have been a constant positive motivational force all of my life. At every turn I always heard the phrase "You can do anything if you put your mind to it." I never really thought much of it. Looking back now, they were right. There were plenty of times when I thought I wasn't good enough or other people told me I wasn't good enough, but they have always been steadfast in their support and belief in my ability. I whole heartedly appreciate all they have done for me over the years.

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To my SJ friends, we are a diverse group bound together by a common bond. Some of us undertook this monumental task of graduate study, and some undertook the monumental task of starting a family. You guys have always been supportive and able to lend an ear when needed, and quick to offer support when things weren't going well. You have also been a source of competition and motivation in the graduate arena and in life. I hope a day comes very soon, when we can sit around the fire and say, "Hey, no one is in school anymore."

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and went new places. A lot of us met our wives and husbands, and some of us even started families. We were able to have intelligent conversations about everything, science included. Above all we were able to lend each other support from common experience; I know helped me through some tough times when none of my experiments worked. I hope the last few from our class finish soon.

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Abstract

Human papillomavirus infection is the cause of more than 99% of cervical cancer cases. The current vaccine is ineffective therapeutically; highlighting the need for continued papillomavirus research. One avenue that could be explored in this regard is the function of the papillomavirus E2 regulatory proteins. HPV E2 represses expression of the viral E6 and E7 oncoproteins. Reintroduction of E2 into cervical carcinoma cells results in growth arrest and cellular senescence. Understanding the mechanism of how E2 regulates the early promoter may be key to developing new therapeutic and prophylactic vaccines.

Here, we describe regulation of E2 through acetylation and possibly through direct interaction with a novel cellular interacting protein, RINT1. Histone acetyltransferase (HAT) proteins have been demonstrated to interact with Bovine Papillomavirus (BPV) and Human Papillomavirus (HPV) E2 proteins as well as enhance E2 dependant transcription luciferase reporter plasmid containing E2 binding sites. We demonstrate that HATs p300, CBP, and pCAF are limiting for E2 dependant transcriptional activation and that each protein functions independently. We have also identified that BPV-1 E2 is a substrate for acetylation by p300. Mutants of E2 that cannot be acetylated on lysines 111 or 112, display abnormal transcriptional phenotypes. Cells deficient in p300 display similar transcriptional defects that are intensified by CBP depletion. We propose that acetylation of BPV-1 E2 is necessary for transcriptional activation.

Acetylation generates a binding site through which a co-factor may interact via a bromodomain. Regulation of E2 dependent transcriptional activation through a post-transcriptional modification represents a novel method through which BPV-1 controls gene expression.

We also present evidence for a direct interaction between BPV-1 E2 and the cellular factor RINT1. This interaction does not appear to be critical for transcriptional regulation; however, several other functional pathways are indicated by the cellular complexes in which RINT1 functions. Some of these, such as ER/Golgi vesicular transport and hTERT independent telomere maintenance, are pathways in which E2 has no known role. Further investigation into regulation and consequences of E2 acetylation and the biological significance of the interaction between E2 and RINT1 could prove important in understanding the complex role of E2 in papillomavirus infection.

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List of Abbreviations

ANOVA	Analysis of variance
BPV	Bovine papillomavirus
Brd4	Bromodomain containing protein 4
C127	Papillomavirus negative mouse mammary tumor cell line
C127-A3	A derivative of C127 cells that are transformed with a BPV-1 genome that contains mutations within the E2 reading frame resulting in three serine to alanine mutations within the hinge region of the E2 protein. The phenotype of these mutations is higher E2 protein level in the cell due to the lack of phosphorylation induced destabilization.
C33a	A spontaneously immortalized cell line derived from cervical epithelia. These cells do not contain papillomavirus DNA.
CBP	CREB binding protein, a histone acetyltransferase similar to p300
ChIP	Chromatin immunoprecipitation
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
CoA	Coenzyme A
CREB	Cyclic AMP response element binding
CTD	Carboxy terminal domain
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
E2R	E2 repressor
E2RE	E2 response element
ER	Endoplasmic reticulum
EV	Epidermodysplasia verruciformis
GNAT	Gcn5-related N-acetyltransferases
HA	Hemagglutinin
HAT	Histone acetyltransferase
hCHLr1	Human CHL1 related 1, a human cohesion establishment factor that is involved in tethering BPV-1 DNA to host chromatin.
HDAC	Histone deacetylase
HPV	Human papillomavirus
hTERT	Human telomerase reverse transcriptase
ID13	A derivative of the C127 cell line that is transformed with wild type BPV-1 genome.
IP	Immunoprecipitation
LCR	Long control region
LMB	Leptomycin B

MME	Minichromosome maintenance element
MYST	A family of acetyltransferases named for the members MOZ, Ybf2/Sas3, Sas2, and Tip60.
NLS	Nuclear localization signal
NTD	Amino terminal domain
Ori	Origin, usually origin of replication
pCAF	p300 and CBP associated factor
pRB	Retinoblastoma protein
PV	Papillomavirus
QPCR	Quantitative real-time PCR
RINT1	Rad50 interacting protein 1
RKO	A colon carcinoma cell line that does not express the histone acetyltransferase p300
RPE-1	A retinal pigment epithelial cell line that has been immortalized by exogenous expression of the hTERT protein.
RTPCR	Reverse transcriptase polymerase chain reaction
Sf9	An insect cell line derived from *Spodoptera frugiperda* (Fall Armyworm) used for expression of recombinant proteins following baculovirus expression.
siRNA	Short interfering RNA
SNAP	Soluble N-ethylmaleimide-sensitive attachment protein
SUMO1	Small ubiquitin like modifier
SV40	Simian virus 40
TAD	Transactivation domain
t-SNARE	Target-associated SNAP receptor
URR	Upstream regulatory region
VLP	Virus like particle
v-SNARE	Vesicle-associated SNAP receptor

CHAPTER I

Introduction

Papillomaviruses and Human Disease

Papillomaviruses (PV) are small non-enveloped viruses that infect dividing cells in the basal epithelia. Viral genetic information is encoded on a double stranded circular DNA genome of between seven and eight kilobases (kb). Despite a small genome size, PVs exhibit a high level of diversity. 189 PV types have been identified as of a report in 2010 with 120 types infecting humans, 64 infecting non-human mammals, 3 infecting birds, and 2 types infecting reptiles (17). Virus types are categorized by sequence divergence of the L1, E6, and E7 reading frames (17, 201). Viruses with greater than 60% L1 nucleotide sequence identity are grouped into genera designated by Greek letters (17). Unique virus types are defined as having greater than 10% L1 sequence divergence from any other type (39). Human papillomaviruses (HPV) are clustered into five (alpha, beta, gamma, mu, and nu) out of the existing 29 genera (17). Each virus type exhibits high species and tissue specificity (31). It is thought that this specificity is due to viruses co-evolving with their hosts and that restriction may involve viral interactions with host specific factors (169). Transmission generally occurs from direct contact with infected tissues; however, PVs are relatively robust in the environment owing to a non-enveloped capsid. This is illustrated by their ability to retain 30% infectivity following dehydration for seven days (155) and thus transmission via inanimate intermediaries is possible.

Human papillomavirus (HPV) infection of epithelial tissue causes an abnormal proliferation of basal cells into the upper layers of the epithelium. This manifests

as a benign tumor or wart. Infection can take place in both cutaneous and mucosal tissue. Cutaneous warts are common in older children and young adults with prevalence of up to 33% in primary school children (195) and only 3.5% in adults (18). These lesions typically regress spontaneously due to immune clearance; however, in some cases they can persist for years if untreated.

Epidermodysplasia verruciformis (EV) is a rare autosomal recessive skin condition characterized by the abnormal susceptibility to cutaneous, beta-HPVs (most often HPV-5). Papillomavirus infection in EV patients results in persistent warts that progress to carcinomas in about half of the cases (90). EV is now thought to be due to a primary immunodeficiency (140). Homozygosity mapping of three families, led to mapping of a predisposition locus on chromosome 17q25.3 and subsequent identification of two adjacent genes (EVER1 and EVER2) that segregated with the disease (151). These genes were found to code for ER resident transmembrane proteins that complex with the ZnT-1 zinc transporter (111). The authors hypothesize that the susceptibility of EV patients to beta-PV infection is related to the E5 protein. They find that the EVER/ZnT-1 complex is inactivated by the HPV-16 E5 protein; beta-HPVs do not encode E5, and are restricted by the EVER/Znt-1 complex (111). The zinc imbalance caused by the lack of the EVER/Znt-1 complex in EV patients results in reduced production of proinflammatory cytokines (111) which may account for the high prevalence of carcinoma in HPV-5 EV associated lesions.

Clinical and subclinical infection of mucosotropic HPVs in the genital tract accounts for one of the most prevalent sexually transmitted diseases in United States and the world (9, 190). Viruses of this category are further subdivided into low risk and high risk groups based on the clinical severity of disease. Infection with low risk viruses produces genital condylomas or warts. These lesions, like cutaneous warts, are generally cleared by the host immune system. Infection with viruses of the high risk group also has a high probability of clearance; however, persistent infection with high risk viruses such as HPV-16, HPV-18, or HPV-31, may progress to a carcinoma. Further, persistent infection with high risk HPV types is the definitive cause of cervical carcinomas with greater than 99% of cases involving infection with HPV (198).

High risk HPV types are also found with high incidence with other mucosal cancers. Studies have found HPV associated with cancers of the oropharynx, anus, vulva, vagina, and penis at a prevalence of between 36% and 93% of all recorded cases (60). Of those associated with HPV, a much higher proportion of those cancers contained types HPV-16 and HPV-18 (86% to 95%). A higher prevalence than even cervical cancer (76%) (60). Several observations were made based on the prevalence and demographic data presented. First, the incidence of non-cervical HPV related cancers represents a higher proportion of total HPV related cancers in regions where adequate cervical screening procedures, such as Pap smear, are in place. Second, there appears to be equal incidence of HPV related non-cervical cancers in male and female

populations, and finally oropharyngeal cancers are more prevalent in men and make up a substantial population of the total HPV related cancers, second only to cervical (60).

In 2008 there were estimated to be 529,000 new cases of cervical cancer worldwide (52). This makes it the third most common cancer in women and the seventh overall. The number of diagnoses increased from the previous report; however, increased screening is probably the cause for this. 85% of the total incidence of cervical cancer was in developing countries and 88% of the total deaths from cervical cancer were also in these regions. There were 275,000 deaths worldwide which was equal to the number reported in 2002 (52). The staggering prevalence in the developing world is not unsurprising. The probability of infection increases with the number of sexual partners and incidence of cervical cancer is higher in regions where screening procedures are lacking or absent. Prior to development of screening programs in the 1960's and 1970's the incidence in Europe, North America, and Australia were as high as those in developing countries today (144).

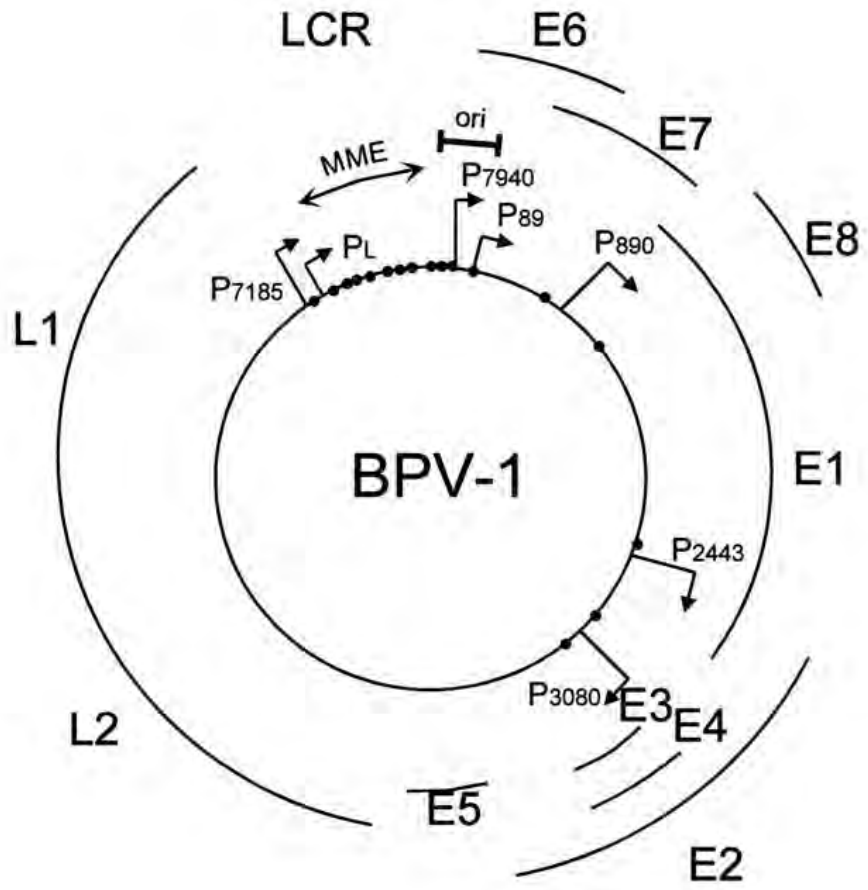
The recent release of two vaccines to HPV may result in a decrease in incidence in cervical cancer in all populations. Gardasil®, released by Merck was raised from virus like particles (VLP) from HPV types 6, 11, 16, 18 and Cervarix™, by Glaxo-Smith-Kline was raised against HPV types 16 and 18. Both vaccines performed well in the trials under the prescribed course demonstrating effective

prevention of lesions in immunized subjects for a period of four years (170). However, these vaccines have limited cross type prophylactic efficacy and are not effective therapeutically (160). Despite their shortcomings, the trial reports indicate that there would be a health benefit from vaccination, although this benefit would not be inexpensive. Each dose would cost $130 per injection or $390 for the entire series (170). This cost would mean that uninsured or under-insured individuals would most likely not be able to see the benefits from such a vaccine. Additionally, the associated cost and the availability for distribution would likely prevent a benefit in the developing world where it is most needed. The financial burden associated with distribution of the current vaccine and the ineffectiveness for treating infected patients therapeutically highlights the need for continued investigation into new therapies and treatments for HPV and cervical cancer.

Papillomavirus Infection

Papillomavirus genomes from different virus types are similar in their size as well as genomic organization. Each genome consists of an average of nine open reading frames encoding early genes (E1, E2, E4, E5, E6, E7, and E8) and late genes (L1 and L2) (Fig 1.1). These gene sets are grouped on opposite sides of the viral genome and are separated by approximately 1 KB of non-coding sequence known as the long control region (LCR) or upstream regulatory region (URR). The LCR contains several promoters controlling early and late gene expression, a constitutive transcriptional enhancer element, the viral origin of

Figure 1.1 Diagram of the BPV-1 genome BPV-1 genomic organization showing the early genes on the right side of the genome, and the late genes on the left. The intervening sequence is called the long control region (LCR), it contains several early promoters, the late promoter, a minichromosome maintenance element and the viral origin of replication. Figure from reference (173), used with permission.


LCR
E6
ori
E7
MME
P7940
P89
E8
PL
P890
P7185
L1
BPV-1
E1
P2443
P3080
E3
E4
L2
E5
E2

replication, and the minichromosome maintenance element. The icosahedral capsid consists of 72 L1 capsomers (11). The minor capsid protein L2 is present at one thirtieth the abundance of L1 and is largely internal (179). L1 alone is capable of forming VLPs; however, L2 is also incorporated upon its expression (72, 98, 216). Papillomaviruses infect the dividing cells in the basal epithelium. The virus is thought to gain access to these cells through wounding of the skin. Initial attachment is thought to be through heparin sulfate proteoglycans (HSPG) (62, 92). Studies have shown that attachment and infection can be blocked by treatment with heparinase, soluble heparin, or sulfated polymers such as carrageenans (28, 62, 92). Studies show that virion attachment is not on the epithelial cell surface but on the basement membrane that has been exposed through wounding (154). One hypothesis is that PVs have evolved to specifically bind to HSPGs on the basement membrane to allow preferential interaction with the dividing basal epithelial cells during wound healing. Attachment to the basement membrane exposes the capsid to cleavage by furin protease which cleaves the minor capsid protein L2 (153). Cleavage of L2 induces a conformational change in L1 that exposed a new region of L1 that interacts with a second receptor on the epithelial cells (161).

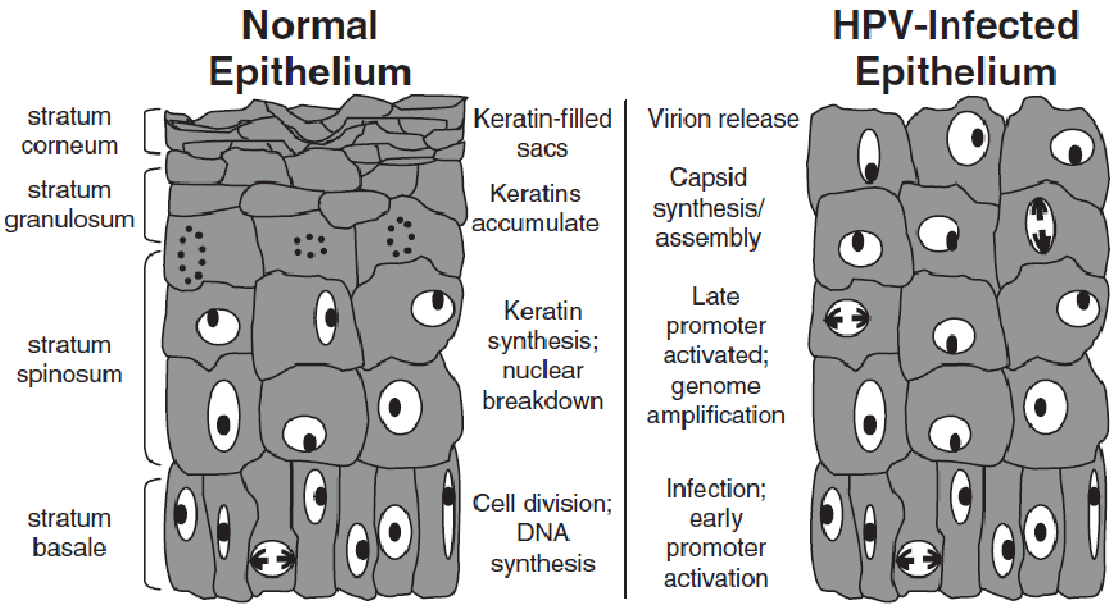
Following initial attachment to the basement membrane or basal epithelial cells, there is a long delay (12-24 hours) prior to detection of productive infection (36). There is a great deal of controversy about the mode of internalization between different virus types and even between different reports on the same virus type.

Studies have demonstrated that internalization of some viruses is clathrin dependent (23, 38, 78, 108), others enter via caveosomes (23, 175), others argue for a hybrid of these pathways (108). One recent study using siRNA mediated depletion and dominant negatives for both clathrin and caveolar-endocytosis claims that HPV internalization is independent of both clathrin and caveolin and might be internalized via a novel pathway involving tetraspanin-enriched microdomains (178). Internalization is followed by trafficking into late endosomal compartments (38) where L2 is required for endosomal escape (94). Finally, through a poorly understood mechanism, the viral genome and L2 are trafficked through the cytosol, probably by dynein microtubule motors, and into the nucleus (54, 164). Once in the nucleus L2 and genomes are localized to promyelocytic leukemia (PML) rich ND10 domains (37) where primary transcription commences.

Productive Virus Life Cycle

The papillomavirus life cycle is tightly linked to the differentiation program of the epithelial cells where infection occurs (Fig 1.2). As mentioned previously, initial infection takes place in the basal stem cell layer of the epithelium. These cells divide and become transit amplifying cells, which are capable of several more rounds of division prior to differentiation (200). These two groups of cells contain the only actively dividing cells within epithelial tissue. After an initial burst of early

Figure 1.2 The papillomavirus life cycle is linked to differentiating epithelia. Papillomaviruses infect through wounding to the basal epithelial stem cells. There, they replicate approximately once per cell cycle. As cells differentiate and move up into the stratum spinosum, E6 and E7 maintain the cell in a proliferative state which allows the virus to amplify its genome and activate late gene expression. Further up in the stratum granulosum, the capsids are assembled with genomes and in the stratum corneum, cells and virus are lost to sloughing. Figure from reference, used with permission (77).


Normal Epithelium
HPV-Infected Epithelium
stratum corneum
stratum granulosum
stratum spinosum
stratum basale
Keratin-filled sacs
Keratins accumulate
Keratin synthesis; nuclear breakdown
Cell division; DNA synthesis
Virion release
Capsid synthesis/ assembly
Late promoter activated; genome amplification
Infection; early promoter activation

gene expression, the virus begins initial genome amplification where it increases copy number to approximately 50 to 100 genomes per cell (93). It then continues expressing early proteins necessary for genome replication and the viral genome replicates approximately once per cell cycle as a stable extra-chromosomal element or episome (110). Viral early proteins E1 and E2 are both required for viral replication and E2 is necessary for episomal maintenance (69, 193). Replication of the BPV-1 genome *in vitro* requires only the viral E1 protein (20), although replication is more efficient with the addition of E2 (21). The roles of E1 and the E2 DNA binding regulatory factor in replication and maintenance will briefly be discussed here. The role of E2 in transcriptional regulation will be discussed at length elsewhere.

E1 protein is a nuclear phosphoprotein that encodes ATPase and helicase activity (24, 168). It specifically recognizes the viral origin of replication (194), although at low affinity. Efficient recruitment of E1 to the viral origin requires complex formation with the viral E2 protein which acts as a specificity factor (21). It is thought that the nonspecific DNA binding activity of the helicase domain necessitates E2. E2 interacts though its N-terminal domain to the helicase domain on E1. This interaction inhibits the ability of the helicase domain to interact with DNA (180). Following E2/E1 dimer association with the origin, additional E2/E1 complexes are added until an E1 tetramer is present. E2 is lost through an ATPase dependent step and the tetramer converts to a head to head

double trimer which begins to melt and unwind DNA. E1 continues polymerizing until a double hexamer forms on the origin (165).

The mechanism by which papillomaviruses maintain stable copy numbers in dividing cells is relatively unclear. It is known that papillomaviruses replicate by a "relaxed" random choice method, each genome is replicated on average once per cell cycle. The level of replication may take several factors into account, including cell specific factors and the expression level of E1 (80). Another factor may be the phosphorylation status of E1. Phosphorylation of E1 by cyclinA/cdk and cyclinE/cdk complexes is important for E1 localization (41, 82). Shuttling of E1 out of the nucleus during S-phase may control the level of replication (82).

In addition to replication of the genome and maintenance of genome copy number, segregation of the genome into daughter cells in mitosis also requires the E2 protein (87, 173). The mini-chromosome maintenance element, which consists of a series of six E2 binding sites within the BPV-1 LCR, is responsible for tethering the viral genome to the host cell chromosomes through E2 (147). Several groups have identified adapter proteins which serve to tether E2 to the host DNA. One report indicates that the double bromodomain containing protein Brd4 may be such a protein (210). A subsequent report from our group demonstrates that the cellular chromatid cohesion establishment factor hCHLr1 interacts with several E2 proteins from HPV types 11 and 16 as well as with BPV-1 and tethers BPV-1 genomes to the host chromosomes (143). This

indicates that interaction of E2 with chromosomes is complex and may not consist of a one to one interaction with one adaptor protein. Further, several different adaptors may be used to tether different viruses. One report indicates that HPV type E2 proteins may localize to the spindles instead of the chromosomes during mitosis (196).

Transit amplifying cells are committed to differentiation. After several more divisions, one daughter cell moves upward from the basal into the suprabasal cell layers (200). Here, cells withdraw from the cell cycle and begin to differentiate (200). Papillomaviruses do not encode a DNA polymerase or any other replicative factor. As the cell begins to differentiate and withdraw from the cell cycle, the viral oncoproteins, E6 and E7, are required to induce the cell to continue proliferating and expressing factors that are needed for viral genome replication (53, 58).

The main function of the HPV E7 protein in the productive viral life cycle is the binding and degradation of pRB and its related pocket protein family members p107 and p130 (48, 163). Interaction with pRB is mediated through the LxCxE motif in conserved region 2 of E7 (32). pRB and its family members regulate cell cycle progression through their interaction with the E2F family of transcription factors. E2F proteins activate transcription of many genes that control cellular DNA replication. During normal cell cycle progression, pRB is phosphorylated by cyclin/cdk complexes at the beginning of S-phase. This phosphorylation results

in the release of E2F which is then able to activate S-phase genes (49, 201). In the context of HPV infection in differentiating tissue, cells are withdrawing from the cell cycle and pRB is no longer phosphorylated. E7 abrogates this need through degradation of pRB inducing cell cycle reentry (32). In addition to degradation of pRB, an E7 interaction with histone deacetylases (HDAC) has been shown to increase transcriptional activation of E2F targets (125). E7 interaction with cyclin/cdk2 complexes (129, 189) and the cyclin dependent kinase inhibitors (CKI) $p27^{kip1}$ (211) and $p21^{cip1}$ (55) all promote cell cycle progression.

The major function of the papillomavirus E6 protein is primarily to bind and degrade the tumor suppressor p53 (159). Aberrant reentry into the cell cycle in the absence of mitogens and signaling causes cellular stress which increases levels of p53 and induces p53 dependent cell cycle arrest or apoptosis (81, 142). Expression of E6 counteracts this response. E6 forms a complex with p53 and a cellular E3 ubiquitin ligase, E6AP (84). E6AP, which does not interact with p53 in the absence of E6, induces its poly-ubiquitination and subsequent proteosomal degradation (83, 85). Another important role for E6 is the activation of telomerase through its catalytic subunit hTERT (99, 131). E6 can interact with the cellular transcription factor c-myc and recruit it to the hTERT promoter increasing hTERT expression (197). Chromosomal telomeres shorten on every cell division eventually resulting in cellular senescence (123). Activation of

telomerase through E6 abrogates this process extending the life of the dividing cells.

While expression of E6 and E7 is beneficial to the replication of papillomaviruses in differentiating cells, these two proteins are the primary causes of cancer in HPV infected cells. Expression of E6 and E7 is controlled by the viral regulatory factor E2 (through a mechanism discussed in a later chapter). In carcinomas, the HPV genome is often integrated into the host DNA (35). This occurs in genetically fragile areas in host chromosomes (188) and generally results in disruption of the E2 open reading frame (91, 157). Integration and loss of E2 expression results in increased E6/E7 mRNA stability and loss of E2 mediated inhibition of immortalization (91, 157).

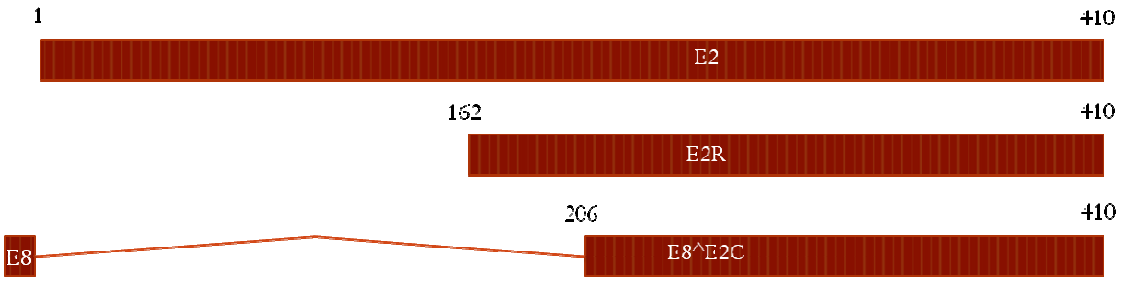
As cells progress upward through the stratum spinosum, differentiation triggers the viral late promoter which induces expression of the capsid proteins L1 and L2. The virus also transitions from maintenance replication mode to vegetative genome amplification. In the stratum granulosum cells undergo nuclear envelope break down and the virus assembles capsids and packages the genome. Finally, in the superficial layer of the stratum corneum cell function ceases and the cells and virus are sloughed off with environmental contact (77, 86, 124).

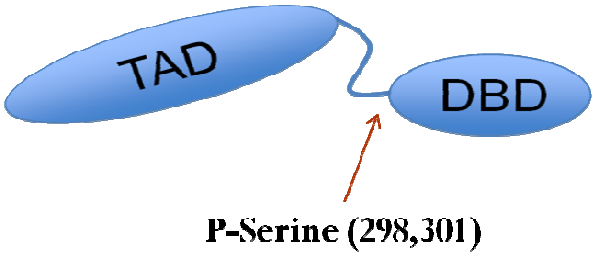
Viral Transcription and the E2 Protein

Like replication, transcription of the viral genome depends upon the differentiation state of the infected epithelial cell. It also depends upon the presence of a variety of cellular transcription, splicing and RNA processing factors (124). Gene expression from the early promoter is controlled on a basal level by a number of cellular transcription factors including TFIID, Sp1, Oct1 and AP1 (34, 73, 158, 186). Papillomavirus transcription is relatively complex, owing to multiple promoters, and multiple and alternate splicing pattern of each transcript. Seven promoters and more than 20 polycistronic mRNAs have been identified in BPV-1 transformed cells (2, 10). Each transcript has potential to code for multiple proteins; however some expressed proteins are undetectable in cells. In addition to binding sites for cellular transcription factors, the LCR also contains specific sites for the viral E2 protein.

The papillomavirus E2 protein is a regulatory factor that is involved in every stage of the productive viral life cycle (50, 124). The E2 open reading frame of BPV-1 encodes a 48 kDa phosphoprotein (106). This protein contains two functional domains (Fig 1.3). The N-terminal 216 amino acids of BPV-1 E2 represent the transactivation domain (TAD) (61). This domain is responsible for most of the protein interaction between E2 and other viral and cellular factors. The functional consequence of many of these interactions and the mechanisms of E2 dependent transcriptional regulation will be discussed in a later chapter. The C-terminal 124 amino acids represent the DNA binding domain (4, 61). Between

Figure 1.3 Diagram of BPV-1 E2 domain organization. The BPV-1 E2 protein is approximately 48 kilodaltons and contains 3 domains. An N-terminal transactivation domain and a C-terminal DNA binding and dimerization domain separated by a flexible hinge region. Two shorter forms are expressed which contain the C-terminal domain but a shortened, non-functional N-terminal domain. Two phosphorylation sites at serines 298 and 301 are present in the hinge region.





P-Serine (298,301)

the two domains is a relatively unstructured and flexible hinge region (61). Through interaction with DNA and cellular factors, E2 can either activate or repress the viral early promoter including its own expression (128). In addition to full length E2, two other forms of E2 are found in transformed cells. These shorter proteins, called E2 repressor (E2R) and E8^E2C are 31 and 28 kDa respectively. E8^E2C is an alternative splice product containing eleven amino acids from the E8 reading frame spiced to the 204 amino acids from the C-terminus of E2 (106). E2R is transcribed from a start site at within the E2 reading frame and encodes amino acids 162-410 (107). Both of these proteins lack a complete N-terminal TAD and negatively regulate the viral early promoter.

E2 interacts through its DNA binding domain with the specific sequences (ACCGN4CGGT) within the viral LCR (4). One of the major differences between BPV-1 and HPV type genomes is the number and location of these sites within the genome. BPV-1 contains 17 total E2 binding sites, of which 12 are located within the LCR (120) whereas HPV-16 encodes only four sites. The E2 protein binds to DNA as a dimer (128, 149) which primarily occurs through its C-terminal DNA binding domain, although dimerization has been reported in the TAD as well (100). N-terminal dimerization is thought to induce DNA looping in order to stabilize distal enhancer elements within the initiation region (5). E2R and E8^2C which contain intact DNA binding domains may also interact with and compete for binding site occupancy with full length E2. E2 is also capable of forming

heterodimers with either of the two shorter forms resulting in a repressive complex (46, 135).

E2 function appears to be regulated by post-translation modification. Specifically, phosphorylation of BPV-1 E2 has been reported to have a number of effects on its roles in the viral life cycle. Inhibition of phosphorylation on serine 301 by mutation to alanine induces replication to levels 20 fold higher than wild type E2 (127). Another report demonstrated that while mutation of several phosphorylation sites increased replication in transient assays, mutant genomes were defective for transformation and stable maintenance (118, 119). More recent evidence indicates that phosphorylation does not affect the interaction with mitotic chromosomes (14). Phosphorylation of serine 301 by casein kinase II reduces the overall stability of E2 causing it to be targeted for proteosomal degradation (59, 146). HPV-16 E2 has been demonstrated to be modified by the small ubiquitin like modifier 1 (SUMO1) protein. Mutation of the sumoylation site on E2, K292, resulted in decreased levels of transcriptional activity (205). It was later shown that increases in overall cell sumoylation levels coincided with increase in E2 protein level (204). This was shown to be through inhibition of ubiquitination and thus proteosomal degradation. The authors hypothesize E2 will be stabilized in differentiating tissue where host sumoylation levels are increased (204).

Preface

A portion of this work will be submitted for publication

Acetylation of BPV-1 E2 on a Conserved Amino Terminal Lysine is Necessary for Transcriptional Activation

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CHAPTER II

The Role of Histone Acetyltransferase Proteins in BPV-1 E2 Dependent Transcriptional Regulation

Introduction

Acetylation and Histone Acetyltransferase Proteins

Protein acetylation is one of the most common modifications in eukaryotes. Co-translational N^{α}-terminal acetylation occurs in 85% of eukaryotes with varied outcomes (148). Less common, but more biologically important, is post-translational acetylation of the ε-amino group of internal lysine side chains. Lysine acetylation, which was initially described with respect to acetylation of histone tails, was thought to generally increase gene expression (3, 76). This led to the idea that the reduction of the positive charge through lysine acetylation decreased the electrostatic interaction between histones and DNA leaving the DNA more accessible for transcription factors to bind (97). The consequences of histone acetylation proved to be more complex; subsequent experiments demonstrated increases in specific lysine acetylation on histones in regions of both transcriptional inactivity and hyperactivity (192). These results indicated that acetylation of specific lysine residues regulated gene expression rather than a net reduction of positive charge.

Further advances were made following identification of histone acetyltransferase proteins. The yeast co-activator Gcn5 was identified as an acetyltransferase in 1995 following development of an assay to detect acetyltransferase activity (27). Subsequently, many known co-activator proteins were found to also encode acetyltransferase activity including p300/CBP, the p300/CBP associated factor

(pCAF), $TAF_{II}250$, which is a subunit of the transcriptional initiation factor TFIID, and Tip60 (12, 132, 141, 208). Acetyltransferase proteins are classified into two major families based on similarities in their catalytic domains. MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) family acetyltransferases, which contains Tip60 are characterized by their signature MYST domain (184), are reported to be involved in long-range/chromosome wide gene regulation. The GNAT super family (Gcn5-related *N*-AcetylTransferase) of acetyltransferases is characterized by the presence, to varying degrees, of four conserved motifs (139); motif A is the most conserved and is responsible for acetyl-CoA recognition and binding (47, 203). The remaining HATs that do not have common structural motifs, including p300/CBP, are grouped into an orphan category (97).

It was noted early that acetylation of histone substrates appears to be non-random (33). Some specificity is directly related to the ability of the HAT's catalytic domain to recognize specific context around acetylated lysines. Analysis of the sequence around acetylated lysines of histone tails led to development of a two step classification (95). First, acetylation sites are grouped into three classes determined by the residue immediately preceding the lysine. Second, each class is further subdivided into two groups determined by sequence similarity of the remaining four flanking residues. The recognition of lysines by each HAT is based on the ability of the HAT to recognize a created surface around the lysine and not necessarily a specific consensus sequence

(95, 97). This may explain the overlapping but not identical sequence preference for p300/CBP and Tip60 (96).

HATs are generally found in complex with other proteins. The function of the catalytic subunit its substrate specificity may be dependent upon the other subunits in the complex. Some catalytic domains of HATs are unable to acetylate substrates unless they are in complex. Specifically, Gcn5 is unable to acetylate histones efficiently in nucleosomal context; however, this is not the case in the multi-protein Gcn5 containing complexes such as SAGA and ADA (67). Complex subunits also confer increased specificity. A model has been proposed that builds upon the two step classification outlined above. While, the two step method identifies the potential substrates *in vivo* based on substrate sequence context (95), the allocation model narrows the specificity using different complex subunits (95). Subunits also commonly encode chromatin binding domains such as bromodomains or Tudor domains among others. These domains recognize acetylated lysine and dimethylated arginine respectively (117). Through the interaction of these domains with modified chromatin, acetyltransferase complexes are recruited to specific areas of promoter or enhancer regions where its acetyltransferase activity is required. The phenomenon of post-translational modification of histones followed by interaction of non-histone proteins specifically to the modified regions became known as the histone code hypothesis; recent evidence has expanded upon this indicating that the code is more like a language (116, 181, 191). Specific

modifications do not simply turn genes on or off; rather, the combinatorial effect of multiple modifications in context regulates gene expression.

In addition to gene regulation through the acetylation of histones, there is mounting evidence that acetylation of non-histone proteins including transcription factors is equally important. The discovery that the tumor suppressor p53 is a substrate for p300/CBP demonstrated the possibility for acetylation of non-histone substrates (71). Subsequent work has identified a number of lysines on p53 that are substrates for p300/CBP, pCAF, and Tip60 (104, 183). Acetylation of specific lysines by different HATs has varied effects on p53 biology. Acetylation of p53 on six C terminal lysine residues (K370, K372, K373, K381, K382 and K386) correlates with increased p53 stability and transcriptional activity (51, 104). Acetylation of K320 on p53 by pCAF favors cell survival by inducing p21 mediated cell cycle arrest (101, 104). In contrast, acetylation of K120 by Tip60 promotes apoptosis (183).

Many other non-histone proteins have been found to be acetylated. Cellular transcription factors, nuclear factors, DNA damage response proteins, and structural proteins have all been shown to be acetylated with various effects on their DNA binding ability, sub-cellular localization, half-life, or their ability to interact with other proteins (63). Interestingly, several viral proteins have also been demonstrated to be regulated by acetylation. The adenovirus E1a 12s protein has been shown to be acetylated by p300/CBP or pCAF on lysine 239,

directly adjacent to its C-terminal binding protein (CtBP) interaction motif (214). Initially, acetylation of this residue was initially thought to specifically inhibit E1a interaction with CtBP (214). It has subsequently been found that it may do so more subtly by destabilizing the molecular interaction surface resulting in a reduction in binding affinity not inhibition of interaction (133). Additionally, acetylation of E1a inhibits its interaction with importin-α3 which enriches acetylated E1a in the cytosol (126). SV40 large Tumor antigen (LTag) has been shown to be acetylated on lysine 697 by CBP (171). Acetylation at this site which is adjacent to the C-terminal Cdc4 phospho-degron (CPD) resulted in the destabilization of LTag (171).

Papillomavirus E2: Activated Transcription and Repression

The role of E2 in viral genome replication and episomal maintenance has been discussed previously. Its specific roles in the regulation of viral transcription are less well understood. While the bovine papillomavirus (BPV) early promoter can and is activated by cellular factors, transcriptional activation is enhanced through expression of the E2 protein. Transfected E2 increases transcriptional activation of BPV promoter elements and artificial reporters containing E2 binding sites in enhancer configuration (176, 177). The BPV-1 LCR has 12 E2 binding sites, four of which are upstream of the major early promoter (p89) and are known as E2 response element 1 (E2RE1) (176). The full length E2 protein strongly activated the promoter through this enhancer element. E2 can activate other promoters within the BPV LCR through nearby E2 binding sites as well. In contrast, the E2

protein is capable of repressing HPV promoters such as those from HPV-16 or 18 that contain only 4 E2 binding sites (16, 185, 187). This repression is thought to be due steric hindrance caused by the proximity of an E2 binding site to the TATA box. E2 bound to this site inhibits formation of the transcriptional preinitiation complex (40, 44, 45). Residues important for the transcriptional activation function of E2 are also required for repression of the early promoter (66). Repression of HPV promoters in cervical carcinoma cell lines result in growth arrest and senescence due to reactivation of the p53 and pRB tumor suppressor pathways (64, 65, 202).

The specific mechanisms by which full length E2 activates and represses the early promoter have not yet been fully characterized. E2 does not have intrinsic enzymatic activity. Therefore, it must act through interactions with cellular transcription factors, co-activators. Many groups have reported interactions of cellular factors with BPV-1 E2 and a variety of HPV type E2 proteins. TFIID, and TFIIB, Sp1 have been shown to interact with E2 and are critical for enhancement of transcription by E2 (152, 158, 209). A functional interaction with these factors indicates a link between basal and E2 activated transcription. E2 has been shown to functionally interact with other cellular factors to regulate transcription. Over expression of the cellular replication and DNA damage response factor TopBP1 is demonstrated to increase transcription and transient replication in cell culture (19). Later it was reported that depletion of TopBP1 redistributes E2 into higher molecular weight nuclear complexes. The authors claim that TopBP1

tethers HPV-16 E2 and the genome to chromatin (43). poly (ADP-ribose) polymerase 1 (PARP), which is also involved in DNA damage repair, is shown to interact with HPV-18 E2 CTD and enhance its transcriptional activity. This interaction augments the DNA binding of E2 *in vitro* (114).

The double bromodomain protein Brd4, which has been shown to be involved in genome maintenance (210), is also a critical factor for E2 dependent transcription and has been extensively studied in this regard. Mutants of E2 that are incapable of interacting with Brd4 are also transcriptionally defective (167). Transient replication of BPV-1 genomes and transcription of BPV-1 reporters were diminished in mouse C127 cells transfected with a C-terminal domain fragment of Brd4 (88). This fragment is the E2 binding region and competes with full length Brd4 for interaction. Similar results were demonstrated for HPV-16 E2 in C33a cells, although in this human cell line CTD expression had no effect on replication of an ori containing plasmid (166). Additionally, siRNA mediated depletion of Brd4 and transfection of E2 mutants incapable of interacting with this protein resulted in decreased E2 dependent transcriptional activation (166, 167). The interaction between E2 and Brd4 appears to stabilize E2 (56, 112, 215). This may explain some of the transcriptional deficiency in the absence of Brd4. Brd4 is a key factor in the pTEF-b complex which facilitates promoter escape for the RNA polymerase II complex (217). One recent report has indicated that disruption of this interaction by E2 may play a role in mediating transcriptional

repression (207). It is not yet clear if pTEF-b plays a role in E2 dependent transcriptional activation.

E2 also has been demonstrated to interact with the SWI/SNF related chromatin remodeling ATPase Brm (105). This interaction is shown to increase E2 activity specifically on chromatinized templates and increase loading of E2 onto DNA containing E2 binding sites. Two cellular factors: Tax1BP1 (199) and Gps2 (also known as AMF-1) (26), were identified as E2 interacting partners by yeast two-hybrid screen and both have been demonstrated to enhance transcriptional activation in an E2 dependent manner. Tax1BP1 stabilizes E2 protein level which may at least partially contribute to transcriptional enhancement (199). However, the enhancement of E2 dependent transcription by Tax1BP1 and the enhancement observed following co-transfection of Gps2 is likely related to higher order complex formation with the cellular acetyltransferase p300 (145, 199).

p300, CBP, and the GNAT family member pCAF have been shown to interact directly with E2 from BPV-1, and HPV types 16, 18, 11, and 6b (113, 115, 137, 145). These proteins have also been demonstrated to enhance E2 dependent transcription (103, 113, 115, 137, 145). p300, co-expressed with low amounts of HPV-16 E2, can enhance transcriptional activity from the HPV-16 promoter, which is generally repressed in the presence of E2 (103). Gps2 strengthens the interaction between BPV-1 E2 and p300 and the presence of Gps2 increases the

amount of purifiable acetyltransferase activity in co-immunoprecipitations of p300 with E2 (145). CBP was demonstrated to interact with the N-terminal transactivation domain of HPV-18 E2 via its N-terminal KIX domain, which is also the CREB binding region of CBP (115). Over-expression of the CBP KIX domain alone fused to the VP16 activation domain is capable of enhancing E2 dependent transcription (115). Finally, pCAF is known to interact with HPV 6b, 11, 16, and 18 E2 through its N-terminal 390 amino acids (113). Enhancement of E2 dependent transcription by pCAF cooperates with expression of CBP (113). Activation in the presence of both HATs is greater than that observed with either alone. The authors suggest that the two synergize and that pCAF acts in a pathway distinct CBP to activate transcription through E2 (113). The acetyltransferase domains of CBP and pCAF are required for E2 dependent enhancement and acetyltransferase defective mutants of both of these proteins are unable to enhance transcription (113, 115).

The interactions of p300, CBP, and pCAF with E2 and the requirement for acetyltransferase activity suggest a role for these proteins in acetylation of histones in the early promoter region or E2 acetylation in the regulation of E2 dependent transcription. We investigated the requirement of p300, CBP, and pCAF for E2 dependent transcription. Further, we explored the possibility that E2 is acetylated and the biological significance of such a modification. We show that E2 is acetylated by p300 and that the potential for modification on a conserved N-terminal lysine correlates with transcriptional activation and retention of E2 in

the nucleus. These data present evidence for the direct acetylation of E2 as a novel method of regulating its transcriptional activity.

Materials and Methods

Antibodies Primary antibodies used for immunofluorescence, immunoblot, and immunoprecipitation include: BPV-1 E2 B201, Santa Cruz p300 N-15 (sc-583), CBP A-22 (sc-369), pCAF E-8 (sc-13124), Cell Signaling Technology acetyl-lysine (9441) and Sigma Aldrich Actin (A-2668). Secondary antibodies for immunofluorescence include Alexafluor 488 and 555 (Invitrogen), and for immunoblot include horseradish peroxidase conjugates (Jackson Labs).

Cells and Transfections RPE-1 cells were cultured in a 1:1 mix of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 Medium (Invitrogen). RKO cells, C127, ID13 cells, and C127-A3 were cultured in DMEM (Invitrogen). ID13 and C127-A3 are both transformed with BPV-1 genomes, the latter containing three mutations within the E2 reading frame leading to increased E2 protein level (119). All cell culture medium was supplemented with penicillin/streptomycin solution (Invitrogen) as well as 10% fetal bovine serum (Atlas Biologicals). Transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's specifications. All DNA, siRNA, and transfection reagents were diluted in Optimem serum free medium (Invitrogen) in the absence of antibiotic.

Chromatin Immunoprecipitation Cells were plated onto 10cm culture dishes, for endogenous immunoprecipitation, cells were prepared the following day for chromatin immunoprecipitation (ChIP) according to protocol (Upstate Cell

Signaling/Millipore), otherwise cells were transfected the following day and prepared for ChIP 24 hours post transfection. Briefly, cells were cross-linked with 1% Formaldehyde (Sigma Aldrich) for 10 minutes at 37°C. Cross-linked cells were then lysed in 1% SDS. Lysates were disrupted using a Branson S-450D sonifier equipped with a microtip probe. Lysates were subjected to five, five second pulses at 30% amplitude alternated with 30 second rests. Following sonication, lysates were diluted to 0.1% SDS and aliquoted for immunoprecipitation with several antibodies. 1% of the original lysate was reserved for input. Antibodies were captured using magnetic Dynabeads (Invitrogen). Following successive washes with Low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 500mM NaCl), LiCl wash buffer (0.25M LiCl, 1% NP-40, 1% deoxycholic acid, 1mM EDTA, 10mM Tris-HCl pH 8.0), and TE (10mM Tris-HCl pH 8.0, 1mM EDTA), DNA-protein complexes were eluted from beads using 1% SDS and cross-links were reversed by adding NaCl to a final concentration of 0.2M and heating samples to 65°C for no less than four hours. DNA was then purified using QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol. Purified samples were amplified by PCR using BPV-1 LCR primers (Table 2.1) and separated on 1% agarose gel.

Immunofluorescence Cells were plated on collagen coated coverslips (BD Biosciences) and transfected the following day with expression plasmids. 24

Table 2.1 RNA and DNA Oligonucleotide Sequences

Type	Name	Sequence	Purpose
siRNA	Human p300	CAGAGCAGUCCUGGAUUAG	Depletion
siRNA	Mouse p300	Ambion Catalog # s116226	Depletion
siRNA	Human CBP	AAUCCACAGUACCGAGAAAUG	Depletion
siRNA	Mouse CBP	Ambion Catalog # s64378	Depletion
siRNA	Human/Mouse pCAF	UCGCCGUGAAGAAAGCGCA	Depletion
DNA	BPV-1 LCR Sense	AAAGTTTCCATTGCGTCTGG	ChIP
DNA	BPV-1 LCR Antisense	GCTTTTTATAGTTAGCTGGCTATTTT	ChIP
DNA	BPV-1 E6 Sense	ATGGACCTGAAACCTTTTGC	QPCR
DNA	BPV-1 E6 Antisense	CAGCCTTCCCGAATTACAAC	QPCR
DNA	BPV-1 E2 Sense	AGGCTGGGGCTGACGGAACT	RTPCR
DNA	BPV-1 E2 Antisense	GCTCTGATGGGACCGCAGGC	RTPCR
DNA	p300 Sense	CTTTTACATGCTCACAAGTGCCAGC	RTPCR
DNA	p300 Antisense	GCTAGGGTTTCCAAGCCCAACT	RTPCR
DNA	CBP Sense	TCCCAAGCACTGAATCCACAAGCA	RTPCR
DNA	CBP Antisense	TCAAGGTCTCCGCCAGCACA	RTPCR
DNA	pCAF Sense	GAAGGCGCAGTTGCGCTCTGCT	RTPCR
DNA	pCAF Antisense	GCAAGGGCATGGCTACAGCTTCGAC	RTPCR
DNA	Beta Actin Sense	GGGAAATCGTGCGTGACATTAAG	QPCR
DNA	Beta Actin Antisense	TGTGTTGGCGTACAGGTCTTTG	QPCR

hours after transfection, cells were fixed using 3.7% paraformaldehyde (Electron Microscopy Sciences) diluted with phosphate buffered saline (PBS) for 30 minutes, and permeabilized using 0.2% Triton X-100 diluted with PBS for 10 minutes. Cells were then blocked overnight in 5% normal goat serum (Invitrogen), 1% bovine serum albumin (BSA), and 0.05% Triton X-100 in PBS and then incubated for one hour each with primary and then secondary antibodies diluted in blocking buffer. Coverslips were washed three times with 0.05% Triton X-100 in PBS following both primary and secondary antibody incubations. Coverslips were mounted on glass slides using ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cells were then visualized and images were collected using a Leica TSC SP2 AOBS confocal microscope using Leica Imaging Software.

Immunoprecipitation and Immunoblot RPE-1 cells were plated on 10 cm tissue culture dishes. The following day cells were transfected with a combination of pCG vector, E2, or Gps2s. 24 hours post transfection, cells were harvested by rinsing with phosphate buffered saline (PBS), scraping into a small volume of PBS for transfer to an Eppendorf tube. Cells were lysed using IPLB (50mM Tris-HCl pH 8.0, 0.1mM NaCl, 20mM NaF, 10mM KH_2PO_4, 1% Triton X-100, 10% Glycerol, 0.1mM DTT, 2mM PMSF, and Complete protease inhibitor cocktail Roche). Lysates were clarified by centrifugation at 15,000 x g for ten minutes. Lysates were the diluted 1:1 with IPBB (50mM Tris-HCl pH 8.0, 0.1mM KCl, 0.1mM EDTA, 0.2% NP-40, 0.5 µg BSA, 2.5% Glycerol, 0.1mM DTT, 2mM

PMSF), specific antibodies were added and complexes were formed with gentle agitation overnight at 4^{o}C. Protein A or Protein G sepharose beads (Invitrogen) were added the following day and complexes were collected for 1 hour. After extensive washing with IPWB (0.1mM Tris-HCl pH 8.0, 0.1mM NaCl, 0.2% NP-40, 0.1mM DTT, 2mM PMSF) complexes were separated by SDS-Polyacrylamide Gel Electrophoresis (PAGE) and membranes were probed for target proteins. For each individual lysate, 5% of the total volume used for each immunoprecipitation used was reserved for use as input protein. This sample was separated by SDS-PAGE with the immunoprecipitated complexes for reference of protein expression.

Cells for immunoblot analysis were plated onto six-well dishes, transfected the following day with either expression plasmid or expression plasmid and siRNA. 24 hours following transfection, or 48 hours following siRNA transfection, cells were lysed using 2% SDS in 50mM Tris-HCl pH 8.0 and 1mM dithiothreitol (DTT). Lysates were then homogenized by passing through a QIAshredder spin column (QIAGEN) and protein concentration was estimated using BCA protein assay (Thermo Scientific). Equal amounts of protein were loaded and separated by SDS-PAGE. Gels were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore), blocked and then probed with specific antibodies. Signal was detected by chemiluminescence (Thermo Scientific).

***In Vitro* Acetylation Assay** 200ng of protein substrate was mixed with 50ng of acetyltransferase in a 10 µL reaction containing 40mM Tris-HCl pH 8.0, 75mM potassium chloride (KCl), and 10 µM Acetyl CoA (Sigma Aldrich). Each reaction was held a 30^{o}C for one hour and stopped by the addition of SDS-PAGE sample buffer and increasing the temperature to 95^{o}C for five minutes. Proteins were then resolved by SDS-PAGE, transferred to PVDF membrane, and probed for acetylated substrates using an anti acetyl-lysine antibody (Cell Signaling Technology). Duplicate samples were stained for total protein using Coomassie Brilliant Blue R250 (Fischer). Substrates tested included BPV-1 E2, p53, and histone protein purified from HeLa cells, acetyltransferases included p300, pCAF, and Gcn5. Gcn5, p53, and BPV-1 E2 were purified from bacteria, pCAF and p300 were purified from baculovirus infected Sf9 cells, and histone proteins were purified from HeLa cells.

Luciferase Reporter Assay Cells were plated onto six-well dishes and transfected in triplicate the following day with luciferase reporter, expression plasmids, and siRNA. Cells were lysed 24 hours after transfection or 48 hours after siRNA transfection by directly applying Reporter Lysis Buffer (Promega) to each well and submitting lysates to one freeze thaw cycle. 30 µL of each lysate was added into duplicate wells of a CulturPlate-96 (Perkin Elmer). 100 µL of Luciferase Assay Reagent (Promega) was added to each well just prior to reading on an EnVision Multilabel Plate Reader (Perkin Elmer). Data was averaged from duplicate wells and means were calculated from each

experimental triplicate. Means were averaged from at least 4 independent experiments and error bars represent the standard error of the mean (SEM). Wild type E2 transcriptional activation in the presence or absence of control siRNA was set at 100% and each experimental value was calculated as a percentage of wild type or control. One-way ANOVA with either Dunnet's or Bonferroni's post-hoc analysis was performed with GraphPad Prism version 5.01 for Windows.

Mass Spectrometry and Data Analysis BPV-1 E2, acetylated *in vitro*, was resolved by SDS-PAGE and the gel was then stained with Coomassie Brilliant Blue R250 (Fischer). The band corresponding to BPV-1 E2 was excised, de-stained and prepared for digestion. The band was then split and digested with trypsin, chymotrypsin, or GluC overnight at 30^{o}C. Peptides from each sample were injected using a NanoAquity Auto Sampler (Waters, Inc.) onto a Symmetry C18 trapping cartridge (Waters, Inc.). Peptides were separated by in-line gradient elution onto a 75 µm internal diameter × 10 cm column packed with BEH 130 stationary phase (Waters Inc), using a linear gradient from 3% to 90% solvent B where A= 2% acetonitrile and B= 98% acetonitrile both containing 0.1% formic acid and 0.01% trifluoroacetic acid. During the gradient elution, data dependant scans were performed with 8 scan events per cycle consisting of one full MS from m/z 400 – 2,000 followed by product ion scans (collision induced dissociation= 35%) on the 10 most intense ions in the full scan. Precursor ions used for product ion scans were dynamically excluded for 30 seconds. Proteins

were identified from the product ion spectra using the SEQUEST (Thermo Scientific) and X!Tandem search engine across the entire Swiss-Prot database (7), the results from each search were combined using Scaffold v3.00 (Proteome Software, Portland, Oregon, USA).

Plasmids and siRNAs pGL2-E24BS contains 4 E2 binding sites up stream of an SV40 promoter which drives luciferase expression. Expression plasmids used include pCG-E2, pCMV β-p300, pRSV-CBP, and pCI-pCAF. All E2 lysine to arginine mutants were generated with the Quickchange II site directed mutagenesis system (Agilent Technologies) using pCG-E2 as a template. ΔNco1 BPV-1 genome does not express E2 resulting from a deletion within the E2 reading frame (143). siRNAs directed toward p300, CBP, and pCAF were purchased from Ambion (mouse p300 and CBP catalog numbers s116226 and s64378, all other siRNAs were custom designed. Sequences are presented in table 2.1).

Quantitative Real-time PCR and RT-PCR Purified DNA for quantification of ChIP assay was added in triplicate to wells of an iQ 96-well real time PCR plate (Bio-Rad) and amplified using a Mastercycler ep *realplex* thermal cycler (Eppendorf) with BPV-1 LCR primers listed above. A four point, tenfold dilution series was prepared from input DNA of each individual lysate for a reference curve. Ct values of each immunoprecipitated DNA sample were then compared to the appropriate reference curves and calculated as a percentage of input DNA.

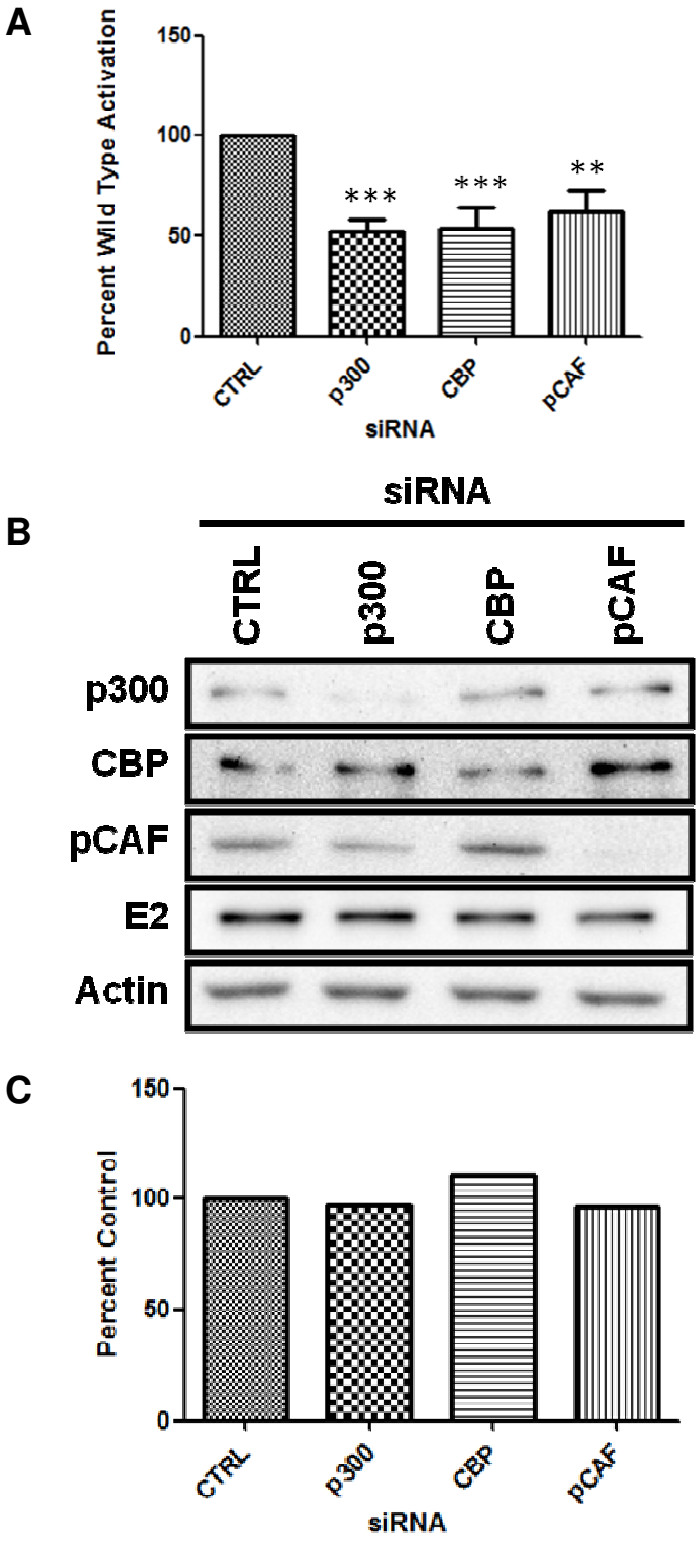
RNA for analysis of gene expression was purified by RNeasy kit (QIAGEN). RNA was then reverse transcribed using ImProm-II reverse transcription system (Promega). cDNA was added in triplicate to wells of an iQ 96-well real time PCR plate (Bio-Rad) and amplified using a Mastercycler ep *realplex* thermal cycler (Eppendorf) with E6 and beta actin primers (Table 2.1). Primer efficiencies were obtained by performing a standard curve titration and then used to relate the Ct values by the Pfaffl method. Two-way ANOVA was performed with Bonferroni's pos-hoc analysis using GraphPad Prism version 5.01 for Windows. Depletion of each HAT was confirmed by performing RT-PCR analysis on identical template cDNA using oligos listed in table 2.1.

Results

p300, CBP, and pCAF are Necessary for E2 Dependent Transcription

Over expression of histone acetyltransferase (HAT) proteins, specifically p300, CBP, and pCAF, has been reported to enhance E2 dependent transcription (113, 115, 137, 145); however, little is known of the biochemical consequences of these protein interactions, and specifically how these proteins affect E2 dependent transcription. To characterize the role of HATs in transcription, E2 responsive luciferase reporter plasmids were co-transfected with BPV-1 E2 and either control siRNA or siRNA targeting p300, CBP, or pCAF. Activation of the reporter by E2 in the presence of control siRNA was nearly 80 fold greater than in the absence of E2, which is consistent with previous reports (26). siRNA mediated depletion of either p300 or CBP resulted in approximately 50% reduction of reporter activity compared to cells transfected with control siRNA (Fig 2.1a). Similarly, depletion of pCAF resulted in an approximate 40% reduction of reporter activity (Fig 2.1a). Protein depletion was confirmed by immunoblot analysis and a 50% or greater reduction in protein level was observed for each protein in the presence of corresponding siRNA (Fig 2.1b). p300, CBP, and pCAF are general transcriptional cofactors; as a result, depletion of these proteins may result in unforeseen consequences on transcription from the E2 expression construct. To address this concern, E2 protein level was examined in the context of each siRNA transfected and determined to be unaffected (Fig 2.1b and c). Significant decreases in reporter activation following

Figure 2.1 p300, CBP, and pCAF are independently necessary for E2 dependent transcription. **A** RPE-1 cells were transfected with a luciferase reporter plasmid containing 4 E2 binding sites, E2, and control siRNA or siRNA directed toward p300, CBP, or pCAF. Luciferase activity was detected 48 hours post transfection. Results are presented as a percentage of control. One way ANOVA was performed with Dunnet's post-hoc analysis comparing knockdown to control, ** $p<0.01$ and *** $p<0.001$. **B** Lysates from transfected cells were prepared for SDS-PAGE and immunoblot analysis was performed to confirm protein depletion. **C** Quantitation of E2 protein level present in **B**. Band intensities were measured and compared to those of actin. Data is presented as a percentage of E2 protein level present in control siRNA transfected cells.


A
Percent Wild Type Activation
150
100
50
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CTRL
p300
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p300
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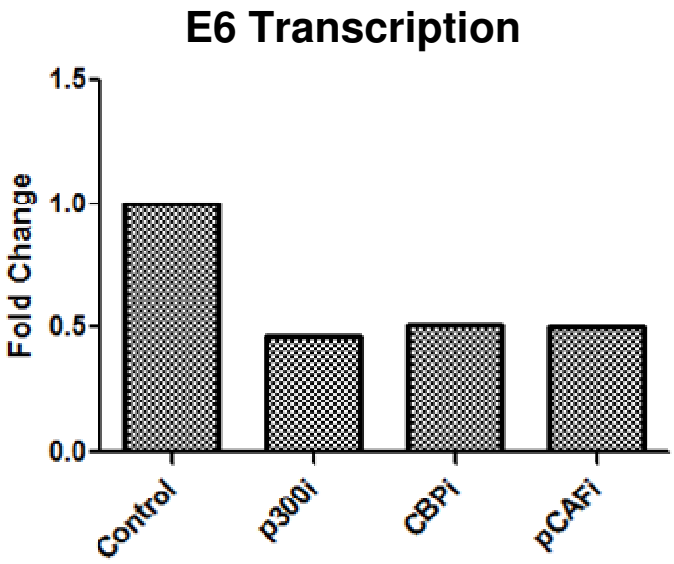
E2
Actin
C
Percent Control
150
100
50
0
CTRL
p300
CBP
pCAF
siRNA

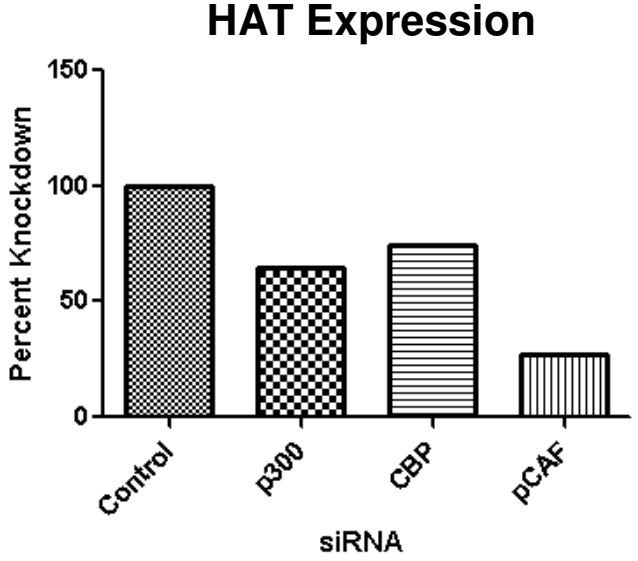
depletion of p300, CBP, and pCAF suggest that each protein is necessary for E2 dependent transcription. While analysis of synthetic reporter activation is informative in revealing the possibility that HATs may be necessary for E2 dependent transcription, this is an idealized situation with four high affinity E2 binding sites upstream of an active promoter. In order to extend these experiments to a physiological setting, the effect of HAT depletion was determined on the expression of the BPV-1 E6 oncoprotein from the viral early promoter. Depletion of p300, CBP, and pCAF, all resulted in at least a 50% reduction in E6 RNA expression as compared to control siRNA transfection (Fig 2.2a). These results are consistent with those observed in the luciferase reporter assay. The drawback of this particular experiment is that while physiological, the role of E2 cannot be determined. To examine this, a mutant genome must be employed that does not express E2. Then the level of E6 expression may be measured after introduction of E2 and compared to that following HAT depletion. In this assay, p300, CBP, and pCAF were amplified by conventional PCR to determine the extent of siRNA mediated knockdown. While pCAF transcript levels were nearly undetectable following siRNA transfection, depletion of p300 and CBP resulted in approximately 50% reduction in transcript (Fig 2.2b). The difference in knockdown efficiency may be attributable to as different set of siRNAs used in this experiment. ID13 cells are of mouse origin, the p300 and CBP siRNAs used in the luciferase reporter assay were directed toward a non-homologous region of the human proteins. The siRNA recognizing pCAF was

Figure 2.2 p300, CBP, and pCAF are necessary for E2 dependent genomic transcription. **A** ID13 cells were transfected with a control siRNA or siRNA directed toward p300, CBP, or pCAF. RNA was isolated 48 hours post transfection and E6 mRNA levels were measured by real-time PCR. Results are presented as a fold change compared to control transfection. Error bars are not included because data is preliminary and the experiment was only performed twice. **B** HAT mRNA was amplified from RNA isolated from siRNA transfected lysates. Intensity of each band was measured and normalized to beta actin. Each bar represents the mRNA expression level of the protein being depleted as a percentage of levels of that mRNA following control siRNA transfection.

A


E6 Transcription
Fold Change
1.5
1.0
0.5
0.0
Control
p300i
CBPi
pCAFi

B


HAT Expression
Percent Knockdown
150
100
50
0
Control
p300
CBP
pCAF
siRNA

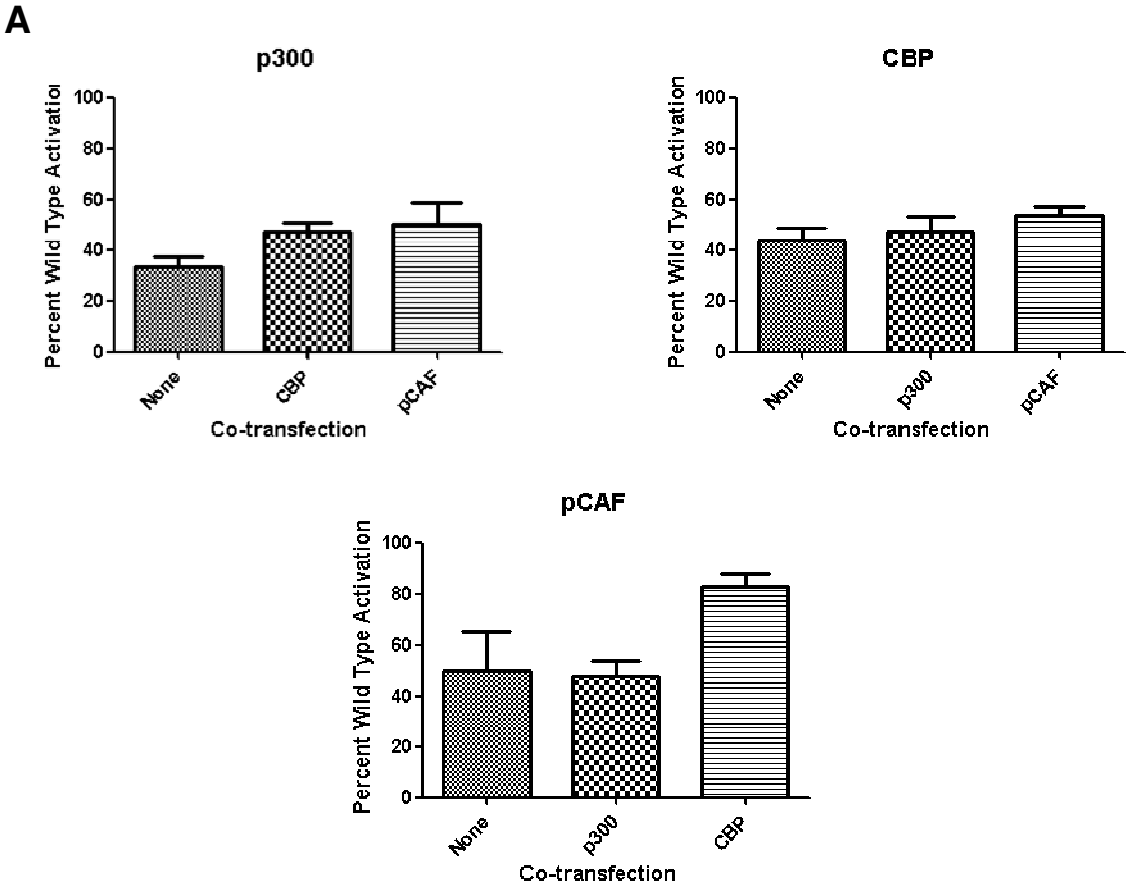
identical to that used in the luciferase assay.

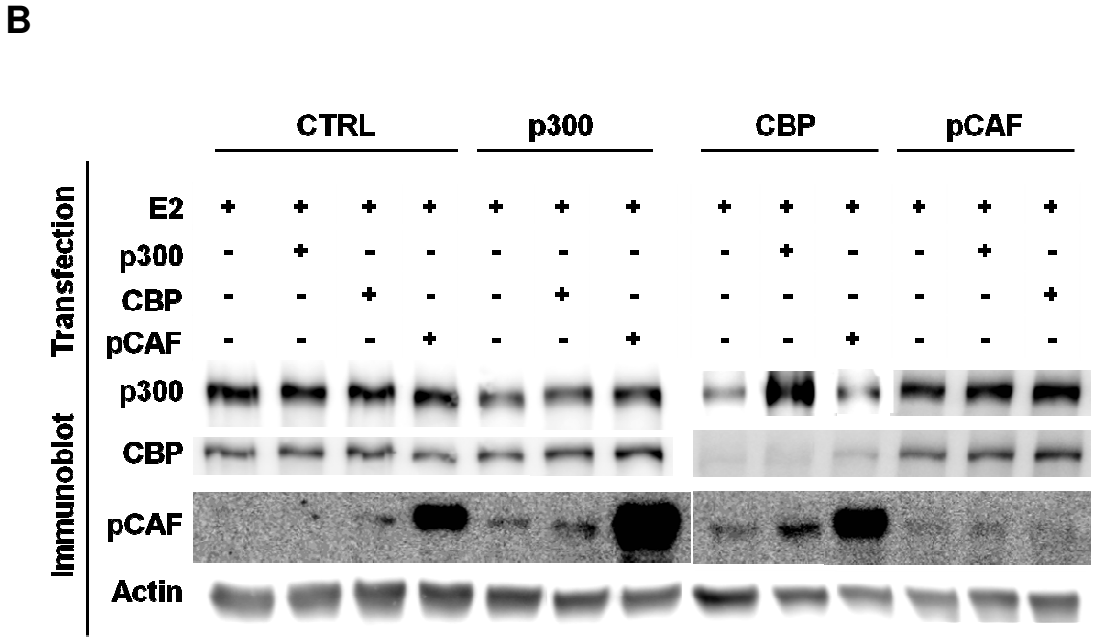
The finding that p300, CBP, and pCAF are each limiting was surprising as there is a large overlap in substrate range for many HATs and particularly for p300 and CBP, which are highly homologous and similar in function (130, 162). The possibility that there is functional redundancy between these proteins was explored by examining the ability of one HAT to restore reporter activation in the context of depletion of another HAT protein. CBP and pCAF were unable to significantly increase transcriptional activation in the context of p300 depletion in RPE-1 cells (Fig 2.3a). This indicates neither CBP nor pCAF can replace the activity of p300 with respect to E2 dependent transcriptional activation. Similarly, neither p300 nor pCAF could replace the transcription activity of CBP and neither p300 nor CBP could replace the transcriptional activity of pCAF (Fig 2.3a). Confirmation of HAT depletion as well as overexpression is presented in figure 2.3b.

The Co-Activator Gps2 Increases Immunoprecipitation with HATs

Gps2 has previously been demonstrated to interact both with E2 and p300 (145). Gps2 facilitates the interaction between p300 and E2 as evidenced by the increase in HAT activity in lysates precipitated with E2 antibodies in the presence of this protein (145). Given the importance of the HATs pCAF and CBP in E2 dependent transcriptional activation, we sought to determine if Gps2 plays a role in these complexes as well. E2 was co-precipitated in increasing abundance by

Figure 2.3 p300, CBP, and pCAF function independently in E2 dependent transcription. **A** RPE-1 cells were transfected with a luciferase reporter plasmid containing 4 E2 binding sites, E2, and p300, CBP, or pCAF were expressed in the presence of siRNA directed toward p300, CBP, or pCAF. Each graph represents one transfected siRNA and results are presented as a percentage of activation in the presence of control siRNA. One way ANOVA was performed with Dunnet's post-hoc analysis comparing each overexpressed protein to vector alone. All comparisons are statistically insignificant. **B** Lysates from transfected cells were prepared for SDS-PAGE and immunoblot analysis was performed to confirm protein depletion and overexpression.


A
p300
Percent Wild Type Activation
100
80
60
40
20
0
None
CBP
pCAF
Co-transfection
CBP
Percent Wild Type Activation
100
80
60
40
20
0
None
p300
pCAF
Co-transfection
pCAF
Percent Wild Type Activation
100
80
60
40
20
0
None
p300
CBP
Co-transfection


B
CTRL
p300
CBP
pCAF
Transfection
E2
p300
CBP
pCAF
Immunoblot
p300
CBP
pCAF
Actin

transfection of Gps2, co-precipitation of E2 by p300 was increased (Fig 2.4). pCAF also co-precipitated a greater amount of E2 following Gps2 expression. Conversely, expression of Gps2 did not appear to have any effect on the co-precipitation of E2 with CBP (Fig 2.4). Gps2 is known to exist in a co-repressor complex in addition to interacting with p300 (213). While this may result in changes in levels of basal transcription, this would not account for increases in association of HATs and E2. Transfection of Gps2 does not affect levels of E2 expression.

p300 and CBP but Not pCAF Interact with the BPV-1 Genome

A previous report has demonstrated that pCAF increases the amount of acetylated histone on a luciferase reporter (113). This may indicate that E2 recruits acetyltransferase proteins to the early promoter to modify chromatin. This report does not directly examine the presence of HAT on the promoter. Here, we address this through chromatin immunoprecipitation (ChIP) of endogenous HAT on episomally maintained BPV-1 genomes. Compared to control immunoprecipitation (IP) and IP in C127 cells, which do not contain BPV-1 genomes, E2 was detected on BPV-1 genomes in ID13 cells (Fig 2.5a). p300 and CBP IP also led to co-precipitation of genomes (Fig 2.5a). Conversely, IP using a pCAF antibody failed to co-precipitate BPV-1 genomes in ID13 cells (Fig 2.5a). The antibody used to IP pCAF in figure 2.5a is the same used in the IP shown in figure 2.4 and all immunoblots for pCAF. These proteins exhibit similar

Figure 2.4 Gps2 facilitates complex formation between E2 and HATs. RPE-1 cells were co-transfected with E2 and either empty vector or Gps2. Cells were lysed and lysates divided for co-immunoprecipitation with endogenous HAT protein. Antibodies used for immunoprecipitation are indicated above the top panel. Precipitates were probed for E2 using B201 antibody. The bottom left panel represents each lysate precipitated with control IgG antibody and the bottom right panel represents 5% of the total lysate added to the immunoprecipitation reaction and * indicates a background band present in the B201 E2 immunoblot.

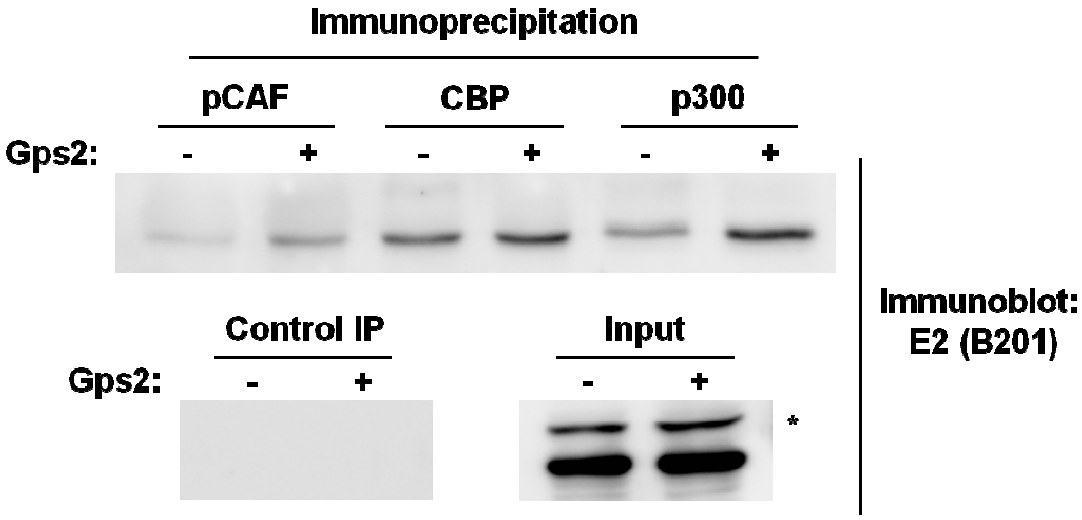
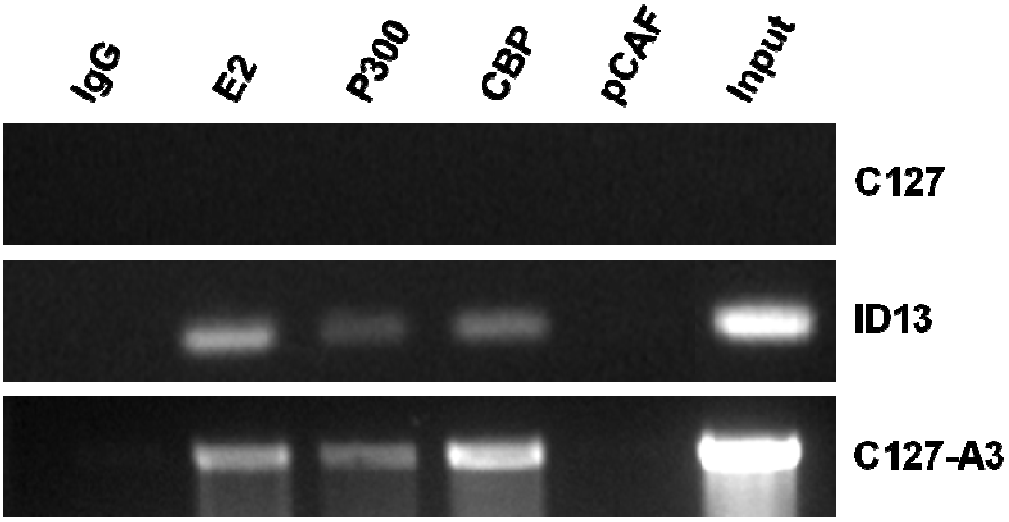
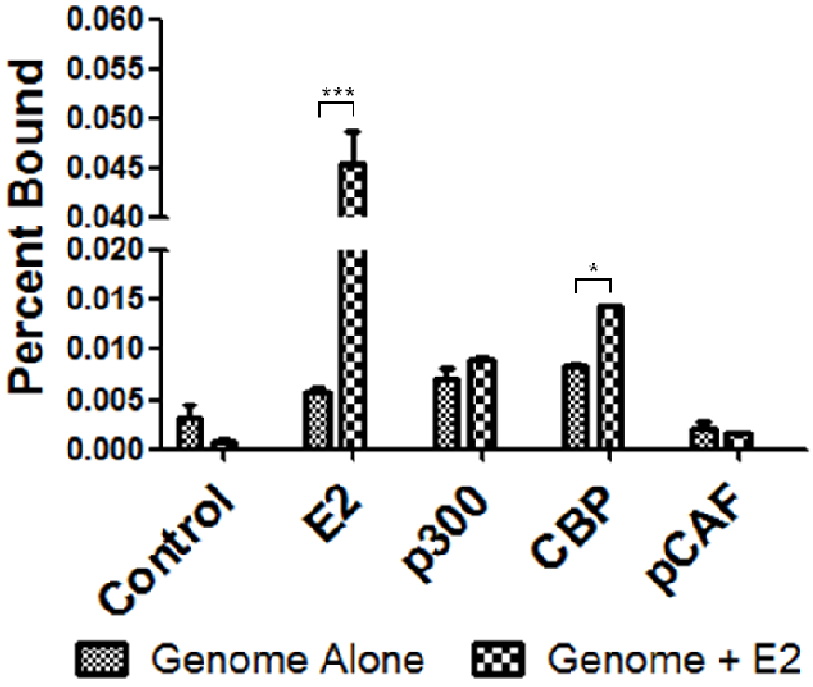

Immunoprecipitation
pCAF
CBP
p300
Gps2:
- + - + - +
Control IP
Input
Gps2:
- + - +
*
Immunoblot:
E2 (B201)

Figure 2.5 p300 and CBP but not pCAF interact with the BPV1 genome. **A** Untransfected C127, ID13, and C127-A3 lysates were prepared for ChIP assay and immunoprecipitated using either control antibody, or antibodies recognizing BPV1 E2, p300, CBP, or pCAF. Precipitated DNA was amplified by PCR. **B** C127 cells were transfected with ΔNco1 genomes in the presence or absence of BPV1 E2. Lysates were prepared for ChIP assay and immunoprecipitated using control antibody, or antibodies directed at BPV1 E2 (B201), p300, CBP, or pCAF. Precipitated DNA was quantified using real time PCR. Two-way ANOVA with Bonferonni's post-hoc analysis was performed comparing genome alone and genome plus E2 for each individual IP, * $p<0.5$, *** $p<0.001$.

A


IgG
E2
P300
CBP
pCAF
Input
C127
ID13
C127-A3

B


Percent Bound
0.060
0.055
0.050
0.045
0.040
0.020
0.015
0.010
0.005
0.000
***
*
Control
E2
p300
CBP
pCAF
Genome Alone
Genome + E2

binding patterns in C127-A3 cells. E2 IP co-precipitates the genome, as does p300 and CBP IP; however, pCAF is still unable to precipitate BPV genomes in C127-A3 cells (Fig 2.5a).

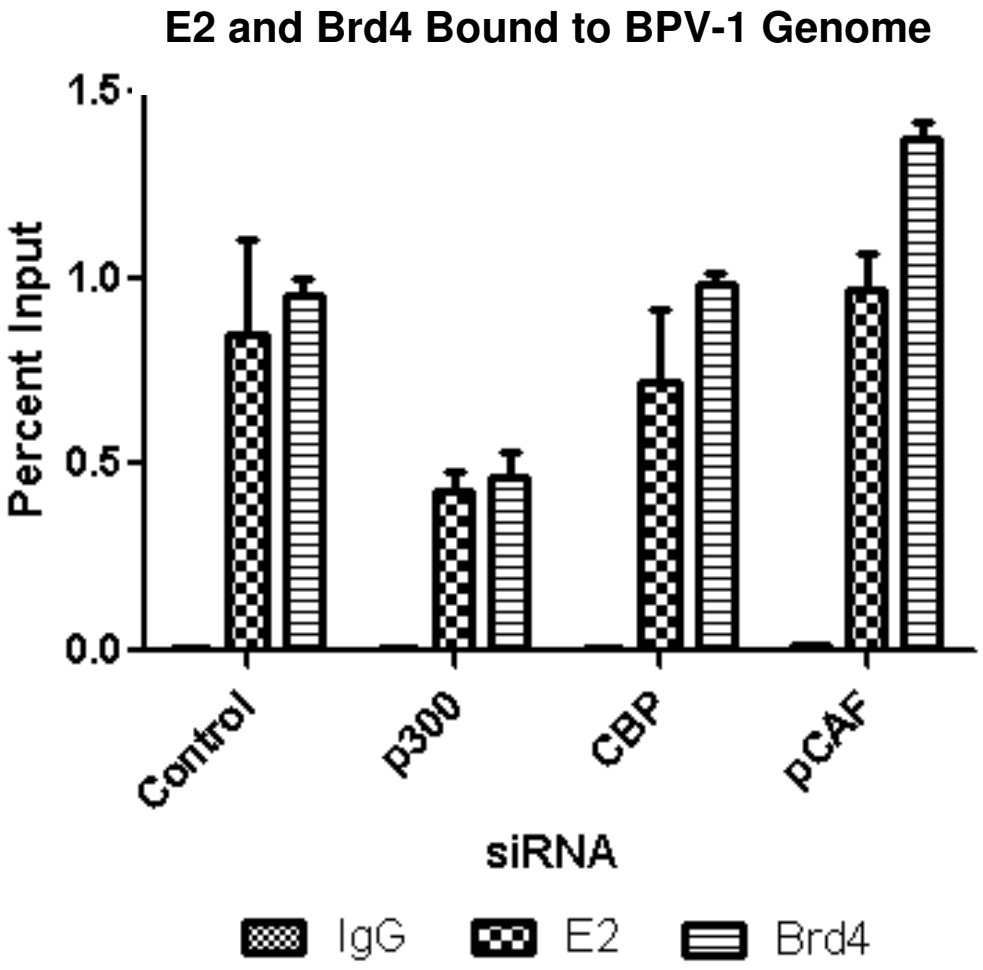
While it is difficult to specifically determine the amount of genome precipitated in each sample, it appears that CBP co-precipitates more genome in C127-A3 cells than ID13 cells (Fig 2.5a). The difference between these two cell lines is a series of serine to alanine mutations in the E2 reading frame that results in higher E2 protein levels in the cell (119). We next asked if E2 specifically recruits either p300 or CBP to the BPV-1 genome. C127 cells were transfected with ΔNco1 genomes with or without co-transfected E2. These genomes do not express E2 and allow exogenous complementation. Compared to control IgG IP, both p300 and CBP were found on the genome in the absence of E2 (Fig 2.5b). Following transfection of E2, a dramatic increase in genome bound E2 was observed as expected (Fig 2.5b). Interestingly, there was also an increase in the amount of genome bound CBP in E2 transfected samples (Fig 2.5b). This increase which was nearly two fold was statistically significant ($p<0.05$). p300 was detectable following expression of E2; however, no significant increase was detected and pCAF levels were below those of IgG control IP in the presence and absence of E2 (Fig 2.5b). While it appears that different levels of each of genome are precipitated by each antibody in the presence or absence of E2, this may not truly be the case. It is difficult to compare IP between different antibodies given varying affinities for each cognate antigen. In this experiment one may only

compare samples in the presence or absence of E2 for each individual antibody. In order to compare between antibodies, an antibody titration must be performed to determine the relative affinities of each antibody, then the one may normalize to ensure equal precipitation of each protein and compare the relative abundances of genome precipitated.

E2 Interaction with DNA is Facilitated by p300

We have now identified at least two HATs that form complexes with the BPV-1 genome. We have presented evidence that suggests CBP may complex with E2 on the genome; it is also possible that p300 is in complex with E2 on the genome as well. We next asked if the critical E2 transcriptional co-factor and acetyl-histone interacting protein Brd4 was recruited into complexes with HATs and E2. Using ID13 cells we transfected control siRNA or that directed toward p300, CBP, or pCAF. We then used quantitative ChIP assay to determine the amount of Brd4 was bound to the LCR. Both E2 and Brd4 were both found to be present on the LCR in after control siRNA transfection (Fig 2.6). Following depletion of p300 we observed a 50% reduction in LCR interaction for both Brd4 and E2 (Fig 2.6). Depletion of CBP did not result in a decrease in occupancy for either protein. Interestingly, depletion of pCAF did not affect the interaction of E2 to the genome; however, it resulted in an increase of nearly 2 fold for Brd4 (Fig 2.6). Depletion of HAT protein for this experiment is consistent with that seen in figure 2.2.

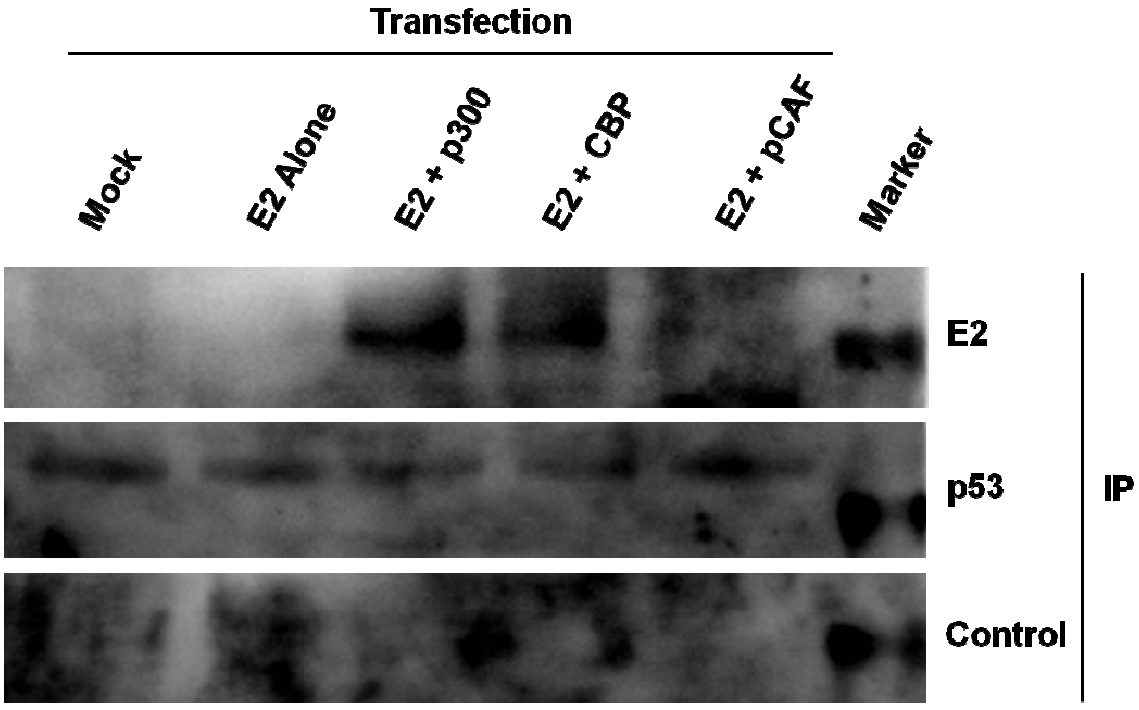
Figure 2.6 p300 facilitates the interaction of E2 and Brd4 to DNA. ID13 cells were transfected with either control siRNA or siRNA directed toward p300, CBP, or pCAF. 48 hours post transfection, cells were crosslinked and immunoprecipitated with control IgG or antibodies to E2 or Brd4. The amount of bound DNA was quantified by real-time PCR. Data is presented as a percentage of input DNA from each precipitated lysate. Statistics are omitted due to the low number of replicates.


E2 and Brd4 Bound to BPV-1 Genome
Percent Input
1.5
1.0
0.5
0.0
Control
p300
CBP
pCAF
siRNA
IgG
E2
Brd4

E2 is Acetylated by p300

Previous studies evaluating the interactions of HATs p300, CBP, and pCAF with BPV-1 E2 have primarily focused on the effects of HAT over expression on transcriptional activation. While these reports provide evidence that HATs affect E2 dependent transcription, little is known about the mechanism. Despite evidence that acetyltransferase activity is necessary for enhancement by CBP and pCAF (113, 115), acetylation of E2 has not been reported. We attempted to detect acetylation of E2 in RPE-1 cells. Acetylated E2 was not detected in mock transfected cells or cells transfected with E2 alone. However, upon over-expression of either p300 or CBP, we were able to detect a band migrating just above the 50 kDa marker (visible in first and last lanes) (Fig 2.7). We were not able to detect acetylated E2 following expression of pCAF in these cells. Acetylated endogenous p53 was detectable in all lanes (Fig 2.7 middle panel) and no visible bands were detected in a control immunoprecipitation (Fig 2.7 lower panel). Although we were able to detect acetylated E2 in RPE-1 cells, these experiments were inconsistent and subsequent attempts to purify acetylated BPV-1 E2 transfected in RPE-1 cells, in ID13 cells which express E2 from episomally maintained BPV-1 genomes, and in RKO cells transfected with FLAG tagged E2 were unsuccessful (data not shown). These experiments were likely unsuccessful due to limitations of new lots of anti-acetyl lysine antibodies used for detection and the possibility of interfering factors *in vivo*. Here, we perform *in vitro* acetylation reactions where each protein was expressed and then

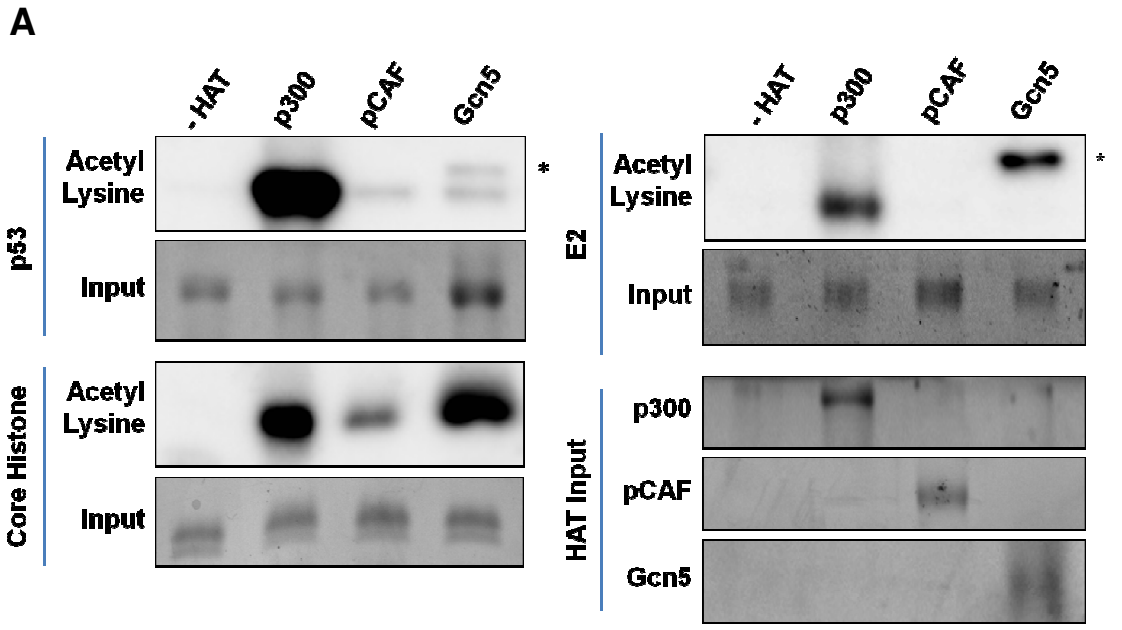
Figure 2.7 E2 is acetylated by p300 and CBP. RPE-1 cells were transfected with empty vector, E2 alone or E2 co-transfected with p300, CBP, or pCAF. Cells were treated with trichostatin A for 12 hours and lysed 24 hours post transfection. Lysates were immunoprecipitated with control antibody or antibodies directed to E2 or p53. Precipitated protein was separated by SDS-PAGE and probed with an anti-acetyl lysine antibody. Each panel represent a separate immunoprecipitation.


Transfection
Mock
E2 Alone
E2 + p300
E2 + CBP
E2 + pCAF
Marker
E2
p53
Control
IP

purified from either bacteria or baculovirus infected Sf9 cells. Purified HAT and E2 proteins were mixed in the presence of acetyl CoA and resulting acetylated E2 was then detected by immunoblot analysis with an acetyl lysine antibody. Histone protein and p53 were used as positive controls for acetylation by p300, pCAF, and Gcn5. We were unable to obtain purified CBP protein at the time of these experiments. Robust acetylation was detected on histone substrates with all of the HATs (Fig 2.8a). p300 mediated strong acetylation of p53 while pCAF and Gcn5 were less active (Fig 2.8a). This may reflect that p300 acetylates p53 on several more lysines than does pCAF or Gcn5 (13, 150) or peptide epitope preference. When BPV-1 E2 was incubated with p300, a strong band corresponding to acetylated E2 was observed. Acetylation of E2 was not detected by immunoblot following incubation with either Gcn5 or pCAF (Fig 2.8a).

E2 lysines specifically targeted by p300 were identified using a proteomic approach. BPV-1 E2 was acetylated *in vitro,* digested, and the resulting peptides were analyzed by mass spectrometry for lysine acetylation. Each peptide was compared to the entire Swiss-Prot database and assembled using both the SEQUEST and X!Tandem search engines. These two utilities directly compare uninterpreted tandem mass spectra to protein databases, using different algorithms, resulting in protein identification. Digestion of BPV-1 E2 with trypsin yielded no acetylated peptides, likely attributed to the inability of trypsin to cleave after acetylated lysines. Analysis of chymotrypsin digestion yielded 97% total sequence coverage and identified eleven acetylated lysine residues on BPV-1 E2

Figure 2.8 E2 is acetylated by p300 *in vitro.* **A** *In vitro* acetylation of purified histone protein (Top left), p53 (Bottom left), and BPV-1 E2 (Top Right), using purified acetyltransferases p300, pCAF, and Gcn5. Acetylation is detected by immunoblot using an anti acetyl-lysine antibody. The higher molecular weight band (*) visible in p53 and BPV-1 E2 acetylation by Gcn5 is due to auto-acetylation of truncated Gcn5. Input for each substrate as well as each acetyltransferase (bottom right) is presented as a duplicate reaction processed in parallel for which the gel was stained for total protein using coomassie brilliant blue. **B** Acetylated peptide sequence coverage of BPV-1 E2 obtained from mass spectrometry. Sequence identified in samples digested with chymotrypsin are underlined and those identified with gluC are highlighted yellow. Detected acetylated lysine residues are in red.


A
- HAT
p300
pCAF
Gcn5
p53
Acetyl Lysine
Input
*
E2
Core Histone
HAT Input
p300
pCAF
Gcn5
B
10
20
30
40
50
60
METACERLHVAQETQMQLIEKSSDKLQDHILYWTAVRTENTLLYAARKKGVTVLGHCRVP
HSVVCQERAKQAIEMQLSLQELSKTEFGDEPWSLLDTSWDRYMSEPKRCFKKGARVVEVE
FDGNASNTNWYTVYSNLYMRTEDGWQLAKAGADGTGLYYCTMAGAGRIYYSRFGDEAARF
STTGHYSVRDQDRVYAGVSSTSSDFRDRPDGVWVASEGPEGDPAGKEAEPAQPVSSLLGS
PACGPIRAGLGWVRDGPRSHPYNFPAGSGGSILRSSSTPVQGTVPVDLASRQEEEEQSPD
STEEEPVTLPRRTTNDGFHLLKAGGSCFALISGTANQVKCYRFRVKKNHRHRYENCTTTW
FTVADNGAERQGQAQILITFGSPSQRQDFLKHVPLPPGMNISGFTASLDF

(Fig 2.8b). Analysis of GluC digestion yielded 89% sequence coverage and nine acetylated lysines, of which three were unique to GluC digestion (Fig 2.8b). In total, 14 of 16 lysines encoded in BPV-1 E2 were found to be acetylated *in vitro*. Nine lysines are in the N-terminal transactivation domain, five in the C-terminal DNA binding domain, and one in the central hinge region. Acetylated lysines were discovered in an average of 22% of each unique E2 peptide identified. The frequency with which each lysine was found to be acetylated ranged from 3% to 60% of the total pool of each unique peptide identified. The low average frequency of acetylation (22%) could explain the difficulty detecting acetylated E2 in mammalian cell culture. A summary of data compiled from chymotrypsin and GluC digests including SEQUEST and X!Tandem correlation scores is presented in Table 2.2.

E2 Lysine Mutations Display Transcriptional Defects

We next sought to investigate the significance of E2 acetylation by p300 in vivo. Six identified lysines were selected for mutation on the basis of sequence conservation in BPV-1 and several HPV types, frequency with which the modified lysine was identified in proteomic analysis, and SEQUEST and X!Tandem correlation scores. Lysines 107, 111 and 112 are part of a reported BPV-1 E2 N-terminal nuclear localization signal (NLS) (172), lysines 111 and 112 are conserved throughout eight HPV types including high risk types 16, 18, and 31 (Fig 2.9a), and lysine 339 is a critical residue that mediates interaction with E2

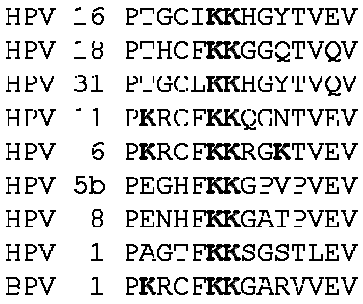
Table 2.2 Proteomic Analysis of Acetylated E2

	Residue	Highest SEQUEST Score	Highest X!Tandem Score	Acetylated Lysines	Total Peptides	Peptide Acetylation Frequency
Transactivation Domain	21	0.94	-	4	12	33%
	25	0.9	-	4	11	36%
	70	0.99	2.07	3	13	23%
	84	0.53	2.49	3	11	27%
	107	1.2	3.19	6	31	19%
	111	0.94	3.12	5	29	17%
	112	1.2	3.19	6	29	21%
	149	0.36	-	1	30	3%
Hinge	226	0.67	-	1	19	5%
DNA Binding Domain	322	0.39	3.5	4	14	29%
	339	0.79	4.09	5	23	22%
	346	2.38	-	3	5	60%
	347	0.31	-	1	5	20%
	391	1.56	2.46	8	24	33%

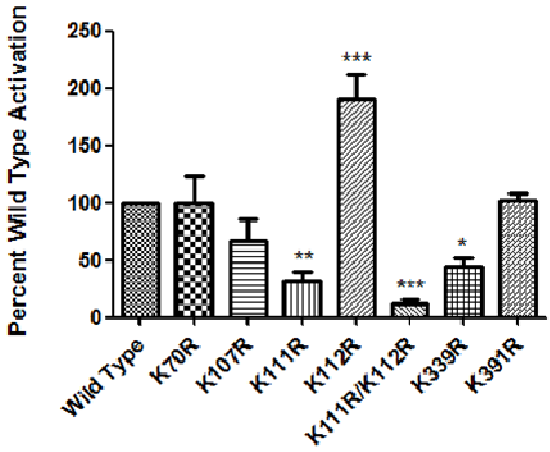
GluC Digest	Chymotrypsin Digest	Both

Figure 2.9 E2 lysine to arginine mutants exhibit transcriptional abnormalities. **A** Sequence alignment around amino acids 106 to 119 of BPV-1 E2. Alignment was prepared using ClustalW multiple alignment tool. **B** RPE-1 cells were co-transfected with an E2 responsive luciferase reporter and either wild type E2 or one of a series of lysine to arginine mutants. Luciferase activity was detected 24 hours post transfection and results are presented as a percentage of wild type E2 activation. One way ANOVA was performed with Dunnet's post hoc analysis comparing each mutant to wild type E2, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **C** Lysates of wild type and mutant E2 transfected RPE-1 cells were prepared for SDS-PAGE and immunoblot analysis was performed to determine the steady state protein level of each E2 expression construct. A longer exposure was required to detect E2 K111R and E2 K111R/K112R (Bottom panel). **D** mRNA from RPE-1 cells transfected with E2 mutants was reverse transcribed and amplified by using E2 or beta-actin primers.

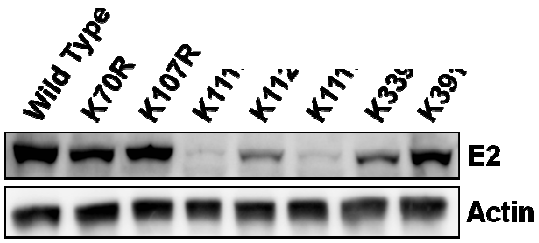
A

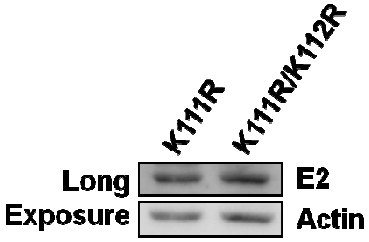

HPV 16 PLGCIKKHGYTVEV
HPV 18 PLHCFKKGGQTVQV
HPV 31 PLGCLKKHGYTVQV
HPV 11 PKRCFKKQGNTVEV
HPV 6 PKRCFKKRGKTVEV
HPV 5b PEGHFKKGPVPVEV
HPV 8 PENHFKKGATPVEV
HPV 1 PAGTFKKSGSTLEV
BPV 1 PKRCFKKGARVVEV

B

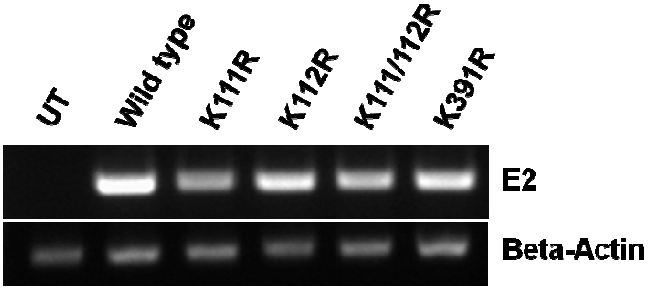

Percent Wild Type Activation
250
200
150
100
50
0
***
**
***
*
Wild Type
K70R
K107R
K111R
K112R
K111R/K112R
K339R
K391R

C


Wild Type
K70R
K107R
E2
Actin


K111R
K111R/K112R
Long Exposure
E2
Actin

D

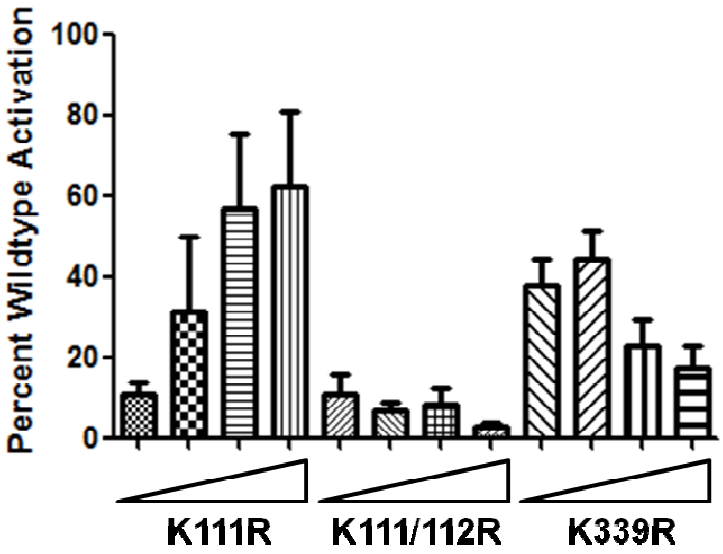

UT
Wild type
K111R
K112R
K111/112R
K391R
E2
Beta-Actin

DNA binding sites (149). Lysines were mutated to arginine to avoid effects related to charge and protein folding. Each mutant was screened for its ability to activate transcription from an E2 responsive reporter and for protein levels.

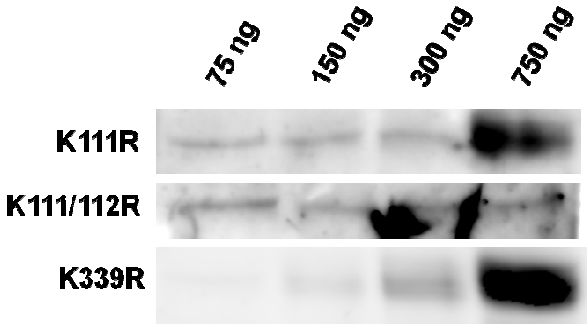
E2 mutants K70R and K391R activated transcription to levels equal to those observed for wild type E2 (Fig 2.9b) despite protein levels approximately 70% of wild type (Fig 2.9c). The K107R E2 mutant protein was present at higher levels than K70R and K391R, 85% of wild type level (Fig 2.9c); however, this mutant was observed to be slightly deficient for transcriptional activation (Fig 2.9b). The K339R E2 mutant was moderately impaired in its ability to activate transcription (Fig 2.9b). This may be partially attributable to decreased protein levels compared to wild type E2, however increasing amounts of K339R mutant transfection resulted in a peak of transcriptional activity at less than 50% of that observed for wild type E2 (Fig 2.10). K111R, K112R, and a double mutation including both residues were expressed to levels between 27% and 40% of wild type levels (Fig 2.9c). Despite its diminished protein level, the K112R E2 mutant activates transcription to levels two fold higher than those observed for wild type E2 (Fig 2.9b). In contrast to K112R, the K111R and K111R/K112R mutants were both impaired in their ability to activate transcription (Fig 2.9b). The expression level of K111R was only partially responsible for the transcriptional deficiency. Transfection of increasing amounts of K111R resulted in a maximum of 60% of wild type activity (Fig 2.10). This was not the case for the K111R/K112R mutant where increasing transfection did not restore any transcriptional activity (Fig

Figure 2.10 Transcriptional dose response for E2 K/R mutants. **A** RPE-1 cells were transfected with a luciferase reporter plasmid containing 4 E2 binding sites, and either 75ng, 150ng 300ng or 750ng of E2 K111R, K111R/K112R, or K339R. Luciferase activity was detected 24 hours post transfection. Results are presented as a percentage of wild type E2 activation (not shown). **B** Lysates from transfected cells were prepared for SDS-PAGE and immunoblot analysis was performed to confirm protein expression.

A


Percent Wildtype Activation
100
80
60
40
20
0
K111R
K111/112R
K339R

B


75 ng
150 ng
300 ng
750 ng
K111R
K111/112R
K339R

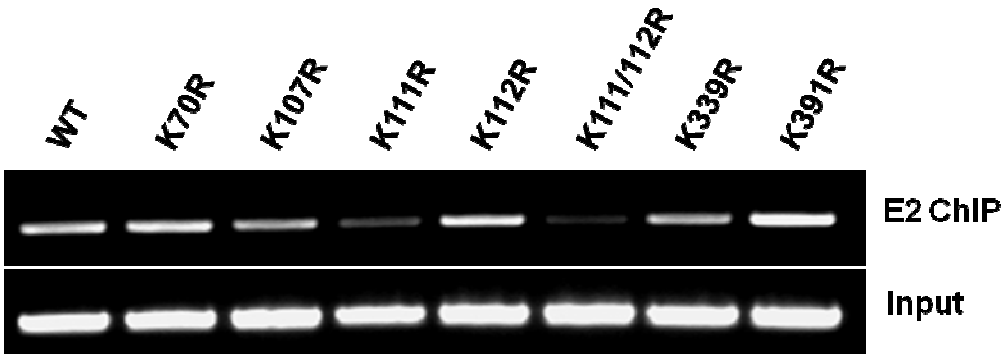
2.10). An immunoblot demonstrating the increasing levels of E2 transfected in the dose response is presented in figure 2.10b. To confirm that the K111R, K112R and K111R/K112R mutant proteins were being expressed, E2 transcript levels were determined by RT-PCR. While all three transcripts were present at levels lower than wild type, they were detectable (Fig 2.9d).

It is known that acetylation of proteins can affect their ability to interact with DNA (63). To examine if E2 acetylation plays a role in its DNA interaction, we transfected C127 cells with the ΔNco1 genome wild type E2 or one of the K/R mutants, and analyzed interaction by ChIP. Wild type E2 interacted with the BPV-1 LCR strongly as expected (Fig 2.11). E2 K70R interacted with DNA nearly as well as wild type. Both K107R and K339R bound DNA about half as well as wild type as determined by visual inspection. K391R actually appeared to bind the LCR better than wild type. This mutation is in a non-conserved region of the C-terminus and is not near the DNA contact or dimerization sites (149). K112R was able to interact with DNA as well as wild type E2. This would be expected given its transcriptional activity. Finally, K111R and K111R/K112R were observed to interact with BPV-1 DNA in lower abundance than wild type E2 (Fig 2.11). These results are consistent with the reduced transcriptional activity and protein expression of these two mutants.

Mutation of Lysines 111 and 112 Induces Cytosolic Mislocalization

A sequence of basic amino acids in the N-terminus of BPV-1 E2 has been

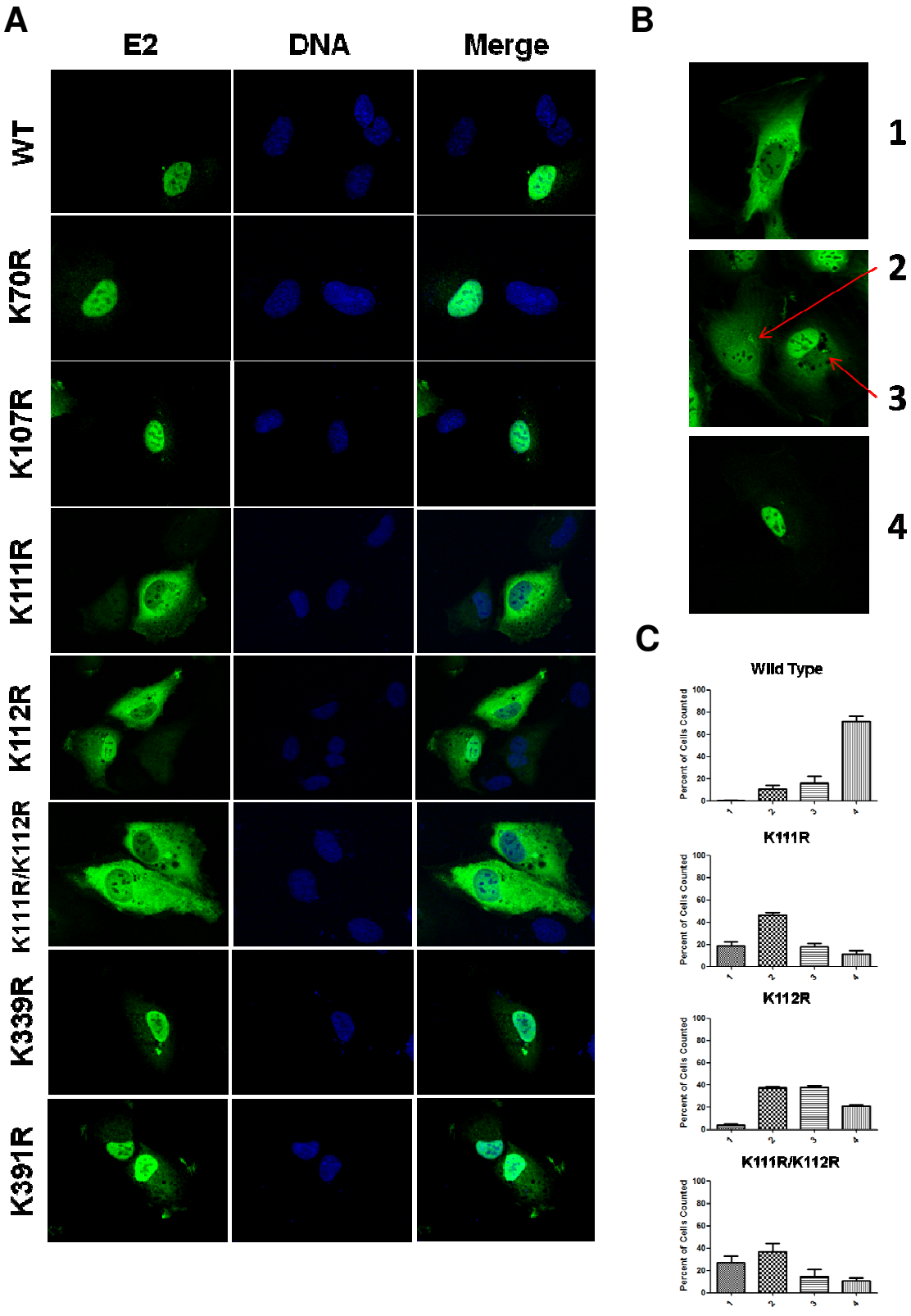
Figure 2.11 Interaction of E2 mutants with DNA. **A** C127 cells were transfected with ΔNco1 genomes and either wild type E2 or the K/R mutant panel. 24 hours post transfection, cells were crosslinked and immunoprecipitated with E2 antibody. Bound DNA was amplified using E2 primers. Input (lower panel) represents 1% of the total lysate DNA used for precipitation.


WT
K70R
K107R
K111R
K112R
K111/112R
K339R
K391R
E2 ChIP
Input

reported to function as a NLS in the absence of the highly conserved C-terminal NLS (172). These two NLS sequences contain four of the six mutated lysines and prompted examination of their localization. The subcellular distribution of four mutants, K70R, K107R, K339R, and K391R were nearly indistinguishable from that of wild type E2; nuclear localization was observed in nearly all cases (Fig 2.12a). The three remaining mutant proteins K111R, K112R, and K111R/K112R deviate from wild type localization and exhibit a spectrum of subcellular distributions ranging from nuclear exclusion to exclusively nuclear (Fig 2.12a). The severity of mislocalization, characterized here as the extent of observed nuclear exclusion, was different for each mutant protein. K112R was observed to be more nuclear and the double mutant K111R/K112R was observed to be primarily excluded from the nucleus. K111R displayed an intermediate phenotype although its localization pattern more closely resembled K111R/K112R than K112R (Fig 2.12a).

Quantifying the extent of mislocalization was necessary to fully characterize these mutants due to the spectrum of localization patterns observed for K111R, K112R, and K111R/K112R. The spectrum was divided into four discrete localization patterns and E2 mutant expressing cells were manually counted and assigned to each category by visual inspection and pre-determined selection criteria (Fig 2.12b). Compared to wild type E2, all three mutant proteins displayed considerably more cytosolic accumulation (Fig 2.12c). K111R and K111R/K112R were consistently found to be evenly distributed throughout both

Figure 2.12 Several E2 lysine to arginine mutants are mislocalized to the cytosol. **A** RPE-1 cells plated on coverslips were transfected with either wild type E2 or one of three lysine to arginine mutants. 24 hours post transfection, cells were fixed and stained using an antibody to E2 (Green) and DNA was visualized using DAPI (Blue). **B** Mutant transfected RPE-1 cells representing the localization of categories 1 through 4 used for quantifying the severity of E2 mutant mislocalization. 1: excluded from the nucleus, 2: even distribution throughout the cytosol and nucleus (indicated by red arrow), 3: diffuse staining with greater intensity in the nucleus (indicated by red arrow), 4: exclusively nuclear staining. **C** The degree of mislocalization was quantified for wild type E2, K111R, K112R, and K111R/K112R. 50 E2 positive cells were counted per coverslip and a minimum of 4 coverslips were counted for wild type E2 and each mutant, each coverslip represents an individual experiment. The degree of mislocalization was scored into four categories as described above. Results are presented as a percentage of total cells counted.


A
E2
DNA
Merge
WT
K70R
K107R
K111R
K112R
K111R/K112R
K339R
K391R
B
1
2
3
4
C
Wild Type
K111R
K112R
K111R/K112R
Percent of Cells Counted

the nuclear and cytosolic compartments (Fig 2.12c). K111R/K112R was found to be excluded from the nucleus more often than either of the other mutants (Fig 2.12c compare second and fourth panels). While expression of the K112R mutant still resulted in significant cytosolic localization, E2 staining was more prevalent in the nuclear compartment (Fig 2.12c). The increase in cytosolic localization for each mutant is not due to Crm-1 mediated nuclear export; treatment of mutant transfected cells with leptomycin B (LMB), which blocks this pathway, had no effect on the subcellular distribution of E2 mutant proteins (Fig 2.13). While manually counting cells allowed us to gauge the level of mislocalization of each mutant, the visual categorization is subjective. In order to more specifically determine the mislocalization of the E2 mutants, quantification of cytosolic and nuclear fluorescence of randomly selected mutant transfected cells would be needed.

HATs Affects E2 Localization

Mutation of E2 at lysines 111 and 112 renders them unable to be efficiently retained in the nucleus. While there is a correlation between acetylation and nuclear retention, the specific role of p300 with respect to E2 localization is still unclear. To address this, the localization of wild type E2 in p300 deficient RKO cells was examined. Following transfection of BPV-1 E2 alone, some E2 was found to be localized to the cytosol (Fig 2.14 Top). The distribution pattern was diffuse throughout both the nuclear and cytosolic compartments, although E2 staining was more intense in the nucleus. Co-transfection of p300 into RKO cells

Figure 2.13 Localization of E2 K/R mutants is unaffected by Leptomycin B. **A** RPE-1 cells were transfected with wild type E2 or E2 mutants. 24 hours post transfection cells were mock treated or treated with 1ng/ml Leptomycin B for 2 hours. The extent of mislocalization was visually scored and data in each category is presented as a percentage of the total number of cells counted. 1: excluded from the nucleus, 2: even distribution throughout the cytosol and nucleus, 3: diffuse staining with greater intensity in the nucleus, 4: exclusively nuclear staining. **B** Representative images of untreated and cells treated with Leptomycin B. Cyclin B is stained red and DNA is labeled blue in the overlayed image.

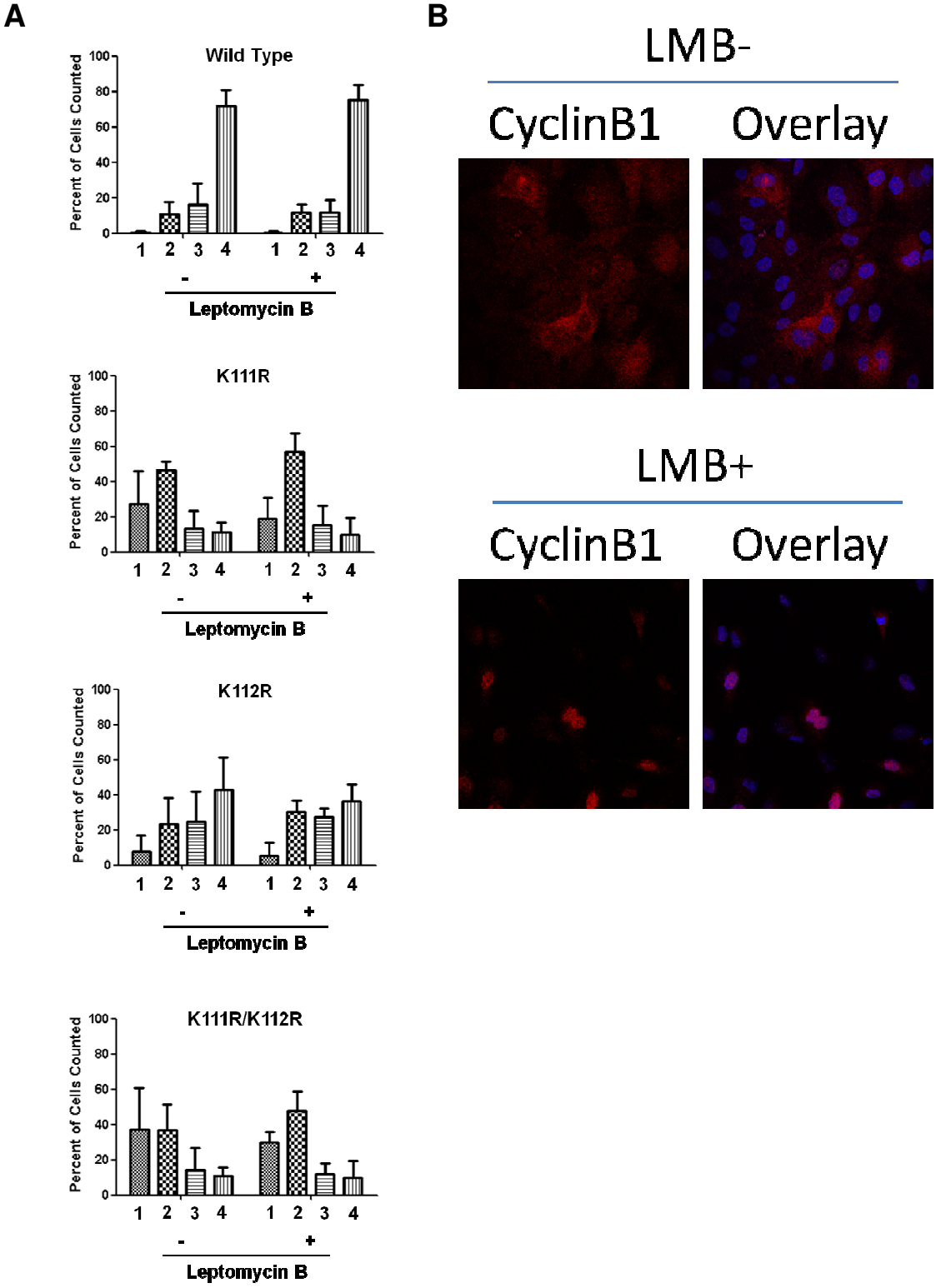
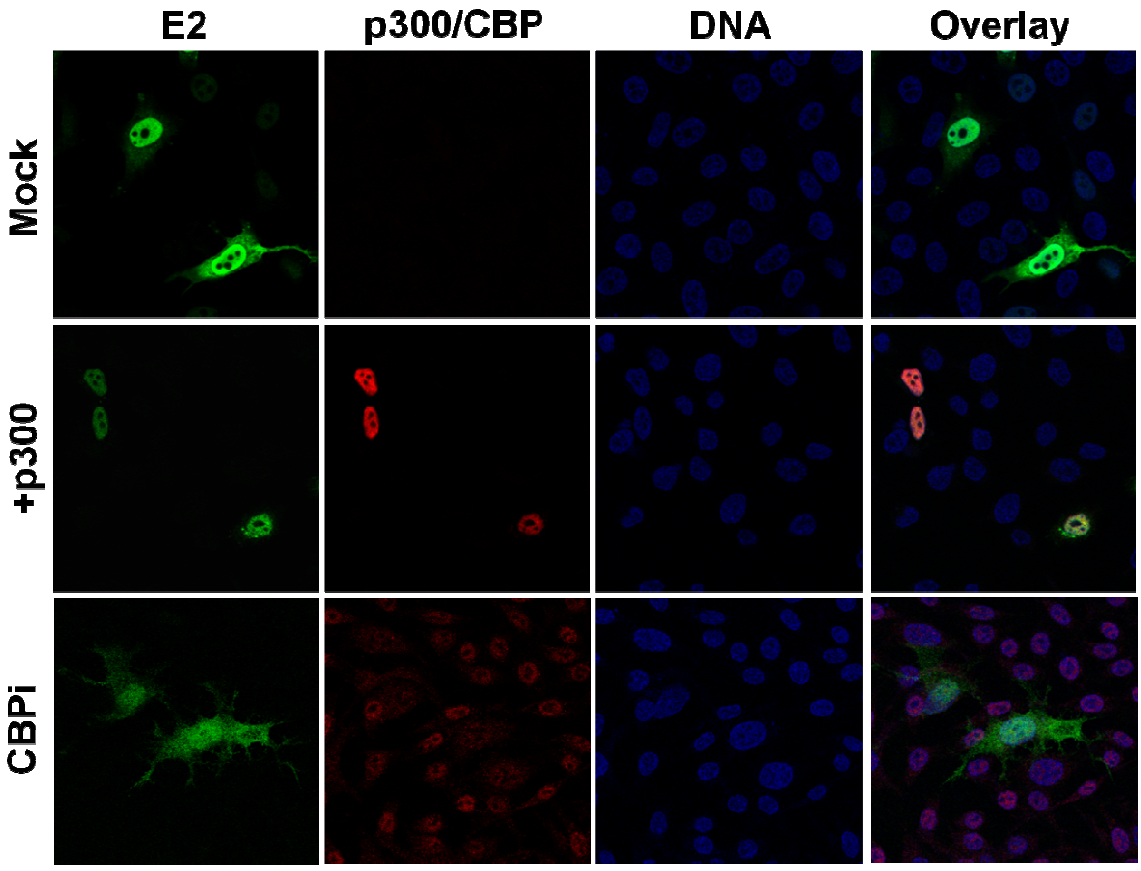

A
Wild Type
Percent of Cells Counted
100
80
60
40
20
0
1 2 3 4 1 2 3 4
- +
Leptomycin B
K111R
Percent of Cells Counted
100
80
60
40
20
0
1 2 3 4 1 2 3 4
- +
Leptomycin B
K112R
Percent of Cells Counted
100
80
60
40
20
0
1 2 3 4 1 2 3 4
- +
Leptomycin B
K111R/K112R
Percent of Cells Counted
100
80
60
40
20
0
1 2 3 4 1 2 3 4
- +
Leptomycin B
B
LMB-
CyclinB1
Overlay
LMB+
CyclinB1
Overlay

Figure 2.14 E2 is mislocalized to the cytosol in cells deficient in p300 and CBP. RKO cells plated on coverslips were transfected with wild type E2 and either a p300 expression construct, empty vector or siRNA to CBP. 24 hours post transfection, cells were fixed and stained using an antibodies to E2 (Green) and p300 or CBP (Red), DNA was visualized using DAPI (Blue).

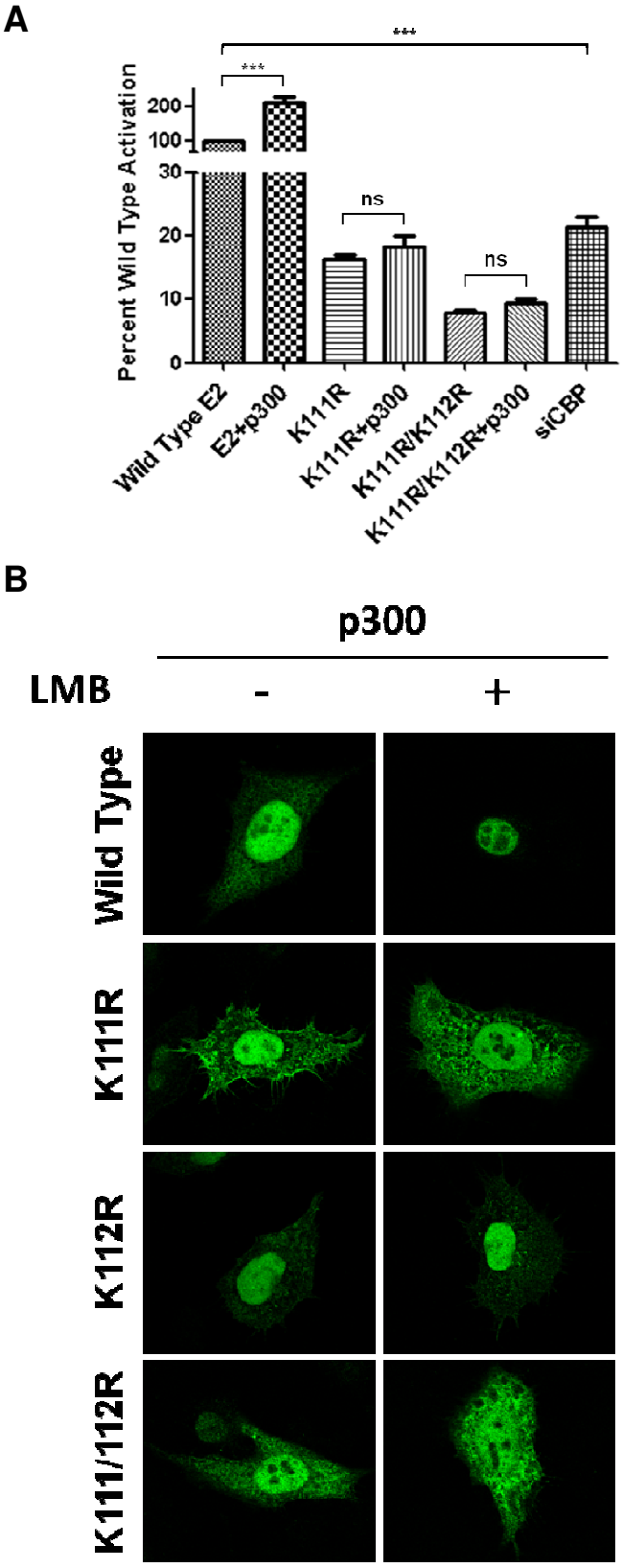

E2
p300/CBP
DNA
Overlay
Mock
+p300
CBPi

with BPV-1 E2 almost completely restored nuclear E2 localization (Fig 2.14 middle). The requirement of CBP to fully activate E2 dependent transcription coupled with the observation that CBP increases acetylation of E2 led us to ask if CBP could compensate for E2 with regard to affecting the localization of E2. To address this we transfected siRNA directed toward CBP into RKO cells. We observed that depletion of CBP resulted in a dispersed E2 localization pattern (Fig 2.14 bottom). E2 staining was even throughout the nucleus and cytosol. This is similar to the patterns observed of E2 K111R and K112R.

p300 is Not Limiting for E2 K/R Mutant Transcription and Localization

It is possible that the transcriptional and localization phenotypes observed for E2 K111R, K112R, and K111R/K112R were not due to the inability of p300 to interact with and acetylate E2. To address this we attempted to restore transcriptional activity by over expression of p300. Co-expression of p300 with wild type E2 enhances activation of E2 dependent transcription in RKO cells to an average of two fold higher than in the absence of p300 (Fig 2.15a). E2 K111R or K111R/K112R exhibited an 80% reduction in transcriptional activity as compared to wild type E2. Furthermore, after introduction of p300 there was no significant increase in transcriptional activity of either K111R or K111R/K112R (Fig 2.15a). The subcellular distribution of the E2 K111R, K112R, and K111R/K112R mutants was also examined in RKO cells. Diffuse distribution patterns similar to wild type E2 were observed for all mutants and reintroduction of p300 into cells transfected with E2 mutants had no effect on E2 localization

Figure 2.15 Reintroduction of p300 is unable to rescue transcriptionally defective E2 mutants. **A** RKO cells were transfected with an E2 responsive luciferase reporter, either wild type or mutant E2, and either a p300 expression construct or empty vector. Wild type E2 sample was also co-transfected with control siRNA for comparison to siCBP transfected sample. Luciferase activity was detected 24 hours post transfection. Results are presented as a percentage of wild type E2 activation. One way ANOVA was performed with Bonferonni post hoc analysis comparing all means *** $p<0.001$. The differences between K111R plus and minus p300 and K111R/K112R plus and minus p300 are not statistically significant. **B** RKO cells transfected with wild type E2 or E2 mutants, in the presence or absence of p300 were prepared for immunofluorescence and stained for E2 (green).


A
***
***
Percent Wild Type Activation
200
100
30
20
10
0
ns
ns
Wild Type E2
E2+p300
K111R
K111R+p300
K111R/K112R
K111R/K112R+p300
siCBP
B
p300
LMB
-
+
Wild Type
K111R
K112R
K111/112R

(Fig 2.15b). Interestingly, in comparison to mutant transfected RPE-1 cells, these cells appeared to be less severely mislocalized. RPE-1 cells are immortalized by expression of hTERT, while RKO cells have defective p53 and p300. There could also be additional genetic lesions in these cells that may contribute to the observed differences in localization.

The ability of E2 to activate transcription in RKO cells which do not express p300 is further evidence that there may be redundancy. To investigate this we transfected RKO cells with siRNA to CBP. Compared to control, transcriptional activation by wild type E2 was dramatically reduced upon CBP depletion (Fig 2.15a). This reduction was statistically significant ($p<0.001$) and was comparable to levels observed of E2 K111R and K111R/K112R (Fig 2.12a).

Discussion

The cellular acetyltransferases p300, CBP, and pCAF, have been reported to interact with E2 proteins from several papillomavirus types. While the significance of these interactions has not been fully characterized, it is known that these proteins are capable of enhancing transcription in an E2 dependent manner (113, 115, 145) and that the acetyltransferase activity of CBP and pCAF is required for transcriptional enhancement (113, 115). We show that physiological levels of p300, CBP, and pCAF are necessary for E2 dependent transcriptional activation and that each protein appears to have independent functions in this process. Several possibilities exist regarding the mechanism through which p300, CBP, and pCAF affect E2 dependent transcription. First, E2 may recruit one or more of these proteins to the viral promoter to acetylate chromatin. This is likely to be true, as both p300 and CBP interact with the BPV-1 LCR and CBP is enriched on the LCR in the presence of E2. pCAF, however, was not found on the BPV-1 genome. Second, acetyltransferases, especially p300 and CBP, may form and stabilize higher order transcriptional complexes. Specifically, p300, CBP, and pCAF have all been demonstrated to be necessary for activation of the interferon β enhancer where p300 and CBP form a scaffold for recruitment of ATF/JUN, NF-κB, and other factors (30). Here, we demonstrate complex formation between the co-factor Gps2 and p300 and pCAF. These complexes could have a variety of implications including dynamic regulation of acetylation. Gps2 is also known to be in the N-CoR complex with

HDAC3 (213). Dynamic switching between p300 and HDAC3 could be control the histone acetylation status at the BPV-1 promoter. While all of these proteins are likely to form large complexes, the requirement for the acetyltransferase activity of CBP and pCAF for enhancement excludes the possibility that these proteins function as scaffolds exclusively. Finally, E2 itself may be a substrate for acetylation which we demonstrate here.

Acetylation of E2 by p300 presents a novel method of regulating papillomavirus transcription. We report that 14 of 16 lysines in E2 are acetylated *in vitro*. While this is suggestive that E2 is capable of being acetylated, it is unlikely that all of these lysines are acetylated under physiological conditions given that some of them are not on the surface of the protein. We have screened several mutants with respect to biological significance of this potential modification. Half of the mutants tested including K70R, K107R, and K391R were not significantly different from wild type E2 with respect to transcription or subcellular distribution. While transfection of the K339R had no effect on E2 localization, its transcriptional activity was reduced to approximately 50% of wild type activity and this defect was not entirely due to lower expression level or inability to interact with DNA; it has been demonstrated previously that this mutant is competent to interact with DNA containing E2 binding sites (172). The remaining mutants including K111R, K112R, and K111R/K112R exhibited aberrant phenotypes in transcriptional activation and localization. All three of these mutants were detectable by immunoblot; however, we were unable to detect increasing protein

levels following a dose response of E2 K111R/K112R. This mutant may be shuttled to the cytosol and degraded. K111R/K112R mutant protein levels are never high, and transcriptional dose response confirms this. Further, it is also possible that higher levels of this mutant are toxic to cells. This could result in preferential selection of lower expressing cells in a pooled lysate experiment such as immunoblot. The reduced protein levels of K111R, K112R, and K111R/K112R are partially due to reduced expression from the CMV driven constructs. This is consistent with previous reports for transcriptionally defective E2 mutants that are unable to enhance their own expression from plasmids (75). We have also found that mutation of lysine 111 alone and together with lysine 112 resulted in significant decreases in transcriptional activation. In contrast, mutation of lysine 112 alone resulted in an enhancement of transcription. All of these mutations affected the localization of E2 to varying degrees.

While it appears that acetylation of lysine 111 is necessary for transcription the result of acetylation at lysine 112 is not as straight forward. It is possible that acetylation of lysine 112 may either promote an inhibitory conformational change or promote an interaction with a transcriptional repressor and mutation of this residue to arginine relieves this repression. Conversely, acetylation of this residue may also inhibit interaction with a co-activator. We speculate that E2 acetylated at lysine 111 interacts with a co-factor, such as Brd4 through its bromodomain. Acetylation at lysine 112 may inhibit this interaction resulting in reduced transcription. Sequence preference of the HAT catalytic domain for

acetylation of lysine 111 over lysine 112 may explain the enhanced transcriptional activity of K112R. The inability of p300 or CBP to acetylate E2 on lysine 112 may enhance acetylation at lysine 111 resulting in increased interaction with Brd4 and enhanced transcription.

Interaction studies previously identifying the region on E2 to which Brd4 binds were carried out using a small peptide of Brd4 and were also done *in vitro* (1). Under these conditions E2 would not be acetylated and an interaction between Brd4 and acetylated lysine 111 would not be discovered. It may be that Brd4 interacts with E2 across all three alpha helices as previously described as well as lateral to alpha helix three where lysines 111 and 112 are exposed. Brd4 was shown to interact with E2 in a heterotetrameric complex (1). The Brd4 peptide contacts two different regions on the alpha helices of the E2 N-terminal domain. Although the structures of several functional domains including both bromodomains and the C-terminal domain which interacts with E2 have been solved, the full length protein has not been crystallized and thus the exact folding of this molecule is unclear. One Brd4 molecule may contact two regions of E2, or a second Brd4 molecule may contact E2 from a distinct E2-Brd4 heterodimer.

Interaction of Brd4 and E2 through acetylated lysine 111 serves multiple functions. First, Brd4 interaction with E2 is required for E2 dependent transcriptional activation (1, 166, 167). Second, as a consequence of the interaction E2 is retained in the nucleus where it may enter another

transcriptional or replication complex. Inability of Brd4 to interact with non-acetylated E2 explains the transcriptional phenotypes. We show that E2 is acetylated by p300 and likely by CBP as well. Depletion of either of these two proteins would result in reduced interaction with Brd4, decreasing transcriptional activation. There may be partial redundancy between p300 and CBP with regard to transcription which may, along with incomplete knockdown, explain why depletion of either of these proteins individually results in only 50% transcriptional reduction. This hypothesis is supported by 80% reduction in E2 dependent transcriptional activation following depletion of CBP in RKO cells where p300 is not expressed. The residual transcription here may be attributable to inefficient depletion of CBP, shown in figure 1 to be approximately 50%. We did not perform double or triple knockdowns in this study; these experiments would be useful in determining the contributions of each of p300 and CBP. Transcription in the absence of p300 and CBP in RKO cells is comparable to the activity of E2 K111R or the double mutant K111R/K112, further suggesting that acetylation of these residues is necessary for E2 dependent transcriptional activation.

Interaction with other factors including Brd4 may tether E2 into transcriptional and replication complexes. Loss of this interaction as with E2 K111R, K112R, or K111R/K112R, would disrupt this tether allowing E2 to be lost to the cytosol. The subcellular distribution of K111R, K112R, and K111R/K112R was nearly identical to that observed of wild type E2 when it was transfected into cells deficient for p300. This correlation indicates that acetylation of lysine 111 and possibly lysine

112 by p300 may be necessary to efficiently retain E2 in the nucleus. These results are consistent with localization E2 N-terminal deletions which lack lysines 111 and 112 (172). Localization of E2 may be regulated in part through acetylation of these residues; however, the lack of complete inclusion or exclusion of the mutants and that leptomycin B had no effect on localization indicates that the defects observed are likely not due to nuclear export, and that reduction in NLS strength through the lysine to arginine mutation, or passive diffusion of E2 through the nuclear pore complex may be responsible. This suggests that instead of these modifications directly controlling the localization of E2, its mislocalization may result from the inability of E2 to be retained in the nucleus when it is not in an active complex. One may speculate that this is a method of regulating E2 protein levels within the nucleus and the cell.

The lack of complete exclusion from the nucleus observed upon transfection of the E2 K111R/K112R mutant and the residual transcriptional activity suggests that acetylated lysine 111 alone does not mediate interaction with Brd4 bromodomain. Another factor or perhaps an additional modification may be necessary to control E2 dependent transcription. A possible target on BPV-1 E2, aside from the previously described Brd4 interaction region (1), is lysine 107. The E2 mutant K107R, while observed to be primarily nuclear also accumulated in the cytosol at a low frequency (data not shown), consistent with localization of an E2 deletion mutant lacking amino acids 101 to 110 (172). Additionally, this mutant exhibited a small deficiency in transcriptional activity. While

bromodomains have high affinity for most acetylated lysines, they do exhibit context sequence preference (121, 136). The interaction of a bromodomain with acetylated lysine prefers a pair of acetylated lysines with several spacer residues between them (42, 134). This would indicated that acetylation of both lysine 107 and 111 would result in a higher affinity interaction. Further characterization of K107R was not pursued because the phenotype was not dramatically different from wild type. While acetylation at lysine 107 does not appear to have a dramatic effect on E2 transcription by itself, perhaps interaction with Brd4 would be stronger in combination with acetylation of lysines 111.

We have outlined here a potential role for the cellular acetyltransferase p300 and potentially CBP in E2 dependent transcription. A specific role for pCAF in E2 dependent transcriptional control was not identified in this study. This HAT was not found to interact with BPV-1 DNA. This does not rule out its interaction altogether due to its tendency to form higher molecular weight complexes with p300 and CBP. It is possible that the sensitivity of the ChIP assay was not high enough to detect pCAF in this manner. While data indicates that p300, CBP, and pCAF may function independently, there does appear to be some redundancy. CBP may also be capable of acetylating E2 given the functional overlap between this protein and p300. Additionally, the reduction in transcriptional activation in RKO cells following CBP depletion suggests that CBP may be compensating for p300 in these cells. Acetylation of E2 by pCAF or Gcn5 may require a second factor to facilitate complex formation or may only occur on a small subset of

available lysines. A more sensitive assay may be required to definitively eliminate pCAF in this capacity.

p300, CBP, and pCAF may each play multiple roles during E2 dependent transcriptional activation and a sequence of events including acetylation of E2 by p300 and E2 dependent chromatin modification by several of these proteins may be necessary to fully activate viral transcription. Additionally, acetylation of two lysines within the N-terminus of E2 control transcriptional activation likely through regulation of co-factor entry into complexes. This study presents a novel method of regulating E2.

CHAPTER III

Characterization of a Novel Interaction Between BPV-1 E2 and RINT1

Introduction

Many cellular proteins have been identified as E2 interacting proteins. These cellular interaction partners have provided a wealth of information about E2 activity. Several E2 interacting proteins and the significance of their interactions have already been discussed. Here the E2 interacting protein RINT1 and the biological complexes in which it exists will be further described.

RINT1-Rad50 Complex

Rad50 interacting protein 1 (RINT1) was identified in a screen for interacting proteins of the human structural maintenance of chromosomes (SMC) family member Rad50 (206). RINT1 is encoded by 792 amino acids and has a molecular weight of 87 kDa. There is a series of leucine heptad repeats at the N-terminus of RINT1, but no other functional domains have been identified. Homozygous deletion of RINT1 results in mice that die early in development; embryos fail to develop past day E5.5 (122). Blastocysts taken from RINT1 knockout embryos failed to expand after four days of culture indicating that RINT1 is essential for proliferation early in embryogenesis. Further, 81 % of RINT1 heterozygous mice were observed to develop multiple tumors over an average lifespan of two years. Taken together these data indicate that RINT1 may be a tumor suppressor gene.

RINT1 was found to be expressed in a variety of cell types and throughout the cell cycle; however, its interaction with Rad50 appears to be limited to the G2/M

phases (206). Rad50 is a highly conserved protein that has been demonstrated to be critical for cellular proliferation. Its presence in complex with hMre11 and NBS1, indicates a role in DNA damage repair, specifically double strand breaks. The interaction between RINT1 and Rad50 and more specifically the temporal nature of this interaction suggests that RINT1 may also play a role in the DNA damage response. Cell lines were developed that stably express a truncation mutant of RINT1 which lacks the N-terminal 256 amino acids. This protein is still able to interact with Rad50 and therefore acts as a dominant negative. Following exposure of these cells to ionizing radiation, there is a marked decrease in bromodeoxyuridine (BrdU) incorporation indicating that the Rad50-RINT1 complex was not affecting the G1/S cell cycle checkpoint (206). To examine a possible role for this complex in the G2/M checkpoint cells were again exposed to ionizing radiation and a mitotic index was calculated. As compared to cells expressing a vector alone, those expressing the truncated RINT1 had a significantly higher mitotic index indicating that this protein in complex with Rad50 may be important for G2/M cell cycle control (206). In addition to its role in DNA damage repair, Rad50 has been implicated in cell cycle control specifically in complex with the tumor suppressor Brca1. RINT1 may be present in this complex or function independently in cell cycle control.

RINT1-p130 Complex

RINT1 has subsequently been found in several other protein complexes in diverse cellular processes. The pocket protein p130, which is related to the

tumor suppressor pRB, has been implicated in the suppression of telomere lengthening in the absence of telomerase (57). In order to further investigate its role in this process, a screen was performed to identify p130 interacting proteins, one of which was determined to be RINT1 (102). Subsequently, RINT1 was determined to interact with p130 *in vitro* and *in vivo* and preferentially with the hypophosphorylated form of p130, which is the active form that binds to E2F family transcription factors and inhibits cellular proliferation (102). RINT1 interacts very weakly with other pocket protein family members pRB and p107 (102). p130 was also found to complex with Rad50 and this interaction appears to be RINT1 dependent (102). Finally, depletion of RINT1 results in lengthened telomeres without increasing telomerase activity. This phenotype was dependent on the interaction of RINT1 with p130 (102). The inability of a single point mutant of RINT1 that is incapable of interaction with p130 to prevent telomere lengthening strengthens the argument that RINT1 is a tumor suppressor.

It is not known currently what role the RINT1-p130 complex may play in papillomavirus biology; however, p130 like its pocket protein family member pRB, interacts with the papillomavirus oncoprotein E7 (156). E7 was found to preferentially bind to p130 over pRB and p107 in co-immunoprecipitation experiments (174). While pRB is expressed throughout the cell cycle, p130 expression is limited to quiescent and differentiating cells where high and low risk E7 proteins promote S-phase reentry by targeting p130 for degradation (59, 156, 212).

RINT1-Syntaxin-18 Complex

RINT1 was also identified in a screen for proteins that interact with human syntaxin-18 (79). Syntaxin-18 is an endoplasmic reticulum (ER) localized target membrane associated soluble N-ethylmaleimide-sensitive attachment protein receptor (t-SNARE) that is involved in membrane trafficking between the Golgi and ER. t-SNAREs specifically interact with vesicle associated v-SNAREs directly leading to membrane fusion between these two membrane compartments. Zw10 and p31, a kinetochore associated protein and another SNARE protein respectively, were also identified in this screen as syntaxin-18 interacting proteins (79). RINT1, Zw10, and p31 were found to form a sub-complex that remained together following dissociation from syntaxin-18. Over-expression of Zw10 or disruption of the interaction between Zw10 and RINT1 results in the disruption of vesicular trafficking between the ER and the Golgi (79).

The syntaxin-18 complex was further characterized in a series studies identifying several other proteins that are present and appear to be critical for the stability of the ER, Golgi and membrane dynamics. First, the BH3 only protein BNIP1 was determined to link the p31, Zw10, RINT1 sub-complex to syntaxin-18 (138). RINT1, which interacts directly with Zw10, was also shown to be critical for recruiting Zw10 into this sub-complex (8, 138). Transfection of a dominant negative truncation of RINT1 consisting of the N terminal 219 amino acids or siRNA mediated depletion of RINT1 result in defects in vesicular transport

between the ER and Golgi concomitant with a redistribution of Zw10 from the ER(8). Immunoprecipitations using antibodies to syntaxin-18, p31 or BNIP1 were unable to efficiently co-precipitate Zw10 in RINT1 depleted cells (8). Conversely, the same antibodies were able to co-precipitate RINT1 in Zw10 depleted cells (8). siRNA mediated knockdown of RINT1 or over-expression of an N-terminal fragment of RINT1, which was shown to interact with Zw10, resulted in the redistribution of Zw10 from the ER and disruption of vesicle transport(8).

While the nature of the syntaxin-18 complex was becoming clearer, at least one key question remained. It is known that there are sub-complexes (8, 138) and that RINT1 and Zw10 interact directly (79), but how these two proteins are recruited into the complex was unknown. Neither interacts directly with p31, syntaxin-18, or BNIP1 (79, 138). Neuroblastoma-amplified gene (NAG) was identified as a link between the SNARES (p31, syntaxin-18) and the peripheral proteins (RINT1, Zw10) (6). NAG was found to interact with both p31 and RINT1/Zw10 through its N and C termini respectively (6). Further, p31 was unable to immunoprecipitate RINT1 or Zw10 in cells depleted of NAG (6). Finally, depletion of NAG caused both RINT1 and Zw10 to dissociate from the ER (6). This phenotype is similar to that observed of Zw10 upon depletion of RINT1 (8). Taken together these data indicate that NAG links the peripheral RINT1 and Zw10 sub-complex to the core SNARE complex including syntaxin-18 and p31.

While initial finding indicated the importance of the syntaxin-18 complex in anterograde transport, subsequent work has since proven their requirement for retrograde transport and that the initial reported defects in anterograde transport were indirectly caused by an inhibition of retrograde trafficking from the Golgi to the ER (182). Zw10 and RINT1 were shown to be downstream effectors of a small GTPase, Rab6. It was found that depletion of Rab6, which is a Golgi associated regulator of retrograde trafficking, suppressed the effects of Zw10/RINT1 depletion mediated Golgi dispersal (182). The authors suggest a model by which Rab6 initiates the recycling of Golgi proteins to the ER where the syntaxin-18 complex including Zw10/RINT1 receive the cargo and tether it to the ER membrane for fusion (182).

In addition to this role in the recycling of Golgi proteins, RINT1 appears to have an important role in the maintenance of Golgi and centrosomal integrity through the cell cycle. RINT1 is found to co-localize to the centrosome throughout the cell cycle (122). It is known that depletion of RINT1 induces a disruption of the Golgi though the defective vesicular recycling pathway (122). However, depletion of RINT1 also results in severe defects in Golgi and centrosomal dynamics throughout mitosis. Loss of RINT1 results in prolonged mitosis, and eventual cell death (122). Additionally, there is an increase in the number of observable centrosomes in RINT1 depleted cells suggesting a role for RINT1 in faithful centrosomal duplication (122). This aberrant centrosomal duplication directly led to an increase in the number of spindle poles in mitosis and

subsequent chromosomal instability (122). Pericentriolar localization is thought to be critical for proper Golgi disassembly in mitosis and subsequent reassembly (29, 74, 89). Depletion of RINT1 also results in a disconnection of the Golgi from the centrosome (122). It is thought that RINT1 may coordinate faithful segregation of the Golgi and the centrosome during mitosis. The presence of syntaxin-18 and Zw10 in this RINT1 complex in this process is unclear.

Syntaxin-18 has also been found in complex with the BPV-1 minor capsid protein L2 (22, 109). Both syntaxin-18 and L2 were both found to co-localize to the ER (22). This was a surprising result because L2 contains two nuclear localization signals. The interaction domain between these two proteins was determined to involve a highly conserved region from amino acids 40 to 44 (22). Mutation of this domain results in generation of non-infectious pseudovirions (22). These pseudovirions are able to attach and enter the cell; however, they are unable to subsequently interact with syntaxin-18 (22). Further, microinjection of a peptide antibody raised against this interaction domain is capable of neutralizing viral infection as a direct result of interference with the L2 syntaxin-18 interaction (109). The authors suggest that syntaxin-18 and its interaction with L2 is necessary for infection. This interaction takes place after vesicular escape and that syntaxin-18 facilitates trafficking of the L2-genome complex or partially disassembled virions to the nucleus for import (109).

RINT1-E2 Complex

The nature of the interaction between BPV-1 E2 and RINT1 remains unclear. RINT1 was initially identified in this lab not as an E2 interacting protein, but in a screen for proteins that interact with hChlr1 (shortly after its identification in Rad50 and Syntaxin-18 complexes). hCHLr1 has been shown in our lab to tether the viral genome to the host chromatin during mitosis. Our lab and others have also shown that this protein as a key factor in establishing sister chromatid cohesion early in mitosis. In order to further characterize hCHLr1 and its role in cohesion and BPV-1 episomal maintenance, a screen was performed to identify other proteins that interact with hCHLr1. One positive interacting protein was RINT1. RINT1 had already been established to play a role in vesicular transport with binding partners Syntaxin-18 and Zw10. Its interaction with the latter protein, which is also a critical factor for signaling spindle attachment to the kinetochore to the spindle checkpoint proteins, prompted investigation into the possibility that RINT1 interacted with E2. It was thought at the time that RINT1 may form a complex with hCHLr1 and Zw10 to facilitate viral genome maintenance and coordinate tethering with the spindle assembly checkpoint. We were subsequently able to confirm that a 185 amino acid fragment of RINT1 interacted with BPV-1 E2.

Preliminary results from our lab show that RINT1 forms complexes with hCHLr1 and with BPV-1 E2. Here, we characterize the binding domains on E2 to which RINT1 interacts. A panel of transcriptionally active and defective point mutants

was screened to gain insight into the nature of this interaction. Finally we addressed the possibility that RINT1 may be post-translationally modified in complex with E2. We identify that several N-terminal mutants of E2 do not interact with RINT1 or interact very weakly. While we were unable to identify the biological significance of this interaction, we present a foundation for future work in this area. The interaction between RINT1 and E2 may present a link between the viral regulatory protein and the vesicular transport pathway.

Materials and Methods

Cell Culture and Antibodies C33a cells were used in all immunoblot and immunoprecipitation experiments, the cells are from spontaneously immortalized (HPV negative) cervical carcinoma biopsies. They were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (Atlas Biologicals) and penicillin/streptomycin solution (Invitrogen). DNA for transfections was diluted in Optimem Serum Free Media (Invitrogen) and transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen).

Primary antibodies used include BPV-1 E2 B201 from lab prepared stock, RINT1 was detected using 12CA5 anti-HA, 9E10 anti-myc, 2074 anti-RINT1 peptide antibody (raised against peptide CKRPENYFKHIKEA starting at amino acid 720 in the C-terminus of RINT1) or anti-RINT1 as published (79) prepared from hybridoma cell line. Secondary antibody used was horseradish peroxidase conjugated goat anti-mouse IgG (The Jackson Laboratory).

Immunoprecipitation and Immunoblot Analysis C33a cells were plated on 10 cm tissue culture dishes. The following day cells were transfected with a combination of pCG vector, pCDNA3-HARINT1, and E2 or E2 mutants. 24 hours post transfection, cells were harvested by rinsing with phosphate buffered saline (PBS), scraping into a small volume of PBS for transfer to an Eppendorf tube. Cells were lysed using IPLB (50mM Tris-HCl pH 8.0, 0.1mM NaCl, 20mM NaF,

10mM KH_2PO_4, 1% Triton X-100, 10% Glycerol, 0.1mM DTT, 2mM PMSF, and Complete protease inhibitor cocktail Roche). Lysates were clarified by centrifugation at 15,000 x g for ten minutes. Lysates were then diluted 1:1 with IPBB (50mM Tris-HCl pH 8.0, 0.1mM KCl, 0.1mM EDTA, 0.2% NP-40, 0.5 µg BSA, 2.5% Glycerol, 0.1mM DTT, 2mM PMSF), specific antibodies were added and complexes were formed with gentle agitation overnight at 4°C. Protein A or Protein G sepharose beads (Invitrogen) were added the following day and complexes were collected for 1 hour. After extensive washing with IPWB (0.1mM Tris-HCl pH 8.0, 0.1mM NaCl, 0.2% NP-40, 0.1mM DTT, 2mM PMSF) complexes were separated by SDS-PAGE and membranes were probed for target proteins. For each individual lysate, 5% of the total volume used for each immunoprecipitation used was reserved for use as input protein. This sample was separated by SDS-PAGE with the immunoprecipitated complexes for reference of protein expression.

Immunoprecipitated complexes for phosphatase treatment were prepared in the same manner as above. In the place of sepharose beads for collection, protein A or protein B magnetic Dynabeads (Invitrogen) were used to clear the entire volume of buffer from the complexes. Following the final wash the complexes were split to two tubes. NEB buffer 3 was added with 20 units of calf intestinal phosphatase (New England BioLabs) in a total reaction volume of 50 µL. 2 µL of water was added to one reaction of every pair in place of phosphatase. The reactions were incubated at 37°C for 60 minutes and then separated by SDS-

PAGE. The effect of phosphatase treatment was detected by immunoblot using an anti-HA antibody.

In Vitro GST-Pull Down Glutathione S-transferase (GST) fused fragments of E2 were synthesized essentially as described previously (26). *E. coli* strain BL21 (DE3) containing pLysS and pGEX2T-TAD or pGEX2T-DBD were grown up to an optical density at 600nm equaling approximately 0.6. Protein expression was induced overnight at 30^{o}C using 1mM IPTG. Cells were collected, lysed in NETN (100mM NaCl, 0.1mM EDTA, 20mM Tris-HCl pH 8.0, 0.1% NP-40), sonicated in the presence of lysozyme, and lysates were clarified by centrifugation. E2 DBD was purified by incubating with glutathione beads at 4^{o}C overnight. E2 TAD, was purified as above except after clarifying lysate, the supernatant was decanted and the pellet was solubilized with 8M urea. E2 TAD was purified from this lysate by incubating with glutathione beads at 4^{o}C overnight. After extensive washing protein concentration was estimated using Bio-Rad protein assay. A sample of each purified protein was then separated by SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 total protein stain (Fischer Scientific).

In vitro transcription and translation of RINT1 and luciferase were performed using ^{35}S-labeled methionine and TnT Coupled Reticulocyte Lysate system (Promega) as described in the manufacturer's protocol. Templates included pSP65-RINT1 containing a Sp6 promoter and pTNT-Luciferase containing a T7 promoter. 5 µL of each reaction was then incubated with GST-E2 proteins or

GST alone at 4^{o}C overnight. Complex bound beads were then thoroughly washed with NETN with 300mM NaCl. Complexes were then separated by SDS-PAGE, the gel dried, and exposed to a phosphor screen (Fuji). Signal on the phosphor screen was then detected using a Fuji Phosphorimager.

Luciferase Reporter Assay C33a cells were plated in triplicate on 6-well tissue culture plates. The following day, cells were transfected with 150ng pGL2-4BS E2 responsive luciferase reporter, 75ng pCG-E2b wild type E2 expression construct, pcDNA3-HARINT1, or pcDNA-TFIIB. 24 hours post transfection, cells were harvested with Reporter Lysis Buffer (Promega) and subjected to one freeze thaw cycle. 30 µL of each lysate was then added to one well of a CulturPlate-96 (Perkin Elmer). Just prior to detection, 100 µL of Luciferase Assay Reagent (Promega) was added to wells and then reporter expression was detected on an EnVision Multilabel Plate Reader (Perkin Elmer). Means were calculated from each triplicate and error bars represent standard deviation from the mean. Data is presented as a percentage of reporter expression in the presence of wild type E2 alone.

Yeast Two-Hybrid *S.cerevisiae* strain DBY1, which was constructed by inactivation of the TRP1 gene of BGW17a (MATa, leu2, 2-leu2, -11; his4–his519, ade1—ade100,ura3–ura52) (25), was transformed with pBSY72 which is a β-Gal reporter containing E2 specific binding sites. Transformants were selected using yeast minimal media in the absence of uracil, and the process was repeated for

transformation of wild type E2, E2 F87S, E2 55, E2 113, and E2 162 in the YEplac112G vector all containing a tryptophan selection marker. After transformation of RINT1, in the plasmid pACT-RINT1, where it is fused to the Gal activation domain, transformants were selected on appropriate dropout medium and ten colonies were streaked onto selective media containing X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). After development of blue color, the darkness of the hue was visually inspected and scored into three categories based on the strength of interaction. Controls for this assay include, lamin interaction with E2 and RINT1, Gps2 interaction with E2, hCHLr1 interaction with RINT1 and transformation of empty pGBKT7 and pGADT7 vectors.

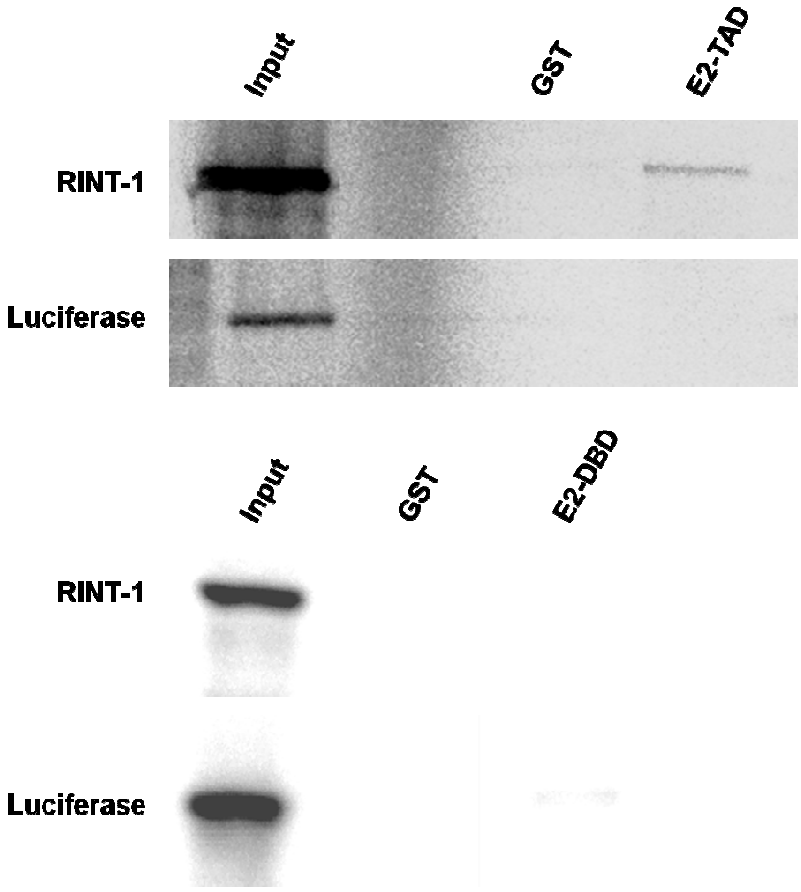
Results

RINT1 Interacts with the Transactivation Domain of E2

While initial yeast two-hybrid experiments show that E2 interacts with a region within the N-terminal half of RINT1 (amino acids 194-379), the interaction surface on E2 has not been characterized. Proteins have been reported to interact with either the N-terminal transactivation (TAD) domain or the C-terminal DNA binding and dimerization domain (DBD). Identifying the domain with which RINT1 interacts with will provide insight to the biological significance of this interaction. The domain on E2 that interacts with RINT1 was probed *in vitro*. Glutathione S-transferase (GST) tagged E2 TAD and DBD fragments were purified from E. *coli* and allowed to interact with isotopically labeled RINT1 or Luciferase control. GST alone was unable to interact detectably with either luciferase or RINT1. Further, labeled luciferase was undetectable or very inefficiently precipitated with GST-TAD or GST-DBD (Fig 3.1) indicating that there is insignificant background in this assay. RINT1 was precipitated following incubation with GST-TAD but not GST-DBD (Fig 3.1).

The domain with which RINT1 interacts on E2 was further characterized using a yeast two-hybrid interaction assay. Several truncation mutants consisting of increasing deletion of the TAD were transformed with an N-terminal truncation of RINT1 which was the original yeast two-hybrid hit. Lamin A/C was used as a negative control and did not interact with RINT1 or E2 (Data not shown). hCHLr1

Figure 3.1 RINT1 interacts with E2 *in vitro.* Purified GST tagged E2 TAD (1-216), E2 DBD (286-410), or GST alone was incubated with ^{35}S labeled RINT1 or Luciferase. E2 was precipitated with glutathione resin and the interaction detected by phosphorimaging. Input represents 20% of *in vitro* translated ^{35}S RINT1 or Luciferase used in reactions.


Input
GST
E2-TAD
RINT-1
Luciferase
Input
GST
E2-DBD
RINT-1
Luciferase

was used as a positive control for interaction with RINT1 and was found to strongly interact (Data not shown). Similarly Gps2, which was used as a positive control for E2 interaction, was found to interact strongly with E2, validating this assay (Data not shown). In addition to wild type E2, the transactivation defective mutant F87S was also used. E2 is able to activate the lactose operon in the absence of an activation domain fused "prey" protein. Wild type E2 and F87S both interacted strongly with RINT1 as expected (Fig 3.2). Deletion of the 54 or 112 amino acids from the N-terminal TAD, resulted in a decrease in interaction with RINT1. This decrease was approximately 50% as determined by visual inspection (Fig 3.2). Further deletion of the N-terminal 161 amino acids disrupted the interaction (Fig 3.2). A summary of the yeast two hybrid results is presented in table 3.1.

RINT1 and E2 Form a Complex *In Vivo*

The interaction between RINT1 and E2 has been confirmed *in vitro* and in yeast. While this strongly suggests the interaction will exist *in vivo* the nature of such a complex and its biological relevance is still unclear. Complex formation between E2 and RINT1 was investigated *in vivo* using mammalian cell co-immunoprecipitation. RINT1 and E2 were efficiently co-precipitated using E2 antibodies in C33a cell lysates where both proteins were transfected (FIG 3.3a). RINT1 was not co-precipitated with E2 antibodies in negative control IPs which included mock transfected lysates and lysates transfected with E2 or RINT1 alone. Cells were also co-transfected with RINT1 and three E2 point mutants.

Figure 3.2 RINT1 interacts with E2 in yeast. *S.cerevisiae* yeast strain DBY1 was transformed with an E2 responsive β-galactosidase reporter, E2, E2 F87L, or E2 truncations, as well as RINT1 fused to the Gal4 activation domain and selected on SD agar plates in the absence of tryptophan, histidine, or leucine. 10 transformants were then streaked onto selective plates containing X-Gal.

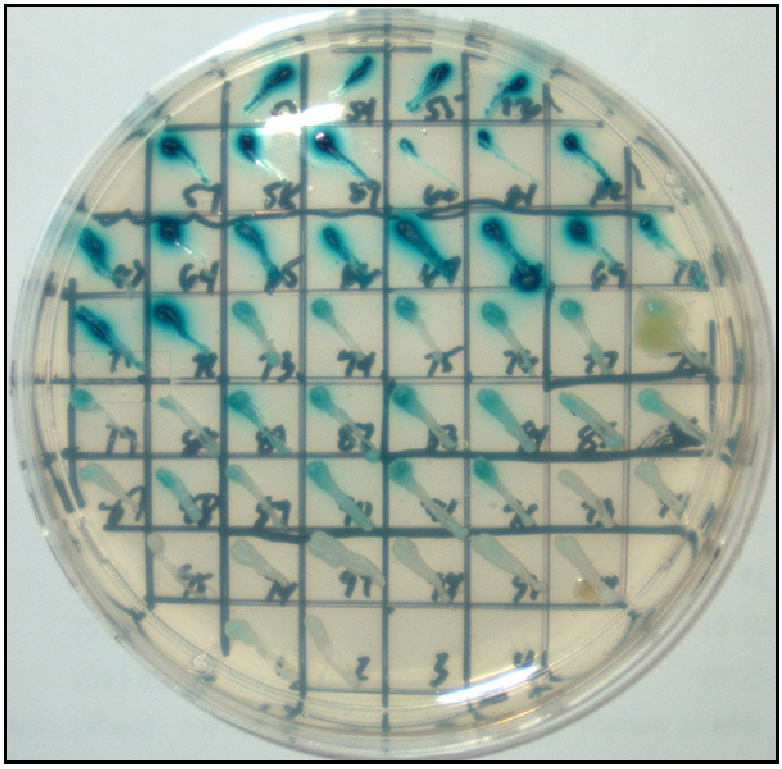
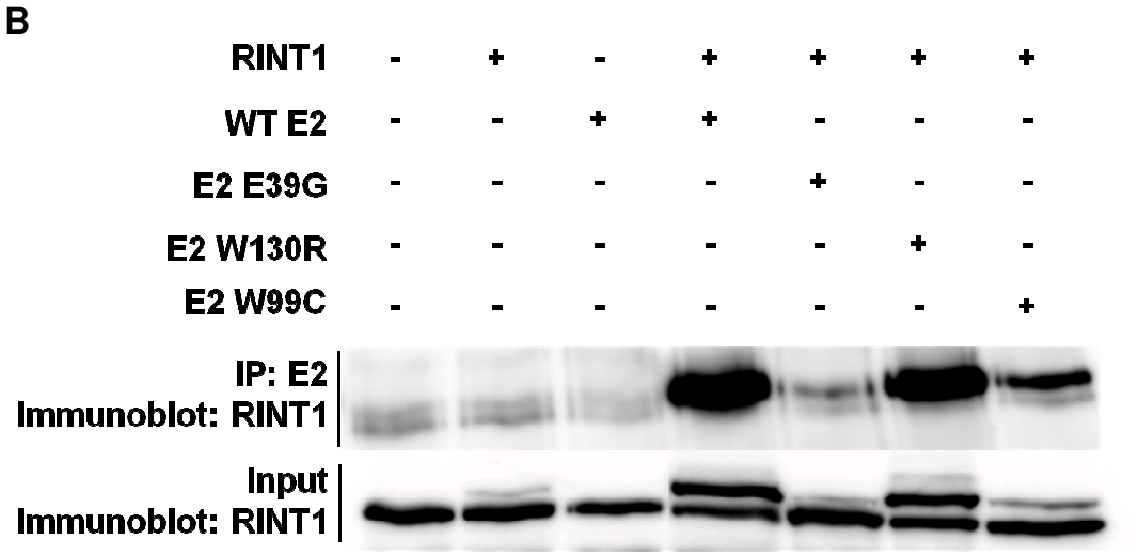


Table 3.1 Summary of Yeast Two-hybrid Binding Assay

Construct	Score
WT	+++
F87L	+++
55-410	+
113-410	+
162-410	-

Figure 3.3 RINT1 interacts with E2 in mammalian cells. **A** C33a cells were transfected with empty vector or a combination of HA-RINT1, wild type E2, or several E2 point mutants. 24 hours post transfection cells were immunoprecipitated with an E2 antibody (B201). Interaction was detected by probing precipitated complexes with an anti-HA antibody to detect RINT1. Lower two panels are 10% input probed for E2 and HA-RINT1. **B** C33a cells were transfected as previously. Protein content of each lysate was estimated prior to immunoprecipitation. The amount of lysate added to each reaction was then normalized to total protein. Complexes were precipitated with an E2 antibody (B201) and probed for RINT1with an anti-HA antibody. The top panel represents input from each lysate.


A
RINT1 - + - + + + +
WT E2 - - + + - - -
E2 E39G - - - - + - -
E2 W130R - - - - - + -
E2 W99C - - - - - - +
IP: E2
Immunoblot: RINT1
Input
Immunoblot: RINT1
Input
Immunoblot: E2


B
RINT1 - + - + + + +
WT E2 - - + + - - -
E2 E39G - - - - + - -
E2 W130R - - - - - + -
E2 W99C - - - - - - +
IP: E2
Immunoblot: RINT1
Input
Immunoblot: RINT1

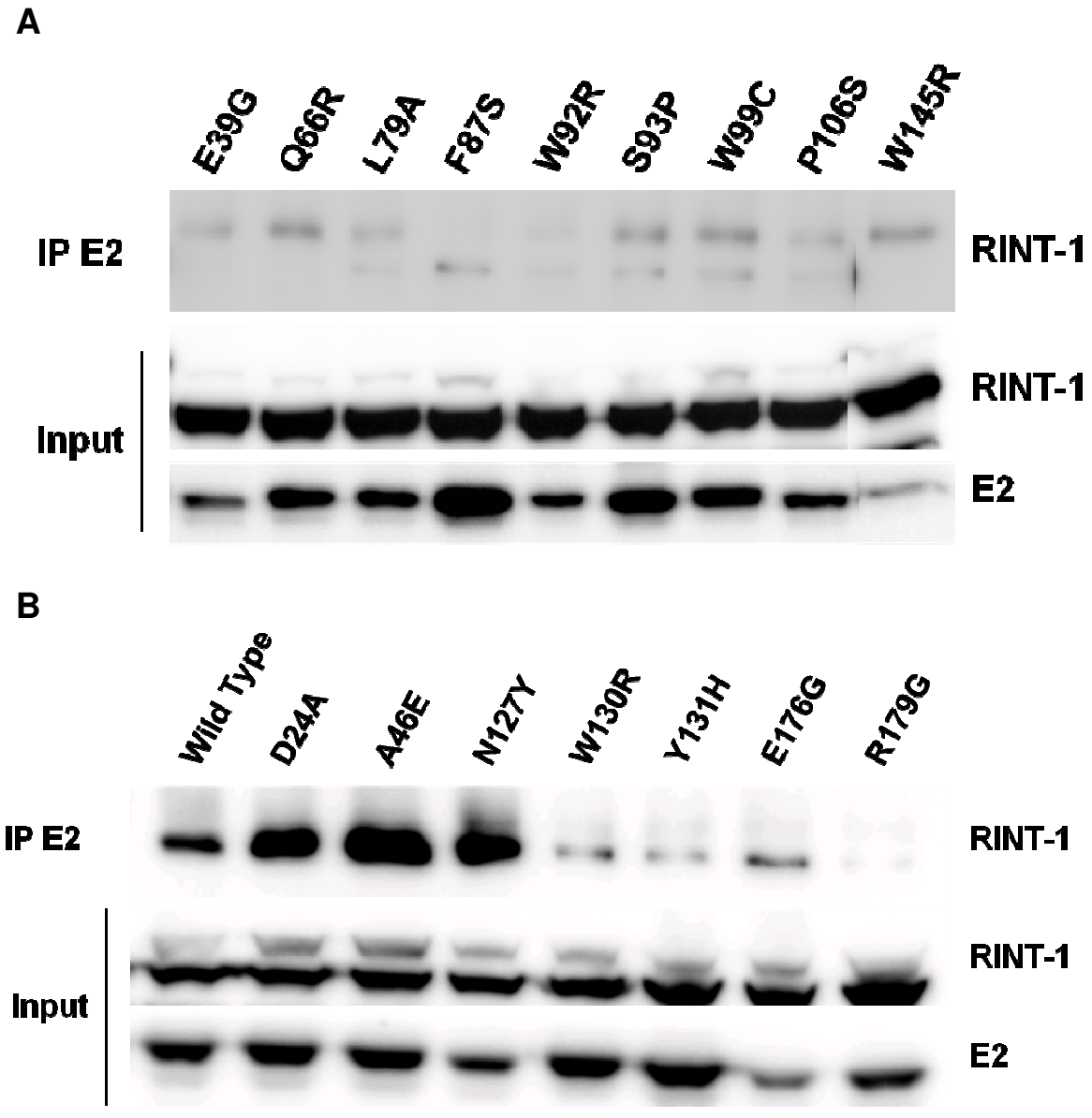
E39G is defective for transcription, but competent for transient replication. This mutant was also used as bait for detection of RINT1by yeast two-hybrid screen. W99C is unable to interact with the general transcription factor TFIIB and is defective for both transcription and replication activity (209). W130R is unable to interact with hCHLr1 and is competent for transcription and transient replication; however, mutant genomes containing this lesion are unable to be maintained episomally (143). Transcriptionally defective mutants E39G and W99C were expressed at lower levels than wild type E2 which is consistent with other transcriptionally defective E2 mutants (25, 70). Additionally, co-expression of these mutants resulted in reduced expression from the CMV promoter driven RINT1 expression construct used in this experiment (Fig 3.3a). This phenomenon is also consistent for co-expression of other transcriptionally defective E2 point mutants. W130R was expressed to levels comparable to wild type E2 and was also able to enhance RINT1 expression. Immunoprecipitation using an E2 antibody efficiently co-precipitated RINT1 in E2 W130R co-transfected cells; however, in cells co-transfected with either of the two transcriptionally defective mutants, E39G or W99C, E2 antibodies were either unable to co-precipitate RINT1 or unable to efficiently co-precipitate RINT1 in quantities detectable over background (Fig 3.3a). Given that transcriptionally defective mutants resulted in dramatically reduced protein levels of E2 and RINT1, we next asked if normalizing the input lysate of the immunoprecipitation for total protein content would allow detection of RINT1 interaction with these two

mutants. The experiment was repeated under the same conditions; however, prior to immunoprecipitation, equal amounts of total protein were separated on a gel and the relative RINT1 protein levels in each lysate were estimated by measuring the intensity of each band on the chemiluminescent image. The volume of each sample lysate used for immunoprecipitation was then calculated based on the ratio of RINT1 protein present in that sample compared to wild type E2 transfected lysates. Following precipitation with E2 antibodies samples transfected with both wild type E2 and E2 W130R were able to efficiently co-precipitate RINT1 (Fig 3.3b). With increase input lysate, RINT1 was also detectably co-precipitated in samples transfected with E39G and W99C. These proteins still appear to only co-precipitate very small amounts of RINT1 as evidenced by the need of a long exposure to visualize RINT1 co-precipitated with E2 E39G (Fig 3.3b).

RINT1 Interacts Preferentially with Transcriptionally Active E2 Mutants

The differential interaction of RINT1 to E2 W130R versus E39G and W99C introduced the possibility that RINT1 may only interact with E2 in transcriptionally active complexes. This phenomenon has also been demonstrated with Brd4 where its interaction with E2 correlates with E2 transcriptional activity (167). We sought to investigate if this was the case with RINT1 by screening a panel of both transcriptionally competent and transcriptionally defective E2 N-terminal point mutants. In accordance with the previous experiment, mutants E39G and W99C were both found to interact with RINT1 (Fig 3.4a lanes 1 and 7). Point mutants

Figure 3.4 Interaction of RINT1 with E2 mutant panel. C33a cells were transfected with RINT1, several **A** transactivation defective point mutants, several **B** transactivation competent point mutants, or wild type E2. 24 hours post transfection cells were immunoprecipitated with an E2 antibody (B201). Interaction was detected by probing precipitated complexes with RINT1 antibody. Immunoprecipitation is the top panel in **A** and **B.** The remaining panels represent 10% input probed for E2 and RINT1.


A
E39G
Q66R
L79A
F87S
W92R
S93P
W99C
P106S
W145R
IP E2
RINT-1
Input
RINT-1
E2
B
Wild Type
D24A
A46E
N127Y
W130R
Y131H
E176G
R179G
IP E2
RINT-1
Input
RINT-1
E2

Q66R, S93P, and W145R were found to interact with RINT1 with similar efficiency to W99C. All of these mutants had similar expression of E2 and RINT1, except for co-transfection of W145R which had noticeably lower protein levels of each protein. Mutants L97A and P106S co-precipitated RINT1 to similar levels as E39G which interacted with RINT1 less efficiently than W99C. Expression of L97A was similar to that of P106S co-expressed RINT1 in these samples was also present at similar levels. Interestingly, E2 mutants F87S and W92R were either unable to precipitate RINT1 or interacted very weakly. F87S was unable to interact with RINT1 despite higher levels of both proteins (Fig 3.4a). The level of co-precipitation despite low levels of RINT1 might suggest that E2 recruits a majority of available RINT1 into complexes. In order to further characterize the interaction between E2 and RINT1 and to possibly identify more residues that are important for this interaction we screened a panel of transcriptionally competent E2 mutants. These mutants are expressed at much higher levels than transcriptionally defective mutants for reasons outlined earlier. Additionally, co-expression of these mutants does not have a deleterious effect on transcription of the RINT1 expression construct. E2 transcriptionally competent point mutants are expressed at similar levels and result in RINT1 levels that are generally equal as well. While transcriptionally negative mutants interacted with RINT1 to nearly equal levels throughout the panel with few exceptions, the transcriptionally competent mutants appear to be clustered into groups that interact with RINT1 at levels higher than wild type: D24A, A46E, and

N127Y, and levels about half wild type levels: W130R, Y131H, and E176G (Fig 3.4b). One mutant, R179G, appears to interaction with RINT1 very inefficiently if at all indicating its importance for interaction with RINT1. A summary of the relative transcriptional activities of each BPV-1 E2 point mutant is presented in Table 3.3.

Post-Translational Modification of RINT1 in E2 Complexes

During investigation into the nature of the interaction between RINT1 and E2, a higher molecular weight banding pattern was consistently observed. RINT1 was initially described to migrate as a doublet the slower migrating band resulting from upstream non-canonical initiation codon (206). Incremental banding is most apparent in samples transfected with wild type E2 (Fig 3.4b), it is also visible in mutant transfected samples as well. This pattern seems to be limited to transcriptionally active mutants and those that are expressed at higher levels (Fig 3.4b). This may suggest that if a post-translational modification is present it may be E2 dependent. A previous report had attempted to detect phosphorylation of RINT1 by treating lysates with calf intestinal phosphatase (CIP). This study was unable to detect phosphorylation; however, we observe more banding in samples over-expressing E2. We attempted to determine if RINT1 is phosphorylated in complex with E2. First, we addressed the possibility that the higher mobility banding is due to antibody background. In all experiments a BPV-1 E2 monoclonal antibody (B201) is used that recognizes an N-terminal fragment of the protein. This antibody is known to recognize several background bands in

Table 3.2 Summary of BPV-1 E2 Point Mutants

Lesion	Transcriptional Activity	Protein Interaction
D24A	++	
E39G	-	
A46E	+++	
Q66R	-	
L79A	-/+	
F87S	-	
W92R	-	
S93P	-	
W99C	-	TFIIB
P106S	-	
N127Y	+	
W130R	++	hCHLr1
Y131H	+	
W145R	-	Gps2
E176G	+	
R179G	+	

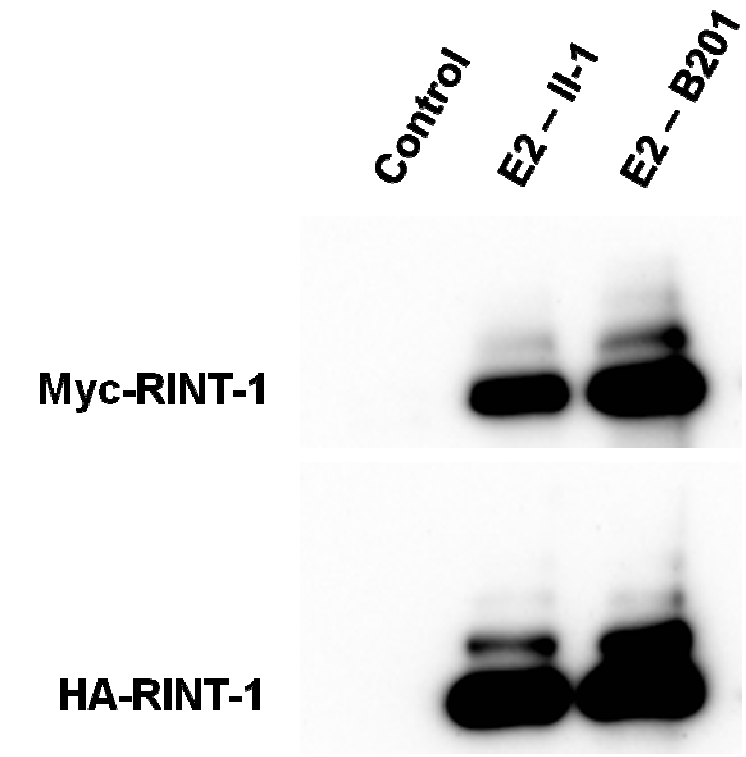
immunoblot experiments. To address this we immunoprecipitated E2 using a rabbit N-terminal polyclonal antibody (II-1) and probed for RINT1 with two separate antibodies. In samples precipitated with both II-1 and B201 antibodies, RINT1 was efficiently co-precipitated. Additionally, higher mobility bands were observed in samples co-precipitated with either antibody. There was also no difference between hemagglutinin tagged or FLAG tagged RINT1 (Fig 3.5a). B201 was used to co-precipitate E2 and RINT1 and the complex was treated on beads with CIP. In RINT1 input immunoblot, co-transfection of RINT1 and E2 resulted in a non-distinct series of bands migrating slower than the expected mobility of RINT1 (Fig 3.5b). Co-precipitation of RINT1 with an E2 antibody clarified these higher molecular weight bands into more than five distinct bands of higher molecular weight than RINT1. Treatment of the sample with CIP did not have any effect on this banding pattern (Fig 3.5b).

RINT1 Does Not Interact with the BPV-1 Genome

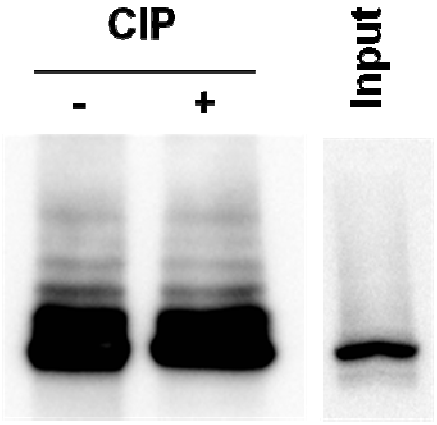
There is no clear suggestion based on RINT1 biology in the literature as to what effects RINT1 may have on E2 function. Many of E2s functions in the viral life cycle are mediated by interaction with the viral genome. Our first step in determining the functional significance of this interaction was to determine if RINT1 interacts with E2 in complex with DNA. To address this, ID13 cells, which episomally maintain BPV-1 genomes, were transfected with a RINT1 expression construct and then ChIP analysis was performed. As expected, E2 interacts

Figure 3.5 E2 co-precipitates higher molecular weight RINT1. A C33a cells were co-transfected with HA-RINT1 or myc-RINT1 and wild type E2. 24 hours post transfection cells were lysed and complexes were precipitated with either rabbit E2 (II-1) or mouse E2 (B201) antibodies. Precipitates were then probed for RINT1 using either anti-HA or anti-myc antibodies. **B** Cells were transfected with HA-RINT1 and the following day immunoprecipitated with E2 B201. Precipitates complexes were split and treated with calf intestinal phosphatase (CIP) or bovine serum albumin (BSA). Precipitates were probed for RINT1 using an anti-HA antibody.

A



B



quite robustly with BPV-1 genomes (Fig 3.6) as detected by amplifying precipitated complexes using BPV-1 LCR primers. However, neither of two RINT1 antibodies used was able to co-precipitate BPV-1 genomes in complex with RINT1 (Fig 3.6).

RINT1 Over-Expression Does not Affect E2 Dependent Reporter Activation

Interaction with the panel of transcriptionally defective mutants did not conclusively determine if RINT1 plays a role in E2 dependent transcriptional activation. Two transcriptionally defective mutants in close proximity do not appear to interact efficiently or with high affinity to RINT1. We addressed the possibility that RINT1 may be a limiting factor in E2 dependent transcription by using a luciferase reporter assay. Upon transfection of wild type E2, an 80 fold increase in luciferase activity was detected (Data Not Shown, Fig 3.7a). Transfection of RINT1 in the absence of E2 showed no increase in luciferase activity over reporter alone (Fig 3.7a). While TFIIB, which has previously been demonstrated to enhance E2 dependent transcription, increased luciferase activity nearly two fold over E2 alone, co-transfection of RINT1 had no effect.

Luciferase activity in the presence of RINT1 was no different than E2 alone (Fig 3.7a). It is possible that over-expression of RINT1 may have a negative effect on E2 dependent transcription or positive effects may be squelched at high levels of RINT1 transfection. To determine if this is the case, we performed a dose response starting at a ten-fold lower concentration than in the previous

Figure 3.6 RINT1 Does not Interact with BPV1 Genomes. ID13 cells were transfected with RINT1. 24 hours post transfection, cells were crosslinked, lysed and immunprecipitated with control IgG, E2 (B201), or one of two RINT1 antibodies rabbit peptide 2074, or anti RINT1 (79). Precipitates were amplified using primers for the BPV-1 LCR.

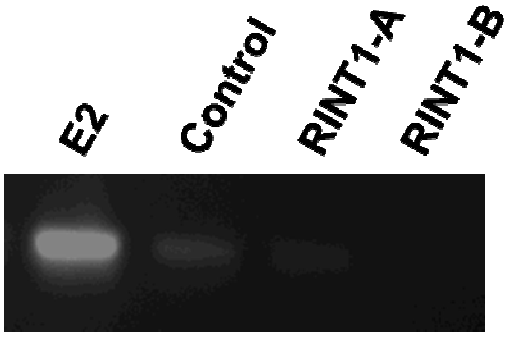
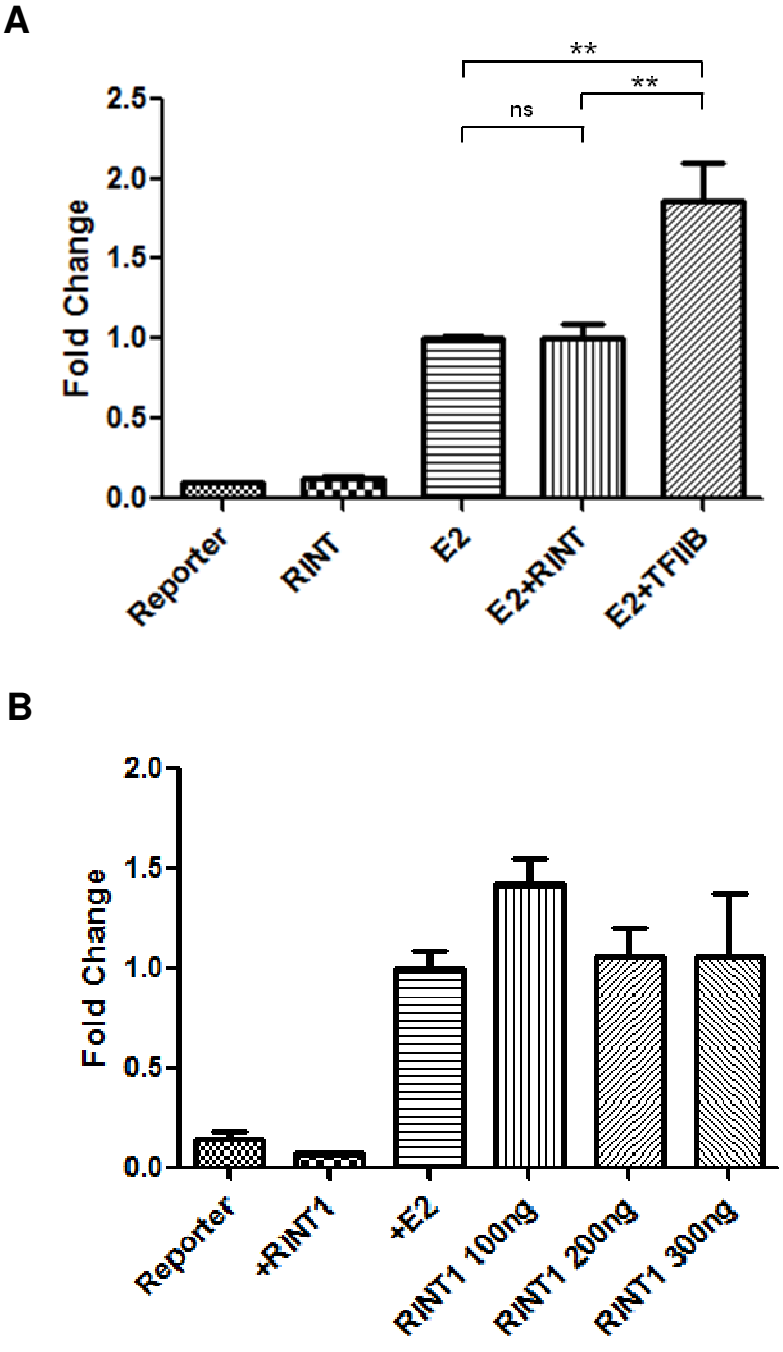

E2
Control
RINT1-A
RINT1-B

Figure 3.7 RINT1 does not affect E2 dependent transcription. **A** and a combination of E2, RINT1, or TFIIB. 24 hours post transfection cells harvested and luciferase detected. One way ANOVA was performed with Bonferoni's post hoc test ** $p<0.01$. **B** C33a cells were transfected with E2 responsive luciferase reporter, E2 and either 100ng, 200ng, or 300ng of RINT1. Luciferase activity was detected 24 hours post transfection.


A
**
**
ns
2.5
2.0
1.5
1.0
0.5
0.0
Fold Change
Reporter
RINT
E2
E2+RINT
E2+TFIIB
B
2.0
1.5
1.0
0.5
0.0
Fold Change
Reporter
+RINT1
+E2
RINT1 100ng
RINT1 200ng
RINT1 300ng

experiment. In contrast to transfection of 1 µg of RINT1, transfection of 0.1 µg led to a 1.5 fold increase in luciferase activity. This increase, however, was not observed in any higher concentrations as both transfections of 0.2 µg and 0.3 µg resulted in no increase over E2 alone (Fig 3.7b). Overexpression is useful in determining if proteins of interest are capable of stimulating a particular pathway, in this case RINT1 in E2 dependent transcription. This does not rule out the importance of RINT1 in E2 dependent transcription and to determine if RINT1 is truly limiting for E2 dependent transcription the effect of RINT1 depletion on reporter activation should be addressed.

Discussion

The papillomavirus E2 regulatory factor functions in nearly every process in the productive viral life cycle. This small protein accomplishes this functional diversity through its interaction with a myriad of cellular factors. Here we discuss RINT1, a protein which we have identified first through a screen for other proteins interacting with the cellular cohesion factor hCHLR1, and then through a directed binding assay with BPV-1 E2. RINT1 also displays diversity with its known complexes and interaction partners, having potential roles in the response to DNA damage (206), vesicular trafficking (6, 8, 79, 182), and hTERT independent control of telomere length (102). While the viral oncogene E7 (59, 156, 212) and the minor capsid protein L2(22, 109) have been demonstrated to interact with some of the common components of these pathways, there have been no reports to date placing either of these proteins in complexes with RINT1. Nor has E2 been shown to be involved in any of the RINT1 dependent processes. The novel interaction between BPV-1 and E2 may reveal a new role for E2 in the viral life cycle.

Identification that the interaction takes place in the N-terminus indicates that E2 may still be able to contact DNA in complex with RINT1. It is not uncommon for E2 to recruit factors to the promoter or replication origin. However, this is unlikely given that we were unable to detect RINT1 interaction on BPV1 genomes. The inability of certain transcriptionally defective mutants to interact with RINT1 suggests that like Brd4, RINT1 may be important for E2 dependent transcription.

If RINT1 is involved in transcriptional activation, its endogenous protein levels may saturate complex formation with E2. This may explain why overexpression does not increase luciferase activity. siRNA mediated depletion of RINT1 and its effect on E2 dependent transcription and other E2 dependent viral functions would be interesting to explore.

Mutation of residues D24, A46, and N127 appear to increase interaction with RINT1. These three residues are not in close proximity in the structure model of the BPV-1 E2 N-terminus and only N127 is within the deletion fragment of E2 that we show is important for RINT1 interaction. These three residues could however, disrupt the interaction of E2 with another protein that inhibits E2 interaction with RINT1. Alternatively, mutation of these residues could promote a stronger interaction with a co-factor which strengthens the E2-RINT1 interaction. In contrast, the remaining transactivation mutants that were tested displayed reduced binding capacity for RINT1 including W130R which is unable to interact with hCHLr1. If RINT1 forms a complex with hCHLr1 and E2, that may explain the reduced binding for the E2 W130R mutant. However, we cannot ignore the possibility that the reduced interaction between RINT1 and W130R, Y131H, and R176G may be a function of the chemiluminescent imaging system which normalizes images to strong band intensities such as N127Y. However, inability to interact with hCHLr1 may also explain the reduced interaction for Y131H as well which is in close proximity to W130R. R176G and R179G are also near the hCHLr1 interaction domain, although their role in interaction with hCHLr1 if any is

unclear. R179G in particular was consistently reduced in RINT1 interaction. This suggests that this mutant may be a useful tool to identify the biological significance of the interaction. Previous studies using this mutation have demonstrated that it is competent for transient replication partially deficient for transcriptional activation (70). Although a role for RINT1 in episomal genome maintenance has not yet been ruled out, the lack of RINT1 interaction with the genome would argue against this possibility.

The presence of multiple bands at incrementally larger molecular weight indicates that RINT1 may be modified in complex with E2. These higher molecular weight bands are not detected in RINT1 input. The study that initially characterized RINT1 has shown that it migrates as a doublet and they suggest that this may be due to initiation at an upstream non-AUG start site (206). While they do not confirm this, they also claim that following treatment with alkaline phosphatase, there was no change in mobility of the slower migrating band which is consistent with our findings here. The incremental ladder that is immunoprecipitated suggests that there may be another type of modification. Several candidates include ubiquitination, neddylation or sumoylation.

The most recent studies on RINT1 biology have converged on the vesicular transport pathway as a critical role for RINT1. This pathway seems incongruous for a viral regulatory factor involved in transcription, replication and maintenance. Immunofluorescent staining of HeLa cells over-expressing both RINT1 and wild

type E2 resulted in ER/Golgi staining for each protein (Data not shown). While this is typical staining for RINT1 as previously demonstrated, BPV-1 E2 is a DNA binding factor that encodes two nuclear localization sequences and is found in the nucleus in almost all cases. It appears that RINT1 may recruit E2 to the ER or Golgi compartments. This staining pattern is similar to what was observed upon transfection of both syntaxin-18 and BPV-1 L2. It is interesting that two viral proteins with distinct functions would localize to the same complex. It is unclear if L2-syntaxin-18 and E2-RINT1 are in complex together at the same time.

The results presented here demonstrate a novel interaction between the cellular factor RINT1 and BPV-1 E2. This interaction appears to be necessary for a novel function of E2. While we were unable to determine the specific function of this interaction, we have demonstrated that RINT1 does not interact with DNA. While this does not exclude a higher order complex formation it does suggest that the interaction may not require the DNA specific activities of E2. Further, we show that RINT1 may be post-translationally modified in complex with E2; it is unclear as to the role of this modification on the function of the E2-RINT1 complex. These results form a basis for further exploration into the functional significance of this interaction.

Conclusions

We demonstrate here a requirement for the histone acetyltransferase proteins p300, CBP, and pCAF for E2 dependent transcriptional activation. Further, we show that despite an overlapping substrate pool, all three of these proteins have unique functions. Our data presented here suggest that there may be two phases of complex assembly on the viral LCR. First, p300 interacts with and modifies either chromatin on the viral genome or E2. This initial modification by p300 facilitates the interaction of E2 to with viral DNA. This is one point of functional specificity for p300; neither CBP nor pCAF are necessary for E2 to efficiently interact with DNA. Second, E2 recruits CBP to the genome. p300 acetylates E2 *in vitro* and it appears that both p300 and CBP acetylate E2 in RPE-1 cells. E2 may be acetylated by p300 primarily and acetylation of E2 by CBP capable of compensating for the loss of p300. It is unclear if E2 is acetylated by p300 after binding to the genome or if acetylation by p300 results in higher DNA interaction efficiency; however, acetylation of E2 is necessary for transcriptional activation. We believe that acetylation of E2 results in the creation of an interaction surface to which a protein containing a bromodomain, such as Brd4, may bind.

The phenomenon of Crm1 independent accumulation of E2 in the cytosol is intriguing. It is possible that acetylation of residues 111 and 112 are necessary for retaining E2 in the nucleus. These two residues are highly conserved which would indicate their importance through papillomavirus evolution. However, at

this time we are unable to draw conclusions about the importance of cytosolic and the mechanism through which it is localized. It may be related to passive diffusion of E2 from the nucleus; however, we cannot yet rule out deficiencies in nuclear import due to a weakened NLS. Alternatively, E2 may be mislocalized as due to an inefficient interaction with a nuclear protein in the absence of acetylation resulting in loss from active transcriptional complexes and subsequent diffusion from the nucleus.

The novel interaction between E2 and RINT1 is interesting as well. While we were unable to generate positive functional data that would have identified the role of this interaction in the viral infection, we were able to rule out transcription. Studies outlining the cellular functions of RINT1 also indicate some interesting possibilities for functional significance. These include some functions that E2 was thought not to participate in such as vesicle trafficking and telomere maintenance.

Functional studies of both E2 acetylation and the interaction between E2 and RINT1 could result in new potential vaccines and therapies. BPV has long been a model system to characterize the mechanism of papillomavirus transcription. This system allows the study of E2 dependent transcriptional activation without the antagonistic effects of early promoter repression as seen in human papillomavirus systems. It has been shown that E2 is still capable of activating transcription from the HPV LCR (103) so many of the effects observed for BPV-1

E2 dependent transcriptional activation may be transferrable to HPV E2 dependent transcriptional activation. These effects may however be limited to differentiation dependent transcription; this report suggests that requirement for a C/EBPα binding site downstream of the E6/E7 transcriptional initiation site and high p300 levels (p300 levels increase in suprabasal cells) are necessary for E2 dependent activation.

Many of the cellularly encoded proteins that interact with BPV-1 E2 are likely to interact with HPV type E2 proteins as well given a high sequence homology. In that regard understanding which proteins interact with BPV-1 E2 and how they affect E2 function may also allow a better understanding of HPV E2 transcription. Further, it has been demonstrated that the function of E2, activation or repression, is at least partly related to the position of the E2 binding sites in relation to the transcriptional start site. Using the knowledge of protein interactions and DNA binding, it may be possible to skew the HPV-16 transcription program to specifically inhibit all viral transcription in basal keratinocytes. This would cause the cell to senesce due to lack of p53 and pRB inhibition by E6 and E7 respectively (65). While this would not clear viral infection it would be an effective method to control pathology. Similarly, inhibition of E2 acetylation through inhibition of its interaction with p300, or direct inhibition of acetylation, would interfere with transcriptional activation. In addition to disruption of transcriptional activation in basal cells, this may also inhibit

expression of late genes in differentiated tissue which would then prevent transmission of the virus.

Given the lack of therapeutic vaccines, further study into the nature of HPV transcription is warranted. Differentiation between activation and repression could lead to therapies that could selectively inhibit viral activation while continuing to repress viral oncogene expression. This in turn could provide an avenue to selectively induce apoptosis in HPV tumor cells without introducing exogenous E2. Several groups have attempted similar approaches by infecting cells with adenovirus expressing E2 proteins (15, 68). These studies demonstrate that selective killing of HPV tumor cells through E2 expression is a promising therapy. The possibility of developing a small molecule that may accomplish this without the need for exogenous introduction of E2 through viral infection is intriguing.

Future Direction

Further characterization of HATs on the viral LCR would be very informative in determining their overall importance in E2 dependent viral transcription. Some of the data presented in this work are preliminary results that need to be repeated several more times and optimized including the quantitative ChIP that demonstrates that E2 DNA binding is p300 dependent. HAT depletion in virally transformed cells including ID13 and C127-A3 was very inconsistent. Optimization of this protocol would generate data for both this ChIP assay, analysis of acetylated Histones on the genome, as well as detection of E6 RNA transcript. This was in progress upon the completion of this work. In addition to these experiments, it would be interesting to determine the timing of early events in E2 dependent transcription. It is unclear how p300 enhances E2 interaction with DNA, it would be informative to use E2 K/R mutants to ask the following questions 1) can E2 still interact with p300? 2) is acetylation of E2 necessary to facilitate E2 binding? And 3) do p300 and E2 interact with DNA as a complex or is the modification of chromatin by p300 sufficient for E2 to interact. The roles of CBP and pCAF are also unclear and more work is needed to determine their unique roles in E2 dependent transcription.

We were able to confirm acetylation of E2 on 14 sites *in vitro*. This is most likely does not happen in cells. It appears through the screening of lysine to arginine mutants that some of these lysines are acetylated *in vivo*. To confirm which lysines are acetylated in a physiological setting, E2 must be purified from

mammalian cells and then acetylation sites identified by proteomic analysis. Subsequent analysis of combinatorial mutations would be informative for understanding the functional significance of E2 acetylation. Additionally, more work must be done to determine what role if any K111 and K112 play in the regulation of E2 localization. It might be interesting to examine the ability of these mutants to be imported into the nucleus. Finally, how does acetylation affect transcription? We postulate here that this is through interaction with a co-factor such as Brd4. Currently, we have no evidence to support this or interaction with any other protein.

Primarily biochemical interaction data was presented here regarding the interaction between E2 and RINT1. First, optimization of RINT1 depletion is critical to determining its role in the viral life cycle. Simple experiments such as depletion of syntaxin-18 might identify if RINT1 interacts with E2 in this complex. If it does, this implicates E2 in the vesicular trafficking. It is known that the minor capsid protein L2 interacts with DNA, syntaxin-18 and is capable of entering the nucleus on its own. Perhaps complex formation between E2 and RINT1 links E2 to Zw10 and dynein, facilitating its transport from the ER membrane. Given that E2 point mutants K111R and K112R are mislocalized to the cytosol, in a possibly ER/Golgi staining pattern, it might be interesting to address a role for these residues in RINT1 interaction. RINT1 interacts with Rad50 in the nucleus in G2/M phase. A time course observing this interaction might indicate if RINT1/E2 is involved in complex with this protein. RINT1 interacts with both Rad50 and

p130 in the maintenance of telomere length by the ALT pathway. Co-precipitation with p130 could help to determine the role played by RINT1 in this process. Additionally, observing telomere length in the presence or absence of E2 and an E2 point mutant that does not interact with RINT1 would be a direct way to answer this question functionally.

E2, RINT1 and hCHLr1 all interact, however their ability to form a ternary complex is unknown. In addition to determination the specific role of the interaction between E2 and RINT1, it would be interesting to identify reasons that RINT1 interacts with hCHLr1 and if this complex interacts functionally with E2. E2 interaction with RINT1 is not dependent on hCHLr1 given that the E2 mutant W130R, which does not bind hCHLr1, is competent for RINT1 binding. However this does not exclude RINT1 as an accessory factor to facilitate loading of E2 and BPV genomes onto chromatin.

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