Regulation of viral and host genomes by high risk human papillomavirus E2 protein in association with cellular factors

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Abstract

Human papillomaviruses (HPVs) are the causative agent in cervical cancer and have been implicated in a rising number of head and neck cancers in both men and women. There are two licensed prophylactic vaccines, both target HPV 16 and 18, the two most common, oncogenic types. However, there are no antiviral drugs for the treatment of HPV infection and disease. Papillomaviruses encode two DNA binding proteins, E1 and E2, which interact with host cell proteins to induce replication. Two essential cellular factors for viral transcription and replication are TopBP1 and Brd4. TopBP1 is a known interactor of HPV16 E2, and is essential for the initiation of DNA replication in eukaryotic cells. Previous studies with E2 mutants have shown that Brd4 is essential for transactivation properties of E2. Brd4 has also been implicated in the regulation of E1-E2 mediated viral DNA replication. However, the role of E2 in regulating the host genome has been less well studied. As attempts at developing a therapeutic vaccine have failed, and current small molecule inhibitors which block the interaction of replication factors, E1 and E2, are not effective across all HPV types due to slight differences in E1-E2 interactions, Brd4 and TopBP1 may present themselves as pan-type specific targets. Blocking the interaction of the host proteins Brd4 and TopBP1 with viral E2 is a viable target for HPV related cancers. This thesis set out to understand how E2 interacts with TopBP1 and Brd4 to regulate the cellular genome as well as the viral genome, to better understand how to terminate the viral life cycle. Two E2 mutants, $E2^{-TopBP1}$ and $E2^{-Brd4}$, which fail to bind TopBP1 and Brd4 respectively, were made to address this question.

Functional assays with E2 wildtype and the two E2 mutants have enhanced our understanding of viral replication. TopBP1 and Brd4 are present at the viral origin of replication in an E1-E2 dependent manner, and optimal initiation of DNA replication is dependent on the interaction with E2. TopBP1 and Brd4 locate into E1-E2 foci and shRNA targeting these proteins destroys these replication foci. However, this has no effect on E1-E2 mediated levels of DNA replication. The results suggest a role for TopBP1 and Brd4 in the initiation of HPV16 E1-E2 DNA replication but not continuing replication, which may be mediated by alternative processes such as rolling circle amplification and/or homologous recombination.

To address the question of how E2 regulates the host, we stably expressed HPV16 and 18E2 wildtype, $E2^{-TopBP1}$ and $E2^{-Brd4}$, in U2OS cells, a p53 positive cell line that tolerates E2 expression. These cells were used in human exon array analysis, to determine which host cellular genes E2 regulates. We determined that HPV16 and 18E2 can regulate cellular genes and a failure to bind either TopBP1 or Brd4 increases the number of cellular genes altered. Overall the results suggest that the levels of TopBP1 and Brd4, which can interact, regulate E2 function and therefore could regulate viral infection.

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Publications

Gauson EJ, Donaldson MM, Dornan ES, Wang X, Bristol M, Bodily JM, Morgan IM. "Evidence supporting a role for TopBP1 and Brd4 in the initiation but not the continuation of human papillomavirus 16 E1/E2-mediated DNA replication". J Virol. 2015 May 1;89(9):4980-91

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Author's declaration

I declare that the work described in this thesis was carried out me personally unles otherwise stated, and has not been submitted in full or in part for consideration for any other degree qualification.

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Definitions/abbreviations

Miscellaneous

5-FU	5-fluoruracil
E2BS	E2 binding site
E6AP	Ubiquitin protein ligase
G418	Geneticin

A

AAD	Activation domain
AAHS	Amorphous aluminium hydroxysulfate
AGW	Anogenital warts
AIN	Anal intraepithelial neoplasia
Akt	Protein kinase B
AP1	Activator protein 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
ATR	ATM- and Rad3-related
ATRIP	ATR-interaction protein

B

BCA	Bicinchoninic acid
BD	Bromodomains
BET	Bromodomain and extraterminal domain
Bp	Base pair
BPV	Bovine papillomavirus
BRCA	Breast cancer susceptibility gene
BRCT	BRCA C-Terminus
BRD	Bromodomain
Brd4	Bromodomain 4
Brg1	BRM(Brahma)- related gene 1
BRM1	Brahma 1
BS	Binding site
BSA	Bovine serum albumin

C

$CaPo_4$	Calcium phosphate
CBP	Calcium binding protein
CDC	Centre for Disease Control
Cdc7	Serine/threonine protein kinase
Cdk2	Cyclin dependent kinase 2
CDK9	Cyclin dependent kinase 9
cDNA	Complementary DNA
cEBP	CCAAT/enhancer-binding protein
CEN	Centromere

C Continued

ChIP	Chromatin immunoprecipitation
CIN	Cervical intraepithelial neoplasia
CRPV	Cottontail rabbit papillomavirus
Cryo-EM	Cryo-electron microscopy
CVT	Costa Rica HPV vaccine trial
CyBP	Cyclophilin B

D

DBD	DNA binding domain
DDR	DNA damage response
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's medium
dsDNA	Double stranded DNA
DTT	Dithiothreitol
DVI	Direct visual inspection

E

E	Early
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ET	Extra-terminal

F

FasL	Tumour necrosis factor ligand
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and Drug Administration

G

GA	Golgi apparatus
GAG	Glycosaminoglycan
GAVI	Global alliance for Vaccines and Immunization
GFP	Green fluorescent protein

H

HaCaT cell	Aneuploid immortal keratinocyte cell line from adult human skin
HATs	Histone acetyltransferases
HBS	Hepes buffered saline
HDACs	Histone deacytlases
HeLa cells	(Henrietta Lacks) human epithelial cells

H Continued

HIV	Human Immunodeficiency Virus
HPV	Human papillomavirus
HR	Homologous recombination
HR- HPV	High-risk Human Papillomavirus
HSPGs	Heparin sulfate proteoglycans
HSV-2	Herpes Simplex Virus 2
hTERT	Human telomerase reverse transcriptase

I

IP	Immunoprecipitation
IPDB	Immunoprecipitation dilution buffer
IPEB	Immunoprecipitation elution buffer
IR-dye	Infrared dye
ISG	Interferon stimulated gene

K

Kb	Kilobase
KNß3	Karyopherin ß3
KRF	Krypton fluoride laser

L

L	Late
LB	Lysogeny broth
LBC	Liquid based cytology
LCR	Long control region
LE	Late endosomes
LLETZ	Large loop excision of the transformation zone
LR-HPV	Low-risk Human Papillomavirus
LSIL	Low-grade Squamous Intraepithelial Lesion

M

mAB	Monoclonal antibody
MCAP	Mitotic chromosome associated protein
MCM	Maintenance proteins
MPL	Monophosphoryl lipid
mRNA	Messenger RNA

N

NaCl	Sodium chloride
NE	Nuclear extract

N Continued

NF1	Neurofibromatosis-related protein
NF-IL6	Transcriptional regulator nuclear factor for interleukin-6
NMR	Nuclear magnetic resonance
ND10	Nuclear domain 10
NP40	Nonylphenoxypolyethoxyethanol

O

Oct1	Octamer binding protein 1
ORC	Origin recognition complex
ORF	Open reading frame
OSCC	Oropharyngeal squamous cell carcinoma

P

p-TEFb	Transcriptional elongation factor
pAB	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ	An acronym combining the first letters of three proteins —**P**ost synaptic density protein (PSD95), **D**rosophila disc large tumor suppressor (Dlg1), and **Z**onula occludens-1 protein (zo-1) — which were first discovered to share the domain
PEF-1	Peflin
PeIN	Penile intraepithelial neoplasia
PI	Pre immune
PID	P-TEFb interacting domain
PML bodies	Punctate structures found in the nuclei of certain cells
PML protein	Probable transcription factor PML is a tumour suppressor protein
PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PODs	PML oncogenic bodies
Poly A	Polyadenylation
POU	The acronym POU is derived from the names of three transcription factors: **P**ituitary-specific Pit-1, **O**ctamer transcription factor proteins Oct-1 and Oct-2 (octamer sequence is ATGCAAAT), neural **U**nc-86 transcription factor from *Caenorhabditis elegans*.
POZ	Pox virus and Zinc finger
PS	Penicillin Streptomycin
PsV	Pseudovirions
PV	Papillomavirus

Q

qPCR	Quantitative PCR

R

Rb	Retinoblastoma

R Continued

Rcf	Relative centrifugal force
RNA	Ribonucleic acid
RNAP II CTD	RNA polymerase II C-terminal domain

S

SCC	Squamous cell carcinoma
SDS	Sodium dodacyl sulfate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIRT-1	Sirtuin-1
SP1	Specificity protein 1

T

TAD	Transactivation domain
TATA box	DNA sequence (cis-regulatory element) found in the promoter region of genes (5'-TATAAA-3' or a variant)
TBE	Tris/Borate/EDTA
TBP	Telomere binding protein
TCGA	The Cancer Genome Atlas
TE	"TE" is derived from its components: Tris, a common pH buffer, and EDTA
TEF-1	Transcriptional enhancer factor-1
TEF-2	Transcriptional enhancer factor-2
TFIID	Transcription factor II D
TGN	Trans-Golgi Network
TLRs	Toll like receptors
TopBP1	Topoisomerase Binding Protein 1

U

UV	Ultra violet

V

VEC	Vector
VIAM	Visual inspection using 3-5% acetic acid and magnification
VILI	Visual inspection using Lugol’s iodine
VIN	Vulvar intraepithelial neoplasia
VLPs	Virus like particles
VSCC	Vulvar squamous cell carcinoma

Y

YY1	Yin Yang 1 is a transcriptional repressor protein in humans

Chapter 1 – Introduction

1.1 History of HPV

Papillomaviruses are small DNA viruses that infect the differentiating epithelium, their characteristic double-stranded DNA structure has been known since 1965 (Crawford 1965, KLUG, FINCH 1965). Papillomavirus genomes have been isolated and characterised from reptiles (Herbst, Lenz et al. 2009) , birds (Terai, DeSalle et al. 2002), marsupials (Bennett, Reiss et al. 2010) and multiple other mammalian species (for recent review see (Bernard, Burk et al. 2010) suggesting an evolutionary history spanning more than 300 million years (Herbst, Lenz et al. 2009) .

In the 1970's it became apparent that there were various sub-types of papillomavirus, due to the availability of molecular techniques. It was established that there were several HPV genotypes; some caused nongenital cutaneous warts (Gissman et al. 1977, Orth et al. 1977), skin lesions of epidermodysplasia verruciformis (EV) (Orth, Jablonska et al. 1978), and other types were responsible for genital warts (Gissman and Zur Huasen 1988,(Orth, Favre et al. 1978)).The spectrum of HPV related diseases has accompanied humans throughout evolution, the HPV types we see in the human population today constitute a set of viral genomes that have co-evolved alongside us (Burk, Harari et al. 2013). Papillomaviruses manipulate the host enzymatic machinery to replicate their genomes, causing the lowest rate of mutations or errors possible. However, occasionally errors occur and these errors have been selected for over time, and are responsible for the differences in papillomavirus genomes and the large heterogeneity of related viral variants detected today (Bernard, Calleja-Macias et al. 2006) (Gottschling, Stamatakis et al. 2007). There are over 160 types of papillomavirus, and the differences in papillomaviruses can be classified by the varying genetic distances between viral genomes (Bernard, Burk et al. 2010, de Villiers, Fauquet et al. 2004). Additionally, a distinct human papillomavirus (HPV) type is defined by at least a 10% difference in the DNA sequence of the L1 open reading frame (ORF) of that of any other characterised type (Bernard, Burk et al. 2010, de Villiers, Fauquet et al. 2004).

Italian physician, Rigoni-Stern (1842) made the first link between sexual contact and cervical cancer. He observed a higher frequency of cervical cancer in married women, widows and prostitutes, and an extremely rare occurrence in virgins and nuns (Rigoni-Stern 1842). 100 years later, Dr. George Papanicolaou established the practice of pap smears in the 1940s as a screening method to detect precancerous and cancerous cervical lesions. However, it was unknown what the cause of cervical cancer was. In the early 1970's the German virologist Harold zur Hausen gained knowledge from medical reports that documented rare events of malignant genital warts (condylomata acuminta) developing into squamous cell carcinomas (zur Hausen 1977). Previous research from Richard Shope in the 1930's showed that infection with papillomavirus caused warts and cancers in rabbits, leading Zur Hausen to believe a similar virus may be responsible for cervical cancer (Shope, Hurst 1933). Originally, Herpes simplex virus type 2 was the viral candidate for the disease, until all attempts to find the viral DNA in cervical cancer biopsies were found to be negative (zur Hausen, Schulte-Holthausen et al. 1974), so this boosted the prospects of papillomavirus being the viral candidate (zur Hausen, Meinhof et al. 1974). Zur Hausen's team began the search in human genital warts, and in 1982 HPV 6 was found in biopsies from three invasively growing giant condylomata acuminta (Buschke-Lowenstein tumours), these tumours are non-metastasising. This was additionally backed up by work from Zachow *et al* in 1982 (Zachow, Ostrow et al. 1982). In the same year, endermodyplasia verruciforms HPV-related bands were demonstrated in some cervical cancer biopsies. This was most likely HPV 10, and has not since been identified in cervical cancer biopsies (Green, Brackmann et al. 1982). HPV 6 was never found in any cervical cancer samples, but it did lead to the discovery of HPV 11. Southern blot hybridisations with HPV 11 DNA allowed for the detection of this DNA in one of 24 cervical cancer samples tested. Using HPV 11 as a probe it was then possible to identify a novel HPV type, HPV 16, which was found to be present in almost half of all cervical cancers (Durst, Gissmann et al. 1983). Closely following the discovery of HPV 16, HPV 18 DNA was identified from cervical cancer biopsies and additionally in cervical cancer derived cell lines, and was detected in around one in five cervical cancer samples (Boshart, Gissmann et al. 1984). Following these primary observations, it was possible to demonstrate HPV16 DNA in typical precursor lesions of anogenital cancer, (Ikenberg,

Gissmann et al. 1983) . A year later papillomavirus was also identified in intraepithelial neoplasias (Crum, Ikenberg et al. 1984).

In the next decade following these primary findings more HPV types were found in cervical cancer biopsies. In 1995 the International Biological Study on Cervical Cancer group was established to understand how the various HPV types affect the human population (Bosch, Manos et al. 1995). Cervical cancer samples were taken from a collective 22 countries; they found HPV in nine out of every ten cervical cancer samples (93%). In 1999, all of the samples were re-tested and this percentage was amended to 99.7%. This gave the strongest evidence to date of a causal link and a specific cancer type (Walboomers, Jacobs et al. 1999).

1.2 HPV involvement in cancer

HPV accounts for 30% of all cancers associated with infections (de Martel, Ferlay et al. 2012) and are associated with cervical carcinoma head and neck cancers and other cancers. There are about 15 oncogenic/ high-risk types of HPV that infect the genital tract. Two most common are HPV 16 and 18. (Stanley 2008). In many developed countries the prevalence of HPV infections in young women is as high as 40-80% and the lifetime probability of ever encountering HPV is as high as 80-90% (Bosch, Broker et al. 2013). Most women will naturally clear the infection, however middle aged women who still carry the virus are at higher risk of developing cancer. An estimated 4-10% of middle aged women are at highest risk. The time period between HPV infection and cancer is about 2 to 4 decades. This makes the initiating infections and precursor lesions of cervical cancer an appropriate target for screening and early detection of the disease.

1.2.1 Cervical cancer

As detailed in 1.1, HPV was found to be a causative agent in cervical cancer. There were more than 530,000 cases of cervical cancer in 2008, 85% of those were in developing countries. The mortality: incidence ratio was 52% and there were 275,000 deaths in 2008, 88% of them were in developing countries (Aggarwal, Batra et al. 2011) (International Agency for Research on Cancer, GLOBOCAN database, 2008). The prevention of cervical cancer requires a multipronged approach involving primary, secondary and tertiary prevention. Primary

prevention includes better sex education about safe sexual practices, and human papillomavirus vaccination, which shall be discussed in more detail in **Chapter 1.3**(Centres for Disease Control and Prevention).

Secondary prevention involves screening asymptomatic patients or carrying out definitive tests in symptomatic patients or screen positive patients to pick up precancerous lesions before they turn into cancer. Implementing screening programmes reduces the incidence of invasive cervical cancer and cancer deaths. There are various screening methods for pre-invasive disease such as: cervical cytology (both conventional and liquid based); direct visual inspection (DVI); direct visual inspection using 3-5% acetic acid (VIA), visual inspection using 3-5% acetic acid and magnification (VIAM), visual inspection using Lugol's iodine (VILI); HPV DNA testing; speculoscopy; and polar probes. Cervical cytology is the most common and globally preferred method for screening. However, the downfall of this method is the frequency of testing and the coverage of the population (Aggarwal 2014). For liquid based cytology (LBC) Pap smear, a cytobrush which rotates 360 degrees five times around the cervix exfoliates the cells. The exfoliated cells are then mixed in a proprietary solution which reduces specimen inadequacy by 80%. However, this proves to be more expensive than traditional Pap tests.

HPV DNA testing is also now available for detecting the presence of HPV DNA. There are currently two types of tests available; one is a nucleic acid hybridisation assay with signal amplification for the qualitative detection of high risk HPV types in cervical specimens (Digene Hybrid Capture 2 High Risk DNA Test (Qiagen, Digene Corporation); Cervista HPV HR Test (Thirdwave Technologies)); the other test is a polymerase chain reaction based assay (HPV DNA Nested Polymerase Chain Reaction Detection Kit (HiFi DNA Tech).

More recently, another test is now becoming established which detects levels of HPV E6/E7 mRNA (PreTect HPV Proofer assay (Norchip) and APTIMA assay (Hologic)). This is a useful assay for gauging the severity of a lesion and offers a predictive method of whether it is likely to progress to cancer.

Tertiary prevention involves vital follow up screens and treatment of women who are in the precancerous stage. Cervical intraepithelial neoplasia (CIN) is a

premalignant lesion that can exist in three stages: CIN1, CIN2, or CIN3 (WHO treatment and prevention). CIN2 and CIN3 are collectively known as CIN2+, and every year 1-2% of women will have CIN2+ which has the potential to progress to cervical cancer. However, in women with HIV this rate is even higher, it increases to 10%. When women test positive during routine Pap smears, the grade of CIN is then determined based on colposcopy, biopsy of suspicious lesions, and then treatment only when CIN2+ lesions have been confirmed. Treatment involves cryotherapy or large loop excision of the transformation zone (LLETZ), which can both be performed in an out patient setting. LLETZ is the most effective treatment as it can remove the entire transformation zone as well as a lesion extending more than 1cm into the endocervical canal, which can be used for histological analysis. Studies have suggested that the deregulation of E6 and E7 expression may be a critical event in determining neoplastic grade, even in the absence of genome integration (Isaacson Wechsler, Wang et al. 2012). Neoplastic grade is determined by the extent of which basal-like cells extend into suprabasal epithelial layers (Jenkins 2007).

In HPV related viral cancers, a characteristic of the cells is integration of the viral genome. Key characteristics of this integration are tandem repeats from single copies to several hundreds of copies of the viral DNA at various random locations throughout the cellular genome. Only a small segment of the viral genome is integrated into the host, this segment contains the regulatory control region or alternatively known as the “Long Control Region” (LCR), and part of the early region, which includes the open reading frames of E6 and E7, which are the two main oncogenes of HPV. These viral oncogenes are transcribed in at least two messenger RNAs initiated at a unique promoter contained within the viral regulatory region. The viral regulatory region for HPV16 and HPV31 is P97 and for HPV18 it is P105, given their names after the genomic sequence with number corresponding to 5’ end of the E6 and E7 messenger RNA. These regulatory regions are located immediately upstream of the E6 open reading frame. In addition to this, another characteristic of integration is the disruption of the E1 and/or E2 open reading frames which are downstream of E7. This integration event leads to a disruption of viral DNA replication. Disruption of E2 leads to transcriptional activation of E6 and E7 oncogenes in cervical cancer (Thierry,

Yaniv 1987). The transcription of E6 and E7 in cervical carcinoma is controlled by cellular factors, due to this disruption in the viral transcription factor, E2.

1.2.2 Head and neck cancer

Men are also at risk of developing HPV positive cancers of the anogenital tract and oropharynx (Brown, White 2010). HPV 16 is the most common high-risk HPV detected in oropharyngeal squamous cell carcinoma (OSCC), this is the only head and neck site with a definite etiological association between persistent high-risk HPV infection. HPV in head and neck cancer probably utilises the same pathways in epithelial cell transformation as established for genital cancer (Rautava, Syrjanen 2012)

As early as the 1940s/50s reports of malignant conversion of recurrent laryngeal papillomas into squamous cell carcinomas (zur Hausen 1977). The demonstration of papillomavirus antigens in premalignant lesions of the oropharynx provided first hints for a possible role of papillomavirus infections in oral squamous cell carcinomas (Syrjanen, Pyrhonen et al. 1983). The first definitive evidence of the involvement of high risk HPV types in oropharyngeal carcinomas came about in 1985 (Loning, Ikenberg et al. 1985). HPV16 was found in three out of 13 carcinomas tested, and one was found to be HPV 11 positive, and other contained HPV 27 DNA. In 2006 Weinberger et al (Weinberger, Yu et al. 2006) demonstrated that out of 61% of HPV 16-positive oropharyngeal carcinomas 23% had a similar expression pattern of p16 as observed in cervical cancers. It has been postulated that a conservative estimate of about 70% of oropharyngeal cancers are caused by high-risk HPV types (**Figure 1.1**).

Patients who have HPV related head and neck cancers are generally younger than those with tobacco related cancers in a similar region. Patients have the same risk factors as those associated with cervical cancer, which include a high number of sexual partners, an early age of first sexual encounter, and prior sexually transmitted infections (D'Souza, Kreimer et al. 2007, Gillison, Koch et al. 1999). In the United States, HNSCC is the sixth most common cancer (American cancer society). It has been shown in clinical trials that HPV+ patients respond better to treatment than those who have HPV- cancers (Rischin, Young et al. 2010, Posner, Lorch et al. 2011, Nichols, Faquin et al. 2009, Hong, Dobbins et al.

2010, Ang, Harris et al. 2010). The differences in the responses to treatment may be in part due to molecular differences between the HPV+ and HPV- cancers (Strati, Pitot et al. 2006, Hafkamp, Speel et al. 2003, Boyle, Hakim et al. 1993). Currently the treatment given to patients with HPV+ and HPV- cancers is the same (Mehra, Ang et al. 2012, Mehanna, Olaleye et al. 2012). However, a change in the clinical treatment of the disease within clinical oncology will be tailored dependent on HPV status of the cancer.

1.2.3 Anal cancer

Anal cancer is uncommon, with age standard incidence rates mostly between 1 and 2 per 100,000 per year. In approximately 80-90% of anal SCC cases high-risk HPV can be detected, HPV16 is accountable for about 90% of HPV positive cases of anal SCC (**Figure 1.1**) (Grulich, Poynten et al. 2012). Studies have revealed that women and men who have sex with men commonly have anal HPV infections, particularly in HIV-infected individuals. Clearance of this infection is common; however, indviduals who show persistant infection are generally those who are HIV-infected. HIV is strongly linked to the persistence and development of anal intraepithelial neoplasia (AIN). The studies that have been conducted in this field show that high-grade AIN is precursor to invasive anal cancer. Women, who have HPV-associated lesions, including CIN3+ and vulvar cancer, also have higher rates of anal cancer (Bosch, Broker et al. 2013).

Anal cancer can be treated using electofulgaration, infrared coagulation, or laser ablation. Those who have high-grade AIN where there may be microinvasive disease or cancer may be treated by excision. Clinical trials using topical therapies for the treatment of high-grade AIN such as 5-fluoruracil (5-FU), imiquimod, and topical cidofvoir have all shown to be promising treatment options (Bosch, Broker et al. 2013).

1.2.4 Penile cancer

Penile cancer is rare in developed nations. In the United States, the incidence is less than 1 per 1000,000 men per year (National Cancer Institute). HPV infection has been associated with penile cancer (**Figure 1.1**) (Del Mistro, Chieco Bianchi 2001, Griffiths, Mellon 1999, Poblet, Alfaro et al. 1999, Frisch, van den Brule et al. 1996), and some observational studies have shown a lower prevalence in men who have been circumcised (Castellsague, Bosch et al. 2002). A higher number of sexual partners in a male's lifetime contributes to the likelihood of being infected with high-risk oncogenic HPV, the clearance of which decreases in men with higher numbers of sexual partners, and this is more rapid with increasing age. Surgical treatment is the best option for local disease, whereas regional disease is usually treated with radical inguinal lymphadenectomy if reselectable. In the case of nonreselectable regional disease and metastases, neoadjuvant cisplatinum-based regimens are the best option. Topical chemotherapy agents such as 5-FU and imiquimod have also proven to be moderately effective first-line therapy for penile cancer.

1.2.5 Vulvar cancer

Vulvar cancer is rare. It is estimated that approximately 3,100 new cases of HPV-associated vulvar cancer are diagnosed in the United States each year (**Figure 1.1**) (Centers for Disease Control and Prevention). Vulvar squamous cell carcinoma (VSCC) is responsible for greater than 90% of malignant tumours of the vulva (Judson, Habermann et al. 2006). Two atiopathogenic pathways for the development of VSCC, one is associated with HPV and one is not (Toki, Kurman et al. 1991, Kurman, Toki et al. 1993, Fox, Wells 2003, McCluggage 2009) . These should be considered two distinct disease types as they both have different epidemiological, clinical, pathological and molecular characteristics.

VSCCs originate in intraepithelial lesions, and precede the development of invasive cancer. VSCC found adjacent to epithelial disorders in around 50-70% of patients. The association of HPV with cases of VSCC varies from 15%-79% in various clinical reports (van de Nieuwenhof, van Kempen et al. 2009, Alonso, Fuste et al. 2011, Riethdorf, Neffen et al. 2004, Pinto, Lin et al. 2000, Carter, Madeleine et al. 2001, Gargano, Wilkinson et al. 2012, Tsimplaki, Argyri et al. 2012, Monk,

Burger et al. 1995, Kim, Thomas et al. 1996, Kowalewska, Szkoda et al. 2010, Garland, Hernandez-Avila et al. 2007, Trimble, Hildesheim et al. 1996).

Vuvlvar intraepithelial neoplasia (VIN) is a term used to designate precursors of VSCC. The presence of HPV DNA in VIN has been detected in 52-100% of the lesions (van de Nieuwenhof, van Kempen et al. 2009, Garland, Hernandez-Avila et al. 2007, Trimble, Hildesheim et al. 1996, Smith, Backes et al. 2009). It has been found that around one-fifth of all VSCCs and over four-fifths of VINs are associated with HPV infection. Despite the strong link with HPV and VINS, most VSCCs arise independently of viral infection. HPV16 is the most common HPV type found in both VIN and VSCCs, although other types are found too, such as 18, 31, 33 and 45 (Santos, Landolfi et al. 2006, Bonvicini, Venturoli et al. 2005). The vaccine has played a vital role in reducing the incidence of VIN in young women (Garland, Hernandez-Avila et al. 2007). Additionally, there is evidence that a vaccination with synthetic long peptides from the HPV16 oncoproteins E6 and E7 has a therapeutic effect on HPV16 positive VIN (Kenter, Welters et al. 2009).

VIN is treated by surgical excision for unifocal disease and lesions which are likely to become invasive. However, this is not optimal for treating multifocal disease. Instead, topical agent imiquimod, and photodynamic therapy have both shown some useful efficacy (~50-60%) in the treatment of high-grade VIN. Vuvlvar cancers are usually treated with surgery for localised disease and a combination of surgery and chemoradiation for nodal metastases. In the case of a very advanced vulval cancer, chemoradiation is the preferred therapy (Bosch, Broker et al. 2013).

Figure 1.1

Cancer site	Average number of cancers per year in sites where HPV is often found (HPV-associated cancers)			Percentage probably caused by HPV	Number probably caused by HPV†		
	Male	Female	Both Sexes		Male	Female	Both Sexes
Anus	1,549	2,821	4,370	91%	1,400	2,600	4,000
Cervix	0	11,422	11,422	91%	0	10,400	10,400
Oropharynx	9,974	2,443	12,417	72%	7,200	1,800	9,000
Penis	1,048	0	1,048	63%	700	0	700
Vagina	0	735	735	75%	0	600	600
Vulva	0	3,168	3,168	69%	0	2,200	2,200
TOTAL	**12,571**	**20,589**	**33,160**		**9,300**	**17,600**	**26,900**

†Individual cells may not sum to total due to rounding.

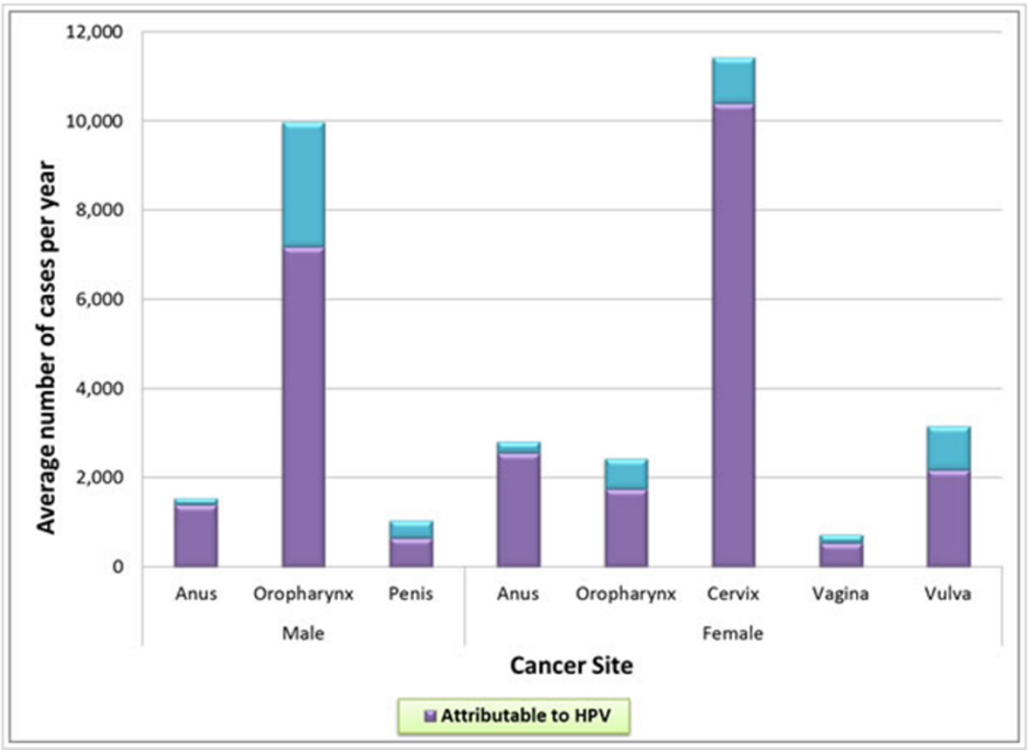


Figure 1.1: Incidence of HPV related cancers.

The table presents the average number of cancers in sites where HPV is often found, and the percentage probably caused by HPV per year.

The graph displays the probable number of HPV related cancer per year in various cancers sites.

(Figure taken from CDC website- Data are all from States meeting USCS publication criteria for all years 2006-2010 and cover about 94.8% of the U.S population.)

1.3 HPV vaccine

Evidence from some of the earliest work carried out on papillomaviruses in the 1930's by Shope, showed that neautralisising antibodies were protective (Shope 1937). In these experiments, Shope systematically infected rabbits with the cottontail rabbit papillomavirus (CRPV) by directly injecting the virus into the muscle, papillomas did not arise on the skin of the challenged rabbits and neutralising antibodies were generated. The rabbits in these experiments were completely resistant to further attempts for the papillomavirus to challenge the epithelium by abrasion. This information suggested that generating neutralising antibodies to viral capsid proteins would be an effective prophylactic vaccine strategy.

Work was also done with the Bovine papillomavirus (BPV), both prophylactic and therapeutic vaccines were developed against BPV-4 (Campo 1994, Campo, Jarrett 1994) These vaccines prevent viral infection and induce early regression of papillomavirus respectively. It was shown that anti-L2 antibodies are responsible for virus neutralisation (Campo, O'Neil et al. 1997). The minor capsid protein L2 and the N terminus of L2 (L2a, amino acids 11-200) are sufficient for protection against infection (Chandrachud, Grindlay et al. 1995).

1.3.1 Clinical trials

In 2002 a pilot controlled clinical trial of a HPV vaccine for HPV 16 only was carried out (Koutsky, Ault et al. 2002). This gave evidence that the vaccine may prevent HPV16 related (cervical cancer) in women who have not come into contact with the virus yet. The trial initially included 2392 women with a median age of 20 years old, 1194 of which received the vaccine and 1198 were given a placebo. Out of these original women included in the study, only 1533 (64 % of the original study cohort) were included in the primary analysis. This exclusion was due largely in part to evidence of HPV-16 infection at enrolment. Women in the study were monitored for approximately 17.4 months after receiving the full three doses of the vaccination. The data provided from this trial showed that this vaccine is a highly efficacious prophylactic against HPV infection. During the clinical trial, 41 women who received the placebo vaccine displayed new cases

of HPV-16 infection, including 9 cases HPV16-related cervical intraepithelial neoplasia. Therefore, the HPV16 vaccine was effective. 99.7 percent of the women in the vaccine study group seroconverted. The geometric mean titer of HPV16 antibodies at month 7 of the trial was 58.7 times higher than the women with serological evidence of natural infection with HPV16 at enrolment. Overall, this study provided strong evidence that this vaccine reduces the incidence of HPV16 infections and related cervical intraepithelial neoplasia, and that immunising HPV16 negative women may reduce their risk of cervical cancer.

Subsequent to this initial study, two prophylactic vaccines were developed that offer protection against multiple HPV types. A bivalent vaccine called Cervarix (GlaxoSmithKline Biologicals, Rixensart, Belgium), which is a bivalent vaccine targeting HPV 16 and 18. A quadravalent vaccine called Gardasil (Merck and Co., Inc, Whitehouse Station, NJ, USA) targets low-risk HPV types, 6 and 11, in addition to high-risk HPV16 and 18. HPV 6 and 11 are responsible for around 90% of anogenital warts (AGW). The viral like particles for Cervarix are produced in insect cells infected with L1 recombinant insect virus vectors (Inglis, Shaw et al. 2006), and Gardasil VLPs are produced in bakers yeast (*Saccharomyces ceravisae*) expressing L1 (Shi, Sings et al. 2007).

Two phase III studies, FUTUREI (Garland, Hernandez-Avila et al. 2007) and FUTURE II (FUTURE II Study Group 2007) , evaluated Gardasil. Two trials evaluated Cervarix, PATRICIA (Paavonen, Jenkins et al. 2007) and the Costa Rica HPV vaccine trial (CVT). Women in the trials were in the 15-26 age range, and all trials were of relatively large participant size (5,500-18,500 vaccinees), and blinded, randomised and controlled. The trials were company sponsored with the exception of the CVT, which was a U.S government sponsored community-based trial, centered in the Guanacaste province of Costa Rica (Herrero, Hildesheim et al. 2008) . Company lead trials were carried out in Europe, North, Central and South America, and Asia Pacific, including Australia. Participants were restricted for selection criteria by their number of sexual partners in order to limit the number of women included in the trial who may have prevalent infections and/or prevalent genital lesions at enrolment. This was a key point as the vaccine trial set out to see if this was a valuable prophylactic for HPV related infection and cancer. The only exception to this was within the CVT and the Finnish participants in the PATRICIA trials, where no such restriction was placed upon the vacinees.

However, in these phase three trials women were not excluded for having a pre-exisiting infection at enrolment, as measured by the presence of genital tract HPV DNA by sensitive PCR-based techniques. Nor were women excluded from the trial if they had been exposed to HPV prior to the trial, as measured by serum antibodies reactive to the VLPs, or in some cases by finding an abnormal cervical cytology at baseline. The reasoning behind this decision was to evaluate the prophylactic efficacy, safety and immunogenicity of the vaccine in women who had prior or current exposure to HPV, and also that the vaccine may have therapeutic activity.

The trials of Gardasil and Cervarix provided evidence that they protected against HPV-16 related types (A9 species), (Brown, Kjaer et al. 2009, Wheeler, Castellsague et al. 2012) . Cervarix demonstrated significant efficacy against HPV31, 33 and 52, whereas Gardasil only showed significant efficacy against HPV31. Cervarix also provided relatively strong protection against HPV45 (79.0%), a HPV18 – related A7 species. Partial protection against HPV45 and HPV31 was also shown in the CVT trial of Cervarix (Herrero, Wacholder et al. 2011). The results gathered by the CVT and PATRICIA trial were in agreement.

Overall, from these clinical trials it was concluded that the vaccine is safe, with only minor injection-site symptoms. Both Gardasil and Cervarix are highly immunogenic, inducing high peak titers of antibodies in virtually all vaccines, and measurable serum antibody responses that persist for years. They offer protection from anogenital infection and subsequent neoplastic disease by the types in the vaccine. The vaccines also induce-partial cross protection against non-vaccine types, which are phylogenitically related. The vaccines do not work therapeutically to induce regression or prevent progression of established infections.

Currently, the duration of the protection offered by the vaccine is unknown. The Cervarix vaccine has shown continued persistence of serum antibodies for up to 8.4 years (Roteli-Martins, Naud et al. 2012) without significant drop in titer after2 years gives hope for the vaccine having continued strong efficacy through the peal years of anogential HPV acquisition and perhaps lifelong.

Work published by David *et al* has predicted that the mean HPV16 and 18 antibody levels will remain well above those associated with a natural HPV infection

for a minimum of 20 years for the bivalent vaccine (David, Van Herck et al. 2009) . Another paper estimates that the duration of detection of vaccine-induced anti-HPV 16 antibodies ranging from 12 years to near life-long in a majority of women following vaccination with HPV16 vaccine (Fraser, Tomassini et al. 2007).

1.3.2 Biology of the HPV vaccine

Since 2006, the bivalent and quadravalent vaccines have been licensed and available in over 100 countries. These vaccines are made of HPV L1 virus-like particle (VLP) that induce high concentrations of neutralising antibodies to L1 (Harper, Franco et al. 2004) (Villa, Costa et al. 2006). The HPV vaccine is delivered intramuscularly, as this leads to a rapid access to the local lymph nodes, which in turn circumvents the immune avoidance strategies of the viral intraepithelial infectious cycle. VLPS induce potent antibody responses in the absence of adjuvant due to their ability to activate both the innate and adaptive immune responses. (Harro, Pang et al. 2001) VLPs are rapidly bound by myeloid DCs and B lymphocytes, and signal via the toll-like receptor (TLR) dependent pathway MYD88, which is essential for B-cell activation and antibody generation in mice and probably humans. (Yang, Murillo et al. 2004) (Yan, Peng et al. 2005). Both of the vaccines that are available commercially have been formulated with adjuvants, these are compounds that enhance immunogenicity. In the quadravalent vaccine, there are VLPs for HPV 6, 11, 16 and 18 and additionally there is proprietry adjuvant amorphous aluminium hydroxysulfate (AAHS). This proprietry adjuvant has been used in many vaccines already on the market without any side effects. The bivalent vaccine contains L1 VLP s for HPV 16 and 18. Plus the adjuvant system AS04, which is composed of aluminium hydroxide and monophosphoryl lipid A (MPL), a modified endotoxin and agonist of TLR4. Immunity provided by vaccines depends largely on the activation of the appropriate antigen-specific CD4+Th2 cells that “help” antigen primed B-cells differentiate into antibody secreting plasma cells and memory B-cells. Under normal circumstances with papillomaviruses infect the epithelium, the differentiation program depends upon signals generated by the recognition of microbial products by pattern recognition receptors such as TLRs expressed on the APCs. The vaccine works by triggering these signals via the adjuvants in them, the adjuvants trigger the innate immune responses that bias to an appropriate adaptive response.

1.3.3 Implementing HPV vaccination

Within the U.S, the President's Cancer Panel released a report urging for there to a greater uptake of the HPV vaccine. This letter to the president outlined three goals to make this possible within the U.S; (1) Reduce missed clinical opportunities to recommend and administer HPV vaccines, (2) Increase parents', caregivers', and adolescents' acceptance of HPV vaccines, (3) Maximise access to HPV vaccination services (President's cancer panel).

All of these steps are in agreement with the published guidelines recommended for a greater communication and uptake of the vaccine published by WHO. At the end of 2012, 45 countries had introduced a HPV vaccination program (WHO). However, most of these countries are developed and have the finances to implement these vaccination programmes. There is still a great need for developing countries to introduce HPV vaccine as part of a national public health strategy, as the greatest burden of cervical cancer is within these countries.

In 2012, Europe saw a vast increase in the number of countries introducing the vaccine the number went from 3 in 2007 to 22 in the beginning of 2012. There are differences in how the vaccine is provided to the public, some countries offer adolescent girls the vaccine at school, whereas other only provide it through health centres and primary care providers. The Global Alliance for Vaccines and Immunization (GAVI) decision to support HPV vaccination in low income countries should increase the implementation of the vaccine, as this is a very costly vaccination and not readily available otherwise. There are also benefits of vaccinating males with the HPV vaccine, such as direct benefits to the vaccinated males themselves as it will prevent male related HPV cancers, also indirect benefits to non-vaccinated females and males via increased herd immunity, and also protection of men who have sex with men.

The World Health Organisation (WHO) has published a second guidebook "Comprehensive cervical cancer control" in December 2014, which outlines the strategies required to prevent and control cervical cancer. The main points highlighted to achieve this are; vaccinate 9 to 13 year old girls with two doses of HPV vaccine to prevent infection with HPV. Two doses of the vaccine have been shown to be just as effective as the three dose schedule, which will make the vaccine

more cost effective. HPV testing is recommended for screening women for the prevention of cervical cancer. Due to the discovery of a causal link between HPV and cervical cancer, molecular tests were developed to identify women who have HPV infections that may lead to cervical precancerious lesions. This testing method can reduce the incidence of cervical cancer within 4-5 years and reduce the mortality due to cervical cancer within 8 years, compared to cytological methods (Bosch, Broker et al. 2013). Following a negative result to testing, women are not required to be rescreened for at least five years, and this offers to be another cost saving method in the prevention of cervical cancer. Finally, WHO urge for there to a greater level of communication about cervical cancer, and reach women at all stages of life, from all levels of the health system.

The vaccine works but it is only prophylactic. Therefore, we still need novel therapeutics. To do this we need a better understanding of the papilloma virus life cycle.

1.4 HPV life cycle

High-risk HPV infects stratified squamous epithelia in the genital tract (**Figure 1.2**). In normal stratified epithelium, cells located in the basal and parabasal layers, attached to the basal membrane, are the only cells that have the potential to proliferate. When these normal cells divide, one daughter cell becomes a new basal cell, while the other will migrate away from the basal layer and launches its differentiation process. The differentiated cells exit the cell cycle and change their gene expression pattern, which eventually results in cell death and desquamation (Jones, Simons et al. 2007). The HPV life cycle is closely linked to the differentiation program of infected epithelium.

1.4.1 HPV genome

The HPV genome can be divided into three different sections: the early region (E) which is approximately 4kb in size and encodes non-structural proteins, a late region (L) that encodes two capsid proteins and is about 3kb in size, and a 1kb noncoding long control region (LCR) that contains a variety of *cis* elements, which regulate viral replication and gene expression (**Figure 1.3**) (Munger, Baldwin et al. 2004).

Figure 1.2

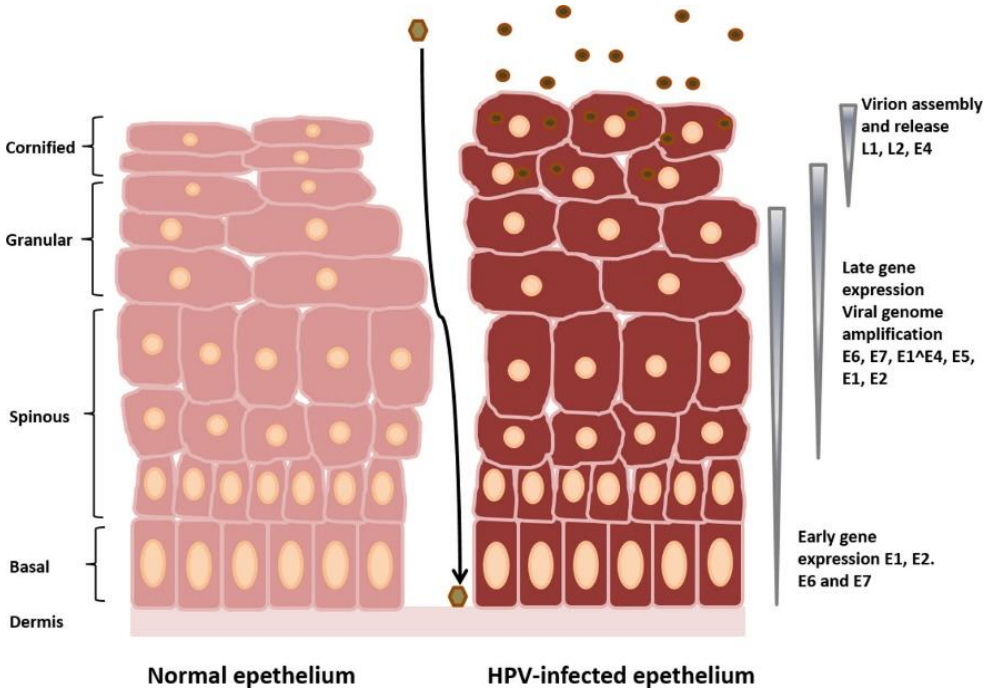
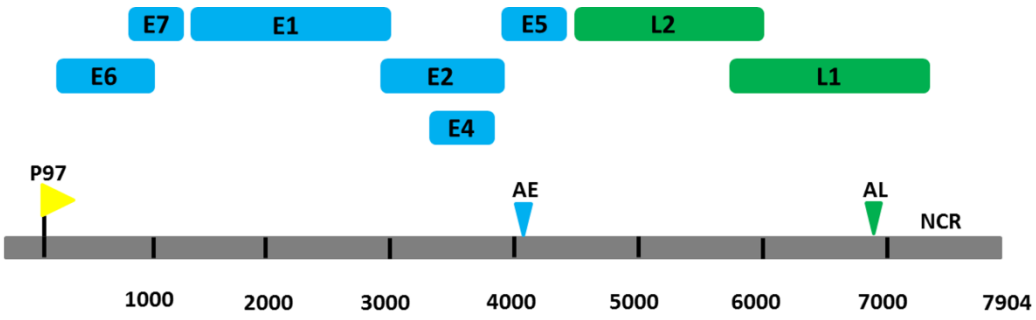


Figure 1.2: Viral life cycle. HPV infection occurs through microlesions in the basal layer of the stratified epithelium. Viral genomes are maintained as episomal DNA in the nuclei of infected cells. The viral lifecycle is closely linked to the differentiation of host cells. As the infected host cells migrate towards the lumen of the cervix, viral proteins are expressed and viral DNA amplified. The late life cycle (productive lifecycle) occurs in the upper layers of the epethelia that are terminally differentiated, and the progenitor virions are released from the cornified keratinocytes. Figure adapted from Human Papillomavirus oncoproteins: pathways to transformation (Moody, Laimins 2010).

Figure 1.3



Early Genes
E6- Binds p53 (transformation)
E7- Binds pRB (transformation)
E1- Replication
E2- Transcription/Replication/ Repression of E6/E7
E4- Viral release/ Disruption
E5- Immune evasion/ Transformation

Late Genes
L2- Minor caspid protein
L1- Major caspid protein

Figure 1.3: Viral genome. Schematic linear representation of the HPV genome. The 8 ORF are shown. Figure adapted from Relevance of infection with human papillomavirus: The role of the p53 tumor suppressor protein and E6/E7 zinc finger proteins (Ruttkay-Nedecky, Jimenez Jimenez et al. 2013).

1.4.2 Viral entry

Papillomavirus particles are small noneveloped icosaheadrons and are about 50-55nm in diameter. 72 pentamers of the major structural protein L1 form the capsid in addition to the minor structural protein, L2, which can vary in amount up to 72 copies. (Buck, Cheng et al. 2008, Baker, Newcomb et al. 1991a). These viral particles mediate transmission and entry through mechanism not currently well understood. Several studies have suggested that virions bound to the cell surface may take several hours to be internalised by endocytic uptake of clathrin coated vesicles and the extended time until infection is established. (Christensen, Cladel et al. 1995, Culp, Christensen 2004, Giroglou, Florin et al. 2001, Schelhaas, Shah et al. 2012, Smith, Campos et al. 2007)

Current work is only beginning to understand the various steps in viral entry and how these steps are linked. Various *in vitro* systems can be utilised for the study of viral entry, by producing surrogate viral particles. Most of the current research on viral entry utilises pseudovirions (PsV), which serve as viral pseudogenomes as they harbour plasmid and encode reporter protein (Buck, Thompson 2007). Expression of the PsV reporter indicates a successful “pseudoinfection”.

The current model for viral entry suggests that PV initially binds to glycosaminoglycan (GAG) chains of heparin sulphate proteoglycans (HSPG) on the cell surface or extracellular matrix (ECM). (Joyce, Tung et al. 1999). Mutational analysis and x-ray crystallography of L1 capsomers have shown that an interaction of PV with heparin at minimally four different sites ((Dasgupta, Bienkowska-Haba et al. 2011, Knappe, Bodevin et al. 2007), and that PV do not require a specific HSPG protein core for binding and infection (Shafti-Keramat, Handisurya et al. 2003, Huang, Lambert 2012).

There may not be one single molecule responsible for the internalisation of the virus. Candidates for such receptors that are all expressed at high levels in the basal epithelium include; CD151, alpha 6 integrin, annexin A2 heterotetramer and tetraspanin (Culp, Budgeon et al. 2006b, Dziduszko, Ozbun 2013, Evander, Frazer et al. 1997, McMillan, Payne et al. 1999, Scheffer, Gawlitza et al. 2013,

Spoden, Kuhling et al. 2013, Woodham, Da Silva et al. 2012, Surviladze, Dziduszko et al. 2012, Spoden, Freitag et al. 2008). All of these are known to associate with HSPG complexes.

The virus is then thought to be delivered to an endosomal compartment which allows for the shedding of L1 capsomers from a subviral particle, and then transported to the Trans-Golgi Network (TGN) where membrane penetration can occur.

Papillomaviruses are trafficked through the endosomal system following endocytosis. In live cells it has been shown that PV associate briefly with Rab5-positive structures (Schelhaas, Shah et al. 2012). It is then beleived that PVs follow the degredative arm of the endosomal system, as PV particles are detected in multi vesicular endosomes and late endosomes (LE)/lysosomes. (Schelhaas, Shah et al. 2012). It is within the endosomes that further structural changes to the PV take place that result in partial uncoating.

The mechanism by which PVs are imported into the nucleus is not yet understood. The L2 protein harbours nuclear localisation signals for karyopherin-mediated import, this may allow for import via nuclear pore complexes (Darshan, Lucchi et al. 2004a, Mamoor, Onder et al. 2012). Another proposed mechanism of entry involves the progression of the host cell through early mitosis, which is required for infection (Pyeon, Pearce et al. 2009). It is not clear which mechanism allows for PV entry, but it is conceivable that the breakdown of the nuclear envelope aids in PV genomes accessing the nuclear lumen. Once PVs enter the nucleoplasm, L2 and the viral genome colocalise at ND10 domains. This is a critical step for the establishment of infection, as early transcription of the virus relies on intact ND10 or expression of the PML protein (Day, Baker et al. 2004). Following infection and nuclear localisation the next step is to activate transcription from the viral genome. This results in expression of viral genes and proteins essential for the life cycle.

1.4.3 Transcriptional regulation of the HPV long control region (LCR)

The LCR can be divided into three functionally distinct regions, called the 5', the central, and the 3' segment. The 5' segment is about 300bp in size and it is bracketed by the termination codon of L1 and E2 binding sites. This segment contains transcription termination and polyadenylation sites for late transcripts and additionally a negative regulator element at the level of late mRNA stability (Furth, Baker 1991, Kennedy, Haddow et al. 1991). The central segment is flanked by two E2 binding sites (#1 and #2), has a size of about 400bp and has been shown to function as an epithelial specific enhancer in HPV 11, 16 and 18 (Chin, Broker et al. 1989, Cripe, Haugen et al. 1987, Gloss, Bernard et al. 1987). The enhancer is also involved in modulating viral gene expression in response to the stage of differentiation the host keratinocyte is in within the different layers of stratified squamous epithelium. These changes are facilitated by the differential binding of transcription factors to their cognate sites in the enhancer, and this in turn will be dependent upon the quantitative, and perhaps qualitative differences in the these transcription factors in a particular cell environment.

Many cellular transcription factors bind in vitro to more than 20 different sites of the HPV 16 enhancer. These include: AP1, cEBP, glucocorticoid receptor, progesterone receptor, NF1, NF-IL6, Oct-1, PEF-1, TEF-1, TEF-2 and YY1 (Chan, Klock et al. 1989) (Chin, Broker et al. 1989, Gloss, Bernard et al. 1987, Chong, Chan et al. 1990, Chong, Apt et al. 1991, Cuthill, Sibbet et al. 1993, Garcia-Carranca, Thierry et al. 1988, Gloss, Chong et al. 1989, Ishiji, Lace et al. 1992, Kyo, Inoue et al. 1993, O'Connor, Bernard 1995, Sibbet, Campo 1990). The 3' side of the central LCR is flanked by the #2 E2 binding site, which is known to be involved in the initiation of replication (Sibbet, Campo 1990, Chiang, Dong et al. 1992, Russell, Botchan 1995), and is also key for the modulation of the transcription of the viral oncogenes, E6 and E7 (Romanczuk, Thierry et al. 1990).

The boundaries of the 3' LCR are marked by the #2 E2 binding site and the E6 gene. The 3' LCR is approximately 140bp in size, and contains a single E1 binding site which identifies the origin of replication. About 5bp upstream of the ATG of E6, and 90bp downstream of the E1 binding site, lies the transcription start site. Within these 90bp a segment of about 45bp contains an SP1 transcription factor

binding site and two E2 binding sites, as well as a TATA box. These four sites are key players in the modulation of E6/E7 promoter activity. The TATA box is required for the binding of TFIID and the establishment of the pre-initiation complex, and the SP1 factor for mediating the long range effect of the enhancer. The E2 sites overlap with the TATA box and with the AP1 site, such that the occupancy by E2 proteins displaces TFIID and the SP1 factor (Bouvard, Storey et al. 1994)(Demeret, Yaniv et al. 1994, Dostatni, Lambert et al. 1991, Gloss, Bernard 1990, Tan, Leong et al. 1994).

It has been proposed from various studies of the HPV promoter that it can switch between transcription initiation and replication functions by depending on the concentrations of E2 available. Binding of SP1 to its site activates the E6/E7 promoter, but not replication. This is due to SP1 blocking E2 binding which would enhance E1-dependent replication initiation. When there are greater concentrations of E2, SP1 is displaced, replication is activated and transcription is partially repressed (Demeret, Yaniv et al. 1994, Tan, Leong et al. 1994). The binding of E2 to the promoter proximal site (site #4) would lead to TFIID displacement and further repression.

1.4.3.1 Host transcription factors

NFI was first identified through its role in the replication of the adenovirus genome, where it binds to the sequence TTGGC(N)6CCAA (de Vries, van Driel et al. 1985, Nagata, Guggenheimer et al. 1983). In HPVs NFI binds to only part of this sequence, TTGGC, however the NFI protein still binds as a dimer, which forms in solution (Mermod, O'Neill et al. 1989). In epithelial cells, where the HPV enhancer is active, the major forms of NFI present consist of dimers made up of the three splice products from the NFI-C gene, CTF-1, CTF-2 and CTF-3. Every HPV LCR contains NFI sites. On average each LCR contains at least 4 sites. In genital HPVs all of these NFI sites occur within the central segment of the LCR, which is only 300-400bp long. The likelihood that this would happen by chance is negligible.

All genital HPV LCRs contain at least one binding site for AP1, with most possessing 3 or 4. Almost all of these sites lie within the central segment of the LCR

in the epithelial specific enhancer. AP1 is heterodimers made up of proteins derived from the Jun, Fos, ATF gene family, recognises DNA motifs related to the sequence TGANTCA and serves as a downstream target of intracellular signalling mechanisms (Vogt, Bos 1990). The AP1 sites that are present in the HPV enhancers may modulate viral gene expression in response to growth factors such as EGF, KRF and tumour growth factors via the protein kinase C pathway. This has been shown through the use of phorbolesters which mimic the signalling pathways and strongly activate E6 and E7 expression. AP1 may also contribute towards epithelial specific activation since the genes that give rise to its subunits are differentially expressed (Thierry, Spyrou et al. 1992).

Oct-1 regulates a number of viral and cellular genes (Ruvkun, Finney 1991), and is a member of the POU factor family (Rosenfeld 1991). All POU factors possess a highly homogenous, bi-partite DNA binding domain. Within this POU domain, an N-terminal POUS subdomain recognises the 5' end of the recognition sequence, ATGCAAAT for Oct-1 (Verrijzer, Kal et al. 1990), and thus is separated by a linker, which varies in length and sequence in different POU factors.

There is an epithelial enhancer and promoter which we don't understand the specificity of. Overall, the effect of their function is to generate an epithelial specific enhancer. There seems to be no one function responsible; rather it can be hypothesized that a combination of functions or post-translational modifications accumulate to generate epithelial specificity.

1.4.4 Three stages of viral DNA replication

There are three stages of viral DNA replication in the papillomavirus life cycle. The first stage is establishment which involves limited genome amplification when a viral particle first enters the host cell. The second phase is genome maintenance, where the viral genome is maintained at a constant copy number in the proliferating basal cells of a papilloma. The maintenance phase requires both genome replication and partitioning. In the third phase of replication, genomes are amplified in differentiated cells to produce progeny virions.

1.4.4.1 Establishment

HPV infection is thought to only be possible through wounds in the proliferative basal layer of stratified epithelium, allowing HPV to penetrate the physical barrier of upper skin layers. Infections are established in the basal compartment of stratified epithelia as this is the only compartment of epithelium which contains cells progressing through the cell cycle. The results from studies also indicate that wound healing might increase efficiency with which the HPV DNA becomes established as a nuclear plasmid in the basal cells, because the basal cells are then in a hyperproliferative stage (Werner, Smola et al. 1994).

Pyeon *et al* presented results using infectious wild type HPV16 virions that showed that events in early prophase segment of mitosis are critical for establishing HPV infection. This was assayed by introduction and expression of HPV encapsidated DNA in nucleus. Pyeon *et al* propose that these events could include nuclear envelope breakdown, cytoskeleton restructuring, and sub-nuclear structure changes as well as the specific expression of one or more genes or gene in combinations in early mitosis (Pyeon, Pearce et al. 2009).

Restructuring of PML oncogenic bodies (PODs) or ND10 and chromatin in early mitosis could be necessary for establishing HPV infection in the nucleus. After the initial steps of HPV infection, PODs are the final destination for HPV DNA, and this is approximately where HPV transcription and replication occurs (Day, Baker et al. 2004) (Van Tine, Dao et al. 2004). During S and M phases of the host

cell cycle, PODs are dynamically restructured, responding to changes in chromatin organisation (Bernardi, Pandolfi 2007). The structural changes of PODs in early mitosis could be essential for HPV DNA localisation to its POD destination during establishment of infection. Additionally, other host mechanisms involved in chromatin restructuring in early mitosis could contribute to viral gene expression.

1.4.4.2 Genome maintenance

Following establishment HPV is maintained at an episomal copy number of around 10-200 copies per cell and the early proteins (E1, E2, E6 and E7) are expressed at low level. (De Geest, Turyk et al. 1993, Stanley, Browne et al. 1989). E1 and E2 early viral genes cooperate to initiate viral DNA replication, whereas E6 and E7 modulate cell cycle regulators to maintain long term replication competence (Frattini, Laimins 1994a, Sedman, Stenlund 1995). These genes are expressed at low levels in undifferentiated cells in order to evade the host immune response and for infection to persist. The low copy number of HPV in basal cell has been proposed based on studies in episomal cell lines derived from cervical lesions. Laser capture methods have been utilised in animal models, looking at benign oral papillomas, showing the copy number is as low as 50-100 copies per cell (Maglennon, McIntosh et al. 2011), but it is likely that there will be variation from lesion to lesion and between different sites.

In order to maintain the viral DNA as an episome, E1 and E2 proteins are expressed (Wilson, West et al. 2002) and to facilitate correct segregation of genomes during cell division (You, Croyle et al. 2004). The establishment phase of the viral life cycle relies on the early proteins E1 and E2. Although, they may not be required for maintenance replication (Kim, Lambert 2002, Angeletti, Kim et al. 2002, Egawa, Nakahara et al. 2012). The E1 protein has been speculated to be able to keep viral DNA replication separate from cellular DNA replication during genome establishment and cellular heli-amplification. (E1 helicase keeps separate from cellular helicases- MCM proteins) (Doorbar 2006, Blakaj, Fernandez-Fuentes et al. 2009).

1.4.4.3 Genome amplification

Throughout the maintenance phase in undifferentiated cells, the viral proteins are expressed at low levels in order to evade the immune response. However, once the HPV-infected cells leave the basal layer they undergo differentiation, which then leads to higher expression of the viral proteins. Higher expression of viral proteins is limited to highly differentiated layers of the epithelium as this circumvents the host immune response (Frazer 2009).

As normal differentiating keratinocytes normally withdraw from the cell cycle, they down-regulate host replication factors (Munger, Baldwin et al. 2004), however, HPV does not encode its own DNA replication enzymes, so must force the cell to stay active to utilise its replication machinery. The viral protein responsible for this task is E7. E7 viral protein overrides pRb's normal negative regulation of the cell cycle that prevents progression to S-phase entry (this is done by associating with the transcription factor E2F). Instead E7 binds to pRb and displaces E2F, regardless of there being other growth factors present replication can take place as this leads to an increase in the expression of proteins needed for DNA replication. E7 can stimulate cell proliferation, but can only do so in a small subset of cells in the parabasal layers during productive infection. To counteract the effect of E7 causing unschedueled entry into S-phase, viral E6 protein prevents the normal response of the cell to cause apoptosis. E6 interacts with p53 as well as other host proteins; such as another pro-apoptotic protein, Bak (Thomas, Banks 1998) and Bax (Li, Dou 2000).

In order to produce infectious virions, papillomaviruses must amplify their genomes and package them into infectious particles. For high-risk types this happens in the mid or upper epithelial layers following the increase of activity of the late promoter. The late promoter resides within the E7 open reading frame. The up-regulation of the late promoter leads to increased expression of the viral proteins involved in viral DNA replication (E1, E2, E4 and E5) without directly affecting expression of the E6 and E7 proteins that are necessary for S-phase entry (Middleton, Peh et al. 2003). In the proliferative compartment viral genome amplification take place, and this requires the expression of the viral early genes,

including E4 (Peh, Brandsma et al. 2004) and E5 (Middleton, Peh et al. 2003), whose roles are not fully understood yet.

In order for viral DNA replication to occur the early E2 protein must bind to the upstream regulatory region. E2 then in turns recruits the viral DNA helicase E1 to the viral origin of replication. The complex that E1/E2 forms is similar to the host replicative complex of cdc6 and MCMs, and this viral complex allows for replication in the absence of these proteins. Throughout the virus life cycle, the levels of the viral proteins is regulated by promoter usage and by differential splice site selection, with an increase in the level of E1 and E2 allowing an increase in viral copy number in the upper epithelial layers (Ozbun, Meyers 1998a). A slight increase in promoter activation during differentiation may lead to an increase in the level of E1 and E2 (also E4 and E5), and a subsequent increase in genome copy number. The newly replicated genomes serve as templates for the continued expression of E1 and E2, which allow for further amplification of viral genomes and in turn the further expression of additional E1 and E2 replication proteins (Middleton, Peh et al. 2003).

1.4.4.4 Mode of DNA replication

Papillomaviruses have regulated and uncontrolled replication phases during their life cycle. Flores and Lambert show in W12 cells a switch in the mode of replication at different stages of the cell cycle. W12 cell lines are naturally derived from infected cervical epithelium and harbour replicating HPV DNA episomes. When W12 cells were undifferentiated in a non-productive like state of the cell cycle, HPV16 DNA replicates primarily by theta structures in a bidirectional manner. Replication was initiated within a bubble located at approximately at nucleotide 100, and terminates between nt 3437 and 4906, where two replication forks converge to a common site. When W12 cells were grown in conditions to promote differentiation the mode of replication switched from theta structure to a rolling circle mode of replication. This was concluded due to the absence of bubble and double-Y shaped DNA RIs, and only the presence of Y-shaped HPV16 DNA RIs (Flores, Lambert 1997).

HPV16 rolling circle replication in epithelial cells relies on the origin of replication, E1 and E2, in the same manner as in bidirectional replication, suggesting that the two modes of replication share the same initiation mechanism. It was proposed that HPV16 rolling circle replication diverges from bidirectional replication by failure of replication termination. In the bidirectional mode of replication, two replication forks, moving from the origin, converge near the point antipodal to the origin. The replication proteins at the forks then dissociate from the DNA to allow for completion of replication. Rolling circle DNA amplification is unidirectional, and one initiation event leads to the generation of multiple copies of the genome. A proposed mechanism for the initiation of rolling circle replication is that E1 fails to dissociate from the DNA on collision of two replication forks, which in turn would displace the 5' end of DNA that has been synthesised by the second replication fork, thus allowing a single stranded circular DNA to act as a continuous template for rolling circle replication (Kusumoto-Matsuo, Kanda et al. 2011).

Alternatively, HPV may utilise homologous recombination (HR) to amplify the HPV genome. Homologous recombination is a DNA repair pathway, and a classical marker of this is Rad51, a recombinase that promotes strand invasion and homologous pairing. Other cellular factors include BRCA1 and pRPA. Recent work has shown Rad51 localised to the core of large replication foci in differentiated HPV31 containing cells (Sakakibara, Chen et al. 2013a, Gillespie, Mehta et al. 2012). The presence of Rad51 is suggestive that there is a switch to a recombination-directed mode of replication. E1 and E2 proteins are capable of inducing replication in these foci (Sakakibara, Chen et al. 2013a). These viral replication foci may contain stalled replication forks or abnormal replication intermediates. Therefore, the presence of HR is required for efficient high fidelity viral DNA synthesis upon differentiation. Or, HPV could utilise these factors to promote replication (Gillespie, Mehta et al. 2012). It has also been suggested that if viral genome amplification happens through rolling circle amplification, then HR may be needed for circularisation of HPV genomes upon cleavage of viral concatemers to allow for packaging into virions.

1.4.5 Virus synthesis

Once viral genome amplification is complete, the two structural proteins L1 and L2 are expressed in the upper layers of infected tissue (Ozbun, Meyers 1998b). The major capsid protein L1 is expressed after L2 allowing the assembly of infectious particles in the upper layers of the epithelium (Florin, Sapp et al. 2002). During virus assembly the L2 protein locates to PML bodies, this may be in association with the transcription factor Daxx (Becker, Florin et al. 2004) and recruits L1 to these nuclear domains. It has been proposed that the nuclear PML bodies may be sites of viral replication (Day, Roden et al. 1998) (Swindle, Zou et al. 1999) and that capsid proteins accumulate here to facilitate packaging. The L2 protein is thought to enhance packing (Stauffer, Raj et al. 1998, Zhou, Stenzel et al. 1993) and viral infectivity (Roden, Day et al. 2001)

Papillomaviruses are not released until the infected cells reach the epithelial surface, their survival may be enhanced if they are shed within cornified squame (Bryan, Brown 2001). It is well documented that papillomaviruses avoid immune detection, by limiting the presentation of viral epitopes to the immune system in the lower epithelial layers (Ashrafi, Tsirimonaki et al. 2002, Marchetti, Ashrafi et al. 2002, Matthews, Leong et al. 2003). The expression of viral proteins can inhibit the expression of differentiation markers which can prevent the formation of normal cornified squames (Doorbar, Foo et al. 1997). A proposed role for the E4 protein is that it may contribute directly to virus egress in the upper layers of the epithelium by disturbing keratin integrity (Doorbar, Ely et al. 1991, Wang, Griffin et al. 2004) and by affecting the assembly of the cornified envelope (Bryan, Brown 2001) (Lehr, Hohl et al. 2004).

1.5 The viral oncoproteins

High risk HPV types cause neoplasia, and there is a definite clear cut role for the Viral E6 and E7 proteins in cell transformation. These early viral proteins drive cell proliferation in the basal and parabsal cell layers (Doorbar 2006).

E6 and E7 proteins are expressed by a bicistronic mRNA (Stacey, Jordan et al. 2000) expressed from the viral promoter (p97). Both of these proteins function to stimulate cell cycle progression (Munger, Basile et al. 2001). Human keratinocytes require both E6 and E7 to become efficiently immortalised ((Hawley-Nelson, Vousden et al. 1989), however E6 alone can immortalise NIH 3T3 and human mammalian epithelial cells ((Joyce, Tung et al. 1999, Liu, Chen et al. 1999). It has been shown in transgenic mouse models that E6 plays an important role in cooperating with E7 in tumour induction at diverse anatomical sites (Lambert, Pan et al. 1993, Shai, Brake et al. 2007, Jabbar, Strati et al. 2010, Shai, Pitot et al. 2010).

1.5.1 E7 protein

E7 proteins are predominantly found in the nucleus and are 100 amino acids in size, and associate with the Rb family of proteins ((Dyson, Howley et al. 1989). The binding of E7 to Rb proteins is mediated through one of three conserved regions, which can be found in all high risk E7 proteins: CR1 at the N terminus; CR2, which contains an LXCXE motif that binds the Rb protein; and CR3, which contains two zinc finger-like motifs. The Rb family of “pocket” proteins includes Rb, which is constitutively expressed throughout the cell cycle; p107, which is synthesised predominantly during the S phase, and p130 which initiates at G0 ((Dyson, Howley et al. 1989, Berezutskaya, Yu et al. 1997, Classon, Dyson 2001). E7 viral protein overrides pRb’s normal negative regulation of the cell cycle that prevents progression to S-phase entry (this is done by associating with the transcription factor E2F1). Instead E7 binds to pRb and displaces E2F1, regardless of there being other growth factors present, replication can take place as this leads to an increase in the expression of proteins needed for DNA replication. E7 protein binds to other host proteins as well as pRB; this includes other proteins required for proliferation, histone deacytelases (Longworth, Laimins 2004), components of the AP-1 transcription complex (Antinore, Birrer et al. 1996) and p21

and p27 cyclin-dependent kinase inhibitors (Funk, Waga et al. 1997). E7 can stimulate cell proliferation, but can only do so in a small subset of cells in the parabasal layers during productive infection. As a result of E7 expression and the disruption of E2F/pRb, cyclin E is expressed, which is necessary for progression into S-phase. In differentiating epithelial cells, the high levels of cyclin-dependent kinase inhibitors (p21cip1 and p27kip1) can lead to inactive complexes of E7, cyclin E/cdk2 and either p21 or p27 (Noya, Chien et al. 2001). The progression into S-phase, stimulated by E7 protein is limited to a subset of differentiated cell with low levels of p21/p27, or where there are high enough levels of E7 to overcome the block to S-phase entry.

1.5.2 E6 protein

The E6 protein is approximately 150 amino acids in size and contains two zinc binding domains with the motif Cys-X-X-cys. The E6 protein has been reported to bind to over 12 different proteins and can be found located in both the nucleus and the cytoplasm (zur Hausen 2002). E6 interacts with p53 as well as other host proteins; such as another pro-apoptotic protein, Bak (Thomas, Banks 1998) and Bax (Li, Dou 2000). E6 is also known to bind to other cellular proteins; these include paxillin, p300/CBP, the putative calcium binding protein E6-BP, and the interferon regulatory factor IRF-3 ((Patel, Huang et al. 1999, Ronco, Karpova et al. 1998, Zimmermann, Degenkolbe et al. 1999).

To counteract the effect of E7 causing unschedueled entry into S-phase, viral E6 protein prevents the normal response of the cell to cause apoptosis. When there is DNA damage p53 becomes activated and induces high levels of p21, this activation leads to cell cycle arrest and apoptosis (Ko, Prives 1996). Viruses have evolved mechanisms to evade the cells apoptotic response, and the activation of apoptotic pathways. This evasion can lead to malignancies, which is why the presence of E6 is a determining factor in the development of HPV related cancers, as this allows for the accumulation of unchecked errors in host DNA. Papillomavirus evades these apoptotic pathways via E6 binding to p53 in a ternary complex with ubiquitin ligase called E6AP (Huibregtse, Scheffner et al. 1991). This leads to the ubiquitination of p53 and the degradation of p53 by the 26S proteasome. This degradation ultimately leads to a significantly shorter half-life of p53, which decreases from several hours to only 20 minutes in keratinocytes

(Hubbert, Sedman et al. 1992, Huibregtse, Scheffner et al. 1993). Alternatively E6 can also down regulate p53 through an interaction with p300/CBP, which is a co-activator of p53 (Zimmermann, Degenkolbe et al. 1999, Lechner, Laimins 1994). This deregulation of p53 means it can no longer carry out its usual cell cycle controls, p53 regulates G1/S and G2/M checkpoints of the cell cycle. This can lead to chromosomal duplications and centrosomal abnormalities (Foster, Demers et al. 1994, Kessis, Slebos et al. 1993, Thompson, Belinsky et al. 1997). E6 mutants in HPV 16 that are unable to degrade p53 retain the ability to immortalise mammary epithelial cells. Also, E6 mutants that retain the ability to degrade p53 have lost the ability to immortalise (Liu, Chen et al. 1999, Kiyono, Hiraiwa et al. 1997).

E6 can also cause cell proliferation in the absensce of E7 through its c-terminal PDZ-ligand domain (Thomas, Laura et al. 2002). High risk E6 protein has a PDZ binding domain, which allows for its interaction with PDZ and in turn E6 can regulate many PDZ targets. Many of these targets are involved in regulation of cell polarity, proliferation and cell signalling. (Javier 2008, Culp, Cladel et al. 2006). The proliferation E6-PDZ binding causes may be sufficient to contribute to the development of metastatic tumours by disrupting normal cell adhesion.

E6 proteins also activate expression of the catalytic subunit of telomerase, hTERT ((Klingelhutz, Foster et al. 1996, Meyerson, Counter et al. 1997, Nakamura, Morin et al. 1997). Telomerase is a four subunit enzyme that adds hexamer repeats to the telomeric ends of chromosomes. The lack of telomerase activity results in a shortening of telomeres with successive cell divisions, which subsequently leads to sensessence (Liu 1999). So when hTERT is reactivated in cancer occurs it leads to reconstitution of telomerase activity (Liu 1999). E6 activates hTERT transcription through Myc and Sp-1 (Greenberg, O'Hagan et al. 1999, Kyo, Takakura et al. 2000, Oh, Kyo et al. 2001, Wang, Xie et al. 1998, Wu, Grandori et al. 1999).

In conclusion, the combination of expression of E7 and E6 in abberant S phase entry and proteins of where cells from host defence mechanisms such as apoptosis.

1.6 The roles of E1 and E2 in replication

The two DNA binding proteins E1 and E2 are required for viral genome replication; E2 protein forms homodimers and binds to 12bp palindromic sequences surrounding A/T rich origin of replication. The origin of replication also contains an E1 binding site (Ustav, Ustav et al. 1991, Ustav, Ustav et al. 1991). E2 recruits the replicative helicase E1 protein to the HPV origin via a protein-protein interaction (Mohr, Clark et al. 1990, Sanders, Stenlund 2001). E1 is the primary replication protein, however, E2 enhances and supports the functions of E1 (Frattini, Laimins 1994a, Mohr, Clark et al. 1990). The E1 helicase forms di-hexameric complex that replicates the viral genome via interaction with host proteins (Sanders, Stenlund 2001). E2 is then displaced, which also displaces nucleosomes from the origin of replication to alleviate repression (Li, Botchan 1994).

1.6.1 Replication protein E1

In 1982, the complete sequence of both HPV1 a and BPV1 genomes revealed a common genetic organisation that included several large open reading frames (Chen, Howley et al. 1982, Danos, Katinka et al. 1982). Following these studies, it was then demonstrated that the putative E1 proteins also shared homology with the SV40 large T antigen (Seif 1984). As the role of the T antigen in SV40 replication initiation had already been established, a similar function for papilloma E1 was predicted (Clertant, Seif 1984). This was established in transformation studies with BPV1, which showed that an intact E1 ORF is required for stable maintenance of the BPV1 genome as a multicopy nuclear plasmid (Lusky, Botchan 1985, Sarver, Rabson et al. 1984, Rabson, Yee et al. 1986).

Genomic E1 ORF sequences are available for over 170 human and greater than 112 non-human papillomaviruses, all of these E1 proteins are predicted to share numerous features with the well characterised BPV1 E1 protein and other helicases (Patel, Picha 2000). E1 proteins vary in size between HPV types, they range from 593 (HPV48) to 681 (HPV10) amino acids with a molecular weight distribution from 67.5 (HPV47) to 76.2 kDa (HPV10). This range in size is due to the mucosal HPVs having additional residues in the N-terminal region, which in turn results in lower sequence similarity for the N-terminal third of E1 proteins than for the remaining C-terminal portion.

E1 proteins recognise the viral origin of replication and have ATPase and 3'-5' helicase activities (**Figure 1.4**) (Hughes, Romanos 1993, Yang, Mohr et al. 1993)(Seo, Muller et al. 1993). E1 recognises AT-rich sequences at the viral origin which are proximal to the start sites of early transcription (Frattini, Laimins 1994a, Frattini, Laimins 1994b, Muller, Giroglou et al. 1997). The binding of E1 onto the viral origin is facilitated by E2 (Frattini, Laimins 1994a, Dixon, Pahel et al. 2000, Lu, Sun et al. 1993, Sun, Lu et al. 1996). E1 then forms hexamers which have a high affinity for DNA (Sedman, Stenlund 1998), the viral DNA is able to pass through the centre of the E1 hexameric ring (Liu, Kuo et al. 1998). With the aid of chaperone proteins, E1 is able to unwind supercoiled DNA. E1 binds to DNA polymerase α, and also help to recruit host cellular replication complexes to viral origin to aid in viral replication (Amin, Titolo et al. 2000, Conger, Liu et al. 1999, Masterson, Stanley et al. 1998).

The crystal structure of the DNA binding domain of E1 has shown an extended loop and α-helix that are important for recognising DNA (Enemark, Chen et al. 2000). The E1 protein has four cyclin kinase binding sites, and E1 binds to both cyclin A and E. When these sites are mutated it greatly impairs E1 replicative abilities (Ma, Zou et al. 1999).

The P-loop of E1 proteins contains one or two prolines (consensus: G-P-P/A-N?D-T-G-K-S) except for HPV16 E1 (GAANTGKS). The 16E1 protein binds ATP weakly, but binding is enhanced by the replacement of the two P-loop alanines with prolines, again implicating this motif in ATP binding (Raj, Stanley 1995). Further to this role of binding ATP, mutational analysis of this motif in HPV11 E1 suggests that the P-loop is critical for both the interaction between E1 and the transcriptional activation domain (TAD) of E2 (Titolo, Pelletier et al. 1999) and for E1-E1 oligomerisation (Titolo, Pelletier et al. 2000). The P-loop may be offering an architectural function for the interaction of E1 and E2, as this interaction does not require ATP (Titolo, Pelletier et al. 1999).Not much is known about the structure of HPV E1 beyond the primary sequence. No definitive information about the secondary structure or tertiary features are known, as E1 has never been crystalised for diffraction analysis. However, there is a crystal structure for BPV1 E1.

Due to the role of E1 in viral replication, it is no surprise it is located in the nucleus of infected cells (Ustav, Ustav et al. 1991, Blitz, Laimins 1991, Santucci,

Androphy et al. 1990, Sun, Thorner et al. 1990). The nuclear localisation of E1 is driven by a sequence in the first 223 amino acids (Sun, Thorner et al. 1990). A small region of (BPV1) E1 (aa 84-166) containing the nuclear localisaiton sequence, KRKVLGSSQNSSGSEASETPVKRRK is necessary for the direct nuclear localisation of fusion proteins (Leng, Wilson 1994).

In addition to its key role in viral replication as a helicase, E1 also plays a role in the regulation of viral transcription and transformation. This is thought to occur through binding to E2, E1 modulates E2s ability to activate certain viral promoters. Studies in BPV1 have shown that E1 negatively regulates the activation of the major early promoter by E2 (Le Moal, Yaniv et al. 1994, Sandler, Vande Pol et al. 1993) and this mechanism may be responsible for the suppression of transformation by E1 (Lambert, Howley 1988, Schiller, Kleiner et al. 1989, Vande Pol, Howley 1995, Zemlo, Lohrbach et al. 1994). E1 also has a direct effect on the host cell cycle (Belyavskyi, Westerman et al. 1996, Belyavskyi, Miller et al. 1994). Reinson *et al* have shown that E1 protein from expression constructs can induce cellular DNA damage when overexpressed. E1 induces double-stranded DNA breaks (DSBs) and activates the ATM-Chk2 pathway, leading to cell cycle arrest in the S and G_2 phases (Reinson, Toots et al. 2013).

Figure 1.4

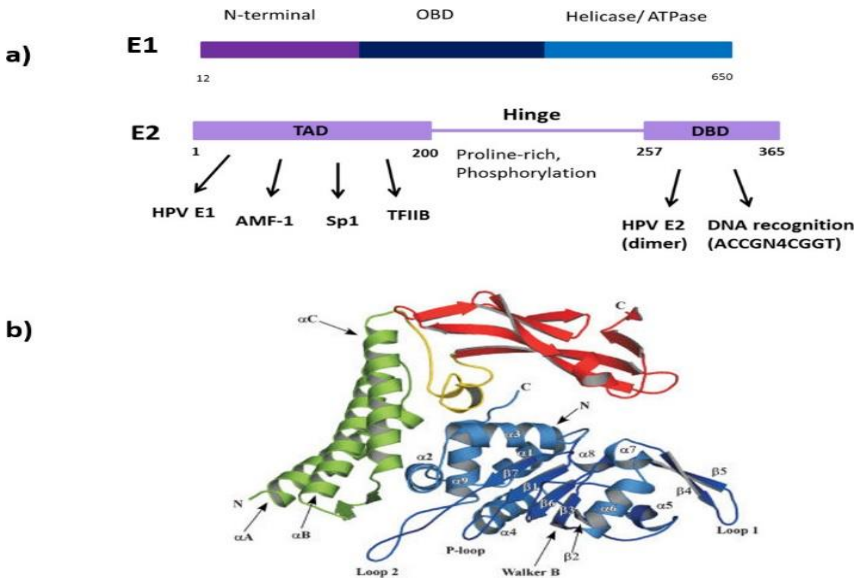


Figure 1.4: X-ray structure of the papillomavirus replication proteins E1 and E2 in complex.

a) **Schematic of HPV E1 and E2 ORFs indicating the amino acid boundaries of key domains.** E1 protein is approximately 650 amino acids in length. The N-terminal contains the nuclear localisation signal. The central portion of E1 contains the origin binding domain, which associates with a series of inverted-repeat sites at the viral origin. The C-terminal contains the helicase domain of E1. ATP stimulates the oligomerisation of E1 and enhances the helicase activity of E1. E1 also interacts with host cell replication factors such as polymerase α to promote bi-directional replication of the viral genome. E2 is approximately 370 amino acids in length. Locations of the functional domains of the E2 protein are indicated. TAD: transcriptional activation domain; DBD: DNA binding domain. The E2 ORF contains a conserved N-terminal activation domain of approximately 200 amino acids. This domain is essential for transcription, replication, and viral DNA segregation. The TAD is connected to the DBD by a flexible proline-rich hinge domain. E2 is a homodimer and interacts with the “ACCGN4CGGT” DNA recognition site, which is conserved throughout the entire PV family.

b) **Cartoon representation of HPV18 E1 and E2 in complex.** E1 is shown in blue. The N-terminal helical domain of E2 is green, the β-strand structural domain is coloured in red, and the linker segment between the two domains is depicted in yellow. This Cartoon was adapted from(Abbate, Berger et al. 2004)

1.6.2 Replication protein E2

E2 is approximately 43kDa in size and functions as a dimer. The full-length E2 protein is expressed from the entire E2 open reading frame (ORF) and has two functional domains which are separated by a hinge region. These are the N-terminal transactivation domain (TAD) which is about 200 amino acids which is linked to the C-terminal DNA- binding/ dimerization domain of about 100 amino acids via a short hinge domain (**Figure 1.4**) (Giri, Yaniv 1988, McBride, Byrne et al. 1989).

The length of the hinge region is variable between the E2s of different papillomavirus genera, but similar within each genus. The E2 hinge region is rich in proline, serine, threonine, glycine and arginine residues, and forms a flexible link between the transactivation and DNA binding domains (Gauthier, Dillner et al. 1991). Although, the hinge region is not involved in transcriptional and replication functions (Winokur, McBride 1992). E2 hinge regions have auxiliary functions such as determinants of intracellular localisation, chromatin binding and protein stability. These functions are regulated by phosphorylation.

The TAD is a protein interaction module that binds through one surface to the viral E1 helicase to promote replication of the genome, and through the opposite surface to cellular transcription factors, including Brd4, to regulate viral genome transcription(Gagnon, Joubert et al. 2009)(Chen, Stenlund 2000). Through crystalisation studies the N-terminus of E2 has been shown to consist of a glutamine rich α-helix packed against a ß-sheet framework (Antson, Burns et al. 2000, Harris, Botchan 1999). E2 dimers bind to E2 binding sites (E2BSs) which have the sequence ACCN6GGT (Androphy, Lowy et al. 1987, Bedrosian, Bastia 1990). In the URR there are 4 E2BSs, and three of these flank the E1 recognition sequences in the origin of replication (Androphy, Lowy et al. 1987, Hirochika, Hirochika et al. 1988, Li, Knight et al. 1989). The C-terminal encodes a DNA binding domains and the crystal structure of this has shown that is has a dimeric ß-barrel structure that bends DNA (Hegde, Grossman et al. 1992).

The crystal structure of (HPV18) E2 activation domain bound to the helicase domain of E1 has been solved (Abbate, Berger et al. 2004). The E1-E2 complex can

be disrupted by mutations in the first N-terminal alpha-helix, or alternatively by antibodies that bind to this region of E2 (Baxter, McPhillips et al. 2005b).

1.7 Additional E2 functions and cellular partners

1.7.1 Transcriptional repression

E2 is a transcription factor, and is a specific DNA binding homodimer harbouring a classical modular structure in three distinct domains (Giri, Yaniv 1988, McBride, Byrne et al. 1989, Dostatni, Thierry et al. 1988). As mentioned previously E2 binding sites are present in multiple copies in different papillomavirus regulatory regions, however, only 4 copies exist in mucosal types (Sanchez, Dellarole et al. 2008). The distances between these sites is well conserved, there are 1 or 2 nucleotides between BS#1 and BS#2, these are the most proximal binding sites to the promoter, there are 64 nucleotides between BS#2 and BS#3 and 320 nucleotides between BS#3 and BS#4. The E2 binding site location relative to the TATA box is also well conserved, involving 3 nucleotides for the majority of the mucosal HPV (**Figure 1.5**).

The three sites proximal to the TATA box are involved in E2 transcriptional repression of the early promoters. The two most proximal sites to the TATA box play the most prominent role (Romanczuk, Thierry et al. 1990, Thierry, Howley 1991, Demeret, Desaintes et al. 1997) . Conversely, these sites are also crucial for the efficient replication of the virus, which involves the viral helicase E1 (Demeret, Le Moal et al. 1995).

Binding of E2 represses transcription through steric hindrance of the interaction with the transcriptional initiation factor TFIID at the proximal TATA box. The work that first described this transcriptional repression mechanism was done by transfecting cells in vivo (Romanczuk, Thierry et al. 1990, Thierry, Howley 1991) and in vitro in cell extract (Dostatni, Lambert et al. 1991), as well as in more recent years with purified transcription factors (Hou, Wu et al. 2000), this indicated that binding of E2 to the HPV regulatory region precludes binding of TBP to the adjacent site.

For transcriptional repression, there needs to be an intact DNA binding domain as well as in the involvement of a competent transactivation domain (Goodwin, Naeger et al. 1998, Nishimura, Ono et al. 2000). The involvement of an intact transactivation domain has been suggested to recruit transcriptional co-repressors. However, this mechanism is not fully understood. One such transcriptional co-repressor is Brd4, which is known for its involvement in mitotic segregation (You, Croyle et al. 2004), could be involved in transcriptional activation and repression, depending on the chromatin context (Schweiger, Ottinger et al. 2007, Wu, Lee et al. 2006a). Another suggested mechanism for repression involves the looping of the regulatory sequences through interaction of the E2 amino-terminal domains, could also be involved in the mechanism of repression (Hernandez-Ramon, Burns et al. 2008)(Hernandez-Ramon, Burns et al. 2008).

Figure 1.5

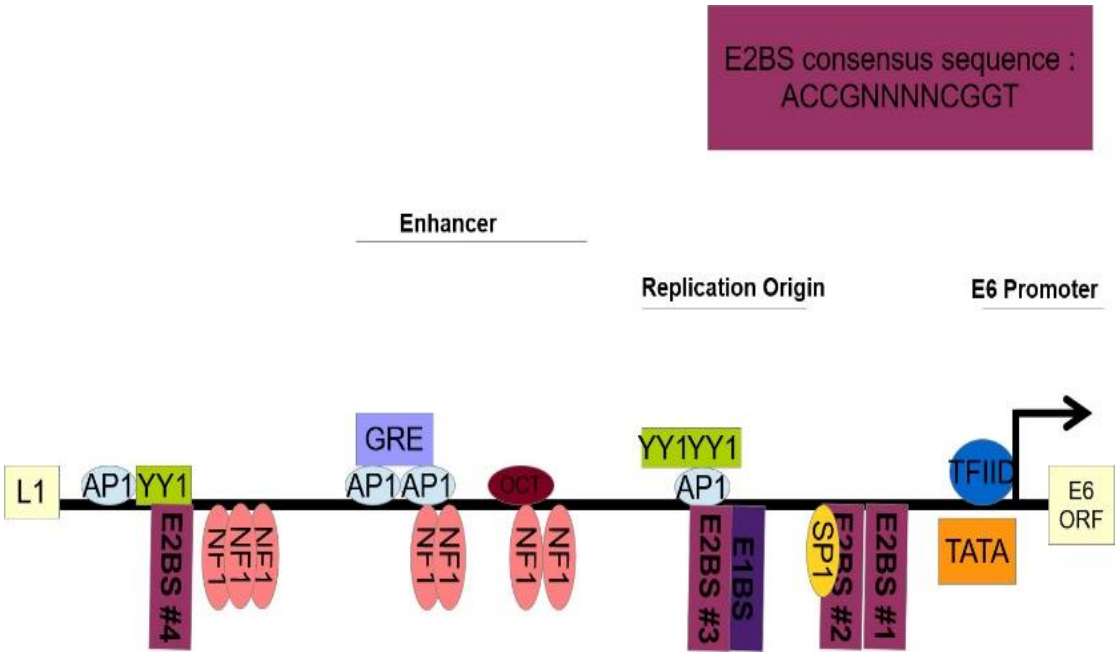


Figure 1.5: Schematic map of the long control region of HPV16. Transcriptional activity is mainly controlled by the LCR. Within the LCR there is a transcriptional enhancer which associates with various cellular transcription factors. Four E2 binding sites are found in the HPV16 LCR, and viral gene expression is regulated by the occupancy status of these E2BSs. Figure adapted from (Muller, Demeret 2012)

1.7.2 Growth inhibition and E2-mediated apoptosis

As well as playing roles in viral transcription and replication, there are a number of reports of additional functions for E2. The pro-apoptotic activity of E2 is one of the first described E2 functions independent of its binding to the viral genome (Desaintes, Demeret et al. 1997) . This role is specific to high risk E2 types. Various assay systems have shown that an overexpression of E2 induces apoptosis, and some of this activity may be related to E2's ability to repress viral gene expression in HPV transformed cells when it is highly expressed, resulting in a decrease in E6 expression and consequent increased levels of p53 (Desaintes, Demeret et al. 1997) . In addition, E2 has also been shown to induce growth arrest in the G1 phase of the cell cycle (Goodwin, Naeger et al. 1998, Hwang, Naeger et al. 1996), as well as to abrogate the mitotic checkpoint (Frattini, Hurst et al. 1997), through both p53 dependent and independent mechanisms.

1.7.3 Mitosis and genome segregation

Papillomavirus infections are usually long lived and the virus requires a specific mechanism to retain the episomal genomes within the dividing epithelial cells. HPV segregates its genome into daughter cells by tethering them to cellular mitotic chromosomes, which ensures that viral DNA is retained in the nucleus after cell division (Bastien, McBride 2000, Ilves, Kivi et al. 1999, Lehman, Botchan 1998, Piirsoo, Ustav et al. 1996).

The E2 protein was first implicated to be involved in segregation of the episomally replicating genome when it was found that multiple E2 binding sites were required in *cis* with the replication origin for long-term genome maintenance (Piirsoo, Ustav et al. 1996). It was established that E2 and the extrachromasomal genome in dividing cells were both tethered to condensed mitotic chromosomes (Skiadopoulos, McBride 1998). The transactivation domain of E2 is responsible for the tethering E2 to chromosomes (Bastien, McBride 2000), which forms a bridge between chromosomes and the genome (Ilves, Kivi et al. 1999). When this interaction is abrogated there is a dramatic loss of viral genomes from infected cells (Lehman, Botchan 1998).

Brd4 has a diffuse cloud like appearance around the condensed mitotic chromosomes (Dey, Chitsaz et al. 2003), however, when E2 is present, both proteins localise in punctate foci (McPhillips, Ozato et al. 2005) E2 stabilises the interaction of Brd4 with host chromatin forming a stable anchor in interphase and mitosis (McPhillips, Ozato et al. 2005). Two conserved residues in the transactivation domain of E2 (R37 and I73) mediate the interaction with Brd4, and mutations in these residues abrogate the interaction of E2 with Brd4 and mitotic chromatin (Baxter, McPhillips et al. 2005a). The DNA binding domain of E2 is required to link the viral genomes to chromatin, but it is not required for the interaction with Brd4 (Abroi, Ilves et al. 2004).

The mechanism of E2-mediated viral genome tethering and E2-Brd4 chromosomal binding is still not fully understood. Alpha-PV E2 proteins only weakly interact with Brd4, they also do not stabilise association of Brd4 with host chromatin and cannot be easily detected on mitotic chromsomes, except in late telophase (Oliveira, Colf et al. 2006, Donaldson, Boner et al. 2007). Due to this difficulty in seeing E2-Brd4 in alpha-papillomaviruses at mitotic foci, it has been proposed that there may be other targets such as the mitotic spindle (Dao, Duffy et al. 2006), a mitotic kinesis-like protein, MK1p2 (Yu, Peng et al. 2007), Ch1R1 (an ATP-dependent DNA helicase important for sister chromatid cohesion) (Parish, Bean et al. 2006) and TopBP1 (Donaldson, Boner et al. 2007).

Work by Stilla *et al* has shown that attachment to genomes alone is not enough (Silla, Mannik et al. 2010); chromatin binding and transactivation functions must cooperate to ensure proper plasmid segregation. BPV1 E2 have been shown to bind to transcriptionally active regions of chromatin through genome-wise ChIP-on-chip studies. It has been proposed that the purpose of this is to ensure that the viral genome is localised to transcriptionally active regions in the nucleus (Jang, Kwon et al. 2009b).

1.8 HPV E2 interaction with host proteins

E2 protein binds to a range of cellular proteins that are required for each of E2s functions. A detailed list of E2 associated proteins can be found in a recent review by Alison McBride (McBride 2013). E2 associated proteins fall into several categories such as transcriptional regulation, RNA processing, apoptosis, cell cycle, nuclear import and protein degredation.

Transcriptional regulation represents the largest category of E2 interacting proteins. E2 binds many basal transcriptional regulators (TAF, TBP, GTF2B) or regulatory transcription factors (SP1, HOXC9, NR4A1, C/EBP). E2 also interacts with co-activators (TMF), which suggests that the transcriptional properties of the virus may rely on the modulation of preinitation complex formation on specific promoters. The best characteristed interaction is between E2 and Brd4, which regulates transcription on the chromatin level. Brd4 binds to acetylated histones and stimulates RNA polymerase II-dependent transcription by recruiting distinct transcriptional regulators (Wu, Chiang 2007b). Failure to bind Brd4 abrogates the ability of E2 to activate transcription (Schweiger, You et al. 2006).

E2 has also been found to regulate transcription through modification of the histone code, by targeting histone-modification factors. E2 interacts with many histone acetyltransferases such as PCAF, EP300, EP400 or CBP (Lee, Hwang et al. 2002, Lee, Lee et al. 2000, Muller, Ritzkowsky et al. 2002, Cha, Seo 2011). Additionally, E2 also interacts with members of the chromatin remodelling complexes, which are involved in the displacement of nucleosomes. NAP1L1 and hSNF5 are both known E2 interactors (Cha, Seo 2011, Rehtanz, Schmidt et al. 2004).

1.8.1 E2 regulation of the host genome

Previous studies of how E2 regulates the host genome to aid progression of the viral life cycle have focused on determining the effects of E2 on specific promoters and biological processes. There is a gap in our understanding of how E2 regulates cellular processes on a global scale.

Two complementary studies have shown that E2 binds to transcriptionally active cellular genes without significantly changing the mRNA levels of the E2-bound cellular genes. This suggests that the binding of E2 to transcriptionally active cellular genes does not have an effect on their activity (Vosa, Sudakov et al. 2012, Jang, Kwon et al. 2009a).

Vosa *et al* 2011 studied the occurrence and functionality of E2 binding sites (E2BS) in the human genome. Using a combination of computational analysis and genome-wide chromatin immunoprecipitation –on-chip analysis with human promoter sequences, specific sites of E2 interaction were identified on chromatin. They found that E2 interacts with E2BSs located in active chromatin regions. E2 and Brd4 were found bound on transcriptionally active promoters, and E2 binding did not correlate with the E2BS in the promoters. BPV1 E2 may bind to active regions to escape silencing, giving an additional role for E2-mediated tethering of viral genomes to host chromatin; partitioning of viral genomes to daughter cells, ensure genomes are retained in the nucleus, and also to ensure that genomes are retained in functionally active nuclear domains to aid in the progression of the viral life cycle (Vosa, Sudakov et al. 2012).

A study by Ramirez-Salazar *et al* overexpressed HPV16 E2 in HPV negative cells (C33a cells) using a recombinant adenoviral vector for use in microarray analysis studies to determine how E2 regulates biological processes (Ramirez-Salazar, Centeno et al. 2011). The main cellular pathway that was regulated by HPV16 E2 expressing cells was WNT. The WNT pathway regulates genes involved in cellular processes such as, apoptosis, proliferation and cell differentiation. Additionally, 16E2 overexpression was also shown to regulate genes from pathways such as PDFG, angiogenesis and cytokine and chemokines mediated inflammation. The cellular processes that were shown to be regulated by E2 expression provide a convenient environment for the replicative cycle of the virus (Ramirez-Salazar, Centeno et al. 2011).

1.9 E5 protein

All α HPVs encode and express E5, however most ß HPVs do not. The E5 ORF can be classified into four different groups depending on genera: alpha, beta,

gamma and delta (Bravo, Alonso 2004), these types correlate with different clinical manifestations, in particular with oncogenic potential (Schiffman, Herrero et al. 2005). E5-aplha protein is encoded by HR αHPV and the E5-gamma and E5-delta proteins are encoded by the low-risk genital HPVs (Garcia-Vallve, Alonso et al. 2005). However, the E5 ORF is often absent in the genome of many of the beta-, gamma- and mu-HPVs, which suggests that it is not an essential protein for the life cycle of HPVs, but may give some extra benefit to the virus with regards to increasing the likelihood of oncogenic transformation following persistent infections. In BPV-4 induced papillomas (Araibi, Marchetti et al. 2004) and HPV-16 induced cervix lesions (Chang, Tsao et al. 2001), E5 has been shown to be expressed in the basal and suprabasal layers.

HPV16 E5 protein is 83 amino acids long. This protein is extremely hydrophobic and located within the membrane and expressed at very low levels. The HPV E5 proteins display weak transforming activity *in vitro*. The first evidence of this transformation capability came from experiments with HPV-6 in mammalian cells, which showed that expression of HPV-6 E5 in established murine fibroblasts lead to anchorage independent growth (Chen, Mounts 1990). The E5 protein is known to interact with many cellular proteins and these interactions are important for the biological activity of E5 and evasion of the immune response. The transforming effects of E5 can be enhanced when it co-operates with other viral oncoproteins. When E5 interacts with E6 it can induce the formation of koilocytes, large cells with cleared cytoplasm and pyknotic nuclei with inconspicuous nucleoli, this is a morphological marker of HPV infection (Krawczyk, Suprynowicz et al. 2008).

1.10 E4 protein

The primary E4 gene product is expressed from a spliced mRNA (E1^E4), in which the first five amino acids of E1 are spliced to the E4 ORF (Chow, Reilly et al. 1987, Doorbar, Parton et al. 1990, Milligan, Veerapraditsin et al. 2007, Wang, Meyers et al. 2011, Ozbun, Meyers 1997). The E4 open reading frame varies in size between papillomavirus types. Although there is some sequence homology between E1^E4 proteins dictated by virus types of similar pathology, sequence homology is found within the amino and carboxy termini of the proteins (Roberts, Ashmole et al. 1994, Roberts, Ashmole et al. 1997).

E4 was originally described as an early gene, although it is not clear that is has any function in the early stages of the life cycle. This original distinction was given to E4 due to where it lies in the viral genome and it is found embedded in amongst early viral genes that are responsible for the regulation of cell cycle entry and genome maintenance (Chen, Howley et al. 1982, Danos, Katinka et al. 1982).

Though extensive studies have been made to investigate the role of E4 in the virus life cycle, its role still remains unclear. Most work focusing on high-risk E4 proteins has come from studies in HPV16, 18 and 31. In these high-risk E4 proteins there is a "leucine-cluster" motif close to their N-terminus, which is important (along with upstream amino acids) for keratin association. This motif is thought to be largely unavailable for cytokeratin binding, and instead associates with the E4 multimerisation-motif located at the C-terminus of the protein (McIntosh, Martin et al. 2008, Bryan, Fife et al. 1998). Prior to the onset of genome amplification, the E1^E4 protein is not very apparent, however, it may be present at very low levels. The large increase in the presence of E1^E4 at around the onset of genome amplification, coincides with the activation of the differentiation-dependent promoter (i.e p670 in HPV16) and subsequently an elevation in E1^E4 transcript levels.

The first evidence of the role of E4 in amplification as opposed to early stages of infection came from work using mutant CRPV genomes that contain translation-termination linkers in the E4 ORF (Peh, Brandsma et al. 2004). These mutant genomes with no E4 reduced the efficiency of genome amplification and the synthesis of capsid proteins (Peh, Brandsma et al. 2004). The role of E4 proteins in the amplification stage and additionally in the synthesis of capsid proteins was then later shown in HPV 16 (Nakahara, Peh et al. 2005), HPV 31 (Wilson, Fehrmann et al. 2005), and HPV 18 (Wilson, Ryan et al. 2007), using organotypic raft culture systems, the presence of which optomises life-cycle completion. The mechanism by which E4 achieves this is yet unknown.

E1^ E4 has been linked to several biological functions within the viral life cycle, such as the disruption of normal cell division. (Davy, Jackson et al. 2002, Knight, Grainger et al. 2004, Nakahara, Nishimura et al. 2002). E1 ^E4 has also been shown to initiate the relocation of PML from multiple intranuclear speckles

(ND10 bodies) to the periphery of E4 inclusions (Roberts, Hillman et al. 2003) (Roberts 2002). Viral replication and virion assembly is proposed to be carried out within ND10 bodies. HPV1 E4 may disrupt of reorganise ND10 domains for efficient viral replication and ultimately increase the efficiency of HPV infection. Additionally, E1^E4 may aid in the release of newly synthesised virions from the upper cells in the epithelium, by a weakening of the cornified cell envelope (Bryan, Han et al. 2000) and also by disturbing the intermediate filament cytoskeleton (Doorbar, Ely et al. 1991, Roberts, Ashmole et al. 1993).

E4 has been shown to be highly expressed in the mid and upper epithelial layers during productive infection (Maglennon, McIntosh et al. 2011, Doorbar, Foo et al. 1997, Peh, Middleton et al. 2002). Due to the high expression of E4 in lesions, it has been suggested that E4 could be a valuable biomarker of active HPV disease (Middleton, Peh et al. 2003, Borgogna, Zavattaro et al. 2012), and at the cervix, E4 may serve as a marker for the severity of the disease (Griffin, Wu et al. 2012, Doorbar, Cubie 2005).

1.11 Capsid proteins

The viral genome is packaged within the major (L1) and minor (L2) capsid proteins that are produced through alternative-splicing mechanisms, which regulates the capsid-ORF transcriptional program (Buck, Cheng et al. 2008, Swindle, Zou et al. 1999, Doorbar, Gallimore 1987, Okun, Day et al. 2001, Buck, Thompson et al. 2005, Kawana, Yoshikawa et al. 1998, Fligge, Schafer et al. 2001, Mole, Milligan et al. 2009).

HPV caspids are composed of approximately 360 copies of the major capsid protein L1, which accounts for 80-90% of total viral protein (Favre 1975). Through hydrophobic interactions L1 proteins rapidly assemble into pentameric capsomers (Kirnbauer, Booy et al. 1992, Kirnbauer, Taub et al. 1993). 72 of these L1 structures that form the capsid are arranged in a in a T=7 icosohedral array through a complex network of hydrophobic interactions and disulphide bonds (Baker, Newcomb et al. 1991a, Culp, Cladel et al. 2006, Trus, Roden et al. 1997, Baker, Drak et al. 1989, Belnap, Olson et al. 1996, Finnen, Erickson et al. 2003, Culp, Budgeon et al. 2006a, Modis, Trus et al. 2002, Ishii, Ozaki et al. 2005, Sapp, Fligge et al. 1998, Li, Beard et al. 1998). The localisation of L2 within this

structure is unknown, nor is its mechanism of assembly, although reports suggest that disulphide bonding may play a role (Belnap, Olson et al. 1996, Baker, Newcomb et al. 1991b). The capsid encases a single, circular, dsDNA genome of approximately 8kb, which associates with histones to form a chromatin-like structure (Fligge, Schafer et al. 2001, Belnap, Olson et al. 1996). The stability of the capsid is increased with the genome, increasing its resistance to environmental stresses such as proteolysis (Fligge, Schafer et al. 2001)

L1 can accommodate up to 72 molecules of the L2, minor capsid protein. Although L1 is only minimally exposed at the surface of mature virion, it emerges from virion during the infectious entry process (Day, Baker et al. 2004)(Day, Gambhira et al. 2008). L1 is also transiently exposed in immature virions (Richards, Lowy et al. 2006).

1.11.1 L1 protein

The papillomavirus major capsid protein, L1, is approximately 55 kDa in size and has the ability to spontaneously self-assemble into a virus-like particle (VLPs). These VLPs make up the exterior surface, which is indistinguishable from the native 60nm non-enveloped papillomavirus virion. Due to the fact assembled VLPs are potent immunogens (Bachmann, Rohrer et al. 1993, von Bubnoff 2012), they have been used in the current VLP-based vaccines, which offer highly effective protection against HPV 16 and 18.

The major protein L1 has a solved crystal structure (Chen, Garcea et al. 2000), which was used for predicting the localisation of L2 within the L1-pentameric shaft through Cluspro docking software (Lowe, Panda et al. 2008). The exterior surface of papillomavirus is made up of 72 knobs of pentameric L1 capsomer. The N- and C-termini of L1 are arranged as extended “invading arms” that form the floor between the capsomer knobs (Modis, Trus et al. 2002, Wolf, Garcea et al. 2010).

1.11.2 L2 protein

L2 is almost 500 amino acids in length, and has an estimated molecular mass of approximately 55kDa. However, when looking at L2 in SDS-PAGE analysis it exhibits an apparent molecular weight of around 64-78kDa (Doorbar, Gallimore

1987) (Jin, Cowsert et al. 1989, Komly, Breitburd et al. 1986, Rippe, Meinke 1989). The L2 protein has numerous functions and interacting partners. L2 facilitates in genome encapsidation, host cell entry, vesicular trafficking of the virus, vesicular escape during infection, nuclear entry and activities within the nucleus. *In silico* analysis confirms interactions of L2 with ß-actin (Yang, Yutzy et al. 2003) and the nuclear pore complex proteins (Holmgren, Patterson et al. 2005, Florin, Becker et al. 2004, Fay, Yutzy et al. 2004, Bordeaux, Forte et al. 2006, Darshan, Lucchi et al. 2004b), and that L2 is required for an infectious virion (Roden, Day et al. 2001).

The amino terminus of the viral L2 protein contains highly conserved cysteine residues (C22 and C28) which are found in all PV types, and form an intra-molecular disulphide hairpin loop rather than bridging with L1. No X-ray crystallographic structures are available for caspids containing L2. Studies have attempted to look at high resolution three dimensional image reconstruction of cryo-electron micrographs of native BPV1, HPV1, or CRPV virions, HPV1 or HPV16 VLPS to visualise the structure of L2 in the capsid (Buck, Cheng et al. 2008, Trus, Roden et al. 1997, Belnap, Olson et al. 1996, Baker, Phelps et al. 1987, Buck, Trus 2012, Hagensee, Olson et al. 1994). Cryo-EM has allowed for us to deduce L2s localisation within the L1 caspid, suggesting that L2 is localised within the L1 caspid, which would be consistent with L2 functioning as a sub-scaffold within the intact virion (Buck, Cheng et al. 2008, Selinka, Florin et al. 2007). Possible reasons for these studies failing to visualise L2 are possibly due to L2 reflecting inadequate incorporation into the capsid, degredation, disorder or lack of symmetry.

1.12 E2 interactors TopBP1 and Brd4

As the papillomavirus genome only encodes a small number of viral proteins, in order to maintain the viral life cycle within the host, each of these proteins interacts with numerous cellular proteins to carry out its life cycle (Muller, Demeret 2012). In order for E2 to carry out transcription and replication roles in the viral life cycle, E2 must interact with host cellular proteins. Two such proteins, TopBP1 and Brd4, are known to play roles in replication and transcription respectively (Schweiger, You et al. 2006, Kumagai, Shevchenko et al. 2010, Makiniemi, Hillukkala et al. 2001).

1.12.1 TopBP1 structure and function

Human topoisomerase IIß binding protein 1 (TopBP1) contains 9 BRCT (Breast Cancer Susceptibility Gene 1 [BRCA1] Carboxy Terminus domains (Yamane et al 1999) which were originally identified within the carboxy terminus of BRCA1 (**Figure 1.6**). TopBP1 was identified in a yeast two-hybrid screen for factors that interacted with Topoisomerase IIß (Yamane, Kawabata et al. 1997). The TopBP1 gene is comprised of 28 exons located on chromosome 3q22.1 and encodes for a 1522 amino acid protein (180kDa) (Karppinen, Erkko et al. 2006, Xu, Leffak 2010, Yan, Michael 2009). TopBP1 is essential for maintenance of chromosomal integrity and cell proliferation. TopBP1 also has many roles which involve; DNA damage response, DNA replication checkpoint, chromosome replication and regulation of transcription (Bang, Ko et al. 2011) (Garcia, Furuya et al. 2005, Jeon, Ko et al. 2011). The critical role of TopBP1 is evolutionary conserved between various organisms including *Drosophila* and mouse. It has been shown that a TopBP1 knockout mouse is embryonic lethal at the peri-implantation stage and TopBP1 deficiency also induces cellular senescence in primary cells (Bang, Ko et al. 2011, Jeon, Ko et al. 2011).

TopBP1 is a scaffold protein capable of making numerous protein-protein interactions through its constituent BRCT domains (**Figure 1.6**). These domains are found in pairs and their interactions are phosphor-specific. This allows for the regulation of their interactions via phosphorylation/dephosphorylation of their respective binding partners through the activity of distinct kinases and phosphates (Yu, Chini et al. 2003). BRCT domains of TopBP1 bring different proteins into temporal complexes, which in turn promote various cellular processes such as DNA replication initiation and checkpoint activation. Human TopBP1 was initially assigned 8 BRCT domains, with BRCT1+2, 4+5 and 7+8 forming pairs. Later an additional BRCT domain was identified (Garcia, Furuya et al. 2005), at the extreme N-terminus. This new BRCT domain has been named BRCT 0, and has since been confirmed by structural studies (Rappas, Oliver et al. 2011).

TopBP1 protein has a transcriptional –activation domain and two surrounding repressor domains and can play a role in regulating transcription. Between amino acids 460-591 lays a transcriptional-activation domain, this region partly con-

tains BRCT4. This is an essential region for transactivation and is rich in hydrophobic amino acids interspersed with acidic residues, this is typical characteristic of transcriptional domains. A transcriptional repression domain located at BRCT2, lies within the transcriptional activation domain (Wright, Dornan et al. 2006). A second repression domain exists within the C terminus of the activation domain, which requires amino acids 586-675. The carboxy-terminal of TopBP1 contains two putative nuclear localisation signals (Going, Nixon et al. 2007, Liu, Lin et al. 2003, Sokka, Parkkinen et al. 2010) and deletion of BRCT7-8 and NLS region of TopBP1 induces cytoplasmic localisation of the protein.

As previously mentioned, TopBP1 contains transactivational-repression domains, and has been proposed as a transcriptional repressor of E2F1 (Wright, Dornan et al. 2006, Liu, Luo et al. 2004), as well as a transcriptional co-activator with HPV16 E2 (Yoshida, Inoue 2004). TopBP1 interacts with E2F1 through the sixth BRCT motif of TopBP1 and N terminus of E2F1 (Liu, Lin et al. 2003, Leung, Kellogg et al. 2010). This induces the ATM-mediated phosphorylation of E2F1 at Ser31 upon DNA damage. E2F1's transcriptional activity is repressed and E2F1 is recruited to DNA damaged nuclear foci (Liu, Lin et al. 2003).

TopBP1 protein is also phosphorylated by Akt. Phosphorylation at Ser1159 induces oligomerisation of TopBP1 through its seventh and eighth BRCT domains, which is important for the interaction with E2F1 and transcriptional repression (Liu, Paik et al. 2006). The E2F1 regulation of TopBP1 involves the phosphoinositide 3-kinase (PI3K)-Akt signalling pathways. This phosphorylation may also prevent association of ATR with TopBP1 after DNA damage, thus inhibiting activation of ATR and G2/M checkpoint proficiency (Pedram, Razandi et al. 2009). Additionally, Akt phosphorylation is also required for the interaction between TopBP1 and Miz1 or HPV16 E2. The interaction between Miz1 and TopBP1 represses transcriptional activity, which implies TopBP1 plays a role in controlling transcriptional factors

TopBP1 also recruits Brg1/BRM (Brahma-related gene 1/ Brahma protein), a central subunit of the SWI/SNF (SWItch/sucrose nonfermentable) chromatin modelling complex, to specifically inhibit E2F1 transcriptional activity. This is critical for E2F1 regulated apoptosis control during S phase and DNA damage. TopBP1

expression is also induced by E2F1 and interacts with E2F1 during G1/S transition. These interactions form a feedback regulation to prevent apoptosis during DNA replication (Liu, Lin et al. 2003).

1.12.2 TopBP1 and DNA damage response

TopBP1 is a multifunctional protein, in response to DNA damage it has many roles such as checkpoint activation at G2/M in response to DNA damage and it also directly inhibits E2F1-mediated apoptosis. TopBP1 can be visualised as foci at sites of DNA damage or replication stress (Makiniemi, Hillukkala et al. 2001). TopBP1 plays a role in bringing DNA damage sensors, mediators and effectors to sites of DNA damage, and can also directly stimulate the kinase activity of ATR (Kumagai, Lee et al. 2006b). Cells have developed DNA damage pathways to maintain genomic stability in response to endogenous and exogenous stress (Aguilera, Gomez-Gonzalez 2008, Ciccia, Elledge 2010, Jackson, Bartek 2009, Branzei, Foiani 2010). Two conserved PI3-kinase-like protein kinases, ATM (Ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) respond to DNA damage to control cell cycle progression and the regulation of other DNA damage responses such as DNA repair and apoptosis. ATM and ATR proteins are serine/threonine-specific kinases of about 300 kDa (Choi, Lindsey-Boltz et al. 2009, Smits, Warmerdam et al. 2010).

The induction of ATR is in response to single stranded DNA (ssDNA) occurring due to replication stress, resected double stranded breaks (DSBs) or other single strand lesions (Biton, Dar et al. 2006, Cimprich, Cortez 2008, Mavrou, Tsangaris et al. 2008, You, Shi et al. 2009). In eukaryotes, DNA damage-induced ssDNA is detected by ssDDNA binding protein complex RPA (Smits, Warmerdam et al. 2010). Once bound to ssDNA, RPA is phosphorylated (Binz, Sheehan et al. 2004, Nasheuer 2010). RPA-coated ssDNA is a critical step for ATR activation. ATR associates with ATRIP (ATR-interaction protein) an 85 kDa protein, which recruits ATR to RPA-coated ssDNA (Ball, Myers et al. 2005, Cortez, Guntuku et al. 2001, Mordes, Nam et al. 2008, Edwards, Bentley et al. 1999). This complex regulates the localisation of ATR to sites of replication stress and DNA damage. ATR activation also requires the activator protein TopBP1 (Mordes, Nam et al. 2008). Before ATR can be activated, the Rad17-RCF checkpoint clamp must be recruited to the junction of RPA-coated ssDNA and double stranded DNA (dsDNA), which

facilitates the loading of Rad9-Hus1-Rad1 (9-1-1) sliding clamp (Zou, Liu et al. 2003). This co-recruitment of ATR and the 9-1-1 clamp establishes a platform for the activation of the ATR pathway (Liu, Shiotani et al. 2011, Lee, Kumagai et al. 2007, Cotta-Ramusino, McDonald et al. 2011).

In response to genotoxic stresses, TopBP1 colocalises and interacts with ATR/ATRIP. The BRCT1 and BRCT2 domains in the amino terminal of TopBP1 bind to phosphorylated Ser373 in the C-terminal of Rad9 (Rappas, Oliver et al. 2011, Lee, Kumagai et al. 2007, Delacroix, Wagner et al. 2007, Kumagai, Lee et al. 2006a)(Smits, Warmerdam et al. 2010)(Takeishi, Ohashi et al. 2010). TopBP1 then binds ATR through its ATR activation domain (AAD), which is located between the sixth and seventh BRCT repeats, in an ATRIP- dependent manner (Kumagai, Lee et al. 2006a)((Mordes, Nam et al. 2008)(Smits, Warmerdam et al. 2010, Takeishi, Ohashi et al. 2010). ATRIP contains a conserved TopBP1 interacting region, required for the association of TopBP1 and ATR and the subsequent TopBP1 mediated triggering of ATR activity (Mordes, Nam et al. 2008)(Smits, Warmerdam et al. 2010)

TopBP1 protein also plays a direct and essential role in the pathway that connects ATM to ATR, specifically in response to the occurrence of DSBs in a genome (Yoo, Kumagai et al. 2007).

1.12.3 TopBP1 and DNA replication

In budding yeast and Xenopus systems, replication initiation has been well studied. During G1 phase the pre-replicative complex (pre-RC) is assembled on the origin of replication. The pre-RC is composed of a number of proteins such as, ORC, Cdt1, Cdc6 components and MCM proteins. The pre-RC is activated at the onset of S-phase by S-phase dependent kinases (S-CDKs) and the Cdc7-Dbf4 protein kinase (DDK). This in turn results in the binding of Cdc45, the conversion of the pre-RC into a pre-initiation complex (Pre-IC), and also the unwinding of DNA at the origin of replication. This then leads to the recruitment of RPA, polα, and polε to form the initiation complex (IC).

The TopBP1 family of proteins (Dpb11 in yeast, Rad4/Cut5 in *S.pombe*, Mus101 in *Drosophila,* and TopBP1 in metazoans) plays an essential role in the initiation of

DNA replication (Masumoto, Sugino et al. 2000, Hashimoto, Takisawa 2003). Depletion experiments in Xenopus indicated that XCut5^{TopBP1} is not required for the loading of pre-replication components, but is needed for the loading of pre-initiation complex components (Cdc45 and RPA) and the DNA polymerases (polα and polε). This is also seen in *S.pombe* (Dolan, Sherman et al. 2004) and *S.cerivsiae* (Araki, Ropp et al. 1992) homologues. In *Saccharomyces cerivisiae,* S-phase cyclin dependent kinase (CDK) promotes initaiton by phosphorylating Sld2 and Sld3, which enhances the binding of Dbp11. Dbp11 then binds origin chromatin and recruits the replisome together with the GINS complex and Cdc45, leading to replication initiation (reviewed in (Zegerman, Diffley 2009, Tanaka, Araki 2010)). In humans, TopBP1 transcript and protein levels increase during S-phase (Makiniemi, Hillukkala et al. 2001, Liu, Luo et al. 2004, Yamane, Wu et al. 2002), which indicates that it plays a role in the initiation of replication. Studies have indicated that the sixth BRCT domain is critical for TopBP1 replication activity, possibly through an interaction with another replication factor (Makiniemi, Hillukkala et al. 2001). TopBP1 and its homologs do not directly bind the origin recognition complex or MCM helicase without recruitment by the phosphorylated RecQ4/Treslin (Kumagai, Shevchenko et al. 2010, Kumagai, Shevchenko et al. 2011) (Sld2/Sld3) (Im, Ki et al. 2009), which regulates replication initiation in a cell cycle controlled manner.

TopBP1, and its Xenopus and yeast homologs, all bind to Polε when overexpressed (Makiniemi, Hillukkala et al. 2001, Mimura, Masuda et al. 2000).

1.12.4 The role of TopBP1 in the viral life cycle

For E2 to function it requires host protein interactions. The Morgan lab aimed to identify these cellular partners in order to provide targets for disruption of the viral life cycle, as well as gaining a better understanding of the viral life cycle, specifically transcription and replication mechanism (Boner, Morgan 2002a). In order to identify these cellular partners, yeast two-hybrid screening was utilised, using the amino-terminus of the viral E2 protein that is essential for mediating transcription and replication. From this screen, TopBP1 was identified as a promising E2 interacting protein, due to its known roles in replication, cell cycle control and response to DNA damaging agents (Yamane, Kawabata et al. 1997, Yamane, Wu et al. 2002)(Makiniemi, Hillukkala et al. 2001). Subsequently,

TopBP1 was shown to interact *in vitro* and *in vivo* with E2 and can regulate the ability of E2 to control transcription and replication (Boner, Taylor et al. 2002).

TopBP1 has been proposed to play a role as a transcriptional regulator. If TopBP1 is overexpressed it co-activates transcription with the HPV16 E2 protein when E2 is bound to target promoters (Boner, Taylor et al. 2002). TopBP1 has also been implicated as being a transcriptional repressor; as TopBP1 can interact with the chromatin modification complex proteins Brg1 (BRM (Brahma)-related gene 1)/BRM1 and represses the transcriptional and apoptotic function of E2F1 (Liu, Luo et al. 2004). A feedback loop is formed as E2F1 positively regulates the TopBP1 promoter (Yoshida, Inoue 2004).

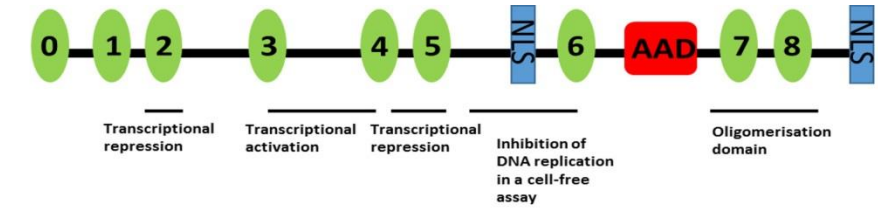
TopBP1 binds to the human papillomavirus early protein E2, and modulates its transcriptional activation, as well as E2F1 transcription factor and the POZ domain factor Miz1 that can be co-regulated by c-Myc. The viral E2 protein regulates viral transcription (Desaintes, Goyat et al. 1999) by binding to specific target sequences through its C-terminus and additionally it recruits transcriptional co-activators such as TBP, TFIIB and p300/CBP via interactions with its amino terminus (Boner, Morgan 2002b). The amino terminal region of TopBP1 has been shown to have transcriptional activation activity in yeast (Makiniemi, Hillukkala et al. 2001), and deletion of this region impairs E2 transcriptional activation.

TopBP1 is proposed to be the mitotic chromatin acceptor for HPV16 E2, the association of E2 with chromatin may play a key role in mediating genome segregation and DNA replication functions of the E2 protein (Donaldson, Boner et al. 2007). Donaldson *et al* showed that an absence of TopBP1 results in a redistribution of HPV 16 E2 into an alternative cellular protein complex, resulting in enhanced affinity for chromatin. This does not significantly alter the ability of E2 to either activate or repress transcription. TopBP1 may also be the mitotic chromatin receptor for HPV16 E2 as it was shown to co-localise on chromatin at late stages of mitosis (Donaldson, Boner et al. 2007).

A non-TopBP1 interacting 16E2 mutant ($E2^{TopBP1}$), which is defective in replication but retains transcriptional function, has been identified (Donaldson, Mackintosh et al. 2012) (amino acids responsible for the E2-TopBP1 interaction are highlighted in **Figure 1.7**). This mutant along with a non-Brd4 mutant shall form

the main focus of this thesis. The transcriptional function with the mutant is compromised at lower levels of input DNA. However, it can activate transcription at almost WT levels when overexpressed. In the case of DNA replication, the optimal function of E2 cannot be regained at higher concentrations of DNA input. Due to the function of E2 not being dramatically impaired in transcription activation and repression function, it is believed that E2 is still binding to Brd4. Additonally, the defects in replication are not due to an altered stability of the E2-TopBP1 protein.

Figure 1.6



BRCT0+1+2	BRCT3	BRCT4+5	BRCT6	BRCT7+8
Rad9 Treslin		MDC1 53BP1 BLM	E2F-1 PARP1 SPBP E2 (BRCTs6-8)	ATR BACH1/Fanc1 RecQ4 TopBP1 P53 Miz1 E2 (BRCTs6-8)

Checkpoint activation
Initiation of DNA replication
DNA repair
Transcriptional regulation

Figure 1.6: TopBP1 domain structure and BRCT domain protein interactions. The diagram of human TopBP1 displays the component domains, highlighting BRCT and AAD (ATR Activation) domains. Human TopBP1 has 9 BRCT domains which are found in pairs. TopBP1 is capable of making numerous protein-protein interactions though these BRCT domains. The table notes these protein-protein interactions, which are colour coded related to function; red for checkpoint activation, blue for initiation of DNA replication, green for DNA repair and purple for transcriptional regulation. This figure was adapted from (Wardlaw, Carr et al. 2014)

Figure 1.7

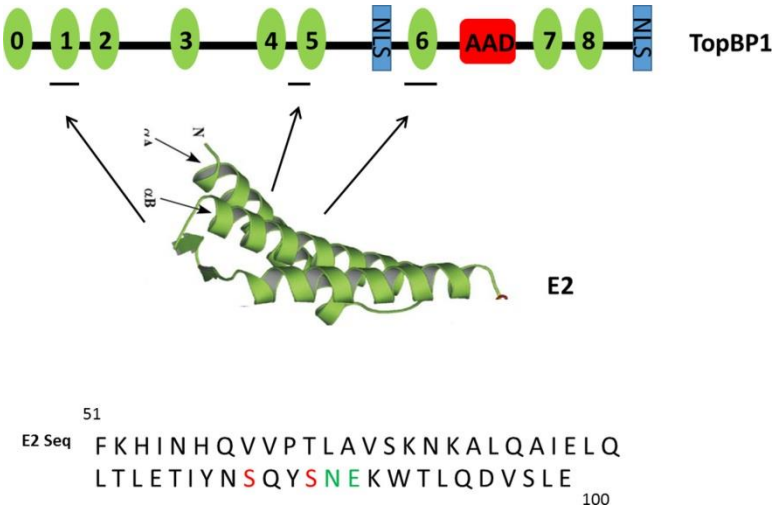


Figure 1.7: TopBP1-E2 interaction. E2 amino acids 50-100 interact with three TopBP1 domains (as highlighted in the figure above, BRCT, 5 and 6). An E2 mutant which fails to interact with TopBP1 has a mutation at amino acids 89-90 (highlighted in green on the E2 sequence). Possible phosphorylation sites (highlighted in red) may also be responsible for the impaired interaction. E2 cartoon was adapted from (Abbate, Berger et al. 2004).

1.13 Bromodomain proteins and disease

Proteins with two tandem bromodomains, followed by an additional extraterminal (ET) domain, constitute the BET subfamily. The BET subfamily of proteins include; mammalian Brd2/Ring3/Fsrg1/Brd3/ Orfx/ Fsrg2/Brd4/MCAP, and Brd5/Brdt, *Drosophila* Fs(1)h, yeast Bdf1 and Bdf2, and corresponding homologues in other species (Denis, Vaziri et al. 2000) (Dey, Ellenberg et al. 2000)(Matangkasombut, Buratowski et al. 2000).

Bromodomain containing proteins are of substantial biological interest, as components of transcription factor complexes and determinants of epigenetic memory. The mammalian bromodomains all display similar gene arrangements, domain organisations, and some functional properties. The bromodomain is a conserved region of approximately 110 amino acids that structurally forms 4 α-helices (α_z, α_A, α_B, α_C) and 2 loops, linking α_z and α_A (ZA loop) and α_B and α_C (BC loop), these loops can bind acetyl-lysine residues in histones in addition to many other proteins (Zeng, Zhou 2002). The mammalian bromodomain proteins all have two conserved N-terminal bromodomains (BD1 and BD2), which are chromatin interaction molecules that recognise lysine residues on histone tails and other nuclear proteins (You, Croyle et al. 2004, Dey, Chitsaz et al. 2003). They are chromatin “readers”; they recruit chromatin regulating enzymes, including “writers” and “erasers” of histone modification, to target promoters and regulate gene expression. The chromatin targeting activity of bromodomain containing proteins allows them to access chromatin and regulate gene activity through cell cycle progression.

The best studied of these bromodomains is Bromodomain-containing protein 4 (Brd4), which is recognized as a critical mediator of normal and disease functions through it’s interaction with both cellular and viral factors. Brd4 is a known target for viral encoded regulators in both human papillomavirus and human immunodeficiency virus. This interaction allows the virus to hijack cellular machinery to facilitate selective viral genom integration during mitosis (You, Croyle et al. 2004, Bisgrove, Mahmoudi et al. 2007). The deregulation of Brd4 is also associated with a number of cancers (acute myeloid leukemia, multiple myeloma, Brukitt’s lymphoma. NUT midline carcinoma, colon cancer). Cancer cells exploit

the host cell's transcriptional and chromatin machinery to propogate their oncogenic gene expression profiles (Wu, Donohoe 2015). Brd4 was initially identified via its association in NUT midline carcinoma (NMC) which is known to affect adjacent parts of the respiratory tract (French, Miyoshi et al. 2003).

The involvement of Brd4 in multiple cancers makes it a valuable therapeutic target. Pharmacological inhibition of BET bromodomain binding with small molecules such as JQ1 and I-BET, interferes with Brd4's requirement for enhancer function and transcriptional elongation in multiple cancers (glioblastoma, lung adenocarcinoma, ALL (acute lymphoblastic leukemia) and MLL (mixed-lineage leukemia (Delmore, Issa et al. 2011, Filippakopoulos, Qi et al. 2010, Rahl, Lin et al. 2010).

1.13.1 Structure and function of Brd4

The ubiquitously expressed Brd4 protein is approximately 200kDa, and was first identified in mouse as mitotic chromosome associated protein (MCAP) as it was first found associated with mitotic chromosomes (Dey, Chitsaz et al. 2003). Brd4 contains two tandem bromodomains (BD1 and BD2) and an extraterminal (ET) domain (Florence, Faller 2001) (**Figure 1.8**). The structures of both Brd4 BD1 and BD2 bromodomains have been solved and consist of 4 alpha helices and 2 loops linking the alpha helices (Filippakopoulos, Picaud et al. 2012, Vollmuth, Blankenfeldt et al. 2009) (**Figure 1.8**). The BD1 domain binds to H3-K5ac, whereas BD2 binds to H4-K5ac and H4-K16ac (Vollmuth, Blankenfeldt et al. 2009). The interactions of the two N-terminal bromodomains of Brd4 with lysine residues on histone tails, allows Brd4 to be retained on the chromatin during mitosis in a range of cell types (Vollmuth, Blankenfeldt et al. 2009). The interaction of bromodomains with acetylated chromatin result in the localisation of BET proteins to discrete locations along the chromosome, where they recruit other regulatory complexes to influence gene expression (Devaiah, Singer 2013).

The association of Brd4 with chromatin persists throughout the cell cycle and is also implicated in cell cycle control (Dey, Chitsaz et al. 2003). The importance of Brd4 for regulating cell cycle control has been demonstrated in various ways, mouse studies injecting anti-Brd4 antibodies into proliferating cells lead to G2/M arrest, overexpression of Brd4 in cultured cells results in G1/S arrest, and severe

knockdown of Brd4 in human cultured cells significantly reduces cell growth (Dey, Ellenberg et al. 2000).

Brd4 also has an extra-terminal domain (ET) which consists of 3 alpha helices and a loop. The ET domain is thought to be a regulatory domain, it has been linked to transcriptional regulation by interacting with several histone modifiers, additionally the ET domain has been found to associate with ATP-dependent nucleosome-remodeling enzymes SWI/SNF and CHD4 (Rahman, Sowa et al. 2011). These interactions imply that Brd4 alters chromatin structure at its occupied sites.

In addition to binding acetylated chromatin, the BD1 and BD2 bromodomains also interact with nonhistone proteins to regulate transcription, DNA replication, cell cycle progression, and other cellular activities. In mouse Brd4, the BD2-containing region has been shown to interact with several cellular proteins, including the cyclin T1 component of human positive transcription elongation factor b (P-TEFb) (Jang, Mochizuki et al. 2005, Yang, Yik et al. 2005), the RCF-140 subunit of human replication factor C (Maruyama, Farina et al. 2002), signal-induced proliferation-associated protein (SPA-1)(Farina, Hattori et al. 2004), and HPV11 E2 protein (Wu, Lee et al. 2006a).

The C terminal domain contains polyserine stretches which are interspersed with glutamate and asprate (SEED) motifs and an unstructured region of about 500 amino acids which is rich in proline and glutamine. This unstructured region of Brd4 is highly phosphorylatable and interacts with P-TEFb and papillomavirus E2 protein. Brd4 promotes transcription by recruiting the transcriptional elongation factor, p-TEFb, to promoters to enhance phosphorylation of the CTD of RNA polymerase II promoters, and additionally by directly phosphorylating the RNA polymerase II CTD (Devaiah, Singer 2013, Jang, Mochizuki et al. 2005).

A region of the Brd4 CTD, termed P-TEFb interacting domain (PID), mediates the activation of P-TEFb from the inactive ribonucleotide complex. The ribonucleotide complex sequesters P-TEFb in a kinase inactive state. The interaction of Brd4 with P-TEFb can modulate the global level of P-TEFb activity in the cell (Wu, Donohoe 2015).

Figure 1.8

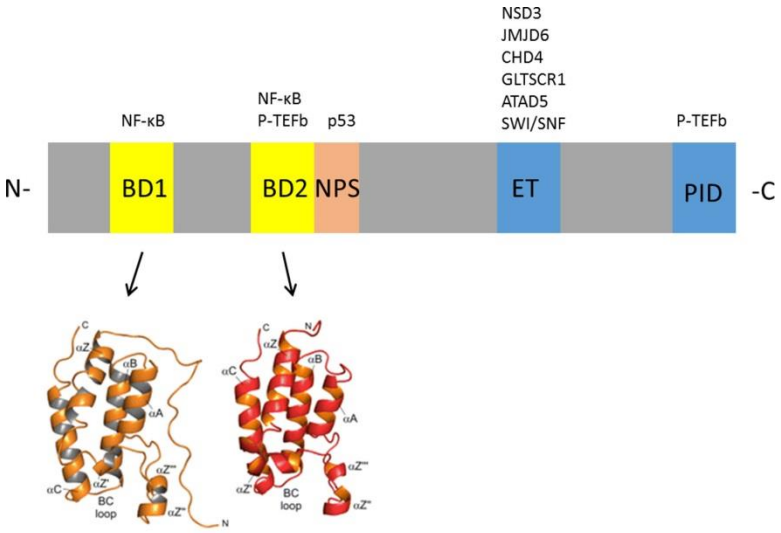


Figure 1.8: Domain organisation of Brd4 and structure of the two bromodomains. Two N-terminal bromodomains (BD1 and BD2) are followed by an extraterminal domain (ET). Protein-protein interactions with each of these domains are listed. Ribbon plot representation of BD1 and BD2 are also highlighted (Image adapted from (Vollmuth, Blankenfeldt et al. 2009)).

1.13.2 The role of Brd4 in the viral life cycle

The cellular chromatin binding protein, Brd4, interacts with the viral E2 protein to function in many processes within the viral life cycle, including viral replication, genome maintenance, and gene transcription. The interaction between Brd4 and E2 was discovered through methods such as proteomic analysis (You, Croyle et al. 2004, Wu, Lee et al. 2006a) and yeast two hybrid screening (Olejnik-Schmidt, Schmidt et al. 2008). E2 was also investigated as a target because the analogous tethering protein in KSHV (LANA) interacts with Brd2 (McPhillips, Ozato et al. 2005, Baxter, McPhillips et al. 2005a) (Platt, Simpson et al. 1999). Brd4 is an essential transcriptional co-activator for all E2 proteins, and is found ubiquitously in all proliferating cells (Houzelstein, Bullock et al. 2002). Brd4 is consistently found to be an interactor with E2 in many HPV types (16,18,31,1,6,8) (Muller, Demeret 2012). When Brd4 interacts with HPV E2 protein it increases the stability of E2 (Lee, Chiang 2009), this interaction may enhance many of E2's functions in the viral life cycle, as well as preventing proteosomal degradation by the E3 ligase cullin-3 (Gagnon, Joubert et al. 2009).

One of the first known roles for Brd4 in papillomaviruses, was the binding of this cellular chromatin component to E2, allowing tethering of the viral episome to host chromosomes in order to facilitate its segregation to daughter cells during mitosis (You, Croyle et al. 2004). Brd4 and other cellular proteins, such as Chr1 (Parish, Bean et al. 2006), have been implicated as being involved in playing the role of chromatin adaptors that facilitate in viral genome segregation during mitosis. Additionally, Brd4 may play an active role in cell cycle progression (Mochizuki, Nishiyama et al. 2008) and cancer development (Crawford, Alsarraj et al. 2008, French, Ramirez et al. 2008) , largely through its ability to modulate gene transcription by recruiting different transcription components to selective target genes.

In luciferase reporter assays where E2 expression stimulates luciferase gene transcription, knocking down Brd4 disrupts the E2-Brd4 interaction and compromises the transcription of the luciferase reporter (Schweiger, You et al. 2006, McPhillips, Oliveira et al. 2006, Senechal, Poirier et al. 2007). Disturbing the E2-Brd4 interaction or knocking down Brd4 also affects the function of the Brd4-E2 interaction to repress transcription in luciferase reporter based repression assays

(Wu, Lee et al. 2006a, Smith, White et al. 2010). Mutagenesis studies of the E2 TAD have shown that substitutions of R37 and I73 affect the binding of HPV 16E2 to Brd4, impairing the ability of E2 to activate transcription (Senechal, Poirier et al. 2007) (**Figure 1.9**). It has also been shown that the E2-Brd4 interaction may be involved in E2 mediated transcriptional repression (Schweiger, Ottinger et al. 2007).

Additionally, in cervical cancer cells where the HPV genome is integrated into the cellular DNA, and subsequently E2 expression is disrupted/lost, Brd4 activates viral oncogene transcription independently of E2 by recruiting P-TEFb to the HPV early promoter. When E2 is reintroduced into the cervical cancer cells, E2 functions to repress viral oncogene expression by interacting with Brd4 and competitively inhibiting the Brd4-P-TEFb interaction (Yan, Li et al. 2010). Helfer *et al* use an E2-responsive reporter assay to demonstrate that Brd4 recruitment of P-TEFb is important for E2-dependent transactivation (**Figure 1.10**). P-TEFb is also recruited to the papillomavirus genome. They also show that Brd4 tethering of E2 to the cellular chromatin is necessary for the transactivation of the E2 responsive reporter. Treatment of cells carrying the papillomavirus genomes with JQ1(+), inhibits Brd4's association with cellular chromatin and in turn reduces transcription of the viral early genes E1, E2, E6 and E7 (Helfer, Yan et al. 2014).

Also more recently Brd4 is thought to play a role in viral replication and the formation of small nuclear foci. In HPV16, Brd4 has been shown to be recruited to active replication origin foci with E2 and E1 and a number of other cellular replication factors (Wang, Helfer et al. 2013). The same R37A/I73A mutation responsible for impairing the transcriptional activation regulatory function of E2 is also found to abrogate the formation of E1-E2 nuclear foci. Additionally, mutagenesis studies and siRNA silencing of Brd4, disrupting the interaction between Brd4 and E2, have both been shown to impair HPV16 replication ability (Wang, Helfer et al. 2013). There is also evidence that Brd4 may recruit DNA damage response proteins to these viral replication foci to aid in replication (shown in human keratinocytes for HPV16). All of these elements together suggest that an established transcription factor may be involved in viral replication.

Figure 1.9

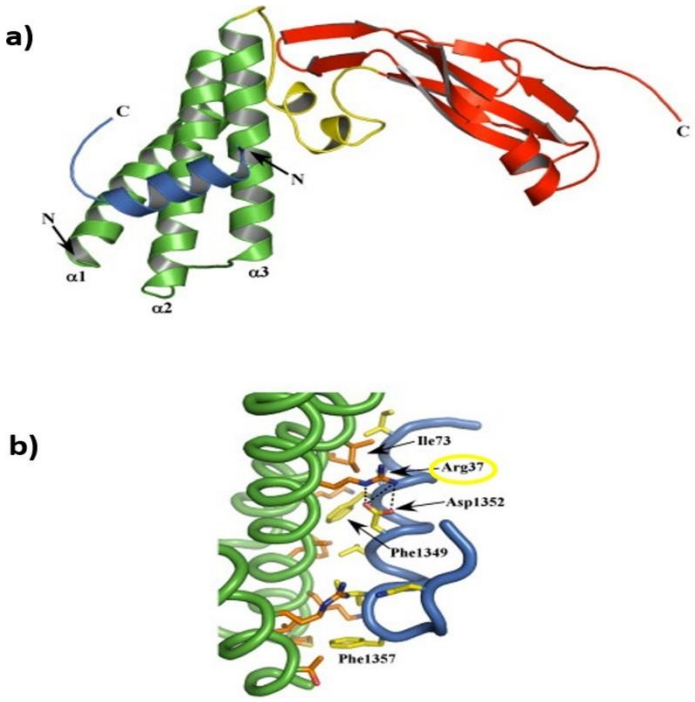


Figure 1.9: Structure of E2-Brd4 complex.

a) Cartoon of the E2-Brd4 complex. Brd4 is depicted in blue. The E2 N-terminal α-helical domain is coloured green, the C-terminal β strand is coloured in red. The linker segment joining these two domains is yellow.

b) As before, Brd4 is coloured blue. E2 is green with orange coloured side chains that make up the interaction surface. Highlighted on the diagram are important residues for the E2-Brd4 interaction (Phe1349 and Phe1357 are there for orientation purposes). The R37A mutation in this thesis is marked with a yellow ring. Figure adapted from Abbate *et al* 2006. (Abbate, Voitenleitner et al. 2006)

Figure 1.10

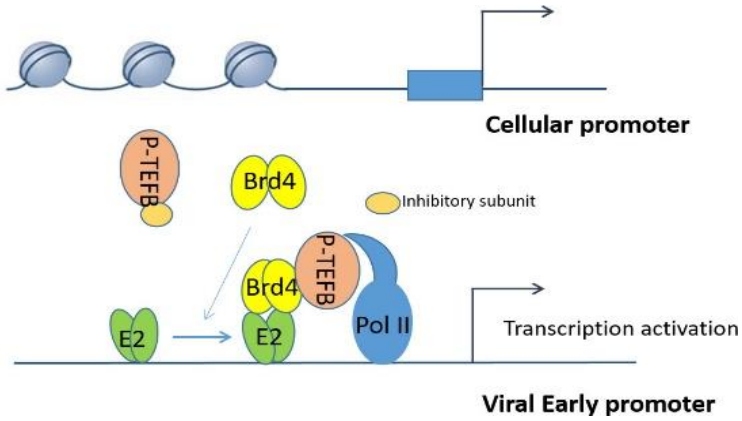


Figure 1.10: Brd4 functions in E2-mediated viral transcription activation. The E2 protein binds to the E2 binding sites upstream of the early promoter as dimer. The C-terminus of E2 interacts with Brd4. The N-terminal bromodomains of Brd4 direct E2 and the viral genome to transcriptionally active regions of the cellular genome. Brd4 forms homodimers and recruits P-TEFb to the viral promoter to phosphorylate the RNA pol II CTD to activate transcription elongation. Figure adapted from Helfer *et al* 2014. (Helfer, Yan et al. 2014)

1.14 Purpose of study

This thesis set out to understand how E2 regulates the viral and host genome by asking the following questions:

(1) What are the effects of E2 mutants that fail to bind TopBP1 and Brd4 on the viral life cycle?

(2) How does E2 regulate the host genome? Do mutant E2 proteins that fail to bind TopBP1 and Brd4 have altered regulation of cellular genes?

Overall, the essential role that E2 plays in the viral life cycle makes it a desirable antiviral target; therefore, we need to fully understand the interaction of this protein with functional cellular pathways. Specifically, we need to gain a better understanding of the viral and cellular proteins utilised by papillomavirus at various stages of DNA replication. We propose targeting E1-E2 mediated viral DNA replication in association with cellular interaction proteins, TopBP1 and Brd4, may provide therapeutic benefits. TopBP1 is well documented for its involvement in replication. However, the role of Brd4 in E1-E2 mediated DNA replication is more controversial. This study utilised two previously characterised E2 mutants which fail to bind TopBP1 and Brd4, to further our understanding of viral replication and the viral life cycle.

Additionally, we wanted to understand how E2 regulates host gene expression. Previous studies have focused on determining the effects of E2 on specific promoters and biological processes. There is a gap in our understanding of how E2 regulates cellular processes on a global scale. A few recent studies have been carried out in HPV negative C33a cells which have been transiently infected with adenovirus, to overexpress 16E2. This model does not tolerate E2 expression well. No study to date has fully investigated the regulation of the host genome by any E2 protein with physiologically tolerated levels of E2. In this study, I have used the U20S cell line for genome studies as it tolerates E2 expression. We hypothesised that manipulation of host gene expression by E2 facilitates infection and the viral life cycle, and may inadvertently contribute to cancer development.

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Antibodies

Abcam Plc

Anti-Sheep IgG horseradish peroxidase linked whole molecule (raised in donkey) - Catalogue Number: Ab6900

Anti-TopBP1antibody, ChIP grade- Catalogue Number: ab2402

Anti-HPV16 E2 antibody (TVG261) 50µg- Catalogue Number: ab17185

1X Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) - Catalogue Number: ab150077

1X Goat Anti-Mouse IgG H&L (Alexa Fluor 55) - Catalogue Number: ab150114

Covance

HA.11 clone 16B12 Monoclonal Antibody- Catalogue Number: MMS-101R-500

Fisher

Anti-mouse IgG peroxidase-linked whole antibody (from sheep) Secondary Antibody, GE Healthcare- Catalogue Number: 45000692

Santa Cruz biotechnology, inc.

Anti-TopBP1 antibody- Catalogue Number: sc-271043

2.1.2 Bacteriology

Invitrogen Ltd

Subcloning efficiency DH5α™ chemically competent cells- Catalogue Number: 18265

Subcloning Efficiency™ DH5α competent cells- Catalogue Number: 18265-017

S. O. C Medium - Catalogue Number: 15544-034

Novagen

KOD Hot Start DNA polymerase- Cat no: 71086.3

Sigma Chemical Co. Ltd

Ampicillin- Catalogue Number: 10047

Worldwide Medical

Kanamycin Sulfate, 50mg/ml solution 20ml - Catalogue Number: 61132047

2.1.3 Cell Lines

C33a cells are derived from a 66 year old female with HPV negative cervical carcinoma, and are defective for both p53 and pRb function. The cells have an epithelial morphology and are grown in continuous culture as a monolayer. C33a cells were obtained from Cancer Research UK (Cancer Research United Kingdom (CRUK), London).

Human Embryonic Kidney 293T (HEK293T) cells are derived from the 293 cell line in which the SV40 T-antigen was inserted. They are p53+ and pRB+, pRB is present but abnormal size, and p53 is elevated. There is a point mutation at codon 273 resulting in an Arg to Cys substitution. Also, these cells are HPV negative. HEK293T cells were a kind gift from Dr. Brian Willet, University of Glasgow.

U2OS cells were cultivated from the bone tissue of a 15 year old female suffering from osteosarcoma. U2OS cells exhibit epithelial adherent morphology, and

have no viruses present. The cells are p53 wildtype and were purchased from CRUK (CRUK, London, United Kingdom).

2.1.4 Chemical and Reagents

Invitrogen Ltd

Agarose (ultrapure electrophoresis grade)- Catalogue Number: 41025

Roche Biosystems

Complete Protease Inhibitor Cocktail Tablets - Catalogue Number: 12481800

Life Technologies

RNaseOUT™ Recombinant ribonuclease inhibitor- Catalogue Number: 10777-019

MicroAmp Fast Optical 96-Well Reaction Plate, 0.1mL- Catalogue Number: 4346907

4-12% Tris Glycine gels- Catalogue Number: EC60355BOX

TAQMAN TAMRA probe 6k PMoles- Catalogue Number: 450025

RNase A (20mg/ml)- Catalogue Number: 12091021

Sigma

siRNA Universal Negative Control #1- Catalogue Number: SIC001-10NMOL

Protein sepharose beads 5ml- Catalogue Number: P9424-5ml

Protein A sepharose beads 5ml- Catalogue Number: P9424-5ml

Formaldehyde solution- Catalogue Number: F8775-4X25ML

$CaCl_2$ Calcium chloride solution- Catalogue Number: 21115-250ml

Bicinchonoinic Acid (BCA) solution- Catalogue Number: B9643

Ethidium Bromide- Catalogue Number: E-1510

Fisher

10X Phospho buffer saline (PBS) 4L- Catalogue Number: BP3994

Glycerol- Catalogue Number: BP2291

VWR

Phenol:Chloroform:Isoamyl Alcohol- Catalogue Number: 101174-118

Ponceau S Stain Proteomics Grade 50ML - Catalogue Number: 97063-652

Protein G Mag sepharose 500µl- Catalogue Number: 89129-076

Protein A Mag sepharose 500µl- Catalogue Number: 89129-078

Ethanol 200 proof- Catalogue Number: 89125172

Greiner Cellstar T75 flask- Catalogue Number: 8205085

Greiner Cellstar T175 flask- Catalogue Number: 82050878

Methanol- Catalogue Number: BDH1135-4LP

USB Affymetrix

Water, RNase-free, DEPC treated, MB grade- Catalogue Number: 70783 100mL

VectorLabs

Vectashield Hardset Mounting Medium 10ml- Catalogue Number: H-1400

Agilent

Brilliant III Ultra-Fast QPCR Master Mix- Catalogue Number: 60080

Bioline

Proteinase K, 100mg- Catalogue Number: 37037

Alfa Aesar

Triethanolamine- Catalogue Number: L04486

Ethanolamine- Catalogue Number: A11697

2.1.5 Enzymes

New England Biosytems

DpnI- Catalogue Number: R0176L

Exonuclease (E.coli) - Catalogue Number: M0206L

2.1.6 Kits

Qiagen

QIAprep Spin Miniprep Kit (50)- Catalogue Number: 27104

QIAfilter Plasmid Midi Kit (25)- Catalogue Number: 12243

Invitrogen

PureLink Quick Plasmid DNA Maxiprep Kit- Catalogue Number: K2100-07

PureLink Quick Plasmid DNA Miniprep Kit- Catalogue Number: K2100-10

2.1.7 Miscellaneous

Genesee Scientific

Olympus 10µl reach barrier tip- Catalogue Number: 24-401

Olympus 200µl reach barrier tip- Catalogue Number: 24-412

Genesee Scientific continued

Olympus 1000µl reach barrier tip- Catalogue Number: 24-430

VWR

Mag rack 6- Catalogue Number: 89129-096

Worldwide Medical

1.5 ML Microcentrifuge tubes 500/pack- Catalogue Number: 41021009

5X7 film- Catalogue Number: 41101002

15ml Centrifuge tube flat top rim seal cap- Catalogue Number: 41101002

Sigma

Silicone grease bayer- Catalogue Number: 85403-1EA

USA Scientific

100mm cell culture dish 300/cs - Catalogue Number: CC7682-3394

2.0ml Seal-Rite tubes, graduated, flat cap, natural- Catalogue Number: 16202700

Fisher

Corning #4488 Pipette, 10ML, 200 per case- Catalogue Number: 7200574

Corning #4489 Pipette, 25ML, 200 per case- Catalogue Number: 7200575

2.1.8 Molecular Weight Markers

Thermo Scientific

Spectra MC BR protein ladder 6X250 µl- Catalogue Number: 26634

2.1.9 Plasmids

E2- described in (Bouvard, Storey et al. 1994)

Ptk6E2- used previously in (Donaldson, Mackintosh et al. 2012)

HPV18LCR- used previously in (Donaldson, Mackintosh et al. 2012)

pOriM- described in (Taylor, Morgan 2003a)

HA-E1 - a kind gift from Mart Ustav and described in (Kadaja, Sumerina et al. 2007)

Addgene

P5188 pSUPER-shRNA-Brd4 (CT)- Catalogue Number: 24746

2.1.9.1shRNA

Short hairpin RNA (shRNA) against TopBP1 and the appropriate negative control were expressed from the pBABE-puro plasmid and have been previously described (Jurvansuu, Raj et al. 2005). shRNA against Brd4 was expressed from pSUPER and had the following targeting sequence: GCGGGAGCAGGAGCGAAGA (Addgene plasmid 24746) (Zheng, Schweiger et al. 2009)

2.1.10 Cell Culture

Gemini bioproducts

G418 solution 20 mL (Geneticin) 50mg/mL- Catalogue Number: 400113

Life Technologies

Trypsin-EDTA .05% - Catalogue Number: 25300054

Lipofectamine 2000 reagent 1.5 ML- Catalogue Number: 11669019

Lipofectamine LTX, 1ML - Catalogue Number: 15338100

DMEM 500ML - Catalogue Number: 11885092

FBS- Catalogue Number: 10437028

Ibidi

25 Culture Inserts for self-insertion, sterile, in a 10cm transport dish- Catalogue Number: 80209

VWR

Greiner 6-well Tc treated polystyrene plare with lid, sterile, individually wrapped, 100/case- Catalogue Number: 82050-842

145X20mm TC treated dish CS120- Catalogue Number: 82050-598

Fisher

Cloning cylinders 6X8 125/CSCL - Catalogue Number: 09-552-20

Penicillin-streptomycin solution, 100X; 100mL- Catalogue Number: SV30010

Fisher continued

Cell Scraper Small 23cm 50/pk- Catalogue Number: 1256558

Neuvitro corporation

German coverslip - Catalogue Number: GG-12-1.5-pre

2.1.11 Primers

Qiagen

QuantiTech primer assay for the following genes:

MAGEC1, ENG, FAR2, APBA2, SH3PXD2B, GDF15, RNF144B, TMPRSS15, NFE4, GTSF1, ADAMTSL1, C6orf15, CLDN4, HOXB2, LRRC15, SERPINA1, SLN, NRID2, ANO1, IFGL1, SPRR2A, EPHA4, SEMA6D, PBX1, TXNIP, DHRS2, ATP8B1, MFAP5, COL6A3, IFIH1, IFI35, DDX58, OAS2, CD14, IFTM1, IFIT3, OAS1, IL8, MX1, IFIT1, IFI44L, IFI27, IFNK.

Invitrogen custom primers

pOri Forward and Reverse primers

Forward - 5’ ATCGGTTGAACCGAAACCG ‘3

Reverse- 5’ TAACTTCTGGGTCGCTCCTG ‘3

Primers continued

Integrated DNA Technologies

Site directed mutagenesis primers:

HPV18 E2 E94V

Forward- 5’ GCACAAAGTGCATACAAAACCGTGGATTGGACACTGCAAGACAC 3'

Reverse- 5’ GTGTCTTGCAGTGTCCAATCCACGGTTTTGTATGCACTTTGTGC 3’

HPV18 E2 R41A

Forward- 5’ CAGTATTGGCAACTAATAGCTTGGGAAAATGCAATATTCTTTGC 3'

Reverse- 5’ GCAAAGAATATTGCATTTTCCCAAGCTATTAGTTGCCAATACTG 3’

HPV16 E2 N89YE90V

Forward- 5’ CAATATATAACTCACAATATAGTTATGTAAAGTGGACATTACAAG 3'

Reverse- 5’ CTTGTAATGTCCACTTTACATAACTATATTGTGAGTTATATATTG 3’

HPV16 E2 R37A

Forward- 5’ CAATATATAACTCACAATATAGTTATGTAAAGTGGACATTACAAG 3'

Reverse- 5’ CTTGTAATGTCCACTTTACATAACTATATTGTGAGTTATATATTG 3’

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Agarose Gel Electrophoresis

1% agarose gels were prepared by dissolving 1g of agarose per 100ml of 0.5% TBE buffer (10 x TBE: 900mM Tris base, 900mM boric acid, 25mM EDTA, pH8.0) using heat. For 200ml solution 5µl (10mg/ml) ethidium bromide was added, and the solution cooled at room temperature, before pouring into the assembled gel cast containing the appropriate sized comb. When the gel had cooled and set it was then placed into the electrophoresis tank, and submerged in 0.5% x TBE buffer. For site directed mutagenesis, 5 µl of PCR product mixed with 5µl of 1 x loading buffer (10 x loading buffer : 65% (w/v) sucrose, 10mM Tris-HCl Ph 7.5, 10mM EDTA, 0.3% (w/v) bromophenol blue). For the confirmation of DNA shearing, 10 µl of DNA and 5µl of loading buffer (as above) were mixed. Samples were loaded onto gel and run alongside a 1kb marker at 90V before gels were visualised by UV light and photographed on the GeneFlash system.

2.2.1.2 DNA purification using phenol chloroform

Restriction digests, sequencing, replication and ChIP assays were purified using phenol:chloroform to remove all residual contaminants. Equal volume of phenol:chloroform:isoamyl alcohol (24:25:1 v/v/v) was added to sample and vortexed for 2 minutes until cloudy. The sample was then centrifuged for 15 minutes at 3293 rcf at room temperature. This resulted in the separation of the aqueous and organic phases; the top aqueous phase was transferred to a fresh 1.5 ml tube and the previous steps repeated. The bottom organic phase was discarded.

2.2.1.3 Ethanol precipitation of DNA

Following purification using phenol:choloroform DNA was precipitated using ethanol precipitation. 1/10 the sample volume 3M sodium acetate, pH 5.2, and 2 x the sample volume of 100% EtoH, was added to the sample and vortexed for 2 minutes. Samples were then incubated at -20ºC for 2 hours or overnight. After samples were incubated they were centrifuged at 3293 rcf for 30 minutes to pellet the DNA. Supernatant was removed leaving the pellets remaining in the tube. Pellets were washed with 1 x the sample volume of 70% EtoH, centrifuged at 3293 rcf for 15 minutes. Supernatant was then removed and pellets allowed to air dry. The precipitated DNA was then resuspended in distilled H_2O.

2.2.1.4 Site directed mutagenesis

Site directed mutagenesis PCR was set up as follows. The PCR reaction contained 5 µl of 10× KOD buffer with 5 µl of 2 mM deoxynucleoside triphosphates, 4 µl of 25 mM $MgSO_4$, 1 µl of dimethyl sulfoxide, forward primer, reverse primer, 50 ng of template DNA, 1 µl of KOD Hot-Start DNA polymerase (Novagen), and distilled H_2O to 50 µl. Reactions were cycled as follows: 94°C for 4 min, followed by 18 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final 72°C for 4 min. After completion of the mutagenic PCR, 10 µl of DpnI was added to the reaction, followed by incubation for 90 min at 37°C. Then, 5 µl of the reaction was transformed into *Escherichia coli* DH5α (following manufacturer's instructions-Invitrogen), and transformed cells were selected on Luria-Bertani/ampicillin plates (50µg/ml). Positive colonies were picked, and plasmids were recovered by using a miniprep procedure (Invitrogen Purelink Quick Plasmid DNA Miniprep kit) and sequenced to determine the mutation. Sequence analysis was carried out using Chromas Lite software and alignments were done using ClustalW. For samples containing the desired mutation the maxiprep procedure was followed to make working plasmid stocks. 100ml of LB broth plus ampicillin (50µg/ml) was inoculated with one bacterial colony and incubated at 37ºC overnight with shaking. Maxiprep was carried out in accordance with Invitrogen PureLink Quick Plasmid DNA Maxiprep kit protocol.

Site directed mutagenesis continued

The primers used for the generation of the N89YE90V mutant were as follows: forward, 5′-CAATATATAACTCACAATATAGTTATGTAAAGTGGACATTACAAG-3′; and reverse; 5′-CTTGTAATGTCCACTTTACATAACTATATTGTGAGTTATATATTG-3′. The primers used for the generation of the R37A mutant were as follows: forward, 5' GACTATTGGAAACACATGGCCCTAGAATGTGCTATTTATTACAAG 3'; and reverse; 5' CTTGTAATAAATAGCTCTAACTAGGGCCATGTGTTTCCAATAGTC 3'.

2.2.1.5 BCA / CuSO4 Assay

In preparation for the assay, bovine serum albumin (BSA) protein standard solutions were made from a stock solution (2mg/ml) in the following concentrations: 80, 100, 200, 400, 1000 and 2000µg/ml. 10µl of each protein standard and protein lysates were loaded onto a 96 well plate, along with 200µl of developing solution (5ml BCA (Bicinchoninic Acid Sigma) 100µl Copper II Suplhate pentahydrate 4% w/v solution (Sigma)). After the protein and BCA solution was mixed it was incubated at 37ºC for 30-60 minutes (or until a change in colour was observed). The absorbance was read using a plate reader to measure the absorbance at 462nm. The absorbance readings of the BSA standards were used to derive a standard curve, from which the sample concentrations were calculated.

2.2.1.6 Western blots

Cells were harvested by trypsinisation and pelleted by centrifugation. Cells were washed twice with phosphate buffered saline then resuspended in 100 µl of lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.8, 150 mM NaCl with protease inhibitor cocktail (Roche Molecular Biochemicals) dissolved in the lysis buffer fresh prior to use) and lysed on ice for 30 min. Lysates were cleared by centrifugation (20,800 relative centrifugal force, 4˚C, 30 min). The supernatant was then removed to a fresh 1.5 ml tube and the cell debris discarded. Protein levels were standardised using BCA Assay (Sigma).

Western blots continued

60 µg of lysate was prepared for SDS-PAGE using the Invitrogen NuPage system (2 µl 10x sample reducing reagent and 5µl 4 x LDS buffer). The lysates were run on 4-12% gradient gels (Invitrogen) at 200 V for 1 hour and proteins were transferred onto nitrocellulose membranes using the wet-blot method (Biorad).

ECL method:

Nitrocellulose membranes were then blocked in blocking solution (PBS, 0.1% Tween, 5% nonfat milk powder) for 1 hour at room temperature. Membranes were incubated with TVG261 mouse monoclonal anti-HPV16 E2 (ab17185) at a dilution of 1:5,000, (anti-HA (1:1,000), Brd4 (1:500) or TopBP1 (1:5,000)) overnight at 4 degrees and followed by a one hour incubation with secondary antibody (anti-Mouse Ig, Horseradish Peroxidase linked) (GE Healthcare) was then diluted to 1: 50,000 with PBS-T and 5% milk and added to the membrane which was incubated at room temperature for 90-120 minutes. The membrane was then developed using ECL-Plus (Amersham Biosciences) to detect the horseradish peroxidase conjugate and the membrane exposed to film.

Odyssey method:

Membranes were blocked in Odyssey blocking buffer (1:1 diluted with PBS) for one hour at room temperature. After blocking the membranes were incubated with 1:1000 rabbit anti HA-tag antibody to detect E1 (ab9110), and 1:5000 (1:1000 for shRNA blot) mouse TVG261 antibody to detect HPV 16E2 (ab17185), overnight at 4°C. Membranes were washed with 0.1% PBS-T before incubation with Odyssey secondary antibodies diluted 1:20,000 (Goat anti-mouse IRdye 800cw, 827-08364 and Goat anti-rabbit IRdye 680RD, 926-68171) for one hour at room temperature. Following secondary incubation membranes were washed 5 times for 5 minutes with 0.01% PBS-T before infrared scanning using the Odyssey Li-Cor imaging system.

2.2.1.7 Stripping membranes

In order to reprobe a membrane with an antibody recognised by the same secondary antibody, membranes were stripped to remove all traces of the previous antiobody. The membrane was submerged in 0.2M NaOH and incubated for 10 minutes at room temperature with gentle agitation. The membrane was then washed twice with PBS-T (0.1% if using Li-Cor imaging system) for 10 minutes at room temperature, and then blocked for one hour in either 5% milk PBS-T or Odyssey blocking buffer, before adding the desired antibody.

2.2.1.8 Crosslinking beads with antibody

Agarose A beads were washed overnight in PBS at 4ºC. The following morning PBS was removed from the beads and replaced with one bead volume of dilution buffer (PBS+ 1mg/ml BSA) and rotated for 10 minutes at 4 ºC. Supernatant was then removed and replaced with 1 bead volume of TopBP1 rabbit antibody in dilution buffer (1µl of 1mg/ml stock in 1ml dilution buffer (1:1,000)), and rotated at 4 ºC for 1 hour. The supernatant was disgarded and beads washed in 1 volume of dilution buffer for 5 minutes at 4 ºC. Following this the incubation the supernatant was disgarded and beads were washed in 1 volume PBS and the supernatant then disgarded. Cross-linking reagent was made fresh (13mg/ml DMP), 1ml of DMP stock was added to 1 ml wash buffer (0.2M triethanolamine in PBS) and vortexed. 1 volume of DMP solution was added to the beads and incubated for 30 minutes at room temperature. Supernatant was then discarded and beads were washed in wash buffer for 5 minutes at room temperature. Supernatant was then discarded. DMP solution incubations were repeated two more times, including the wash step. Following this, 1 volume of quench buffer (50mM ethanolamine in PBS) was added to the beads and rotated for 5 minutes at room temperature. The supernatant was discarded and the quench buffer incubation step was repeated. Beads were then washed with PBS and supernatant discarded. Beads were then washed with 1 volume of elution buffer (1M glycine pH3) for 10 minutes at room temperature.

Crosslinking beads with antibody continued

Supernatant was discarded and elution buffer incubation step repeated. Finally, beads were then washed three times with NP40 lysis buffer supplemented with protease inhibitors.

2.2.1.9 Co Immunoprecipitation

For CoIP, 200µg of protein lysate (C33a cells) was incubated with cross-linked beads overnight at 4 ºC. The following morning the beads were washed 5 times with PBS. On the final wash all PBS was carefully removed from the beads with a fine tip pipette. The beads were then prepared for SDS-PAGE using the Invitrogen NuPage system (2 µl 10x sample reducing reagent, 5µl 4 x LDS buffer and 10µl lysis buffer). The lysates were run on 4-12% gradient gels (Invitrogen) at 200 V for 1 hour and proteins were transferred onto nitrocellulose membranes using the wet-blot method (Biorad). The membrane was then prepared for exposing using the ECL method. Western blots were probed with Brd4 and TopBP1 antibodies (1:500 Brd4 antibody and 1:5,000 TopBP1 antibody).

2.2.1.10 RNA extraction

$1x10^6$ stably transfected U20S clones were plated out on 100mm plates. The following day cells were washed 2x with PBS. 600µl of buffer RLT from the Qiagen RNeasy kit was added to each plate and incubated at room temperature for about 5 minutes, cells were then scraped from the plate and buffer RLT/cell mix was added to a Qiashredder column (Qiagen) and centrifuged following the manufacturers instructions to homogonize the sample. The Qiagen RNeasy protocol was then followed to extract RNA from the U2OS cells stably expressing E2. The DNA was removed using DNase treatment (Qiagen) on column.

2.2.1.11 cDNA synthesis

cDNA was made using the Finnzymes (Thermo Scientific) DyNAmo SYBR Green 2-Step qRT-PCR kit (F-430S, F-430L), following the protocol provided.

2.2.1.12 Sybr Green qPCR

Sybr green real-time qPCR was used to validate the Human Exon array results (DyNAmo SYBR Green qPCR Kit with ROX, Cat no: F-400RL), using primers designed by Qiagen, (Qiagen QuantiTech primer assay). The house keeping gene GAPDH was used as an endogenous control alongside the Vector, no E2 expressing U20S cell line to normalise the results, using the ΔΔct method.

2.2.1.13 Transcription activation/ repression assay

2X 10^5 C33a or 293T cells were plated out on a 60-mm plate and transfected 24 h later using the calcium phosphate technique. The next day cells were washed, and 24 h following the wash cells were harvested. The cell monolayers were washed twice with phosphate-buffered saline and then lysed with 300 µl of reporter lysis buffer (Promega) at room temperature for 10 minutes. After incubation plates were scraped and the lysate was transferred into a 1.5 ml Eppendorf tube and spun in a refrigerated microfuge for 10 minutes at 4 °C with 3293rcf to clear debris. 80 µl of the supernatant was assayed for luciferase activity using the luciferase assay system (Promega). To standardise for cell number a BCA protein assay was carried out, the RLU from the luciferase assay was divided by the protein concentration of that sample. All results were normalised to the ptk6E2 reporter plasmid and the final result expressed as fold difference compared with ptk6E2 (or 18LCR for repression assay). pGL3CONT, which contains the SV40 promoter and enhancer driving expression of the luciferase gene, was always included in a parallel transfection to confirm efficient transfection. The assays shown are representative of at least three independent experiments carried out in duplicate.

2.2.1.14 DNA replication assay

DNA replication assays were carried out by plating out 6×10^5 C33a or 293T cells in 100-mm^2 plates. The following day cells were CaPO4 transfected with 100 pg of pOriM, 1µg of E1 and 10ng- 1µg of E2 wild-type and mutant plasmids. For shRNA replication assays the same transfection process was followed expect for the addition of 1µg TopBP1 and Brd4 shRNA and control shRNA (plasmids from Addgene). The following morning cells were washed twice in PBS then 48 hours later low molecular weight DNA was harvested in Hirt solution (10mM EDTA, 0.5% SDS). Samples were processed for qPCR-detected transient replication assay as previously described (Taylor, Morgan 2003b).

2.2.1.15 Chromatin Immunoprecipitation (ChIP) assay

A 100mm^2 dish of 60% confluent C33a cells were transfected with 1µg of pOriM, 1µg of E1 and 10ng- 100ng of E2 wild-type plasmid, using the CaPO4 method. The following day cells were washed twice with PBS and transferred to 150mm^2 dishes. 48 hours post transfection cells were cross linked with 1% formaldehyde at room temperature for 15 minutes. The cross linking reaction was stopped using 0.125M glycine. Cells were harvested by scraping and then lysed in 1.5X cell pellet volume cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% NP40, 10mM sodium butyrate, 50 µg/ml PMSF, 1X complete protease inhibitor). Cells were incubated on ice for 10 minutes then nuclei collected by centrifugation at 105 rcf at 4˚C. Cells were then resuspended in 1.2ml of nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, 10mM sodium butyrate, 50 µg/ml PMSF, 1X complete protease inhibitor) and incubated on ice for a further 10 minutes, then diluted in 0.72ml of immunoprecipitation dilution buffer (IPDB) (20mM Tris-HCl pH 8.1, 150 mM NaCl, 2mM EDTA, 1% Triton X-100, 0.01% SDS, 10mM sodium butyrate, 50 µg/ml PMSF, 1X complete protease inhibitor). The sample was sonicated using a water bath sonicator (Diagenode Bioruptor 300) till chromatin was sheared to <400bp. Shearing efficiency was tested by running 10µl of sample from each cycle condition of sonication (18-24 cycles) on a 1% DNA gel. Prior to running the gel, each sample was treated with 100µg proteinase K overnight, and purified using phenol:chloroform extraction.

Chromatin Immunoprecipitation (ChIP) assay continued

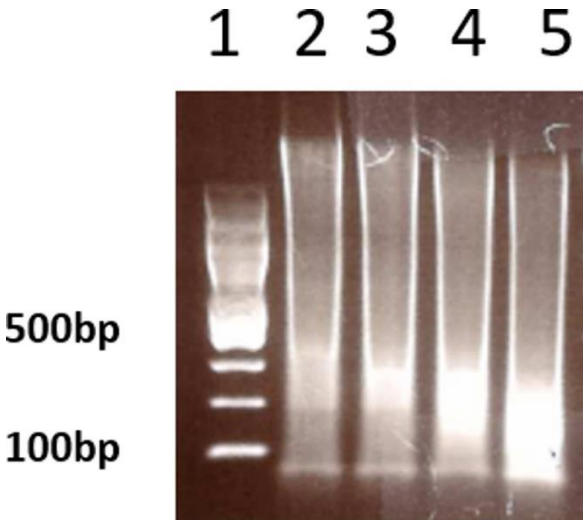


Figure 2.1: Sonication to shear chromatin. An example of a DNA gel (1%) showing the size of chromatin after shearing. Lane one is the DNA ladder, with 500bp and 100bp highlighted. Lanes 2 to 5 display the product of 18, 20,22, and 24 cycles of sonication respectively. Each cycle represents 30 seconds on (at full power) and 30 seconds off.

Chromatin was measured using a nanodrop and 100µg of chromatin was used per antibodyexperiment. The antibodies used were as followed, per IP; 2µl of a sheep anti-HPV16 E2 (amino acids 1-201) prepared and purified by Dundee Cell Products, UK; 2µg of rabbit anti-HA for detecting HA-E1 (AbCam, ab9110); 2µg rabbit anti-Brd4 (Bethyl, A301-985A1003); 2µg of mouse anti-TopBP1 (Santa Cruz, sc-271043). The antibodies and chromatin were incubated along with 20µl of a slurry of A/G magnetic beads (washed in IPDB) (Thermofisher scientific; product number 26162). Chromatin, bead and antibody slurry was incubated rotated at 4˚C overnight. The following day beads were washed with 750µl IP wash buffer 1 (x2) (20 mM Tris-HCl pH 8.1, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), then with IP wash buffer 2 (x2) (10 mM Tris-HCL pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholic acid) and TE pH 8.0 (x1 or x2). Chromatin was then prepared for qPCR by eluting the immune complexes from the beads by adding 250µl IP Elution buffer (IPEB) (100 mM $NaHC0_3$, 1% SDS) and 10µg RNase A; and incubated at 65˚C for 30 mins; beads were separated from the supernatant leaving the ChIP DNA; DNA was incubated for 6 hours to overnight at 65˚C. 100µg of proteinase K was added following this incubation and incubated for 2 hours at 55˚C or overnight at 45˚C. Taqman qPCR using pOriM primer and probe

set was used to quantify the levels of E2, E1, TopBP1 and Brd4 at the HPV origin of replication (Taylor, Morgan 2003a).

2.2.1.16 Immunofluorescence staining and imaging

Cells were plated at a density of 2 X 10^5 cells/well on acid-washed coverslips and simultaneously transfected with the indicated plasmids (Lipofectamine 2000 commercial protocol). At 24h or 48h post-transfection, cells were fixed in 4% formaldehyde and permeablised with 0.2% Triton X-100. Cells were blocked in 1% FBS and then stained with the indicated primary and secondary antibodies. Coverslips were mounted in Vectashield mounting medium containing DAPI. Images were collected with a Zeiss LSM710 laser scanning confocal microscope and analyzed using Zen 2009 LE software.

2.2.2 Tissue Culture

2.2.2.1 General growth

All cell culture work was conducted under strict sterile conditions inside a flow hood (Class II Microbiology Safety Cabinets, Gelaire BSB4). Cells were maintained in Dulbecco's modified eagle medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% (v/v) penicillin/streptomycin mixture (Invitrogen Life Technologies) at 37°C in a 5% CO2/95% air atmosphere. Cells were passaged 1 in 10 every 3 days.

2.2.2.2 Making stable cell lines

4x 10^5 U2OS cells were plated onto 100mm^2 plates. The following day cells were transfected using calcium phosphate, with 1µg of plasmid DNA, plasmid vectors encode neomycin resistance (G418) for selection in mammalian cells. 48 hours post transfection, cells were lysed with 0.5% trypsin EDTA (Invitrogen), and re-plated at several dilutions (1:5, 1:20 and 1:50) and fed with DMEM media containing G418 (Geneticin) at a concentration of 0.75mg/ml. Cells were monitored and re-fed every 3-4 days with fresh G418 media, for 14 days post the initial G418 treatment. Distinct "islands" of surviving cells are seen; drug resistant clones appear in 2-5 weeks. Clones were transferred using standard techniques,

using cloning cylinders, to 6 well plates, and cultures were maintained in G418 medium. Candidate clones were then grown in 75cm^2 flasks, and lysates made from pelleted cells using our cell lysis buffer with a protease inhibitor mixture (0.5% Nonidet P-40, 50 mM Tris, pH 7.8, 150 mM NaCl). The presence of E2 was tested using western blot technique.

2.2.2.3 Growth Curve

2 x 10^5 cells were seeded in 100mm plates in triplicate and grown in complete DMEM medium, cells were trypsinised and counted using a hemocytometer. This process was repeated three times in three day intervals. Growth curves of two separate sets of U20S vector and 16E2 wildtype clones were plotted on a log scale from the successive cell counts, 0, 3, 6 and 9 days.

2.2.2.4 Wound healing assay

To determine cell migration, “wound-healing” (scratch) assays were used. Cell culture inserts (Ibidi, cat # 80209) were attached to 6 well plates and seeded with 5 x 10^4 cells in each side of the cell insert divide. This was done in replicate for each Vector and 16E2^{-WT}, E2^{-Brd4}, and E2$^{-TopBP1}$ U2OS clone. Cells were grown for 24 hours until each side of the chamber was confluent. The cell insert was the removed and pictures taken at time points 0, 16 and 24 hours (Zeiss, Axio-vert 200M microscope and Axiocam). The width of the cell free gap was approxi-mately 500 microns (+/- 50 microns) at time 0 hours.

2.2.3 Genomics

2.2.3.1 Human exon array

Three independent polyA+ RNA preparations were made and converted to cDNA (**as described in 2.2.1.11**) and analysed using Affymetrix Human Exon Array. 1.5µg of RNA was required to perform the array. The quantity and quality of the RNA was analysed using a 210000 Agilent bioanalyser (Jing Wang, SHWFGF). The

DNA was prepared using GeneChip cDNA synthesis and amplification kit (Affymetrix) and analysed using Affymetrix Exon array software by Dr Pawel Herzyk. Analysis of the microarray core data was performed using Partek genomics suite.

2.2.3.2 Ingenuity Pathway Analysis (IPA)

Gene data sets from the human exon array with a fold-change of >1.5, were uploaded into Ingenuity Pathway Analysis software (IPA). IPA was used to analyse gene data sets from the human exon array to allow us to look at the functions and networks that E2 may be affecting. List of the raw data uploaded into IPA can be found in the appendices section.

2.3 Contributions

Data by Mary Donaldson as follows:

Figure 3.7.1 Localisation of E1 and E2 into nuclear foci

Figure 3.7.2 TopBP1 and Brd4 are recruited to E1-E2 foci

Figure 3.7.2 Knock down of TopBP1 and Brd4 disrupts E1-E2 DNA replication foci

Plasmids and cell lines made by:

Edward Dornan made HPV16 cell line sin U20S (bar second R37A clone).

The Peter Howley lab kindly gave us the HPV18 E2 plasmid.

TopBP1 and R41A single mutations made in HPV18 by Mary Donaldson and Edward Dornan.

N89YE90V HPV16 E2 mutation made by Mary Donaldson.

Chapter 3 – Mutants of HPV16 and 18E2 compromise viral transcription and replication.

As discussed in **Chapter 1.13**, the viral protein E2 links most of its functions to direct interactions with host cellular factors, either to support viral genome expression, segregation, or replication cycle (Muller, Demeret 2012). TopBP1 has been shown to interact *in vitro* and *in vivo* with E2 and can regulate the ability of E2 to control transcription and replication (Boner, Taylor et al. 2002). Also, an essential transcriptional co-activator for all E2 proteins is Brd4, which is found ubiquitously in all proliferating cells (Houzelstein, Bullock et al. 2002).

E2 mutants that impair the binding of both TopBP1 and Brd4 have been identified and shown to compromise the transcription ability of papillomavirus E2 (Donaldson, Mackintosh et al. 2012)(Schweiger, You et al. 2006). A previous study in the Morgan lab showed that a non-TopBP1 interacting 16E2 mutant, $E2^{-TopBP1}$ has compromised replication potential but retains transcriptional ability (Donaldson, Mackintosh et al. 2012). The $E2^{-TopBP1}$ mutant has an N89Y and an E90V mutation, amino acid 90 is a conserved glutamic acid in almost all E2 proteins from all HPV types. A Brd4 mutant ($E2^{-Brd4}$) R37A (arginine to alanine at position 37) in E2 compromises interaction with Brd4 and transcriptional activation (Schweiger, You et al. 2006). The role of Brd4 in regulating viral replication is unclear. Reports have demonstrated two different phenotypes of the $E2^{-Brd4}$ mutant in replication; a 50% decrease in replication properties of the $E2^{-Brd4}$ mutant (Wang, Helfer et al. 2013), or suggesting that Brd4 is not involved in DNA replication by E2 (Baxter, McBride 2005). Our current studies were designed to further characterise these E2 mutants with our functional assays in various cell lines.

3.1 Expression of HPV16 E2 and E2 mutants.

Plasmids expressing HPV16 E2 had mutations successfully introduced using site-directed mutagenesis (as described in **Methods 2.2.1.4**) so they could be used for functional analysis (primer sequences in **Materials 2.1.11**). These mutations were originally described in the following papers (Donaldson, Mackintosh et al. 2012, Schweiger, You et al. 2006). From this point onwards the E2 mutants shall

be referred to as $E2^{-TopBP1}$ and $E2^{-Brd4}$. **Figure 3.1** shows a western blot of the expression of all HPV16 E2 mutants created for this study in C33a keratinocytes. Lane 1 shows a non-E2 cell control, lane 2 shows that 16 $E2^{-WT}$ is more strongly expressed than either $E2^{-TopBP1}$ or $E2^{-Brd4}$, lanes 3 and 4 respectively.

3.2 Transcription function of E2 and E2 mutants in 293T cells

Transcriptional activation studies were carried out to determine the transcriptional activation properties of the E2 mutants generated compared to wildtype E2. The 293T cell line was chosen for initial characterisation of the mutants.

Figure 3.2 shows the transcriptional activation properties of $E2^{-WT}$, $E2^{-TopBP1}$ and $E2^{-Brd4}$ in 293T cells. In lanes 1 and 2, it is clear that $E2^{-WT}$ induces transcription activation from a tk promoter with 6 E2 binding sites located upstream and that this activation becomes squelched at higher levels of E2 input (full description of plasmid in (Vance, Campo et al. 1999). Lanes 3 and 4 shows that the $E2^{-TopBP1}$ mutant has compromised transcriptional activation capabilities, 8% of $E2^{-WT}$at lower levels of input DNA, but can recover to more than half of $E2^{-WT}$ levels of transcription activation with higher plasmid input. Finally, lanes 5 and 6 show that the $E2^{-Brd4}$ mutant is severely compromised in transcriptional activation at all levels of input plasmid DNA, 22% with 10ng of E2 input and 11% at 1000ng, however it still has some transcriptional activity likely due to residual interaction between E2 and Brd4.

3.3 HPV16 $E2^{-TopBP1}$ and $E2^{-Brd4}$ display compromised replication in 293T cells

To investigate whether E1–E2 mediated DNA replication is affected by point mutations known to impair the ability of E2 to bind to either TopBP1 or Brd4, a real-time PCR detection method was used to measure E1-E2 mediated DNA replication in 293T cells.

In **Figure 3.3** results for the mock transfected, pOri only (plasmid containing viral origin of replication(Taylor, Morgan 2003b)) and HA-E1 expression plasmid

with pOri gives very little background signal in this assay, so only results with E2 are presented. Lanes 1-3 shows that with increasing levels of $E2^{-WT}$ plasmid DNA (10ng, 100ng and 1000ng) the levels of replication also increase. For both $E2^{-TopBP1}$ and $E2^{-Brd4}$ with 10ng of input plasmid (lanes 4 and 7) replication function is severely compromised, 28.7% and 35% respectively, at higher levels of plasmid DNA the $E2^{-TopBP1}$ mutant recovers some replication ability, 65% compared to $E2^{-WT}$ at the highest concentration, the $E2^{-Brd4}$ also recovers replication capabilities at 100ng (130%) and 1000ng (76.5%) of input to a greater extent than the TopBP1 mutant.

Figure 3.1

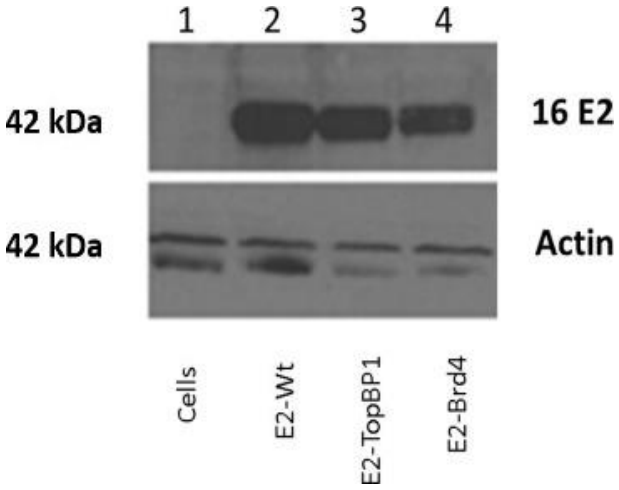
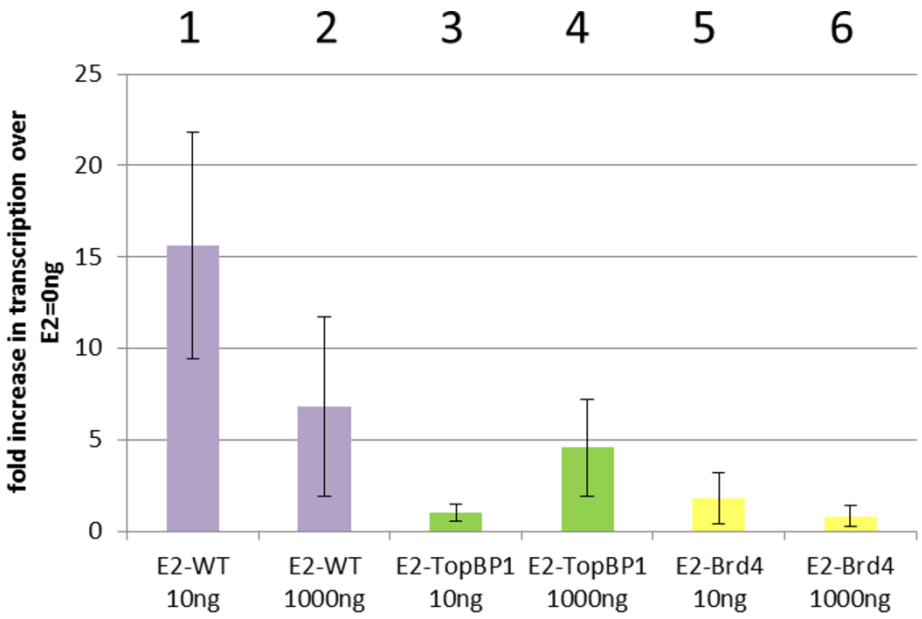


Figure 3.1: Expression of HPV16 E2^{-WT}, E2$^{-TopBP1}$ and E2^{-Brd4}.
C33a cells were transfected with 1µg of each expression plasmid. Cells were harvested and protein prepared for western blot (as described in **Methods section 2.2.1.6**). Western blot was carried out with 50µg of cellular protein extract. A non-E2 expressing control (C33a cell lysate) was also included for comparison to E2 transfected cells. E2 expression was detected using TVG261 antibody. Following E2 expression in the upper panel the membrane was stripped (as described in **Methods section 2.2.1.7**) and re-probed with actin (lower panel) to act as a loading control.

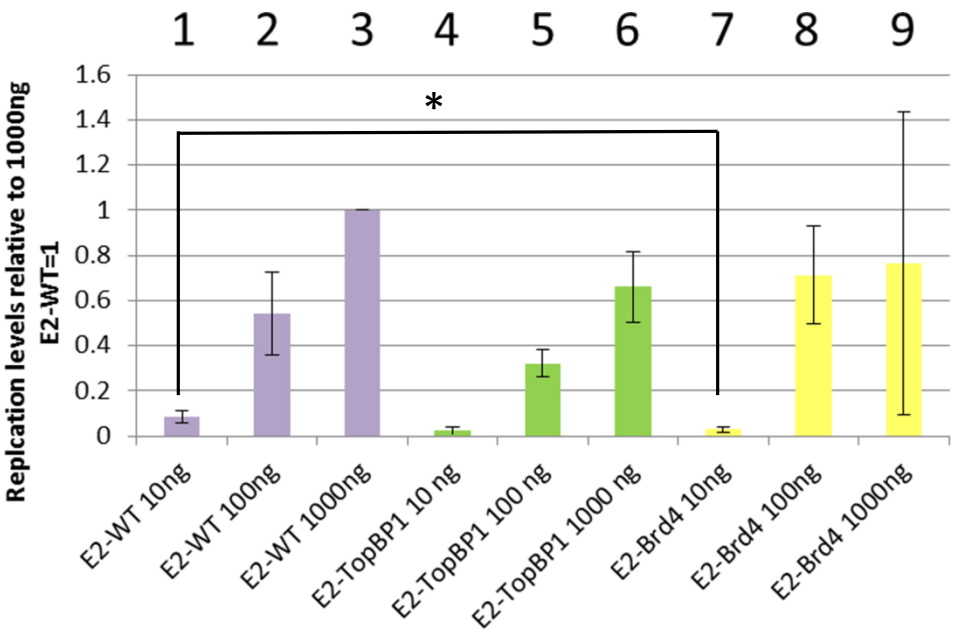
Figure 3.2



Plasmid	10ng	1000ng
$E2^{-WT}$	15.7	6.8
$E2^{-TopBP1}$	1.0	4.6
$E2^{-Brd4}$	1.8	0.8

Figure 3.2: 16E2 Transcription activation in 293T cells. Transcription assays were carried out in 293T cells with input levels of 10ng and 1000ng, along with 1µg of ptk6E2, an E2 reporter containing 6E2 DNA binding sites upstream from a tk promoter driving luciferase (Vance, Campo et al. 1999). Cells were harvested and luciferase and protein assays carried out (as described in **Methods section 2.2.1.5 and 2.2.1.13**). The results are normalised to protein levels in each sample and are represented as fold increase over ptk6E2 levels in the absence of E2. The results are representative of three independent experiments done in duplicate. Bars represent standard error. Fold activation in transcription over that obtained when no E2 was co-expressed is shown in the table below. None of the above results were significant using a student T-test.

Figure 3.3



Plasmid	10ng	100ng	1000ng
$E2^{-WT}$	0.08	0.5	1
$E2^{-TopBP1}$	0.02	0.3	0.7
$E2^{-Brd4}$	0.03	0.7	0.8

Figure 3.3: 16E2 mediated replication in 293T cells. 293T cells were co-transfected with various concentrations of E2 input DNA (10ng, 100ng and 1000ng) along with 100pg of the viral origin of replication (ori) and 1µg of HA-E1. Low molecular weight DNA was harvested using HIRT buffer, and fresh replication assays as described in **Methods section 2.2.1.14**. Results shown are expressed relative to the wild-type E2 levels equaling 1; and are representative of three independent experiments, numbers shown in table below. Bars represent standard error. Significant results from student T-test are displayed on the graph. * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.4 Transcription function of E2 and mutant E2 in C33a cells

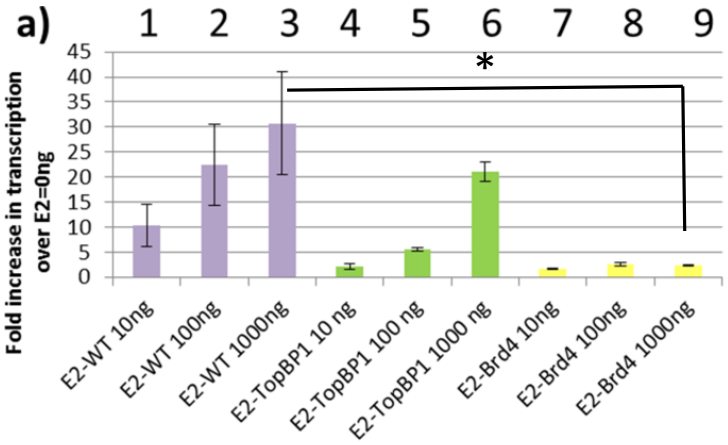
To further determine whether TopBP1 or Brd4 could regulate E2 transcriptional activity, transcription assays were carried out in the C33a cell line. The C33a cell line was selected for studying transcriptional activation by E2 because they are keratinocytes which are derived from a cervical carcinoma devoid of HPV sequences. C33a cells are an extensively used model cell line for the study of HPV E2 protein function. This is the first report of the $E2^{-TopBP1}$ transcriptional activity in C33a cells.

Figure 3.4a shows the transcriptional activation properties of $E2^{-WT}$, $E2^{-TopBP1}$ and $E2^{-Brd4}$ in C33a cells. A range of E2 plasmid concentrations were transiently co-transfected in C33a cells with an E2-responsive reporter construct consisting of six E2 binding sites upstream of the minimal thymidine kinase promoter (ptk6E2) and firefly luciferase gene. The results were averaged and normalised to ptk6E2 in the absence of E2 equalling 1. In **Figure 3.4a** it is clear to see $E2^{-WT}$ induces transcriptional activation from the ptk6E2 promoter in a concentration dependent manner (lanes 1-3), activation increases as plasmid input increases (10ng, 100ng and 1000ng).Lane 4 demonstrates that $E2^{-TopBP1}$ activates transcription, although activation is significantly less than $E2^{-WT}$ (21.5%), lane 5 shows activation at 100ng is only 24.7% of $E2^{-WT}$, however some function is regained when E2 is overexpressed, at 1000ng activation recovers to 68.6% of $E2^{-WT}$. $E2^{-Brd4}$ activation is the most compromised (lanes 7-9), due to Brd4's essential role in transcription. Despite this highly compromised ability to activate transcription from the ptk6E2 promoter, there is still a notable 3-fold increase in transcription over background levels, despite the decrease in activation compared to $E2^{-WT}$ (only 7.7% at highest E2 levels), this 3-fold increase may represent a residual interaction with Brd4.

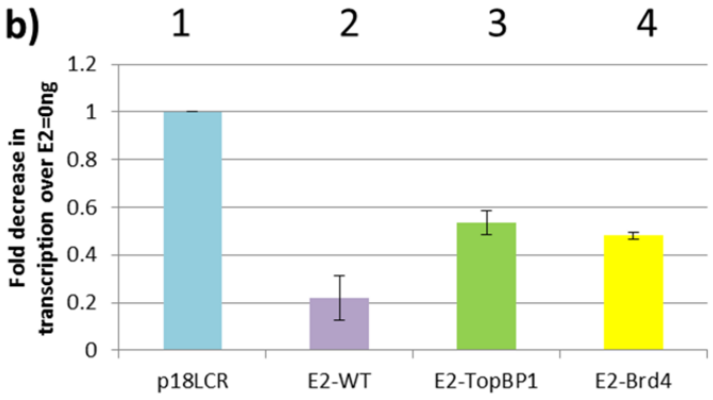
E2 can also act as a transcriptional repressor of papillomavirus control regions, and the ability of $E2^{-WT}$, $E2^{-TopBP1}$ and $E2^{-Brd4}$ to repress the HPV18 long control region (LCR) was determined (**Figure 3.4b**). The HPV18 LCR was used as it has some transcriptional activity, in our hands the 16 LCR doesn't. In lane 1, $E2^{-WT}$ is shown to repress transcription from the HPV18 LCR as predicted, and in lanes 2

and 3 both E2 mutants, $E2^{-TopBP1}$ and $E2^{-Brd4}$ also repress transcription activation, although to a lesser extent than $E2^{-WT}$.

Figure 3.4



Plasmid	10ng	100ng	1000ng
E2^{-WT}	10.4	22.4	30.7
E2$^{-TopBP1}$	2.2	5.6	21.09
E2^{-Brd4}	1.6	2.6	2.4



Plasmid	Control	100ng
18LCR	1	
E2^{-WT}		0.2
E2$^{-TopBP1}$		0.5
E2^{-Brd4}		0.5

Figure 3.4: Transcriptional function of 16E2 in C33a cells.

a) Transcription assays were carried out in C33a cells with input levels of E2 expression plasmids of 10ng, 100ng and 1000ng, along with an E2 reporter containing 6E2 DNA binding sites upstream from a tk promoter driving luciferase. Cells were harvested and luciferase and protein assays carried out. The results are normalised to protein levels in each sample and are represented as fold increase over ptk6E2 out (as described in **Methods section 2.2.1.5 and 2.2.1.13**). The results are representative of three independent experiments done in triplicate. Bars represent standard error. Fold activation in transcription over that obtained when no E2 was co-expressed is

shown in the table below. Significant results from student T-test are displayed on the graph. * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

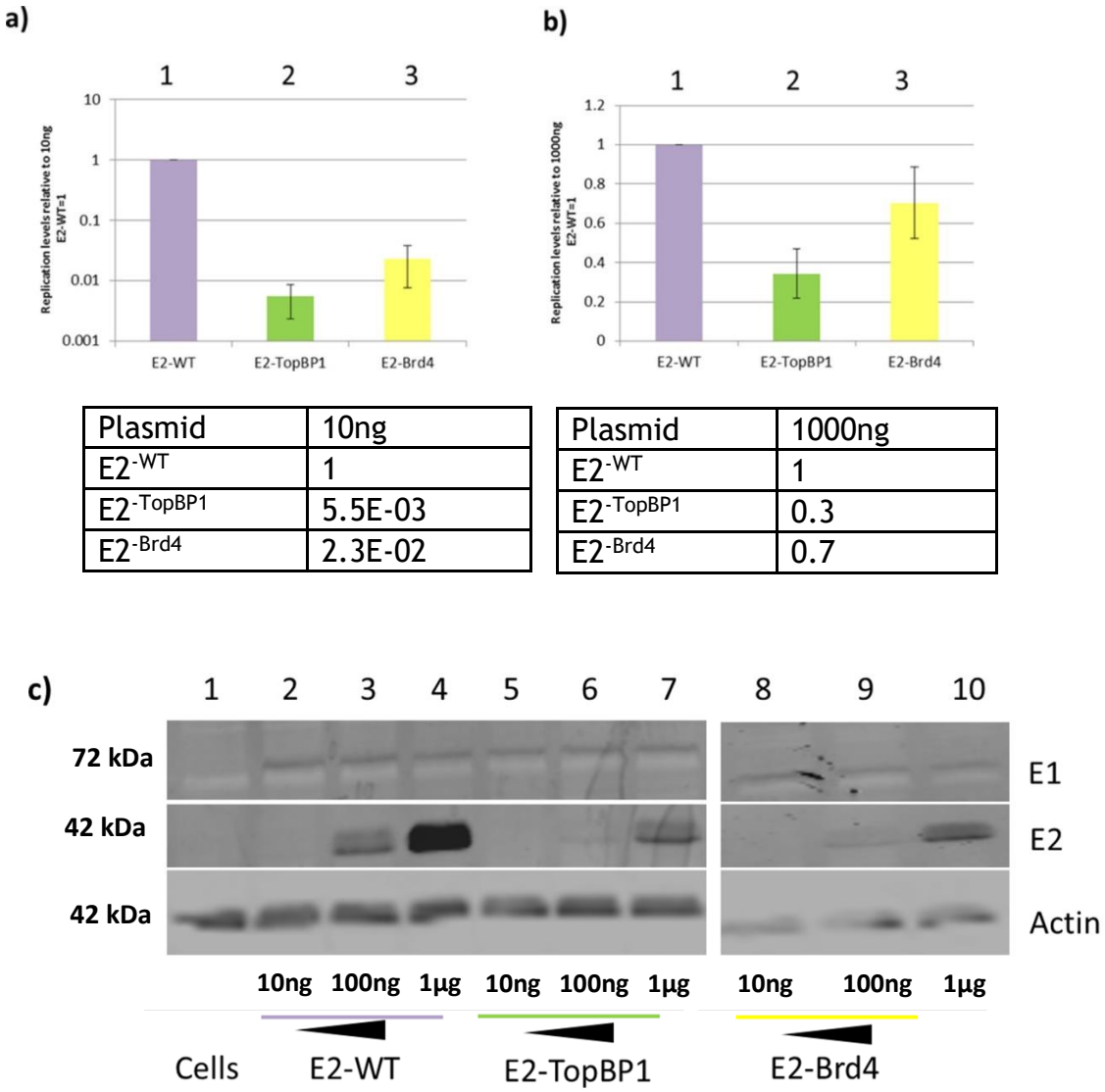
b) Wild-type and mutant E2 plasmids were titrated into C33a cells along with 1µg of pHPV18LCR-luc (long control region) reporter containing the origin of replication. Cells were harvested and luciferase and protein assays carried out. The results are normalised to protein levels in each sample and are represented as fold increase over 18LCR. The results are representative of three independent experiments done in duplicate, numbers are represented in a table below. Bars represent standard error. No significant results.

3.5 HPV 16E2$^{-TopBP1}$ and E2^{-Brd4} have compromised replication in C33a cells.

To further investigate whether TopBP1 and Brd4 are required for E1-E2 mediated DNA replication, replication assays were carried out in the C33a cell line with both low and high levels of E2 input plasmid. Results are shown in **Figure 3.5a** (10ng E2) and **3.5b** (1000ng E2), only results with E2 are presented as background was close to zero. **Figure 3.5a** replication results are presented on log-scale and E2^{-WT} levels set to 1 and the level of replication induced by mutants set relative to this. At low levels of E2 expression plasmid (10ng), E2$^{-TopBP1}$ and 16 E2^{-Brd4} levels of DNA replication are down compared to E2^{WT}, levels of replication are reduced to 0.55% and 2.57% of E2^{-WT} respectively. In **Figure 3.5b** the results obtained with 1000ng of input plasmid are represented. At higher levels of E2 input the mutant phenotype can be overcome, however the mutants do not regain 100% of their replication function. Replication levels for E2$^{-TopBP1}$ and E2^{-Brd4} (lanes 2 and 3) are 34.4% and 70.3% respectively when compared to E2^{-WT}.

Recent studies from the Morgan lab have demonstrated that an interaction between E2 and E1 stabilises the E2 protein, so therefore the expression levels of E2 proteins co-transfected with E1, with E2 input levels from 10ng, 100ng and 1000ng were investigated and shown in **Figure 3.5c**. Lane 1 contains only C33a cell lysate (no E1 or E2 transfection) whereas lanes 2-10 all contain HA-E1 and E2. Lanes 2-4 are transfected with E2^{-WT} increasing in concentration from left to right (10ng, 100ng and 1000ng), lanes 5-6 contain E2$^{-TopBP1}$ increasing in concentration, and lanes 8-10 contain E2^{-Brd4} also increasing in concentration from left to right. The blot shows that E2^{-WT} is expressed at higher levels than either E2$^{-TopBP1}$ or E2^{-Brd4} mutant suggesting that there may be a contribution of E2 protein levels to the compromised DNA replication abilities of these mutants. It should be noted that the mutant expression does not appear stable in this blot and results merely give an indication of this conclusion.

Figure 3.5



Plasmid	10ng
E2^{-WT}	1
E2$^{-TopBP1}$	5.5E-03
E2^{-Brd4}	2.3E-02

Plasmid	1000ng
E2^{-WT}	1
E2$^{-TopBP1}$	0.3
E2^{-Brd4}	0.7

Figure 3.5: E1-E2 mediated DNA replication and expression in C33a cells.

a) C33a cells were transfected with 10ng of E2 input DNA along with 100pg of the viral origin of replication (ori) and 1µg of HA-E1. Low molecular weight DNA was harvested using HIRT buffer and fresh replication assays as described in **Methods section 2.2.1.14**. Results shown are expressed relative to the wild-type E2 levels equaling 1; and are representative of three independent experiments, numbers shown in table below. Bars represent standard error.

b) C33a cells were transfected with 1000ng of E2 input DNA along with 100pg of the viral origin of replication (ori) and 1µg of HA-E1. Low molecular weight DNA was harvested using HIRT buffer and fresh replication assays as described in **Methods section 2.2.1.14**. Results shown are expressed relative to the wild-type E2 levels equaling 1; and are representative of three independent experiments, numbers shown in table below. Bars represent standard error.

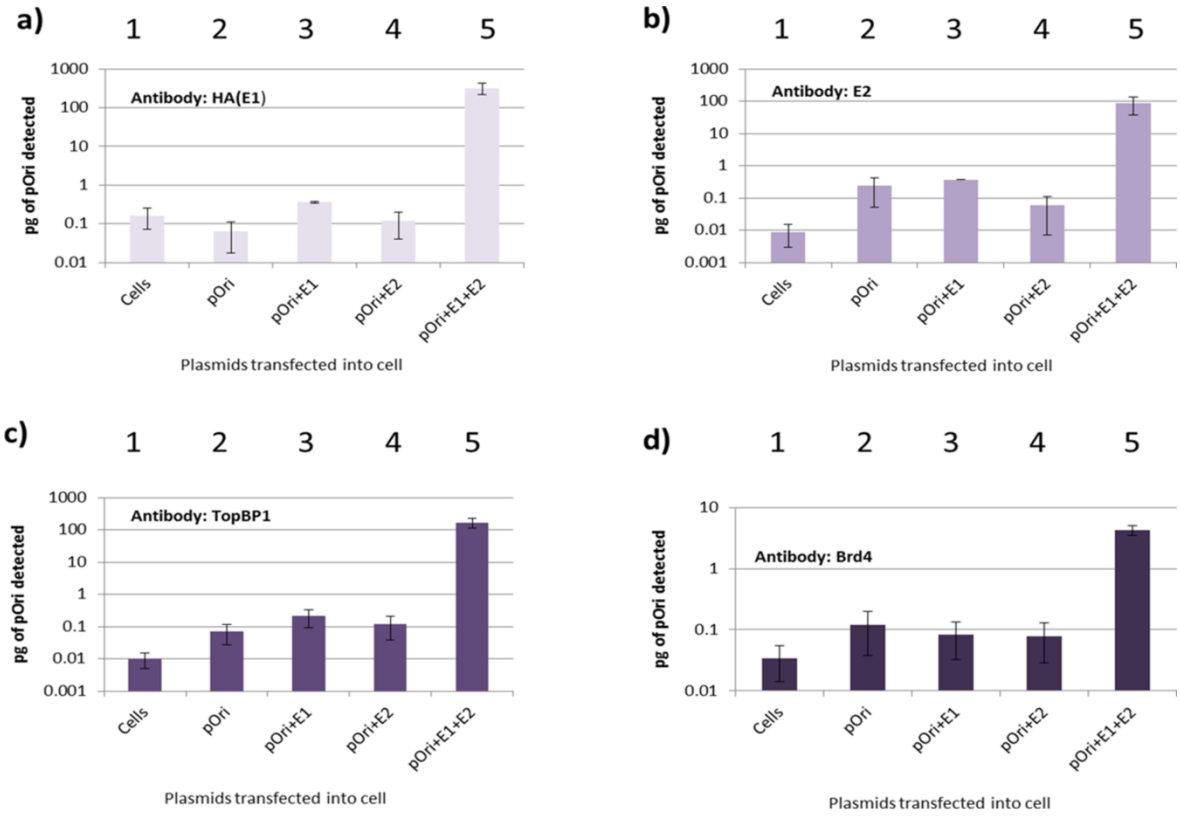
c) C33a cells were co-transfected with 1ug of E1 and $E2^{-WT}$, $E2^{-TopBP1}$ or $E2^{-Brd4}$, titrated at 10ng, 100ng or 1000ng (concentration of plasmid input increases from left to right). Cells were harvested and protein prepared for western blot (as described in **Methods section 2.2.1.6**). Western blot was carried out with 50µg of cellular protein extract. A non-E2 expressing control (C33a cell lysate) was also included for comparison to E2 transfected cells. Actin is shown as a loading control. Following E2 expression in the upper panel the membrane was stripped (as described in **Methods section 2.2.1.7**) and re-probed with actin (lower panel) to act as a loading control. The E2 mutants to appear to be unstable and this may affect the quality of the blot. Therefore, these results present an indication of their expression.

3.6 Brd4 and TopBP1 proteins are recruited to the viral origin of replication in an E1-E2 dependent manner.

Results in **Figures 3.5 a** and **b** suggest TopBP1 and Brd4 are required for optimum DNA replication by E2 in association with E1 in C33a cells. To test whether these host proteins interact with the HPV viral origin of replication directly, ChIP assays were carried out with chromatin prepared from C33a cells that had been transfected with the HPV16 origin of replication along with HA-E1 and E2 (100ng) expression vectors (as described in **Methods section 2.2.1.13**). Averaged results from replicate ChIP experiments are presented on a log-scale as the levels of pOri detected in the control conditions are orders of magnitude lower than when the viral origin is transfected alongside both E1 and E2 plasmids.

The results are graphed in **Figure 3.6.** In **Figure 3.6a,** the results of the ChIP experiment carried out with an HA antibody that recognises the HA-E1 protein is displayed (graphed on a log scale). In lane 1 the result with non-transfected cells is shown, lane 2 has the origin plasmid only transfected while lanes 3 and 4 have the HA-E1 and E2 expression plasmids transfected respectively. Transfecting either HA-E1 or E2 with the origin of replication alone (lanes 3 and 4) gives no signal, and highlights both E1 and E2 are required together at the viral origin of replication. Neither are present in the absence of the other. In lane 5, cells were co-transfected with pOri, E1 and E2 plasmid DNA. This condition clearly shows an increase in signal of several orders of magnitude. **Figure 3.6b** shows ChIP carried out with an E2 antibody; again only a signal is detected when pOri plus HA-E1 plus E2 expression plasmids are transfected, E2 is located at the origin in an E1 dependent manner (lane 5). This result demonstrates that E2 recruitment and/ or stabilisation at the viral origin of replication is assisted by the presence of E1. **Figure 3.6c** shows TopBP1 at the origin in an E1-E2 dependent manner when ChIP is performed with a TopBP1 antibody (lane 5). This is also true for Brd4, results shown in **Figure 3.6d**, lane 5. When the viral origin is combined with HA-E1 and E2 the level of signal increases.

Figure 3.6



Plasmid	E1 antibody	E2 antibody	TopBP1 antibody	Brd4 antibody
Cells	0.162	0.009	0.01	0.034
Ori	0.064	0.236	0.072	0.118
Ori+E1	0.361	0.371	0.211	0.082
Ori +E2	0.119	0.06	0.123	0.079
Ori+E1+E2	321.406	86.545	167.001	4.249

Figure 3.6: ChIP of TopBP1 and Brd4 at the viral origin in an E1-E2 dependent manner. Cells were transfected with nothing (lane 1); 1µg of the viral origin of replication by itself (pOri) (lane 2); along with 1µg of HA-E1 expression plasmid (lane 3); 1µg of pOri plus 1µg of HA-E1 expression plasmid (lane 4); 1µg of pOri plus 1µg of HA-E1 expression plasmid plus 1µg of E2 expression plasmid (100ng) (lane 5). Approximately $1x10^7$ cells were cross linked and chromatin prepared <400bp using sonication method (as described in **Methods section 2.2.1.15**). 100µg of chromatin was immunoprecipitated with 2µg of the appropriate antibody (HA (recognises E1), E2, TopBP1 or Brd4) and the resulting DNA quantified using our pOri taqman qPCR to quantify the levels of pOri DNA in the presence of our proteins of interest. This experiment was done in triplicate and results averaged. Error bars represent standard error. Pg of Pori detected is also shown in a table below.

3.7 TopBP1 and Brd4 are present in E1-E2 induced nuclear foci

E1 and E2 have previously been shown to locate to nuclear foci when co-expressed and some reports suggest this is enhanced by the presence of the viral origin. **Figure 3.7.1a** shows a random example of C33a cells transfected with HA-E1, E2 and pOri. The left panel is HA staining (to detect HA-E1), the middle panel is E2 and the right panel is a merge with a DAPI stained image (all immunofluorescence done by Mary Donaldson). Three distinct staining patterns were observed; large foci (i), punctate foci (ii) and a dispersed appearance (iii) that also looked like co-localisation was occurring. In order to distinguish whether pOri influences the distribution of these nuclear foci three independent experiments and 50 random images were taken. Large, punctate and diffuse patterns were counted in these images and the results shown for E2 in **Figure 3.7.1b** and for HA-E1 in **Figure 3.7.1c**; cells that displayed expression of both proteins were counted. The results showed that the presence of pOri enhanced the numbers of large foci containing cells and a reduced number of cells with a dispersed phenotype. This is suggestive that the large foci are replication foci.

To determine whether TopBP1 and Brd4 are located in these foci, they were stained for either TopBP1 or Brd4 and co-stained for E1 as a marker for the E1-E2-pOri replication foci. **Figure 3.7.2a** shows images of TopBP1 and **Figure 3.7.2b** shows images of Brd4; which show that both of these proteins are located in the replication foci containing E1 and E2. Additionally, both proteins in the absence of E1-E2-pOri has dispersed nuclear expression patter (No image shown).

3.8 shRNA knockdown of TopBP1 and Brd4 destroys E1-E2-pOri nuclear foci but has no effect on DNA replication.

Mary Donaldson investigated whether knocking down TopBP1 or Brd4 would have an effect on the formation of nuclear foci. **Figure 3.8.1a** shows co-transfection with shTopBP1 (i) and co-transfection with shBrd4 expressing plasmids (ii), which shows no replication foci are formed when TopBP1 or Brd4 is knocked down. In this experiment only HA-E1 was stained for but E2 and the pOri plasmids were

co-transfected and there is no TopBP1 detected in foci of any cell. **Figure 3.8.1b** shows that no replication foci were formed and Brd4 cannot be detected. These experiments were repeated multiple times with no change in results and the absence of foci was observed after only 24 hours post transfection.

As TopBP1 and Brd4 are present at the origin of replication, we hypothesized that knocking down the expression of these proteins may effect E1-E2 mediated DNA replication. To test this replication assays were carried out with increasing concentrations of E2 input plasmids (10ng, 100ng and 1000ng) in the presence and absence of shRNA plasmids targeting either TopBP1(as described in **Methods section 2.2.1.14,** details of shRNA in **Materials section 2.1.9.1**) (**Figure 3.8.2a**) or Brd4 (**Figure 3.8.2b**). There does not appear to be any change between the control and TopBP1/Brd4 shRNA.

Figure 3.8.2c shows a western blot demonstrating shRNA knockdown of Brd4 (TopBP1 shRNA knockdown has been shown in a previous publication (Donaldson, Boner et al. 2007). Lane 2 shows Brd4 expression in the absence of shRNA and lane 1 clearly shows a reduced level of Brd4 expression when cells are treated with 1µg Brd4 shRNA. Lane 3 is a cell control. This shows us our shRNA works and the replication result is true, knockdown of TopBP1 and Brd4 has little to no effect.

Figure 3.7.1

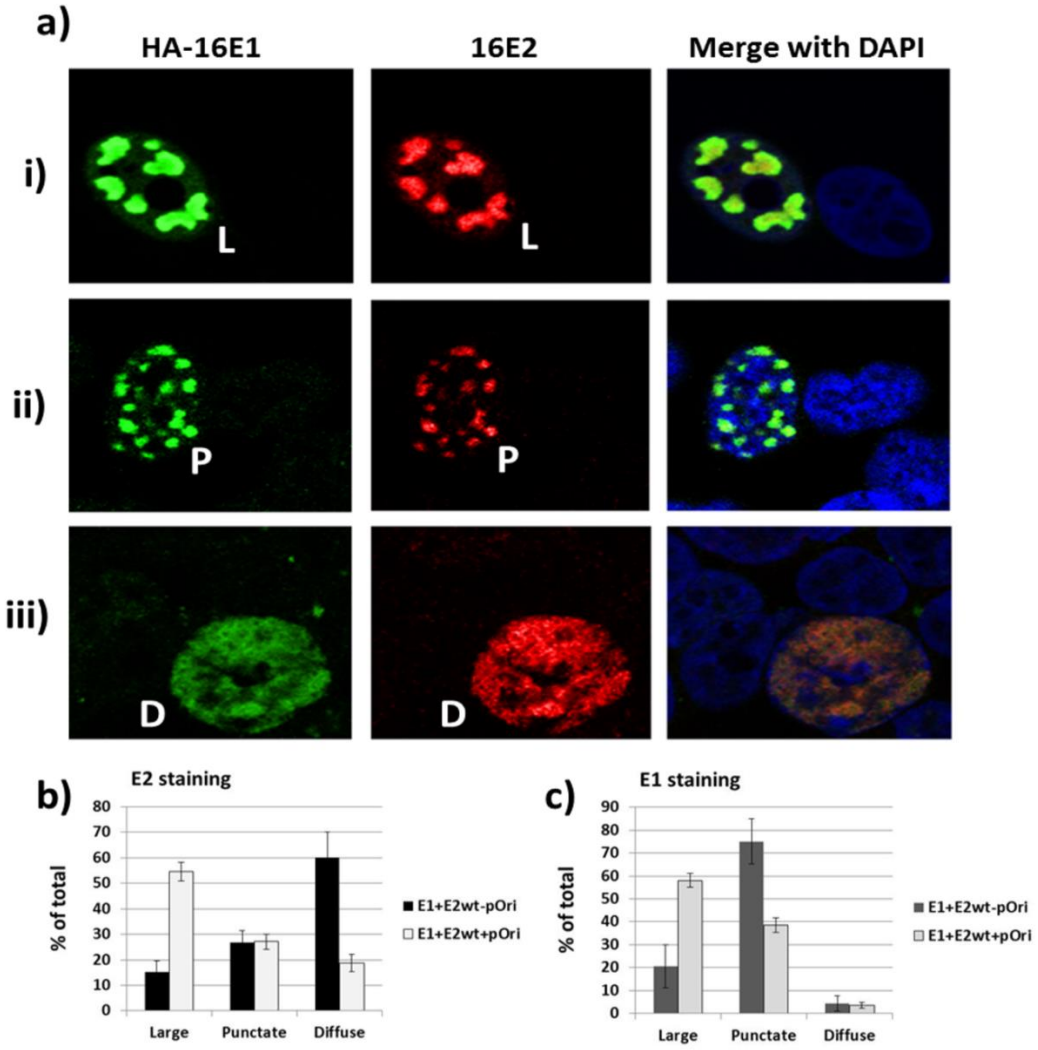


Figure 3.7.1. Localization of E1 and E2 into nuclear foci.

a) The panel of images demonstrates examples of three types of staining: i) Large; ii) Punctate; iii) dispersed. All staining was carried out in C33a cells transfected with 1µg pOri, 1µg HA-E1 and 1µg E2. HA-E1 or E2 by themselves did not form foci and showed a dispersed nuclear appearance (not shown).

b) Cells staining positive for both E1 and E2 were scored for the types of cell staining observed for E2 in both the absence and presence of pOri. The table displays a summary of three experiments with standard error bars shown.

c) Cells staining positive for both E1 and E2 were scored for the types of cell staining observed for HA E1 in both the absence and presence of pOri. The table displays a summary of three experiments with standard error bars shown. **These results are the work of Mary Donaldson.**

Figure 3.7.2

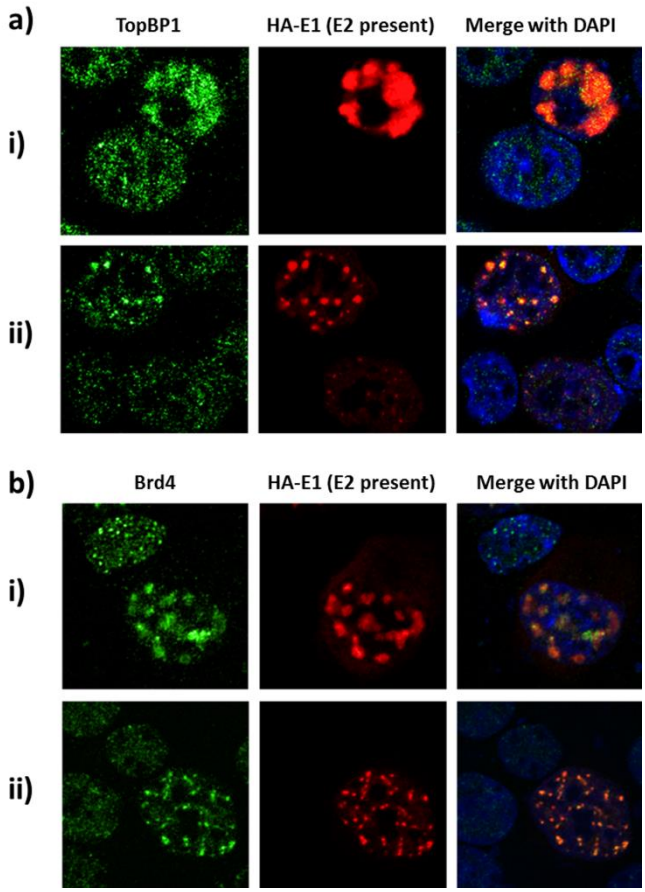


Figure 3.7.2. TopBP1 and Brd4 are recruited to E1-E2 foci.

a) C33a cells were transfected with 1µg pOri, 1µg HA E1, 1µg E2 then stained for TopBP1 (left panel) or HA (to detect HA E1, middle panel); the presence of E1 foci indicated co-expression of E2 (as described in **Methods section 2.2.1.16**). Staining is shown in the context of DAPI staining (right panel). All cells with E1 foci demonstrated recruitment of TopBP1 to these foci, two example panels are shown.

b) C33a cells were transfected with 1µg pOri, 1µg HA E1, 1µg E2 then stained for Brd4 (left panels) or HA (to detect HA E1, middle panels); the presence of E1 foci indicated co-expression of E2 (as described in **Methods section 2.2.1.16)**. Staining is shown in the context of DAPI staining (right panels). All cells with E1 foci demonstrated recruitment of Brd4 to these foci, two example panels are shown. This experiment was repeated with identical results at least three times.

These results are the work of Mary Donaldson.

Figure 3.8.1

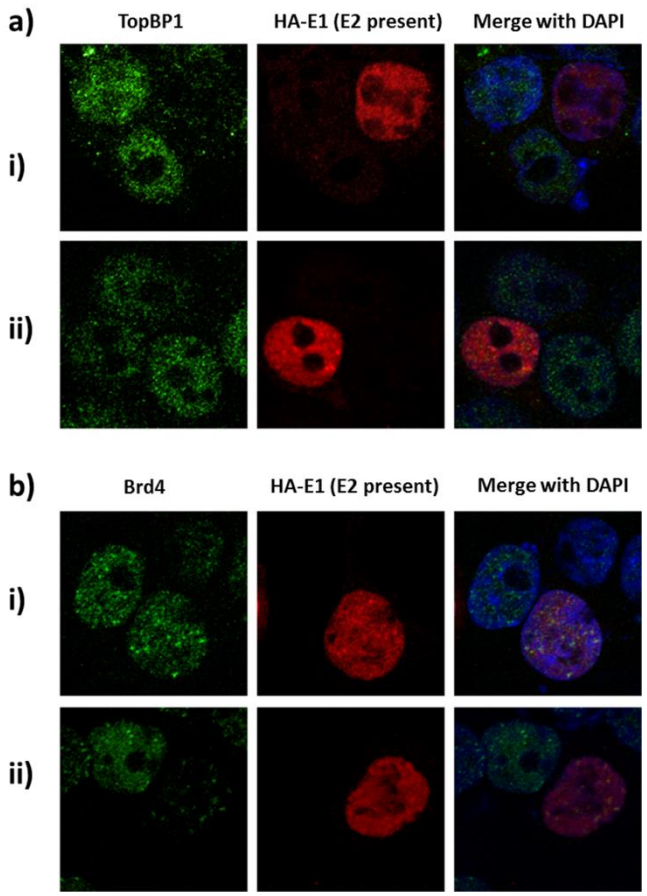


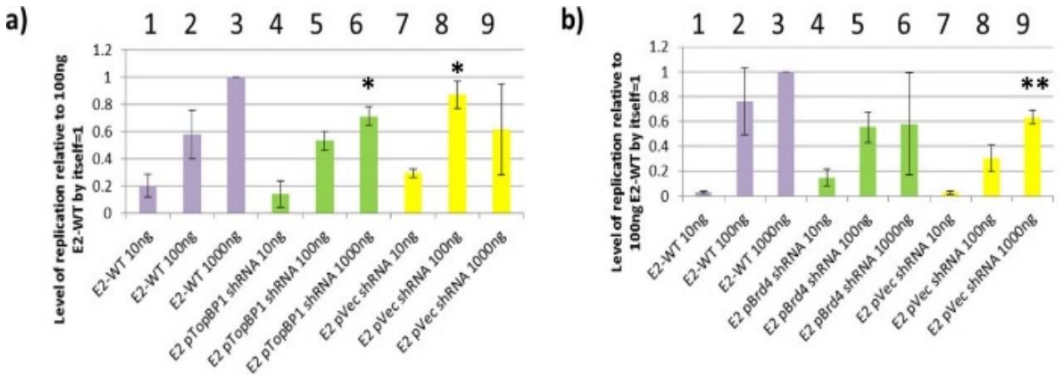
Figure 3.8.1. Knock down of TopBP1 and Brd4 disrupts E1-E2 DNA replication foci.

a) C33a cells were transfected with 1µg pOri, 1µg HA E1, 1µg E2 and 1µg of pShTopBP1 (i) or 1µg pShBrd4 (ii) then stained for TopBP1 (left panels) or HA (to detect HA E1, middle panels) **(**as described in **Methods section 2.2.1.16**, details of shRNA in **Materials section 2.1.9.1)**; staining is shown in the context of DAPI staining (right panels).

b) C33a cells were transfected with 1µg pOri, 1µg HA E1, 1µg E2 and 1µg of pShTopBP1 (i) or 1µg pShBrd4 (ii) then stained for Brd4 (left panels) or HA (to detect HA E1, middle panels) (as described in **Methods section 2.2.1.16**, details of shRNA in **Materials section 2.1.9.1**); staining is shown in the context of DAPI staining (right panels). This experiment was carried out two times with identical results, the knock down of TopBP1 or Brd4 always ablated the E1-E2-pOri induced foci.

These results are the work of Mary Donaldson.

Figure 3.8.2



Plasmid	10ng	100ng	1000ng
E2 WT	0.2	0.6	1
E2 pTopBP1 shRNA	0.1	0.5	0.7
E2 pVec shRNA	0.3	0.9	0.6

Plasmid	10ng	100ng	1000ng
E2 WT	0.03	0.8	1
E2 pBrd4 shRNA	0.1	0.6	0.6
E2 pVec shRNA	0.03	0.3	0.6

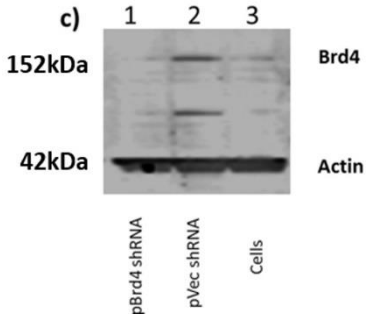


Figure 3.8.2: shRNA against TopBP1 and Brd4 has no effect on replication.

a) C33a cells were co-transfected with various concentrations of E2 input DNA (10ng, 100ng and 1000ng) along with 100pg of the viral origin of replication (ori) and 1µg of HA-E1, also 1ug of TopBP1 shRNA or vector shRNA control (as described in **Methods section 2.2.1.14**). HPV16 E2 and mutants DNA replication was tested by quantifying pOri levels, results shown are expressed relative to the wild-type E2 levels equalling 1; and are representative of three independent experiments. Bars represent standard error. Significant results from student T-test are displayed on the graph. * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

b) C33a cells were co-transfected with various concentrations of E2 input DNA (10ng, 100ng and 1000ng) along with 100pg of the viral origin of replication (ori) and 1µg of HA-E1, also 1ug of Brd4 shRNA or vector shRNA control (as described in **Methods section 2.2.1.14**). HPV16 E2 and mutants DNA replication was tested by quantifying pOri levels, results shown are expressed relative to the wild-type E2 levels equalling 1; and are representative of three independent experiments. Bars represent standard error. Significant results from student T-test are displayed on the graph. * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

c) Brd4 shRNA western blot. C33a cells were transfected with 1µg of either a control vector for Brd4 shRNA or Brd4 shRNA and harvested 48 hours post transfection, and pellets lysed using NP40 buffer (as described in **Method section 2.2.1.6**). The knockdown of Brd4 using shRNA was examined using western blot. TopBP1 shRNA has been previously shown in a western blot in (Donaldson, Boner et al. 2007).

3.9 The role of Brd4 and TopBP1 in HPV18 E2 transcription and replication functions

Plasmids expressing HPV18E2 (HA-tagged) had mutations successfully introduced using site-directed mutagenesis so they may be used for functional analysis (primer sequences shown in **Materials section 2.1.11**). Amino acid 90 is a conserved glutamic acid in almost all E2 proteins from all HPV species, mutating either amino acid 90 or 89 has been shown to disrupt the E2-TopBP1 interaction (Donaldson 2012). Mutations made in HPV18 E2 correspond to the mutants made in HPV16 E2, as 18E2 has an extra 4 amino acids at the beginning of its sequence compared to 16E2 **(Figure 3.9)**, the R37A mutant becomes R41A in HPV18, and the N89YE90V mutant becomes T93YE94V. Ed Dornan made a T93YE94V mutant in 18E2 to make a comparison to the N89YE90V 16E2 mutant. T93YE94V was shown to replicate poorly, thus, we decided to analyse whether making a single mutation in 18E2 (E94V) would have the same functional consequence as "E" glutamic acid is a conserved amino acid.

Figure 3.10 demonstrates that all of the plasmids made express E2 successfully when transfected into C33a cells.

Figure 3.9

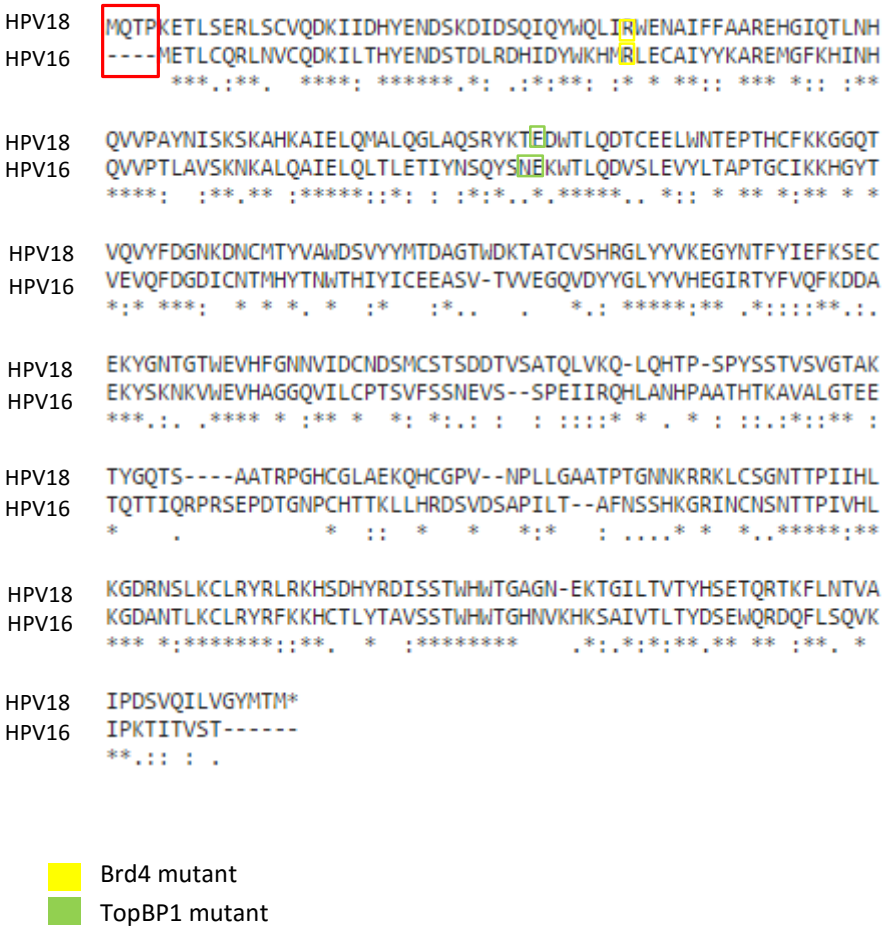


Figure 3.9: HPV18 has 4 extra amino acids at the beginning of its sequence compared to HPV16. Sequences of both HPV16 and 18 were put into Clustal W to align the sequences based on similarity. The red box highlights the 4 amino acid difference between 16 and 18. E2-TopBP1 and E2-Brd4 mutants of both HPV16 and 18 are indicated by green and yellow boxes respectively.

Figure 3.10

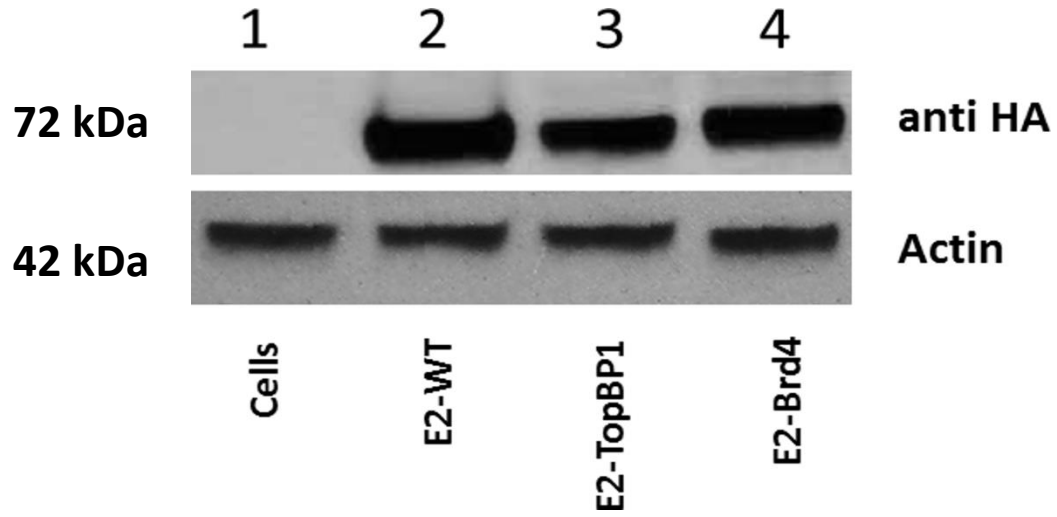


Figure 3.10: Expression of HPV18 E2^{-WT}, E2$^{-TopBP1}$ and E2^{-Brd4}. C33a cells were transfected with 2µg of each expression plasmid. Cells were harvested and protein prepared for western blot (as described in **Methods section 2.2.1.6**). Western blot was carried out with 50µg of cellular protein extract. A non-E2 expressing control (C33a cell lysate) was also included for comparison to E2 transfected cells. HPV18 E2 was detected using anti HA antibody. Following E2 expression in the upper panel the membrane was stripped (as described in **Methods section 2.2.1.7**) and re-probed with actin (lower panel) to act as a loading control.

3.10 Transcription properties of 18E2 in 293T and C33a cells.

Plasmids expressing each protein were transiently co-transfected into 293T and C33a cells with an E2-responsive reporter construct consisting of six E2 binding sites upstream of the minimal thymidine kinase promoter (ptk6E2) and firefly luciferase gene. The results from three independent experiments were averaged and normalised to ptk6E2, which in the absence of an E2 expression vector there is very little reporter activity.

Figure 3.11a shows 18E2 mediated transcription activation from the tk6E2 promoter in 293T cells and **Figure 3.11b** shows HPV18 E2 mediated transcription in C33a cells. In both 293T and C33a cells, $18E2^{-WT}$ induces transcriptional activation from a tk promoter (lanes 1-3), and this activation increases as the concentration of E2 input increases (500ng, 1000ng and 2000ng). Unlike $16E2^{-WT}$, higher concentrations of input DNA do not "squelch" activation of $18E2^{-WT}$. The ability of the two E2 mutants to activate transcription is severely compromised compared to $E2^{-WT}$, although Brd4 is slightly more compromised than TopBP1. This defect is more pronounced with increasing levels of E2. In **Figure 3.11a**, $E2^{-TopBP1}$ (lanes 4-6) has compromised replication at lower levels of E2, 26.8% of $E2^{-WT}$ and does not regain transcriptional activation at higher levels of E2 input as there is decrease to 19% of $E2^{-WT}$ at 1000ng. $E2^{-Brd4}$ (lanes 7-9) can activate transcription to 19% of what $E2^{-WT}$ levels at low concentrations of E2 and this activation decreases to 14% at higher concentrations (1000ng). In Figure **3.11b**, $E2^{-TopBP1}$ replication (lanes 4-6) drops from 31% of $E2^{-WT}$ at 10ng to 15% at 1000ng. The same is true for $E2^{-Brd4}$ (lanes 7-9) which drops from 34% compared to $E2^{-WT}$ at 10ng to 12.6 % at 1000ng of E2 input.

3.11 18E2 wild-type and mutants cannot repress transcription

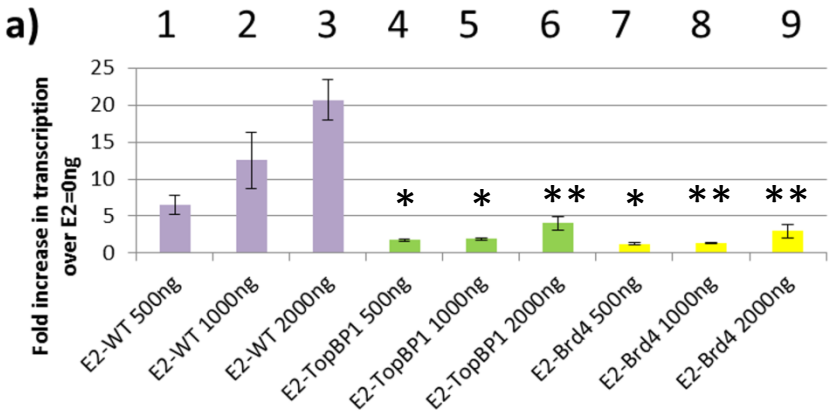
HPV E2 can act as a transcriptional repressor of papillomavirus control regions, and the ability of 18E2 and mutants to repress the HPV18 long control region (18LCR) was tested in **Figure 3.12**. Repression assays were carried out in C33a cells, and the results were averaged and normalised to 18LCR. 18E2 wildtype

and mutants cannot repress transcription from the HPV18 LCR **(lanes 1-9)**. 18E2 does work in C33a cells as transcriptional activation in this cell line is similar to results seen in 293T cells, so these results are valid.

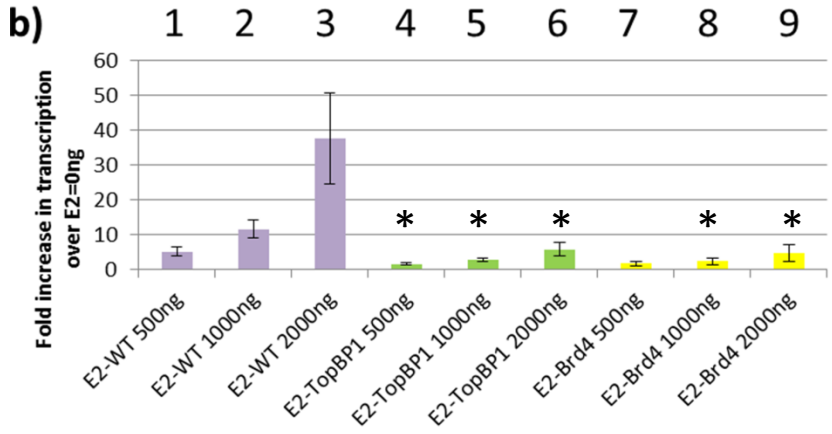
3.12 18 E2$^{-TopBP1}$ has compromised replication and 18 E2^{-Brd4} replicates better than 18 E2^{-WT} in 293T and C33a cells

Replication assays were carried out in 293T and C33a cells to investigate the role of TopBP1 and Brd4 on HPV18 E2 replication function (**Figure 3.13**). Results are presented on a log-scale and E2^{-WT} levels set to 1 and the level of replication induced by mutants set relative to this. In **Figure 3.13a** and **Figure 3.13b**, E2^{-WT} replication levels increase as the concentration of E2 input increases (10ng, 100ng and 1000ng, lanes 1,2 and 3 respectively). In **Figure 3.13a**, at all concentrations of E2 expression plasmid (10ng, 100ng and 1000ng), E2$^{-TopBP1}$ is severely compromised in replication abilities compared to E2^{-WT}, 4.7% of E2^{-WT} at 10ng and 0.1% at 1000ng. However, unlike the results seen with 16E2^{-Brd4}, 18E2^{-Brd4} replication is similar to E2^{-WT} at all concentrations of input plasmid, 66% at 10ng compared to E2^{-WT} and optimal replication ability of the E2^{-Brd4} mutant are observed at 100ng, 145% of E2^{-WT}. In **Figure 3.13b**, lanes 4-6 show replication ability of the E2$^{-TopBP1}$ mutant, which displays compromised replication at all levels of E2 input compared to E2^{-WT} at all concentrations of E2 input, this defect is more pronounced in C33a cells compared to 293T. Similarly the E2^{-Brd4} phenotype is more pronounced in C33a cells, at the optimal level of E2 for replication with this mutant (100ng), E2^{-Brd4} replicates 664.8% greater than E2^{-WT}.

Figure 3.11



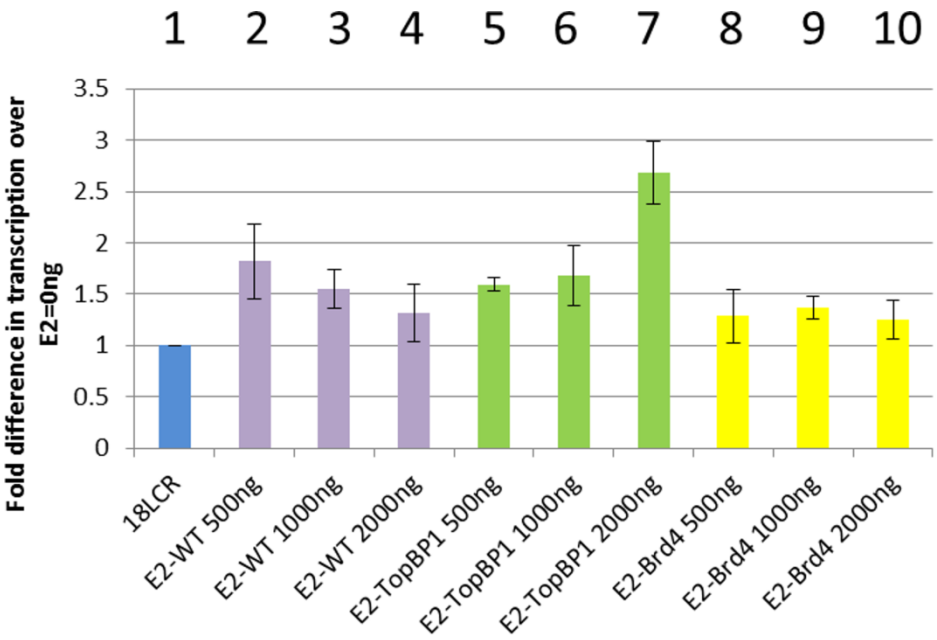
Plasmid	500ng	1000ng	2000ng
E2^{-WT}	6.5	12.5	20.7
E2$^{-TopBP1}$	1.7	1.9	4.0
E2^{-Brd4}	1.2	1.3	3.0



Plasmid	10ng	100ng	1000ng
E2^{-WT}	5.0	11.6	37.5
E2$^{-TopBP1}$	1.6	2.7	5.7
E2^{-Brd4}	1.7	2.3	4.7

Figure 3.11: HPV18 E2 transcription activation in 293T and C33a cells. Transcription assays were carried out in 293T **(a)** and C33a cells **(b)** with input levels of wild-type 18E2 and mutant E2 of 500ng, 1000ng and 2000ng, along with 1µg of ptk6E2, an E2 reporter containing 6E2 DNA binding sites upstream from a tk promoter driving luciferase (Vance, Campo et al. 1999). Cells were harvested and luciferase and protein assays carried out (as described in **Methods section 2.2.1.5 and 2.2.1.13**). The results are normalised to protein levels in each sample and are represented as fold increase over ptk6E2 levels in the absence of E2. The results are representative of three independent experiments done in duplicate. Bars represent standard error. Fold activation in transcription over that obtained when no E2 was co-expressed is shown in the table below. Significant results from student T-test are displayed on the graph. * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3.12

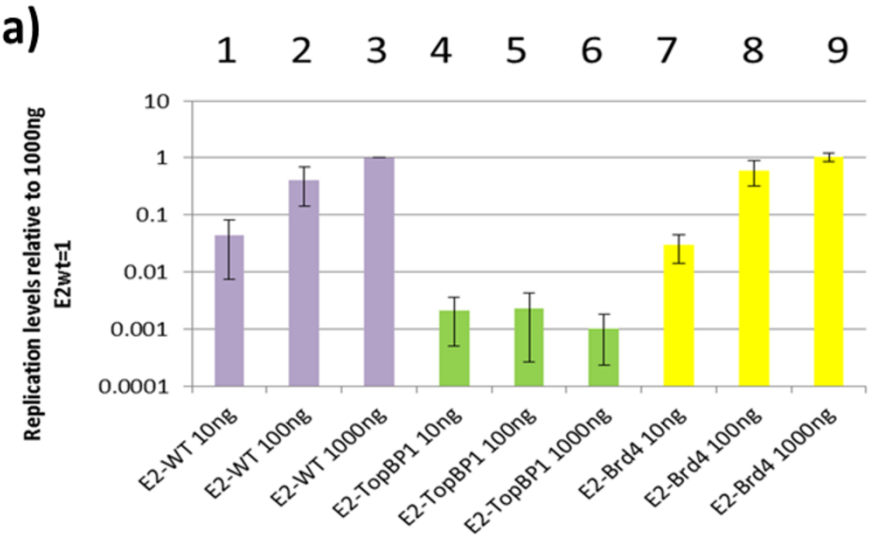


Plasmid	500ng	1000ng	2000ng
$E2^{-WT}$	1.819135	1.552602	1.316991
$E2^{-TopBP1}$	1.593976	1.684541	2.68649
$E2^{-Brd4}$	1.290142	1.374809	1.250008

Figure 3.12: 18E2 cannot repress transcription in C33a cells. Wild-type and mutant E2 plasmids were titrated into C33a cells along with 1µg of pHPV18LCR-luc (long control region) reporter containing the origin of replication (as described in **Methods section 2.2.1.13**). Cells were harvested and luciferase and protein assays carried out. The results are normalised to protein levels in each sample and are represented as fold increase over 18LCR. The results are representative of three independent experiments done in duplicate, values shown in table below. Bars represent standard error. No significant results.

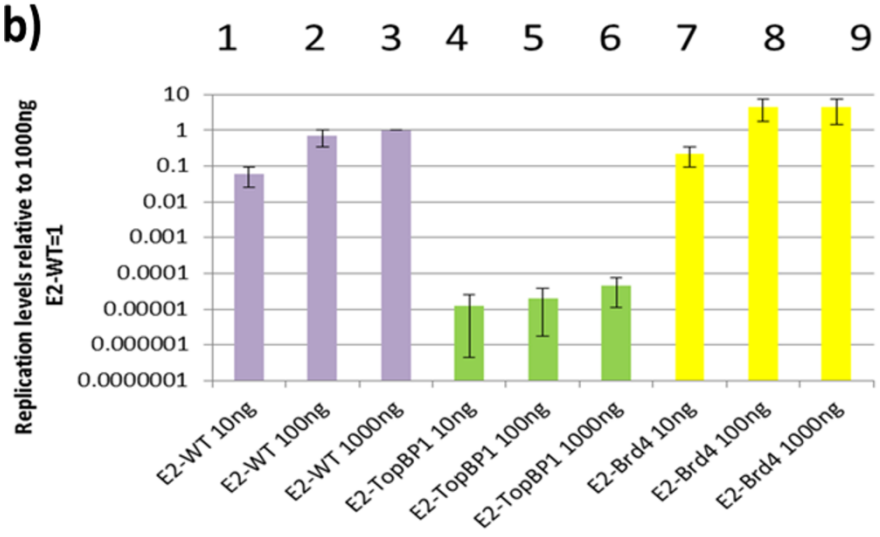
Figure 3.13

a)



Plasmid	10ng	100ng	1000ng
E2-WT	0.044851	0.412256	1
E2-TopBP1	0.002094	0.002275	0.001038
E2-Brd4	0.029856	0.598603	1.029833

b)



Plasmid	10ng	100ng	1000ng
E2-WT	0.058481	0.683942	1
E2-TopBP1	1.26E-05	1.97E-05	4.45E-05
E2-Brd4	0.218031	4.546511	4.328683

Figure 3.13: E1-E2 mediated DNA replication of 18E2 in 293T cells. 293T cells were co-transfected with various concentrations of E2 input DNA (10ng, 100ng and 1000ng) along with 100pg of the viral origin of replication (ori) and 1µg of HA-E1. Low molecular weight DNA was harvested using HIRT buffer and fresh replication assays as described in **Methods section 2.2.1.14**. Results shown are expressed relative to the wild-type E2 levels equaling 1; and are representative of three independent experiments, numbers shown in table below. Bars represent standard error. No significant results.

3.13 Chapter summary and discussion

As described in Chapter 1 the viral E2 protein regulates transcription from and replication of the viral genome, in association with viral and cellular factors. This is an essential for the continuation of the viral life cycle, making E2 a desirable antiviral target. Two essential host cellular factors that E2 interacts with to promote viral transcription and replication are TopBP1 and Brd4.

TopBP1 has been shown to interact *in vitro* and *in vivo* with E2 and can regulate the ability of E2 to control transcription and replication (Boner, Taylor et al. 2002). Previously our lab characterised a mutant of 16E2 which does not bind TopBP1 ($E2^{-TopBP1}$) which has compromised replication in association with E1 but retains the ability to activate transcription, demonstrating that this mutant can bind target sequences and is correctly folded and functionally active in mammalian cells (Donaldson, Mackintosh et al. 2012). The model proposed by the Morgan lab is that the DNA binding proteins E1 and E2 both form a complex with TopBP1, and TopBP1 is involved in loading DNA polymerases onto the E1 helicase in mammalian cells resulting in replication initiation. Brd4 is also known to interact with E2, and this interaction is present in many HPV types (16,18,31,6,8). Brd4 is believed to be part of a "transcription core", made up of a number of host cellular genes, which are essential for transcriptional functions of all E2 proteins. Brd4's involvement in HPV transcription is well documented (Schweiger, You et al. 2006, Schweiger, Ottinger et al. 2007), and its key role in the HPV viral life cycle makes it a valuable target for antiviral therapies.

In this report I determine the functional effects of two E2 mutants, $E2^{-TopBP1}$ and $E2^{-Brd4}$, which fail to bind to TopBP1 and Brd4 respectively. The previous reports investigating the role of Brd4 in viral replication, have failed to keep experimental conditions similar. Different cell types, different E2 proteins, and different expression vectors were all used to quantify DNA replication.

3.13.1 Mutants of HPV16 E2 compromise viral transcription

In **Chapter 3.2 and 3.4**, the effects of two E2 mutants were determined, $E2^{-TopBP1}$ and $E2^{-Brd4}$, which fail to bind TopBP1 and Brd4 respectively, on HPV 16 transcription in two model cell lines.

Transcription results in 293T cells for HPV16 E2 and mutants are in agreement with previous publications (Donaldson, Mackintosh et al. 2012, Schweiger, You et al. 2006), $E2^{-TopBP1}$ and $E2^{-Brd4}$ are compromised in activating transcription, as they only activate transcription from the ptK6E2 promoter 8% and 22% respectively (Figure 3.2). When $E2^{-TopBP1}$ is overexpressed it regains almost wildtype activation, 54% of $E2^{-WT.}$ This suggests that the $E2^{-TopBP1}$ mutant can still interact with Brd4, which is known to be essential for transcriptional function of HPV16 E2 in 293T cells (Schweiger, You et al. 2006). $E2^{-Brd4}$ does not regain this function when overexpressed, which is expected due to its essential role in viral transcription, 11.4% of $E2^{-WT}$. This also confirms that Brd4 plays the more dominant role out of the proteins in the complex for this function.

Results in the C33a cell line show a similar defect, whereas at higher concentrations of E2 input $E2^{-Brd4}$ shows a more severe phenotype compared to $E2^{-TopBP1}$, $E2^{-Brd4}$ only activates transcription by 7.7% of what is seen in $E2^{-WT}$. conditions, compared to 68.7% for $E2^{-TopBP1}$ (Figure 3.4a). The defect displayed by $E2^{-Brd4}$ in transcription activation abilities can be supported by various studies that show the E2 stability is enhanced by Brd4 (Li, Li et al. 2014) and inhibiting the interaction of E2-Brd4 with Brd4-CTD or knocking down Brd4 with siRNA for Brd4 reduces the E2 dependent transcription activation (Schweiger, You et al. 2006), additionally JQ1 (+) treatment (dissociates E2-Brd4 complexes from chromatin) potently reduces transcription (Helfer, Yan et al. 2014).

HPV E2 is known to mediate transcriptional repression as well as activation. The repression of HPV LCR by $E2^{-TopBP1}$ and $E2^{-Brd4}$ is lessened by the failure of E2 to interact with these host proteins. Previously published results from the Morgan lab showed no difference in E2-mediated repression of the $E2^{-TopBP1}$ mutant in 293T cells (Donaldson, Mackintosh et al. 2012). However, the slight differences in repression found in this study may be explained by the use of C33a cells.

Studies from other labs have shown the knockdown of Brd4 in human cells has been shown to alleviate E2-mediated repression of HPV transcription (Wu, Lee et al. 2006b). This is somewhat in agreement with the results observed in C33a cells. It is proposed that there is an acetylation-dependent role of Brd4 in the recruitment of E2 for transcriptional silencing of HPV gene activity (Wu, Lee et al. 2006b).

3.13.2 Mutants of HPV16 E2 compromise viral replication

Previous replication studies have employed a Southern blot technique to quantify levels of replication, which is limited in its ability to measure E1-E2 mediated DNA replication. In this study a more sensitive real-time PCR assay for the detection of E1-E2 replication was utilised, which can detect a wider range of levels of replication than the traditional Southern blot technique.

Previous work from the Morgan lab has shown that the interaction between E2 and the host protein TopBP1 is essential for E1-E2 replication in 293T cells (Donaldson, Mackintosh et al. 2012). The results from assays conducted in this thesis are in agreement with this original finding. $E2^{-TopBP1}$ displays compromised replication compared to $E2^{-WT}$ and this defect is more pronounced at lower levels of E2 input, at 10ng $E2^{-TopBP1}$ only has 28.7% replicative function as $E2^{-WT}$. Function is regained by both mutants when E2 is overexpressed but not to $E2^{-WT}$ levels. This was also seen in C33a cells.

Additionally, in this thesis work, the role of Brd4 in HPV replication was investigated. As well as the known role of Brd4 in viral transcription, new evidence suggests that an interaction between E2 and Brd4 may be required for E1-E2 mediated DNA replication (Wang, Helfer et al. 2013). In HPV16, Brd4 has been shown to be recruited to active replication origin foci with E2 and E1 and a number of other cellular replication factors (Wang, Helfer et al. 2013). Additionally, mutagenesis disrupting the interaction between E2 and Brd4, and siRNA silencing of Brd4, have both been shown to impair HPV16 replication ability (Wang, Helfer et al. 2013).

$E2^{-Brd4}$ was similarly compromised in its ability to replicate at 10ng of input DNA, as the $E2^{-TopBP1}$ mutant. However, at the higher input concentration of 100ng, the ability of the $E2^{-Brd4}$ mutant was greater than $E2^{-WT}$. Due to the highly sensitive nature of our replication assay, we are able to see this defect in $E2^{-Brd4}$ replication, at lower concentrations of DNA input. It should be noted that at higher levels of input DNA, the defect is not so defined and less sensitive techniques such as southern blots will not be able to detect the replication defect.

3.13.3 Brd4 and TopBP1 proteins are recruited to the viral origin of replication in an E1-E2 dependent manner

In our replication assay experiments, the interaction between TopBP1/Brd4 with HPV16 E2 was shown to be required for viral replication. However, it was unknown whether these host cellular factors are recruited to the viral origin of replication along E1 and E2. The interaction of the "complex" at the viral origin of replication was investigated using chromatin immunoprecipitation.

A few novel observations were made about HPV replication using this technique: The first notable feature of HPV replication is that E1 and E2 require each other to be present at the origin of replication, neither one is expressed in the absence of the other. Previously, it was believed that E2 would recruit E1 to the viral origin of replication (Ustav, Ustav et al. 1991). Perhaps, E2 does not bind the viral replication origin until it is complexed with E1. Alternatively, it could be suggested that once E1 is recruited to the viral origin there is an enhanced interaction of E2 with viral DNA. The mechanism by which these proteins are recruited the viral origin remains unclear.

The second novel finding from ChIP experiments is that TopBP1 and Brd4 locate to the viral origin of replication in an E1-E2 dependent manner. This result confirms that both TopBP1 and Brd4 are required for HPV replication. It may also suggest that TopBP1/Brd4 complex could act as a chaperone to bring the viral E1-E2 proteins to the viral origin. Further optimisation of the ChIP technique is required to test whether this is true for the E2 mutants, $E2^{-TopBP1}$ and $E2^{-Brd4}$.

3.13.4 shRNA knockdown of TopBP1 and Brd4 destroys E1-E2 nuclear foci but has no effect on DNA replication

In **Chapter 3.7** Mary Donaldson showed that TopBP1 and Brd4 are present in E1-E2 induced nuclear foci when co-expressed with the viral origin of replication, in C33a cells. The E2 mutants, $E2^{-TopBP1}$ and $E2^{-Brd4}$ were then tested for their ability to form nuclear foci in **Chapter 3.8**. Mary Donaldson clearly demonstrated that the ability of these E2 mutants to form nuclear structures was compromised. This defect is particularly noticeable at lower concentrations of E2 expression, giving a correlation between nuclear foci formation and DNA replication (as seen in **Figure 3.5a**).

To follow up from this finding of TopBP1 and Brd4 being present in these replication foci, it was decided to see if the role these host proteins is critical for the initiation of viral replication. E1-E2 mediated DNA replication initiated in conjunction with the host proteins TopBP1 and Brd4 is not affected by shRNA knockdown of either TopBP1 or Brd4. However, knocking down TopBP1 or Brd4 completely destroyed the formation of E1-E2-pOri nuclear foci formation. Perhaps the interaction of TopBP1 and Brd4 are required for the initiation of replication but once initiated they are no longer needed, which would explain by knocking down TopBP1 and Brd4 after initiation of replication had no effect.

Supporting evidence for this argument comes from a recent report by (Sakakibara, Chen et al. 2013a) who demonstrated that Brd4 is in the heart of replication foci induced by E1-E2 but after initiation Brd4 becomes peripheral to the foci.

3.13.5 Conclusions

Novel findings were made that enhance our understanding of DNA replication by human papillomavirus 16 E1 and E2. (1) Our replication assays showed that $E2^{-Brd4}$ is severely compromised in DNA replication ability which suggests that Brd4 is required for efficient initiation of E1-E2 mediated DNA replication. (2) E1 and E2 co-operate to interact at the viral origin as neither is present at the origin in the absence of the other. Previously it was thought that E2 could bind to the origin of replication independently of E1, but this was not reflected in the ChIP

assay results. We propose that E1 may stabilise the interaction of E2 with the origin allowing for detection in ChIP assays, similar to how E1 stabilises the E2 protein (King, Dornan et al. 2011). (3) ChIP was used to demonstrate the presence of both TopBP1 and Brd4 at the viral origin of replication in an E1-E2 dependent manner. (4) TopBP1 and Brd4 were also shown within E1-E2 foci and shRNA targeting the expression of the cellular proteins destroys these replication foci. (5) Although the replication foci are destroyed by shRNA targeting the expression of TopBP1 and Brd4, the levels of DNA replication are not affected, suggesting that replication is not dependent upon these foci.

Overall, these initial findings indicate that TopBP1 and Brd4 are located in the same cellular complex, and that the TopBP1-Brd4 complex may recruit E2 and the HPV genomes to areas of host chromatin that would stimulate viral replication following entry into S phase. Previous work from the McBride group has shown that E2 and Brd4 are located on similar host regions of chromatin, known for viral genome replication, implicating Brd4 in E1-E2 mediated DNA replication (Jang, Shen et al. 2014). Additionally, E2 also co-localises with TopBP1 in late mitosis, allowing the HPV genome to interact with a number of host replication factors (Donaldson, Boner et al. 2007).

The co-localisation of the E1-E2-TopBP1-Brd4 complex is required for the initiation of replication. If this failed perhaps there would be a mislocalisation of the viral genome to inappropriate locations in the host chromatin, which could affect the quality of the replication. We currently do not understand what will happen to the quality of the replication in these conditions and further work is needed in this area. Also, we do not fully understand the mechanisms of replication used by the virus at different stages within the life cycle. As was demonstrated in **Figure 3.8.2**, the complex is not required for the continuation of replication, only for initiation, this suggests the mode of replication switches.

As described in Chapter 1, homologous recombination is the proposed method of viral amplification throughout the lifecycle (Gillespie, Mehta et al. 2012) (Sakakibara, Chen et al. 2013b), and the virus promotes DNA damage response to promote amplification of the viral genome (Moody, Laimins 2009).The results support this model as it has been shown that both TopBP1 and Brd4 are required

for initiation for viral replication, but not the continuation. Suggesting that Brd4 and TopBP1 are not required for the homologous recombination mechanism of replication. Although, it should be noted TopBP1 has been suggested to be involved in this process (Morishima, Sakamoto et al. 2007) (Germann, Oestergaard et al. 2011).

3.14 HPV18 replication and transcription

A study from the Bodily lab showed that the Brd4 mutant in HPV31 could maintain viral episomes, whereas the same mutant in HPV16 failed to do so (Gauson, Donaldson et al. 2015). This result suggests that there are differences between HPV types and their requirement for Brd4, and possibly TopBP1, for genome replication and establishment. This highlights the need to address questions about viral replication of a specific HPV type in a consistent manner.

As past studies have focused on 16E2, not much work has been done to understand the transcription and replication function of HPV18 E2, in relation to E2's ability to bind TopBP1 and Brd4. Due to the differences seen with HPV31, it was expected that HPV18 may also demonstrate key differences.

3.14.1 Mutants of HPV18 E2 compromise viral transcription

Activation defects are similar for both $E2^{-TopBP1}$ and $E2^{-Brd4}$ mutants in HPV18, $E2^{-Brd4}$ is less compromised in HPV18 than HPV16, this is true at all levels of E2 input, and both model cell lines. This would suggest that Brd4 may not play the most dominant role in HPV18 transcriptional activation. Perhaps HPV18 is complexed with other transcription factors to facilitate effective transcription.

A novel difference between HPV16 and HPV18 is that HPV18 E2 cannot repress transcription, unlike HPV 16E2 which does repress transcription. In HPV18 the failure to repress transcription is independent of its interaction with TopBP1 and Brd4. The differences in the mechanism used by HPV 18 is not yet understood an further studies are required.

3.14.2 Mutants of HPV18 E2 compromise viral replication

As with HPV18 transcription, there have been no previous studies on the effect of 18 E2 mutants on viral replication. These assays were carried out in both 293T and C33a cells.

In HPV18, the $E2^{-TopBP1}$ mutant exhibited severely compromised replication compared to 16 $E2^{-TopBP1}$. As seen in Figure 3.10, $E2^{-TopBP1}$ fails to replicate at all levels of E2 input, the defect worsens as the concentration increases, the replicative ability goes from 4.7% at with 10ng of input and drops to 0.1% with 1000ng of input (in 293T cells). However, in contrast to this 16 $E2^{-TopBP1}$ can still replicate, although at lower levels than $E2^{-WT}$. Perhaps 18E2 relies solely on the interaction with TopBP1 for viral replication, whereas 16E2 may interact with other host proteins to aid in replicative function, such as Brd4. E2 is known to interact with a number of host proteins, however the roles these interactions play is currently understudied. The interaction of E2 with TopBP1 stabilises E2 in S phase explaining the replication defect seen in our assays (Donaldson, Boner et al. 2007).

Despite $E2^{-TopBP1}$ being extremely compromised in HPV18, $E2^{-Brd4}$ can replicate better than $E2^{-WT}$ (**Figure 3.13**), this phenotype is more apparent at higher concentrations of E2, in both 293T and C33a cells. Replication of $E2^{-Brd4}$ is optimal at 100ng of DNA input in both cell lines, 154% in 293T and 664.7% in C33a cells compared to $E2^{-WT}$.

All of these differences seen between HPV16 and 18 E2 transcription and replication function indicates that they interact with host proteins in a different manner to carry out the viral life cycle.

3.14.3 Summary of HPV18 results

Major differences between HPV16 E2 and 18 E2 have been observed in this study; (1) $18E2^{-TopBP1}$ mediated DNA replication is severely compromised compared to 16 $E2^{-TopBP1}$. (2) $18E2^{-Brd4}$ can activate transcription stronger activation of transcription than $16E2^{-Brd4}$. (3) 18E2 does not repress transcription unlike 16E2 which does repress transcription. (4) 16 $E2^{-Brd4}$ is compromised in DNA replication

function in a cell type specific manner. (5) 18 $E2^{-Brd4}$ is not compromised in DNA replication in any cell type.

Further experiments similar to those done with 16 E2 and mutants are required to investigate whether these host cellular factors are playing a different role in viral replication. This emphasises the need to make conclusions about viral replication in a very consistent manner, using the same HPV type, assays, and even concentrations of plasmid in each assay. This also emphasises the need to gain an antiviral target that is pan-type specific to many HPV types. Perhaps TopBP1 offers to be this target?

Chapter 4 – Regulation of human genome expression by HPV16 E2

4.1 Wildtype E2 regulates host genes

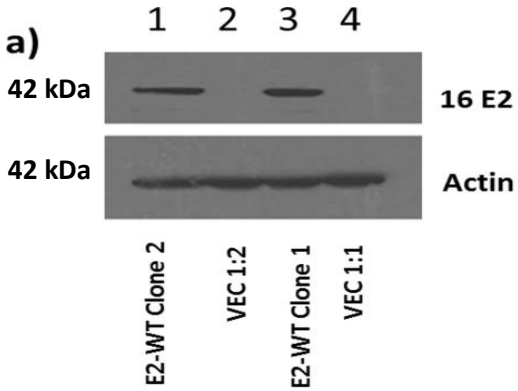
Following the findings of the previous chapter which focused on how TopBP1 and Brd4 interact with E2 to regulate the viral genome, further investigation of how these host proteins interact with viral E2 to regulate the host genome was carried out. The proposed hypothesis is that E2 manipulates host gene expression to facilitate infection and the viral life cycle.

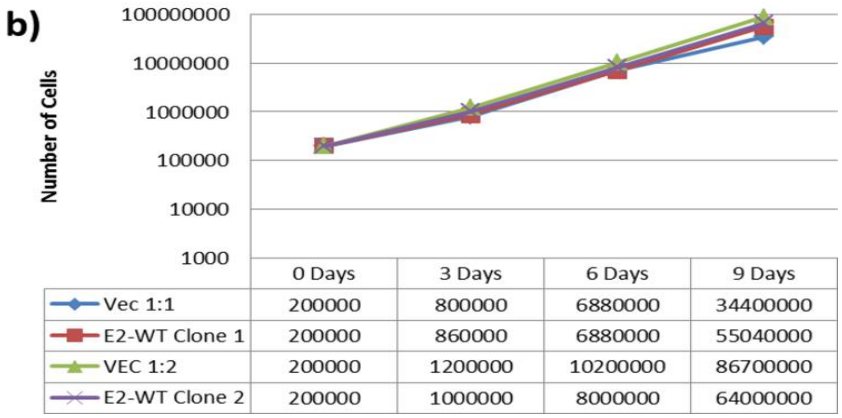
4.1.1 Generation of U20S cells expressing HPV16 E2

To address this question, U20S cells which are derived from an osteosarcoma were chosen as a model system. These cells provide a good model for the investigation of the regulation of the host genome by E2. U20S cells are p53 wildtype and tolerate E2 expression. Similar stable clones expressing E2 have been made previously using the U20S cell line, and are therefore known to tolerate physiological levels of HPV16 E2 (Taylor, Boner et al. 2003). The Ustav lab have also used U20S cells as a model to study the replication of low and high-risk cutaneous HPV (Reinson, Toots et al. 2013, Geimanen, Isok-Paas et al. 2011), as well as analyse the transcriptome of HPV18. They did not observe any changes in the promoter, splice site, or polyadenylation usage in U20S when compared to keritinocytes (Wang, Meyers et al. 2011). U20S cells are a valuable tool for investigating the E2 interaction with the host chromatin and histone modifications, as ENCODE data is readily available for this cell line.

U20S cell lines that stably express $16E2^{-WT}$ were generated for use in gene expression studies, using a similar protocol to that previously published (Taylor, Boner et al. 2003) (as described in **Methods section 2.2.2.2**). Stable colonies following transfection were isolated and expanded and screened for E2 expression (**Figure 4.1.1a**). Growth of these E2 expressing clones was monitored and recorded by carrying out growth curves with two separate clones, which show no difference in the growth rates of non-E2 and E2 expressing U20S cells (**Figure 4.1.1b**).

Figure 4.1.1





	0 Days	3 Days	6 Days	9 Days
Vec 1:1	200000	800000	6880000	34400000
E2-WT Clone 1	200000	860000	6880000	55040000
VEC 1:2	200000	1200000	10200000	86700000
E2-WT Clone 2	200000	1000000	8000000	64000000

Figure 4.1.1: Generating and characterising stably expressing E2 clones.

a) Potential E2 expressing clones were screened by making lysates for western blot analysis (as described in **Methods section 2.2.1.6**). Lanes 1 and 3 show cells which stably express HPV 16E2 protein and lanes 2 and 4 show non-E2 expressing vector controls.

b) $4x10^5$ cells were seeded and counted every 3 days, growth curves were generated from the numbers of cells from each count (as described in **Methods section 2.2.2.3**). The growth curve displays no difference in the growth rate between the E2 and non-E2 expressing clones.

4.1.2 Identifying differentially expressed host genes induced by E2

To investigate the regulation of the host genome by viral E2, three independent polyA+ RNA preparations were made and converted into cDNA (as described in **Methods section 2.2.1.10** and **2.2.1.11**) and analysed using Affymetrix Human Exon Array (as described in **Methods section 2.2.3.1**). From this point forward this shall be known as "array 1". The aim of this study was to identify the cellular genes E2 regulates in the host genome and whether mutations in E2 alter this regulation. Analysis of the array was carried out by Pawel Herzyk using Partek genomics suite software, lists of the top 50 up and down regulated genes can be found in the tables below (**Table 4.1.1). (Supplementary Table S.1 displays full list of genes regulated by 16 E2 wildtype ≥ 1.5-fold).**

From the analysis; 74 genes were found to be differentially regulated 2 fold or greater (p-value ≤0.05) when E2 was compared with the vector control (non-E2 expressing); 33 up-regulated and 41 down-regulated. Of the list of 74 E2 regulated genes, ten of the altered genes were selected to validate the array results in two independent clones of E2 (**Table 4.1.2** displays validation). Sybr green real-tume qPCR was used to validate the array gene expression results. The house keeping gene GAPDH was used as an endogenous control alongside the vector, no E2 expressing U20S cell line to normalise the results using the ΔΔct method. In clone 1, 7 out of 10 gene changes validated while in clone 2, 10 out of 10 tested validated. This demonstrates that the results generated from the exon array screen were predictive of actual fold gene changes.

Ingenuity Pathway Analysis (IPA) was used to further analyse the 74 genes (as described in **Methods section 2.2.3.2**). 5 networks were found and the functions were associated with the presence of cancer, reproductive system disease, gene expression and DNA replication and repair (**Table 4.1.3**).

Table 4.1.1

Up-regulated by E2	Fold-change	Down-regulated by E2	Fold-change
HIST1H2BM	71.9508	GTSF1	-302.705
HIST1H3H	70.7304	NFE4	-24.794
MAGEC1	40.1869	TMPRSS15	-11.624
SLN	14.5889	SLC14A1	-5.94492
HOXB2	10.631	C1orf85	-4.48436
TNFAIP6	4.86318	CD33	-4.1363
ENG	4.85397	CLIC2	-3.9316
GNG11	4.11702	FBXL13	-3.60857
FAR2	3.70124	PTGFR	-3.59376
ZNF788	3.3983	CSTA	-3.49077
APBA2	2.99782	NLRP5	-3.33859
SERPINA3	2.74046	DNAJC18	-3.31614
C10orf72	2.5905	BMPER	-3.27652
GFPT2	2.53867	HBE1	-3.07671
SH3PXD2B	2.50361	RNF144B	-3.04874
FAP	2.47498	GDF15	-2.98979
LRRCC1	2.36516	LOC79015	-2.97446
ARHGDIB	2.35827	HSD17B8	-2.75366
SNTB1	2.25035	HIST1H3E	-2.69962
EML1	2.23335	MLH3	-2.64666
ARHGAP11A	2.23306	NLRP4	-2.60899
CCDC99	2.16172	TRIML2	-2.55713
FGD4	2.1616	CD68	-2.47599
ZNF271	2.15217	ACYP1	-2.43839
MTMR10	2.11787	PSG9	-2.41357
CALHM2	2.11199	HLA-DPA1	-2.38118
HNMT	2.09265	GNGT2	-2.37174
GOLGA8B	2.08224	FST	-2.35479
AQR	2.05191	GCNT2	-2.3148
NFIA	2.02545	GRAMD3	-2.29601
ZNF770	2.02363	PGAP2	-2.20441
ZADH2	2.01373	RPL23	-2.20367
RTKN2	2.00087	IL1RAPL1	-2.18097
CLEC2B	1.98705	OR10A3	-2.14773
RAB27B	1.97895	GPR65	-2.14222
MYO5B	1.97609	AURKC	-2.13613
TMEM55A	1.96272	ZNF300P1	-2.10876
IMPACT	1.92209	ME3	-2.06315
ZNF738	1.90705	ADRB2	-2.05351
TRPS1	1.88341	PSG4	-2.04519
TTC39C	1.87129	ZFP90	-2.02583
ATPBD4	1.86744	DPYSL4	-1.9764
FAN1	1.86327	TOX	-1.96981
B4GALNT3	1.85095	LTBP2	-1.96341
STK38L	1.83674	IL6	-1.95904
ZNF365	1.82942	CARS	-1.90807
CASC5	1.81003	EIF2B2	-1.89313
FAM60A	1.79363	AOC2	-1.88615
C8orf40	1.79137	MDGA2	-1.88515
NUP210	1.78893	WNT2B	-1.87542

Table 4.1.1: Genes regulated by HPV16 E2 in U20S cells. Human exon array analysis was used to generate lists of genes regulated by HPV16 $E2^{-WT}$. Pawel Herzyk carried out Partek analysis to sort the raw data into lists of genes that had a fold-change (F.C) of ≥ 1.5 and a p-value of ≤0.05. The genes represented in this table display a transcriptional level which has been increased or decreased by the levels indicated. The results are validated in the same clone used for the microarray study, as well as an additional clone to verify the findings.

Table 4.1.2

Gene Name	Fold-change in Array	Fold-change clone 1	Fold-change clone 2
MAGEC1	40.19	190.7 ± 197.63	594.2 ± 522.29
ENG	4.85	11.7 ± 3.90	6.61 ± 11.79
FAR2	3.7	3.78 ± 1.26	2.81 ± 1.28
APBA2	2.99	6.83 ± 11.15	27.09 ± 33.27
SH3PXD2B	2.5	1.93 ± 0.27	3.9 ± 4.43
GDF15	-2.99	-1.24 ± 0.53	-3.47 ± 2.44
RNF144B	-3.05	-1.29 ± 1.10	-6.81 ± 0.84
TMPRSS15	-11.62	-6.22 ± 2.75	-14.32 ± 5.25
NFE4	-24.79	-16.22 ± 6.97	-10.31 ± 3.37
GSTF1	-302.71	-2413.91 ± 1449.10	-2,723.91 ± 1940.0

Table 4.1.2: Validation of genes regulated by HPV16 E2. The table displays the fold-change relative vector (no E2) of 5 up and 5 down-regulated genes that were regulated by 16 $E2^{-WT}$ in the human exon array. Validation was done in two separate clones in triplicate, standard error is shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Table 4.1.3

Network	Associated network functions
1	Protein synthesis; protein degradation; hereditary disorders
2	Tissue morphology; cancer; reproductive system disease
3	Gene expression; cancer; renal and urological disease
4	Hematological system development and function; organismal development; cell morphology
5	Cellular assembly and organization; DNA replication, recombination, repair; gene expression

Table 4.1.3: Top 5 16E2 regulated pathways from Ingenuity Pathway analysis. The 74 $E2^{-WT}$ regulated genes (≥ 2-fold) were subjected to IPA and 5 networks of genes were identified. The table lists the predicted functions with the IPA generated networks. (**Genes associated with this table are shown in Supplementary Table S.2 and p-values associated with functions can be found in Supplementary Table S.13**).

4.1.3 Regulation of cell motility by E2

A number of genes from the microarray gene expression analysis are implicated in cell movement and motility (**Table 4.1.1**). SH3PXD2B, FDG4, FAP and ARHGDIB were all up-regulated in the microarray analysis (**Table 4.1.1**) and may modulate the migration of E2 expressing cells.SH3PXD2B encodes for an adapter protein that is required for podosome formation and is involved in cell adhesion and migration of various cell types (Bogel, Gujdar et al. 2012). FDG4 is involved in the regulation of of the actin cytoskeleton and cell shape, and promotes migration of nasopharyngeal carcinoma cells infected with EBV (Liu, Chen et al. 2012). FAP is thought to be involved in the control of fibroblast growth or epithelial-mesenchymal interactions during development, tissue repair, and epithelial carcinogenesis (Kelly, Huang et al. 2012). ARHGDIB is a RhoGDP dissociation inhibitor that can regulate metastasis, potentially by downregulating adhesion (Griner, Theodorescu 2012).

To investigate how E2 effects cell movement, wound healing assays were carried out over a 24 hour time period in two separate E2 and non-E2 expressing clones. After 24 hours of wound healing only the non-E2 expressing cells had successfully closed the wound, whereas the cells expressing E2 struggled to do so (**Figure 4.1.2**), despite there being no difference in the rates of cell growth, as seen in **Figure 4.1.1a**. To further understand the differences in cell movement, videos were made to monitor the movement over 24 hours, also with two separate clones (available to view online in the following paper (Gauson, Windle et al. 2014)) From the videos it is clear to see that E2 expressing cells have impaired movement, they move in large sheets of cells and do not move unidirectional like non-E2 expressing cells. This accounts for their inability to close the wound in the same time frame as the non-E2 cells.

Figure 4.1.2

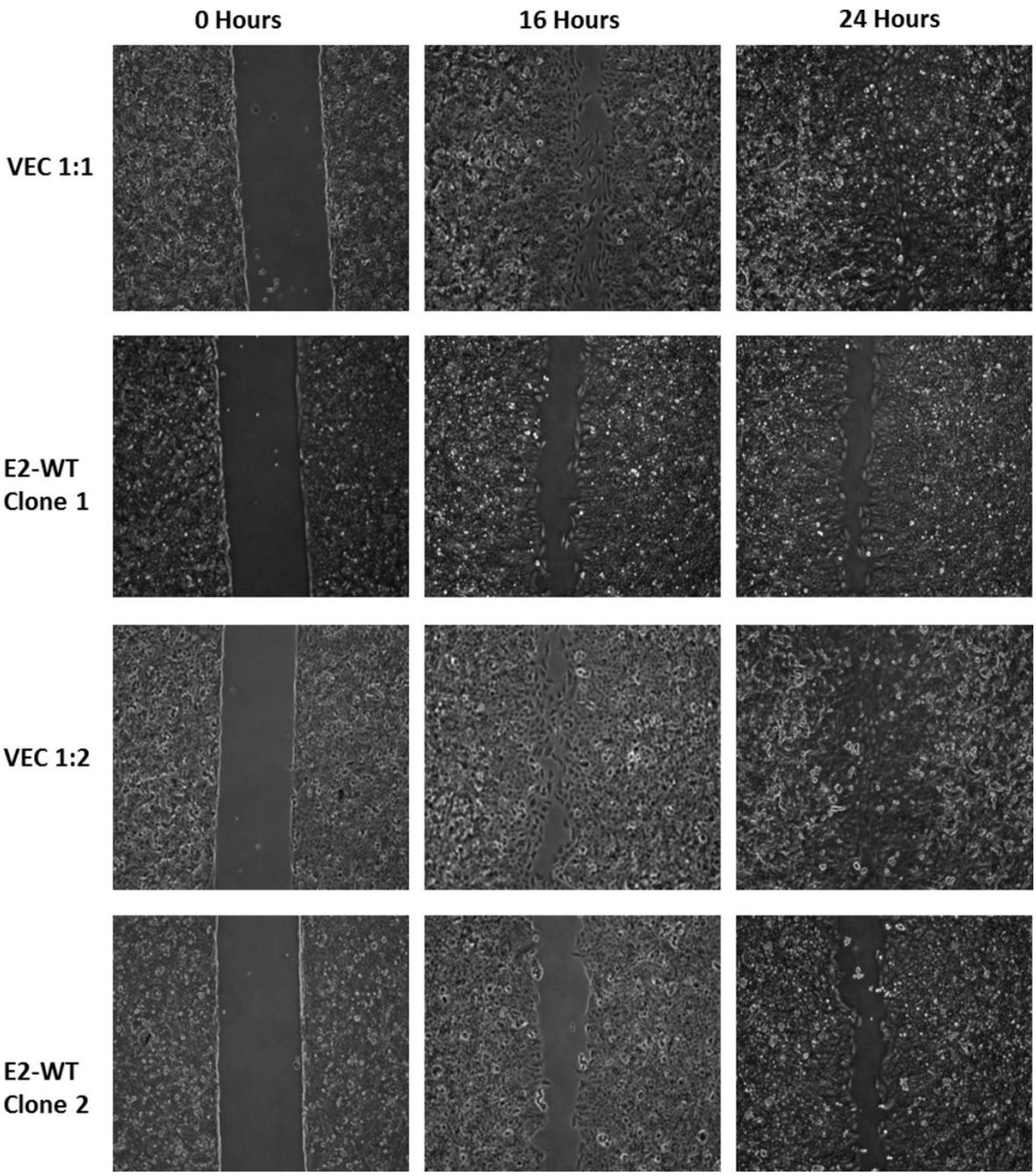


Figure 4.1.2: Wound healing and cell movement of HPV16 E2. $5x10^4$ cells were seeded into cell chambers and grown for 24 hours. 24 hours after seeding the chambers were removed and movement of E2 expressing cells vs non E2-expressing cells was monitored by microscopy and images were collected at 0, 16 and 24 hour time points (as described in **Methods section 2.2.2.4**). Two separate clones for each condition were monitored. Both $E2^{-WT}$ clones clearly show a defect in movement and their ability to close the gap in the 24 hour time period, whereas both no E2 clones have closed the gap by the 24 hour time point.

Figure 4.1.3

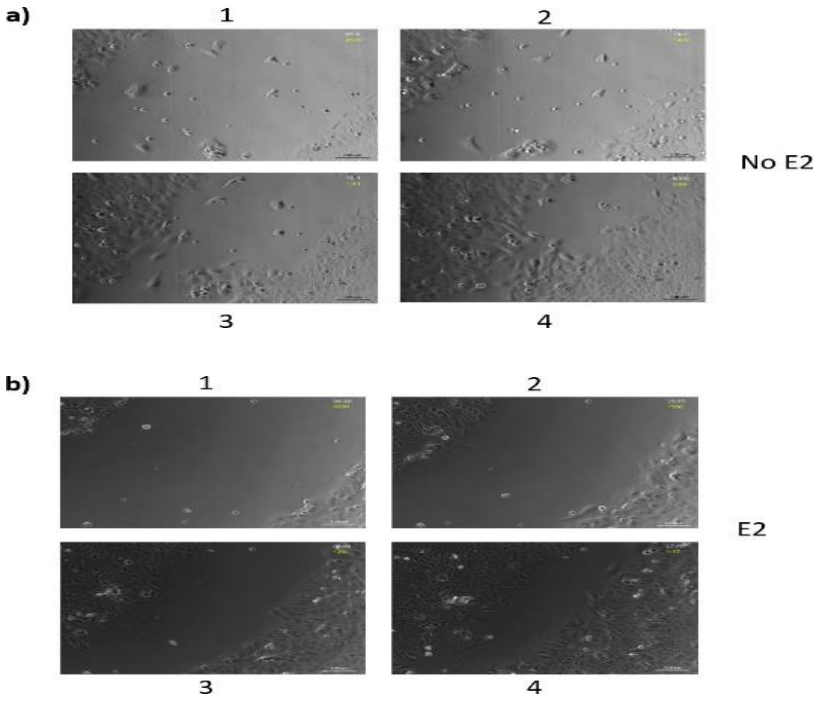


Figure 4.1.3: Still images of E2 and non-E2 expressing cells movement.

a) Cell movement by U2OS Vec (No E2 expression) cells. A monolayer of U2OS cells was scratched with a 20µl pipette tip to create a “wound”. The movement of the cells to heal the “wound” was followed over a 24 hour period. There is random movement of the cells at the leading edge to move forward into the empty space created by the scratch.

b) Cell movement by U2OS E2 expressing cells. A monolayer of U2OS cells was scratched with a 20µl pipette tip to create a “wound”. The movement of the cells to heal the “wound” was followed for 24 hours as before. The E2 expressing cells are more coordinated and tend to move into the gap created by the scratch together in sheets. Note this assay did not measure the size of the “wound”, but only demonstated the mechanism of movement of these cell lines.

4.2 The role of Brd4 in E2 regulation of host genes

As demonstrated in Chapter 3, Brd4 is a key player in HPV transcription and E2 mutants that fail to bind Brd4 have severely compromised transcription activation capabilities, in both 293T and C33a cells. Additionally, Brd4 is also involved in HPV16 E1-E2 mediated replication, and is likely required for the efficient initiation of viral replication. As Brd4 plays key roles in the viral life cycle we wanted to address the question of how E2 regulates the host genome in conjunction with Brd4, and would E2's regulation of the host genome change if it fails to interact with Brd4. This may allow us to better understand how E2 regulates the viral life cycle as well as how E2 regulates viral and host gene regulation.

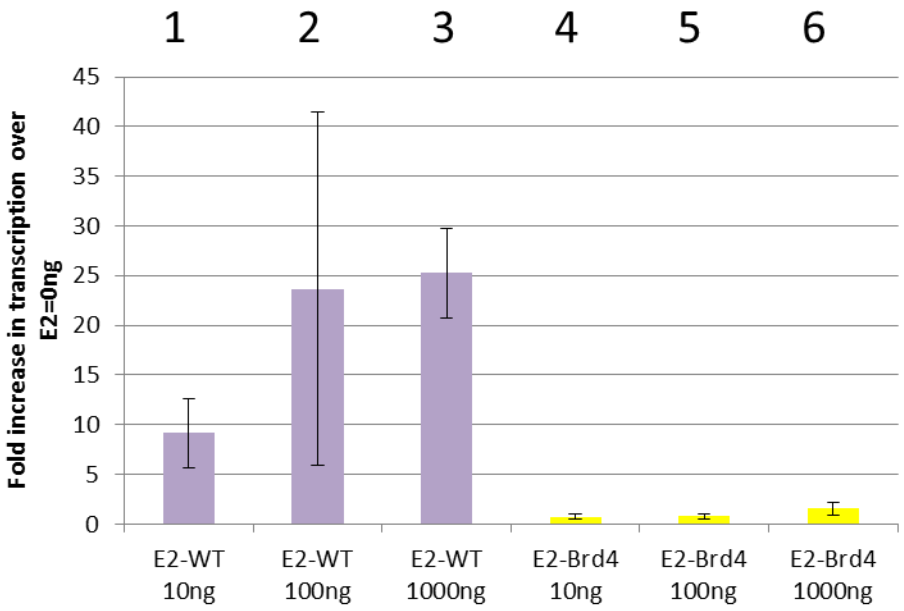
4.2.1 $E2^{-Brd4}$ fails to activate transcription in U20S cells

To address this question a human exon array study that mirrors the previous study in $16E2^{-WT}$ was set up, this shall be known as "array 2" from this point forward. Firstly, the ability of the $E2^{-Brd4}$ mutant to activate transcription in U20S cells was tested. The results demonstrate that $E2^{-Brd4}$ is severely compromised in its ability to activate transcription from the ptk6E2 promoter, at all levels of E2 DNA plasmid input (**Figure 4.2.1**). This is similar to the results previously obtained in 293T and C33a cells in Chapter 3 (**Figure 3.2 and 3.4**).

4.2.2 Generation of U20S cells expressing $16E2^{-Brd4}$

This allowed us to utilise the U20S model to answer the question of how the interaction of Brd4 and E2 affects the regulation of the host genome. To do this, U20S cells that stably express $E2^{-Brd4}$ were generated in the same way as $E2^{-WT}$ cells (as described in **Methods section 2.2.2.2**). Expression of potential clones was tested using western blot analysis, as seen in **Figure 4.2.2a**. The growth of these cells was also monitored by a growth curve, and no significant difference in growth rate was seen between $E2^{-Brd4}$, $E2^{-WT}$ and non-E2 expressing U20S cells (**Figure 4.2.2b**).

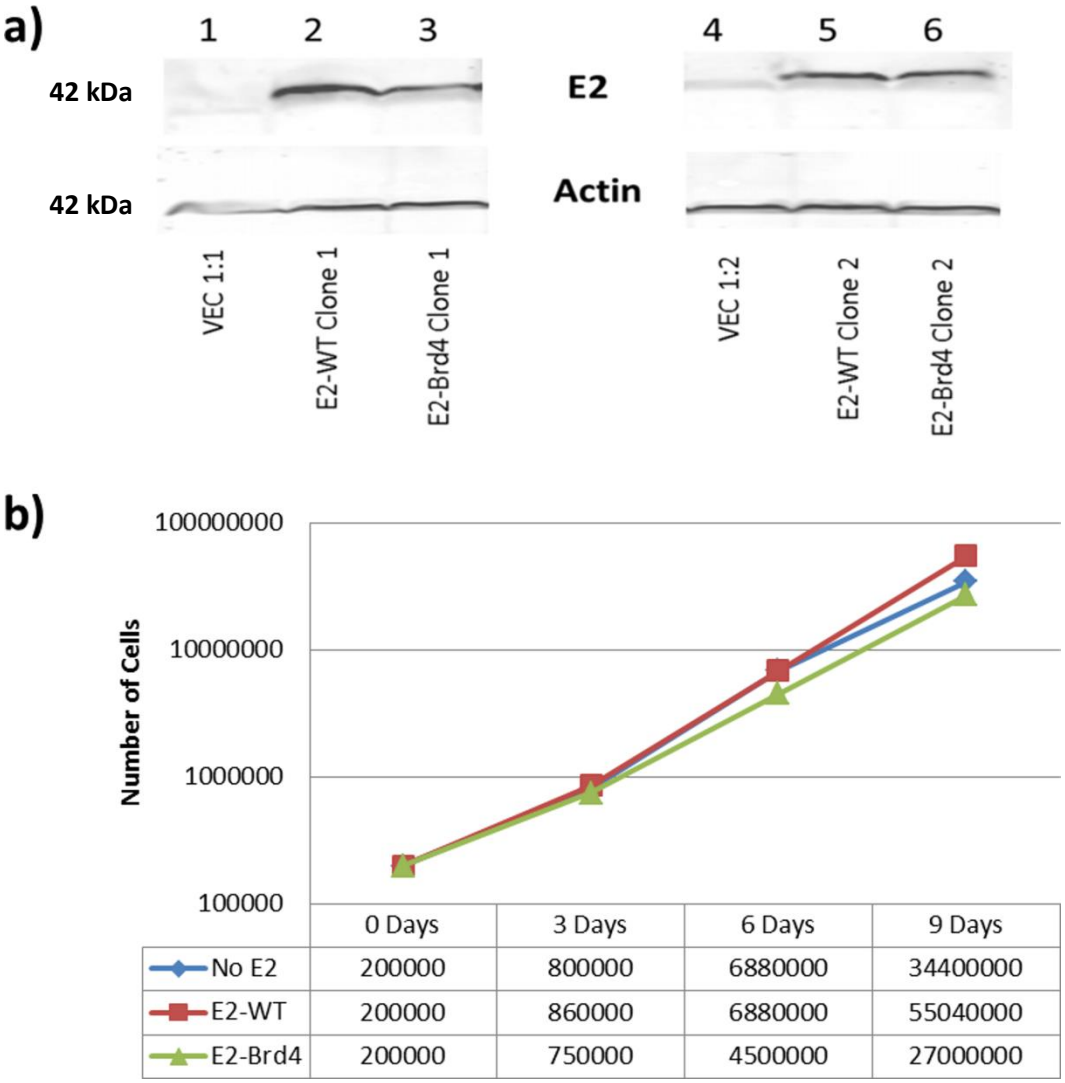
Figure 4.2.1



Plasmid	10ng	100ng	1000ng
$E2^{-WT}$	9.15	2.37	25.28
$E2^{-Brd4}$	0.75	0.81	1.56

Figure 4.2.1: $16E2^{-Brd4}$ Transcription activation in U20S cells. Transcription assays were carried out in U20S cells with input levels of 10ng, 100ng and 1000ng, along with an E2 reporter containing 6E2 DNA binding sites upstream from a tk promoter driving luciferase (Vance, Campo et al. 1999). Cells were harvested and luciferase and protein assays carried out (as described in **Methods section 2.2.1.5 and 2.2.1.13**). The results are normalised to protein levels in each sample and are represented as fold increase over ptk6E2 levels in the absence of E2. The results are representative of three independent experiments done in duplicate. Bars represent standard error. Fold activation in transcription over that obtained when no E2 was co-expressed is shown in the table below. No significant results.

Figure 4.2.2



	0 Days	3 Days	6 Days	9 Days
No E2	200000	800000	6880000	34400000
E2-WT	200000	860000	6880000	55040000
E2-Brd4	200000	750000	4500000	27000000

Figure 4.2.2: Generating and characterising stably expressing E2^{-Brd4} clones.

a) Colonies were picked and expanded after G418 selection. Potential E2 expressing clones were screened by making lysates from the cells and tested for E2 expression using standard western blot procedure (as described in **Methods section 2.2.1.6**).

b) 4x10^5 cells were seeded and counted every 3 days, growth curves were generated from the numbers of cells from each count (as described in **Methods section 2.2.2.3**). The growth curve displays no difference in the growth rate between the E2 and non-E2 expressing clones, E2-Brd4 is also similar. This was done in two individual clones. Only one clone is graphed as the clones grow similarly.

4.2.3 Identifying differentially expressed host genes induced by $E2^{-Brd4}$

A Human exon array study was carried out to look at the differences between non-E2 expressing cells, $E2^{-WT}$ and $E2^{-Brd4}$ ("array 2"). 16E2 was shown to regulate 879 genes, of these genes 407 are up-regulated ≥1.5 fold and 472 are down-regulated ≥1.5 fold. The $E2^{-Brd4}$ mutant, that does not bind Brd4, regulates about 1884 host genes (≥ 1.5 fold), 956 up-regulated and 928 down-regulated (**Table 4.2.4** displays top 50 $^{+/-}$ regulated genes ≥ 2-fold for $E2^{-WT}$). **(Supplementary Table S.3 and S.5 displays the full list of genes regulated by 16 $E2^{-WT}$ and 16$E2^{-Brd4}$ ≥ 1.5-fold respectively).**

When E2 fails to interact with Brd4 there is a notable increase in the number of genes E2 is able to regulate in the host genome. It should also be noted that in "array 2" 16 $E2^{-WT}$ regulates the gene expression of more genes than in "array 1", this is most likely due to the RNA being prepared at different times and conditions may have varied slightly. The overlap of the $E2^{-WT}$ regulated genes from array 1 and 2 corresponds to 58% of the top 50 up-regulated genes in array 1 (**Table 4.1.1**) were similarly regulated in array 2 (**Table 4.3.2**), and 54% of the top 50 down-regulated $E2^{-WT}$ genes in array 1 were similarly regulated in array 2 . This difference may also be contributed to the experiments being carried out at different times.

4.2.4 Validation of $E2^{-WT}$ and $E2^{-Brd4}$ regulated genes

The microarray was validated with primer sets for genes which were up or down-regulated greater than two-fold by both $E2^{-WT}$ and $E2^{-Brd4}$ (**Table 4.2.1 and 4.2.2**). Seven out of 8 and 6 out of 8 genes validated for $E2^{-WT}$ clones 1 and 2 respectively, and 7 out of 7 genes validated for both $E2^{-Brd4}$ clones.

4.2.5 Specific $E2^{-WT}$ genes not regulated by $E2^{-Brd4}$

An additional 6 genes were selected to further validate the array. These genes are specifically regulated by $E2^{-WT}$ (≥ 2-fold). Three out of the 6 $E2^{-WT}$ specific genes screened validated successfully, and are presented on a graph to highlight the difference in regulation between no E2 control, and $E2^{-Brd4}$ expressing clones (**Table 4.2.3 and Figure 4.2.3**). $E2^{-Brd4}$ Clone 1 validated for all six genes, but

$E2^{-Brd4}$ Clone 2 only validated 3 out of six genes (**Table 4.2.3**). This shows that the genes regulated by $E2^{-WT}$ only in the array are specific to $E2^{-WT}$. The figure to accompany the three validated genes demonstrates this (**Figure 4.2.3**).

4.2.6 Similarities between $E2^{-WT}$ and $E2^{-Brd4}$ regulated genes

A table of the top 50 $E2^{-WT\ +/-}$ regulated genes ≥ 2-fold highlights genes similarly regulated by $E2^{-Brd4}$ (grey= similarly regulated in top 50 genes, and green= similarly regulated but not in the top 50 genes) (**Table 4.2.4**). Interestingly, out of the top 50 $E2^{-WT}$ up-regulated genes (≥ 2-fold change), $E2^{-Brd4}$ regulates approximately 14% of these genes. Comparatively, out of the top 50 $E2^{-WT}$ down-regulated genes (≥ 2-fold change), $E2^{-Brd4}$ regulates about 50% of these $E2^{-WT}$ genes. When this comparison is extended to look at all of the genes regulated ≥ 1.5 fold, 42% of the up-regulated 16 $E2^{-WT}$ genes are regulated in a similar manner to $E2^{-Brd4}$. The number of down-regulated genes shared between 16 $E2^{-WT}$ and $E2^{-Brd4}$ is 92%. In this array we have demonstrated that both $E2^{-WT}$ and $E2^{-Brd4}$ are down-regulating the same subset of genes.

Additionally, IPA analysis was carried out to investigate the pathways regulated by the sets of genes found from the analysis of the second array, for both $16E2^{-WT}$ and $E2^{-Brd4}$ (as described in **Methods section 2.2.3.2**). Tables **4.2.4** and **4.2.5** display the top 5 biological functions associated with the top 100 genes regulated by $E2^{-WT}$ and $E2^{-Brd4}$ respetively. Some similarities between $E2^{-WT}$ and $E2^{-Brd4}$ functions are an involvement in cancer, cellular movement, and immune cell trafficking.

Table 4.2.1

Gene Name	Fold-change in array	Fold-change clone 1	Fold-change clone 2
MAGEC1	30.25	541.85 ± 178.85	1964 ± 813.94
APBA2	2.69	33.59 ± 4.52	42.61 ± 15.92
ENG	2.63	10.29 ± 4.99	6.58 ± 2.16
FAR2	2.19	3.02 ± 0.75	2.6 ± 0.26
RNF144B	-3.05	-4.32 ± 2.38	-6.03 ± 5.02
TMPRSS15	-9.06	-1463 ± 697	-981.84 ± 481.84
NFE4	-33.27	-50 ± 8.37	-21.76 ± 2.43
GTSF1	-219.71	-1735.49 ± 218.59	-174188 ± 87637

Table 4.2.1: Validation of $E2^{-WT}$ regulated genes. The table displays the fold-change of 8 up and down-regulated genes that were regulated by 16 $E2^{-WT}$, the fold-change corresponds to array 2. Validation was done in two separate clones in triplicate, and fold-change was calculated relative to VEC1:1.Standard error is shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Table 4.2.2

Gene Name	Fold-change in array	Fold-change clone 1	Fold-change clone 2
MAGEC1	35.05	615.05 ± 226.68	1009.61 ± 219.75
ENG	2.67	13.23 ± 5.64	18.52 ± 5.66
APBA2	2.28	29.66 ± 2.58	62.10 ± 32.80
ATP8B1	-2.28	-3111 ± 2627	-2.77 ± 1.13
TMPRSS15	-7.4	-674.80 ± 325	-19177 ± 6507.25
ADAMTSL1	-8.82	-5.53 ± 0.67	-7.23 ± 0.86
GTSF1	-291.81	-5962 ± 3550	-53282 ± 27525

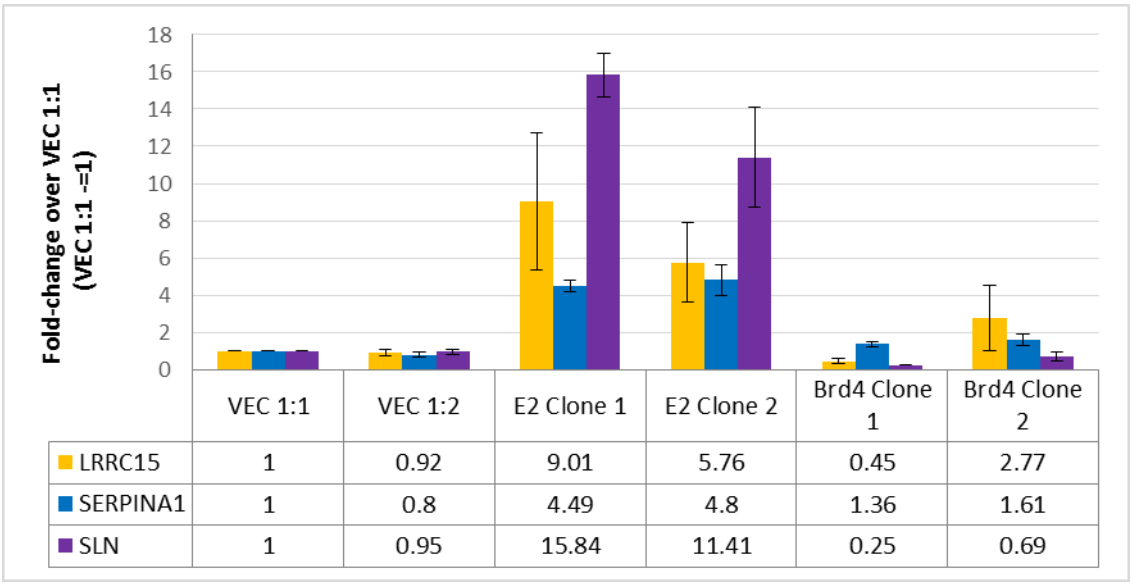
Table 4.2.2: Validation of E2^{-Brd4} regulated genes. The table displays the fold-change of 7 up and down-regulated genes that were regulated by 16 E2^{-Brd4}, the fold-change corresponds to array 2. Validation was done in two separate clones in triplicate, and fold-change was calculated relative to VEC1:1. Standard error is shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Table 4.2.3

Gene Name	Fold-change in Array	$E2^{-WT}$ clone 1	$E2^{-WT}$ clone 2	$E2^{-Brd4}$ clone 1	$E2^{-Brd4}$ clone 2
c6orf15	7.11	3.52 ± 2.3	12.16 ± 3.7	0.39 ± 0.1	7.08 ± 5.2
CLDN4	6.72	28.91 ± 20.2	1704.06 ± 1662.6	0.62 ± 0.1	29.67 ± 28.3
HOXB2	9.14	63.59 ± 34.0	97.77 ± 56.4	1.48 ± 0.5	97.20 ± 58.2
LRRC15	6.62	9.00 ± 3.7	5.76 ± 2.1	0.45 ± 0.1	2.77 ± 1.8
SERPINA1	5.93	4.49 ± 0.3	4.80 ± 0.8	1.36 ± 0.2	1.61 ± 0.3
SLN	20.23	15.84 ± 1.2	11.41 ± 2.7	0.25 ± 0.02	0.69 ± 0.2

Table 4.2.3: Validation of $E2^{-WT}$ regulated genes. The table displays the fold-change of 6 up-regulated genes that were regulated by 16 $E2^{-WT}$ exclusively in the second array. Validation was done in two separate clones of $E2^{-WT}$ and $E2^{-Brd4}$. Fold-change is calculated relative to vector (no E2 control) and standard error is shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Figure 4.2.3



	VEC 1:1	VEC 1:2	E2 Clone 1	E2 Clone 2	Brd4 Clone 1	Brd4 Clone 2
LRRC15	1	0.92	9.01	5.76	0.45	2.77
SERPINA1	1	0.8	4.49	4.8	1.36	1.61
SLN	1	0.95	15.84	11.41	0.25	0.69

Figure 4.2.3: Graph of the validation of 16 E2^{-WT} regulated genes. Three genes exclusively regulated by E2^{-WT} were successfully validated using SYBR green qPCR in two separate clones, in triplicate (as described in **Methods section 2.2.1.12**). The fold-change (over vector control (VEC 1:1)) is indicated below the graph, standard error can be found in Table 4.2.3 above. The graph clearly shows that these genes are E2^{-WT} regulated genes.

Table 4.2.4

Up-regulated by E2	Fold-change	Down-regulated by E2	Fold-change
HIST1H3H	90.7546	GTSF1	-219.713
HIST1H2BM	46.231	NFE4	-33.2709
MAGEC1	30.2533	BGN	-22.9955
SLN	20.2274	KCNIP1	-21.732
HOXB2	9.13427	MIR31	-18.8279
C6orf15	7.1056	MGP	-18.0495
FAM198B	7.06128	KRT75	-17.2035
CLDN4	6.71694	FGF5	-14.8293
LRRC15	6.61625	RIMS1	-14.4025
SERPINA1	5.93304	SEMA3A	-12.5742
LRRC17	5.67212	SULT1B1	-12.5642
CXCL14	5.30661	ESM1	-12.2765
CDK15	4.91096	IL1RL1	-9.54772
SPTLC3	4.61896	CCL2	-8.46037
IL2RB	4.54159	HHIP	-8.32544
LIPH	4.41975	PDCD1LG2	-7.53008
TRIM16	4.35329	CD274	-7.42495
PLAC8	4.3376	MC5R	-7.17821
MGST1	4.26395	DCHS2	-7.10103
UNC13D	4.13402	SEMA3E	-6.75657
CA12	4.07438	ADAMTSL1	-6.52013
SLC12A8	4.00346	KRTAP4-1	-6.49334
LUM	3.96639	ST6GALNAC3	-6.15485
LIMCH1	3.91481	TMEM154	-6.04587
	3.67791	TMPRSS15	-6.0099
LCP1	3.51652	CLDN1	-5.66267
CALHM2	3.42559	MTAP	-5.63307
IGFBP5	3.42263	SLITRK6	-5.55367
KCNJ8	3.41959	VLDLR	-5.38956
GFPT2	3.38769	RAB38	-5.2863
KLK6	3.33344	LPAR1	-5.20042
PAGE2	3.29191	KIAA1797	-5.17872
GPR56	3.26354	KITLG	-4.94164
SPP1	3.22762	KRT34	-4.93765
CDO1	3.20934	FBXL13	-4.75785
PTP4A3	3.20919	FAM180A	-4.7544
TNNC1	3.16845	KCNB2	-4.63984
SERPINF1	3.14816	KIAA1432	-4.60797
GPR45	3.12373	EGLN3	-4.43062
KRT86	3.09044	DENND4C	-4.40059
RASD2	3.0506	AK3	-4.38385
FAM49A	3.04373	ADAMTS5	-4.27239
AQP1	3.03761	TM4SF18	-4.24862
PDGFRB	3.02402	CYP24A1	-4.20666
B3GNT1	3.00978	DPY19L2P2	-4.20305
MAOA	2.98823	KLHL9	-4.19991
CERCAM	2.98504	LGR5	-4.14646
RASSF5	2.97825	CDC37L1	-4.14413
TGM2	2.95568	PGM5	-4.13192
TTC39A	2.95362	AFAP1	-4.12776

Table 4.2.4: Similarities in genes regulated by $E2^{-WT}$ and $E2^{-Brd4}$. Human exon array analysis was used to generate lists of genes regulated by HPV16 $E2^{-WT}$. Pawel Herzyk carried out Partek analysis to sort the raw data into lists of genes that had a fold-change (F.C) of ≥ 1.5 and a p-value of ≤0.05. The table lists the top 50 +/- $E2^{-WT}$ regulated genes ≥ 2-fold. Similarities between the top 50 +/- $E2^{-WT}$ and $E2^{-Brd4}$ regulated genes are highlighted in grey. Genes up and down regulated by both $E2^{-WT}$ and $E2^{-Brd4}$ ≥ 2-fold in the top 50 genes are highlighted in green.

Table 4.2.5

Network	Associated network functions
1	Cellular Movement, Cancer, Endocrine System Disorders
2	Cellular Movement, Immune Cell Trafficking, Hematological System Development and Function
3	Cell Cycle, Tissue Development, Organ Morphology
4	Dermatological Diseases and Conditions, Hereditary Disorder, Lipid Metabolism
5	Cellular Growth and Proliferation, Connective Tissue Disorders, Inflammatory Disease

Table 4.2.5.: Top 5 16E2^{-WT} regulated pathways from Ingenuity Pathway analysis (array 2). The top 100 E2^{-WT} regulated genes (≥ 2-fold) were subjected to IPA and 5 networks of genes were identified. The table lists the predicted functions with the IPA generated networks. (**Genes associated with this table are shown in Supplementary Table S.4 and p-values associated with functions can be found in Supplementary Table S.13**).

Table 4.2.6

Network	Associated network functions
1	Gene Expression, Protein Synthesis, Digestive System Development and Function
2	Cancer, Cellular Movement, Cellular Development
3	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking
4	Amino Acid Metabolism, Molecular Transport, Small Molecule Biochemistry
5	Dermatological Diseases and Conditions, Drug Metabolism, Lipid Metabolism

Table 4.2.6.: Top 5 16E2^{-Brd4} regulated pathways from Ingenuity Pathway analysis (array 2). The top 100 E2^{-Brd4} regulated genes (≥ 2-fold) were subjected to IPA and 5 networks of genes were identified. The table lists the predicted functions with the IPA generated networks. (**Genes associated with this table are shown in Supplementary Table S.6 and p-values associated with functions can be found in Supplementary Table S.13**).

4.2.7 Regulation of cell motility by $E2^{-Brd4}$

As previously described in **Chapter 4.1.4**, $E2^{-WT}$ regulates cellular movement. We decided to test the role of Brd4 in E2 regulated cellular movement by carrying out would healing assays (as previously seen in **Figure 4.1.2**). The $E2^{-Brd4}$ mutant expressing cells was slower than non-E2 expressing cells and faster than $E2^{-WT}$ at wound closing. The wound is more visible at the 12 hour time point and after 24 hours the cells covering the wound are less dense than the non-E2 cell condition. As demonstrated before, the $E2^{-WT}$ expressing cells fail to fully heal the wound after 24 hours (**Figure 4.2.3**). This difference in movement is not due to differences in cell growth (**Figure 4.2.2b**). This suggests a partial phenotype therefore the interaction between E2 and Brd4 is required, in part, for the regulation of all movement.

Figure 4.2.4

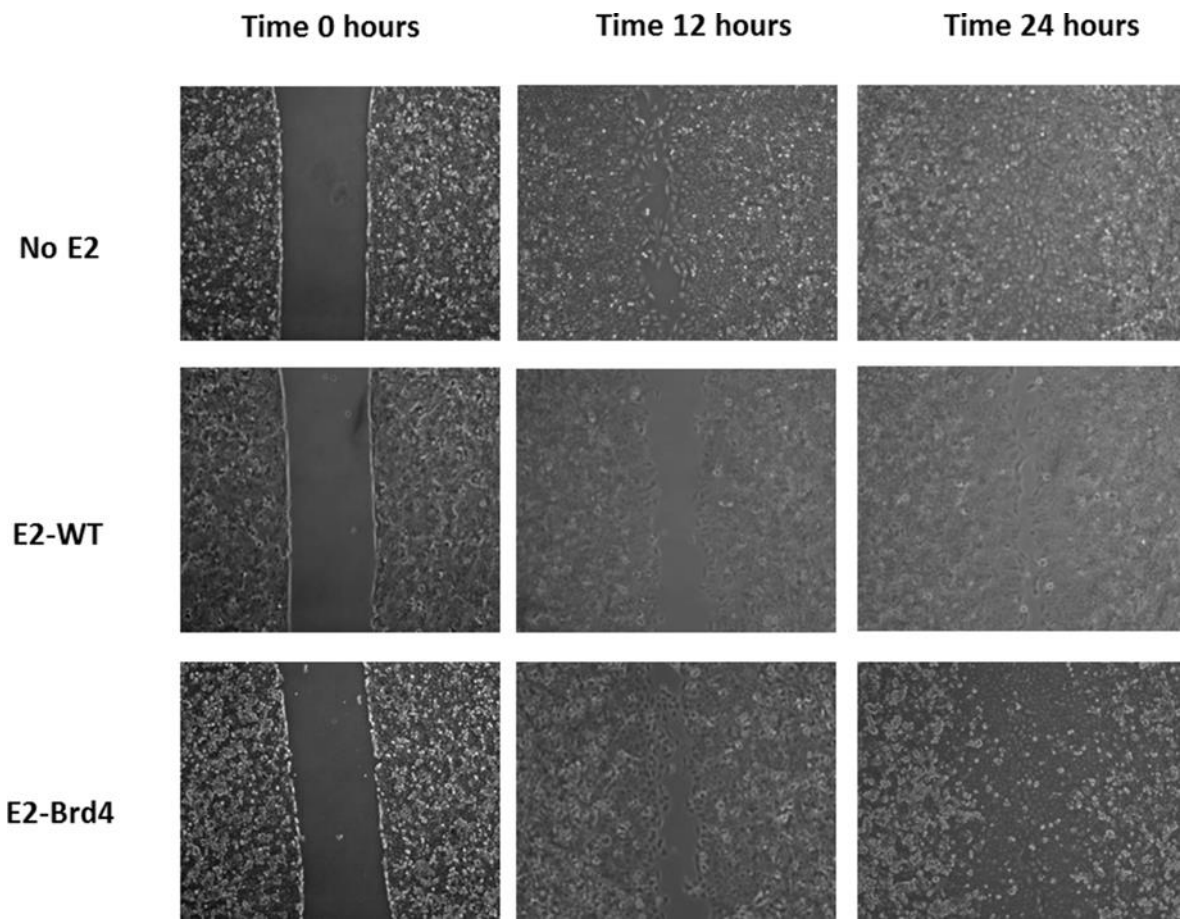


Figure 4.2.4: Wound healing and cell movement of HPV16 E2^{-Brd4}. 5x10^4 cells were seeded into cell chambers and grown for 24 hours. 24 hours after seeding the chambers were removed and movement of E2 expressing cells vs non E2-expressing cells was monitored by microscopy and images were collected at 0, 16 and 24 hour time points (as described in **Methods section 2.2.2.4**). Both No E2 and E2^{-Brd4} clones have closed the gap by the 24 hour time point. E2-Brd4 is slightly slower at closing the wound however, and you can see that the cells covering the wound are less dense than in the non-E2 cell condition.

4.3 The role of TopBP1 in E2 regulation of host genes

Due to the novel finding that Brd4 is not required for E2's down-regulation of host genes, we wanted to ask how TopBP1 is involved in HPV16 E2 regulation of the host genome.

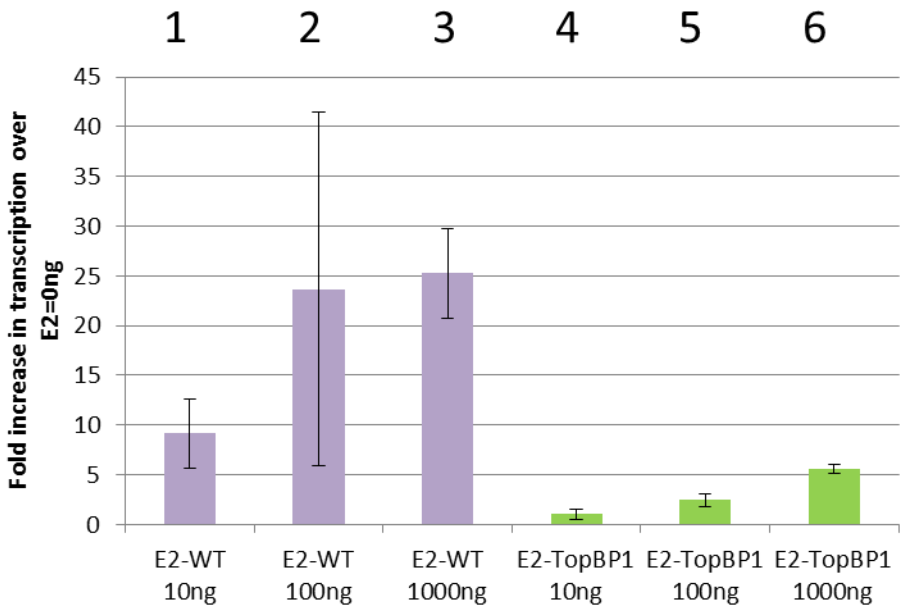
4.3.1 $E2^{-TopBp1}$ is compromised in activating transcription in U20S cells

As demonstrated previously for $E2^{-Brd4}$ (**Figure 4.2.1**), the ability of the $E2^{-TopBP1}$ mutant to activate transcription in U20S cells was tested. The ability of $E2^{-TopBP1}$ to activate transcription compared to $E2^{-WT}$ is compromised (**Figure 4.3.1**). At 10ng of $E2^{-TopBP1}$ input DNA transcription is approximately 11% of $E2^{-WT}$. This increases to 22% with 1000ng of DNA input. These results in U20S cells mirror those previously seen in 293T and C33a cells in Chapter 3.

4.3.2 Generation of U20S cells expressing 16 $E2^{-TopBP1}$

Cells that stably express $E2^{-TopBP1}$ were generated in U20S cells for use in human exon array analysis (as described in **Methods section 2.2.2.2**). Expression of potential clones was tested using western blot analysis as seen in **Figure 4.3.2a**. The growth of these cells was also monitored by conducting a growth curve, which showed that the cells containing the E2-^{TopBP1} mutation grew slower than $E2^{-WT}$ or no E2 cells (**Figure 4.3.2b)**.

Figure 4.3.1



Plasmid	10ng	100ng	1000ng
$E2^{-WT}$	9.15	23.7	25.28
$E2^{-TopBP1}$	1.02	2.44	5.57

Figure 4.3.1: 16 $E2^{-TopBP1}$ Transcription activation in U20S cells. Transcription assays were carried out in U20S cells with input levels of 10ng, 100ng and 1000ng, along with an E2 reporter containing 6E2 DNA binding sites upstream from a tk promoter driving luciferase (Vance, Campo et al. 1999). Cells were harvested and luciferase and protein assays carried out (as described in **Methods section 2.2.1.5 and 2.2.1.13**). The results are normalised to protein levels in each sample and are represented as fold increase over ptk6E2 levels in the absence of E2. The results are representative of three independent experiments done in duplicate. Bars represent standard error. Fold activation in transcription over that obtained when no E2 was co-expressed is shown in the table below.

Figure 4.3.2

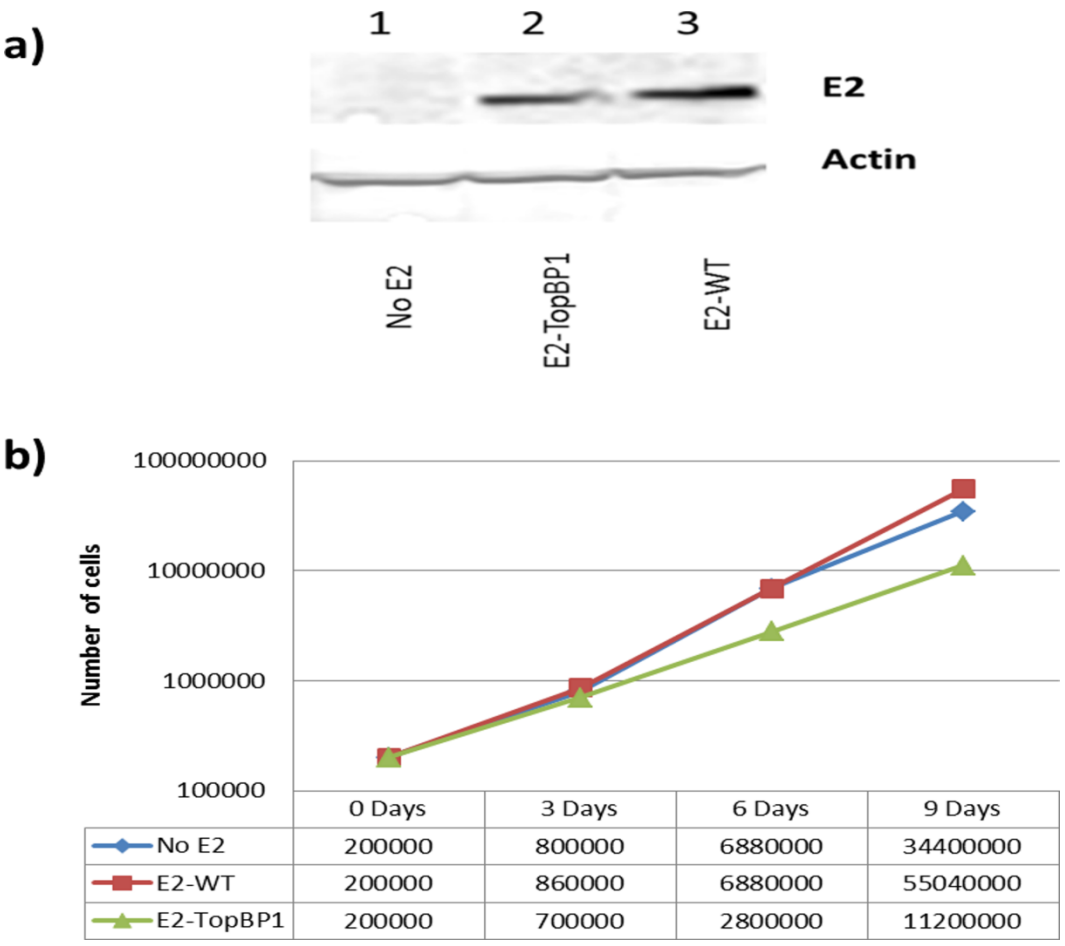


Figure 4.3.2: Generating and characterising stably expressing E2-^{TopBP1} clones.

a) Potential E2-^{TopBP1} expressing clones were screened by making lysates for western blot analysis (as described in **Methods section 2.2.1.11**). Actin control is shown for equal loading.

b) 4x10^5 cells were seeded and counted every 3 days, growth curves were generated from the numbers of cells from each count (as described in **Methods section 2.2.2.3**). The growth curve displays no difference in the growth rate between the E2 and non-E2 expressing clones, but E2-^{TopBP1} grows slightly slower. This was done in two individual clones. Only one clone is graphed as the growth of the two clones was similar.

4.3.3 Identifying differentially expressed host genes induced by E2$^{-TopBP1}$

Human exon array analysis in the U20S model was carried out as previously described, to compare E2^{-WT} and E2$^{-TopBP1}$ regulation of host genes. This was the first set of microarray analysis that we set out to do in the U20S model ("array 1"). It should be noted the results for E2^{-Brd4} were done in a separate human exon array experiment ("array 2"). As the number of genes regulated ≥ 2-fold by E2^{-WT} was smaller in array 1 it was decided to look at all of the genes regulated by E2^{-WT} ≥ 1.5-fold. 16 E2^{-WT} regulates 267 genes ≥ 1.5-fold, out of the 267 genes, 130 are up-regulated and 137 are down-regulated by E2^{-WT}. Comparatively, the E2$^{-TopBP1}$ mutant regulates 1380 genes ≥ 1.5-fold, 683 are up-regulated and 697 are down-regulated. As described before for E2^{-Brd4} mutant, E2$^{-TopBP1}$ regulates a large subset of genes. **(Supplementary Table S.7 displays full list of genes regulated by 16 E2$^{-TopBP1}$ ≥ 1.5-fold).**

4.3.4 Validation of E2^{-WT} and E2$^{-TopBP1}$ genes

Fifteen genes were chosen for validating the array, 10 down-regulated genes and 5 up-regulated genes. The 15 genes fall into three distinct groups; genes regulated by E2^{-WT} only (validation in **Table 4.1.2**), genes regulated by both E2^{-WT} and E2$^{-TopBP1}$ (validation of E2^{-WT} **Table 4.1.2**), and genes regulated only by E2$^{-TopBP1}$. Using SYBR green qPCR to validate genes with a fold-change ≥ 2, and a p-value ≤0.05, the validation agreed with the initial array analysis, genes that were up or down-regulated followed the same trend **(Table 4.3.1).** 5 out of 5 of the up-regulated genes (up-regulated by both E2^{-WT} and E2$^{-TopBP1}$) validated in the E2$^{-TopBP1}$ expressing cells. 4 out of 5 of the down-regulated E2$^{-TopBP1}$genes validated successfully, confirming our array results.

4.3.5 Similarities in genes regulated by E2^{-WT} and E2$^{-TopBP1}$

As Brd4 was shown to not be involved in the down-regulation of many genes that E2 regulates, the role of TopBP1 in E2's regulation of host genes was also investigated. **Table 4.3.2** displays the top 50 +/- regulated genes by E2^{-WT}, and additionally highlights the genes that are regulated in a similar way by both E2^{-WT} and E2$^{-TopBP1}$. In the top 50 E2^{-WT} up-regulated genes, 18% of these were similarly

regulated by $E2^{-TopBP1}$. In the top 50 $E2^{-WT}$ down-regulated genes 16% were similarly regulated by $E2^{-TopBP1.}$ The number of genes regulated in a similar manner increased to 64% for up-regulated, and 46% for down-regulated $E2^{-WT}$ genes when genes not in the top 50 were also included.

Additionally, IPA analysis was carried out to investigate the pathways regulated by the sets of genes found from the analysis of the second array, for both $16E2^{-WT}$ and $E2^{-TopBP1}$. Tables **4.2.4** and **4.3.3** display the top 5 biological functions associated with the top 100 genes regulated by $E2^{-WT}$ and $E2^{-TopBP1}$ respetively. The only similarity in the top 5 associated network functions of $E2^{-WT}$ and $E2^{-TopBP1}$ regulated genes is cancer. The regulation of the host genome is altered when E2 cannot bind to TopBP1.

Table 4.3.1

Gene Name	Fold-change in Array	Fold-change clone 1	Fold-change clone 2
MAGEC1	59.28	344.32 ± 308.96	528.93 ± 522.29
ENG	7.22	16.42 ± 9.31	29.9 ± 11.79
FAR2	6.07	6.43 ± 2.53	2.79 ± 1.28
APBA2	5.25	29.69 ± 16.37	35.7 ± 33.27
SH3PXD2B	2.33	2.08 ± 0.29	7.93 ± 4.43
ADAMTSL1	-2.83	-133.70 ± 90.51	-32.38 ± 18.49
COL6A3	-8.7	1.55 ± 0.76	2.03 ± 0.74
MFAP5	-13.21	-16.37 ± 1.16	-81.42 ± 18.49
ATP8B1	-14.43	-65.87 ± 57.71	-547.79 ± 540.70
DHRS2	-27.01	-26.99 ± 6.77	-275.74 ± 140.47

Table 4.3.1: Validation of genes regulated by HPV16 E2$^{-TopBP1}$. Table displays 5 up-regulated and 5 down-regulated genes fold-change in the microarray analysis. 5 genes are up-regulated by both E2^{-WT} and E2$^{-TopBP1}$ and 5 are down-regulated exclusively by E2$^{-TopBP1}$. Genes are validated in two E2$^{-TopBP1}$ expressing clones in triplicate, standard error shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Table 4.3.2

Up-regulated by E2	Fold-change	Down-regulated by E2	Fold-change
HIST1H2BM	71.9508	GTSF1	-302.705
HIST1H3H	70.7304	NFE4	-24.794
MAGEC1	40.1869	TMPRSS15	-11.624
SLN	14.5889	SLC14A1	-5.94492
HOXB2	10.631	C1orf85	-4.48436
TNFAIP6	4.86318	CD33	-4.1363
ENG	4.85397	CLIC2	-3.9316
GNG11	4.11702	FBXL13	-3.60857
FAR2	3.70124	PTGFR	-3.59376
ZNF788	3.3983	CSTA	-3.49077
APBA2	2.99782	NLRP5	-3.33859
SERPINA3	2.74046	DNAJC18	-3.31614
C10orf72	2.5905	BMPER	-3.27652
GFPT2	2.53867	HBE1	-3.07671
SH3PXD2B	2.50361	RNF144B	-3.04874
FAP	2.47498	GDF15	-2.98979
LRRCC1	2.36516	LOC79015	-2.97446
ARHGDIB	2.35827	HSD17B8	-2.75366
SNTB1	2.25035	HIST1H3E	-2.69962
EML1	2.23335	MLH3	-2.64666
ARHGAP11A	2.23306	NLRP4	-2.60899
CCDC99	2.16172	TRIML2	-2.55713
FGD4	2.1616	CD68	-2.47599
ZNF271	2.15217	ACYP1	-2.43839
MTMR10	2.11787	PSG9	-2.41357
CALHM2	2.11199	HLA-DPA1	-2.38118
HNMT	2.09265	GNGT2	-2.37174
GOLGA8B	2.08224	FST	-2.35479
AQR	2.05191	GCNT2	-2.3148
NFIA	2.02545	GRAMD3	-2.29601
ZNF770	2.02363	PGAP2	-2.20441
ZADH2	2.01373	RPL23	-2.20367
RTKN2	2.00087	IL1RAPL1	-2.18097
CLEC2B	1.98705	OR10A3	-2.14773
RAB27B	1.97895	GPR65	-2.14222
MYO5B	1.97609	AURKC	-2.13613
TMEM55A	1.96272	ZNF300P1	-2.10876
IMPACT	1.92209	ME3	-2.06315
ZNF738	1.90705	ADRB2	-2.05351
TRPS1	1.88341	PSG4	-2.04519
TTC39C	1.87129	ZFP90	-2.02583
ATPBD4	1.86744	DPYSL4	-1.9764
FAN1	1.86327	TOX	-1.96981
B4GALNT3	1.85095	LTBP2	-1.96341
STK38L	1.83674	IL6	-1.95904
ZNF365	1.82942	CARS	-1.90807
CASC5	1.81003	EIF2B2	-1.89313
FAM60A	1.79363	AOC2	-1.88615
C8orf40	1.79137	MDGA2	-1.88515
NUP210	1.78893	WNT2B	-1.87542

Table 4.3.2: Similarities in genes regulated by $E2^{-WT}$ and $E2^{-TopBP1}$. Human exon array analysis was used to generate lists of genes regulated by HPV16 $E2^{-WT}$ and $E2^{-TopBP1}$. Pawel Herzyk carried out Partek analysis to sort the raw data into lists of genes that had a fold-change (F.C) of ≥ 1.5 and a p-value of ≤0.05. The table lists the top 50 +/- $E2^{-WT}$ regulated genes ≥ 1.5-fold. Similarities between the top 50 +/- $E2^{-WT}$ and $E2^{-TopBP1}$ regulated genes are highlighted in grey. Genes regulated in a similar way not in the top 50 genes are highlighted in green.

Table 4.3.3

Network	Associated network functions
1	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
2	Cancer, Tumour Morphology, Hereditary Disorder
3	Inflammatory Disease, Inflammatory Response, Respiratory Disease
4	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function
5	Cardiovascular Disease, Connective Tissue Disorders, Developmental Disorder

Table 4.3.3.: Top 5 16E2$^{-TopBP1}$ regulated pathways from Ingenuity Pathway analysis (array 2). The top 100 E2$^{-TopBP1}$ regulated genes (≥ 2-fold) were subjected to IPA and 5 networks of genes were identified. The table lists the predicted functions with the IPA generated networks. (**Genes associated with this table are shown in Supplementary Table S.8 and p-values associated with functions can be found in Supplementary Table S.13**).

4.3.6 Regulation of cell motility by E2$^{-TopBP1}$

As E2^{-WT} was shown to be involved in cellular movement, it was decided to see if TopBP1 plays a role in this by testing the E2$^{-TopBP1}$ mutant in wound healing assays. Wound healing assays demonstrate that E2$^{-TopBP1}$ is defective in cellular movement in a similar manner to E2^{-WT} (**Figure 4.3.3**). This would suggest that TopBP1 is not required for E2 regulation of cellular movement.

Figure 4.3.3

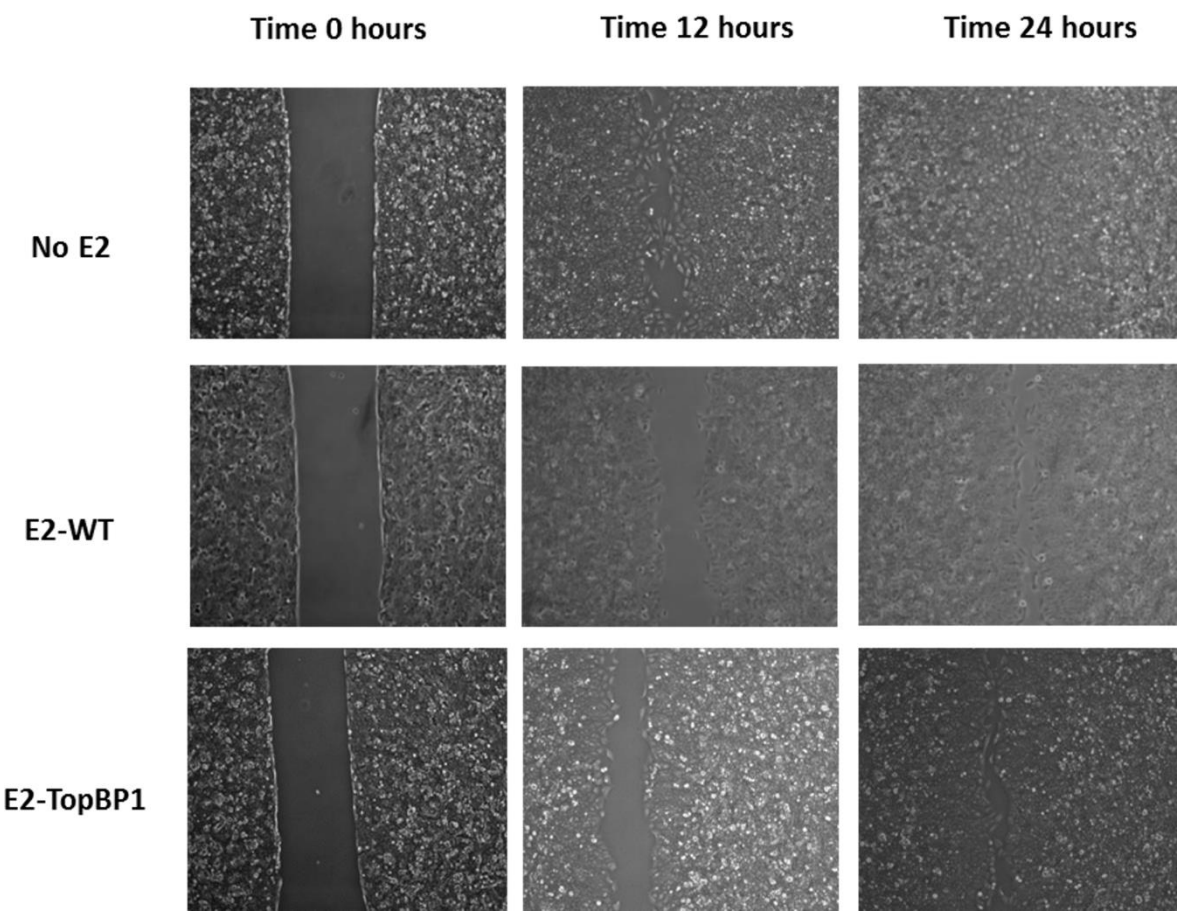


Figure 4.3.3: Wound healing and cell movement of HPV16 E2$^{-TopBP1}$. 5x10^4 cells were seeded into cell chambers and grown for 24 hours. 24 hours after seeding the chambers were removed and movement of E2 expressing cells vs non E2-expressing cells was monitored by microscopy and images were collected at 0, 16 and 24 hour time points (as described in **Methods section 2.2.2.4**). Both E2^{-WT} and E2$^{-TopBP1}$ clones clearly show a defect in movement and their ability to close the gap in the 24 hour time period.

4.4 Wildtype 18E2 regulates host genes

As this analysis focused only on HPV16 E2 regulation of the host genome we decided to extend our studies to investigate how HPV18 E2 regulates host gene expression. As discussed in Chapter 1, HPV16 and 18 are the two most common high-risk HPV types, although HPV18 demonstrates a more severe outcome in cervical cancer than HPV16, adenocarcinoma versus squamous cell carcinoma. Perhaps there are key changes in virus-host gene regulation contributing to this phenotype.

The comparison between 16 $E2^{-WT}$ and 18 $E2^{-WT}$ was carried out in the second human exon array, "array 2". 16 $E2^{-WT}$ regulates 879 genes ≥1.5-fold, 407 up-regulated genes and 472 down-regulated genes. 18 $E2^{-WT}$ regulates 745 genes ≥ 1.5-fold, 469 up-regulated and 276 down-regulated. An overlap of 362 genes were regulated in the same direction in both HPV16 and 18, showing E2 of these two high-risk types must regulate a subset of the same host genes**. (Supplementary Table S.9 displays full list of genes regulated by 18 E2 wildtype ≥ 1.5-fold).**

7 up and down-regulated genes were validated in two separate clones for $18E2^{-WT}$ in triplicate. All 7 genes validated in both clones (**Table 4.4.2**). Validation of 16 $E2^{-WT}$ genes can be found in **Table 4.2.1.**

Similarities between the top 50 up and down-regulated genes in 16 $E2^{-WT}$ and 18 $E2^{-WT}$ are set out in **Table 4.4.3**. 16 and 18 $E2^{-WT}$ share a large overlap in the genes they down-regulate in the host genome. 88% of the top 50 16 $E2^{-WT}$ down-regulated genes are similarly down-regulated by 18 $E2^{-WT}$. Also, 60% of the top 50 16 $E2^{-WT}$ up-regulated genes are also similarly regulated in 18 $E2^{-WT}$.

Table 4.4.1

Gene Name	Fold-change in array	Fold-change clone 1	Fold-change clone 2
MAGEC1	35.05	442.6 ± 122.44	1927 ± 671.13
ENG	2.67	8.7 ± 4.61	16 ± 6.37
APBA2	2.28	15.2 ± 1.82	60.4 ± 31.48
ATP8B1	-2.28	-2.8 ± 0.58	-2.9 ± 0.38
ADAMTSL1	-4.93	-5.6 ± 0.67	-9.4 ± 1.18
TMPRSS15	-7.4	-2317 ± 1148	-400 ± 100
GTSF1	-291.81	-3174 ± 3174	-62421 ± 62421

Table 4.4.1: Validation of genes regulated by HPV18 E2^{-WT}. Table displays the fold-change of 3 up-regulated and 4 down-regulated genes in the microarray analysis. Genes are validated in two clones in triplicate, standard error shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Table 4.4.2

Net-work	Associated network functions
1	Cellular Movement, Cancer, Organ Morphology
2	Cell Cycle, Cellular Development, Embryonic Development
3	Cellular Development, Cellular Growth and Proliferation, Hair and Skin Development and Function
4	Cell-to-cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Traffick-ing
5	Neurological Disease, Cell Signaling, Embryonic Development

Table 4.4.2.: Top 5 18E2^{-WT} regulated pathways from Ingenuity Pathway analysis (array 2). The top 100 18E2^{-WT} regulated genes (≥ 2-fold) were subjected to IPA and 5 networks of genes were identified. The table lists the predicted functions with the IPA generated networks. (**Genes associated with this table are shown in Supplementary Table S.10 and p-values associated with functions can be found in Supplementary Table S.13**).

Table 4.4.3

Up-regulated by E2	Fold-change	Down-regulated by E2	Fold-change
HIST1H3H	90.7546	GTSF1	-219.713
HIST1H2BM	46.231	NFE4	-33.2709
MAGEC1	30.2533	BGN	-22.9955
SLN	20.2274	KCNIP1	-21.732
HOXB2	9.13427	MIR31	-18.8279
C6orf15	7.1056	MGP	-18.0495
FAM198B	7.06128	KRT75	-17.2035
CLDN4	6.71694	FGF5	-14.8293
LRRC15	6.61625	RIMS1	-14.4025
SERPINA1	5.93304	SEMA3A	-12.5742
LRRC17	5.67212	SULT1B1	-12.5642
CXCL14	5.30661	ESM1	-12.2765
CDK15	4.91096	IL1RL1	-9.54772
SPTLC3	4.61896	CCL2	-8.46037
IL2RB	4.54159	HHIP	-8.32544
LIPH	4.41975	PDCD1LG2	-7.53008
TRIM16	4.35329	CD274	-7.42495
PLAC8	4.3376	MC5R	-7.17821
MGST1	4.26395	DCHS2	-7.10103
UNC13D	4.13402	SEMA3E	-6.75657
CA12	4.07438	ADAMTSL1	-6.52013
SLC12A8	4.00346	KRTAP4-12	-6.49334
LUM	3.96639	ST6GALNAC3	-6.15485
LIMCH1	3.91481	TMEM154	-6.04587
	3.67791	TMPRSS15	-6.0099
LCP1	3.51652	CLDN1	-5.66267
CALHM2	3.42559	MTAP	-5.63307
IGFBP5	3.42263	SLITRK6	-5.55367
KCNJ8	3.41959	VLDLR	-5.38956
GFPT2	3.38769	RAB38	-5.2863
KLK6	3.33344	LPAR1	-5.20042
PAGE2	3.29191	KIAA1797	-5.17872
GPR56	3.26354	KITLG	-4.94164
SPP1	3.22762	KRT34	-4.93765
CDO1	3.20934	FBXL13	-4.75785
PTP4A3	3.20919	FAM180A	-4.7544
TNNC1	3.16845	KCNB2	-4.63984
SERPINF1	3.14816	KIAA1432	-4.60797
GPR45	3.12373	EGLN3	-4.43062
KRT86	3.09044	DENND4C	-4.40059
RASD2	3.0506	AK3	-4.38385
FAM49A	3.04373	ADAMTS5	-4.27239
AQP1	3.03761	TM4SF18	-4.24862
PDGFRB	3.02402	CYP24A1	-4.20666
B3GNT1	3.00978	DPY19L2P2	-4.20305
MAOA	2.98823	KLHL9	-4.19991
CERCAM	2.98504	LGR5	-4.14646
RASSF5	2.97825	CDC37L1	-4.14413
TGM2	2.95568	PGM5	-4.13192
TTC39A	2.95362	AFAP1	-4.12776

Table 4.4.3: Similarities in genes regulated by 16 E2^{-WT} and 18 E2^{-WT}. Human exon array analysis was used to generate lists of genes regulated by HPV18 E2^{-WT}. Pawel Herzyk carried out Partek analysis to sort the raw data into lists of genes that had a fold-change (F.C) of ≥ 1.5 and a p-value of ≤0.05. Numbers of genes up and down-regulated by each are shown. The table lists the top 50 +/- 16 E2^{-WT} regulated genes ≥ 2-fold. Genes similarly up or down-regulated by 18 E2^{-WT} are highlighted in grey if they are in the top 50 16 E2^{-WT} genes table. Genes regulated by 18 E2^{-WT} in a similar way which are not in the top 50 16 E2^{-WT} genes are highlighted green.

Results demonstrate that 18E2 can regulate cellular genes and that there are overlapping, but also distinct, gene sets regulated by 16 and 18 E2. To investigate the similarities and differences between 16 and 18, Ingenuity Pathway Analysis (IPA) was used to analyse gene data sets (fold-change of ≥ 1.5) highlighted by microarray analysis (**Tables 4.2.4 and 4.4.2**) (as described in **Methods section 2.2.3.2**). Cell movement and cancer both came up as the number one biological functions in IPA anaylsis for HPV16 E2 and HPV18 E2. Additionally, both 16 and 18E2 are involved in pathways regulating the cell cycle, immune cell trafficking and cellular growth and proliferation. These functions may be essential for successful HPV infection. Yet, there are also distinct differences between the two high-risk types. **(Supplementary file S.10 lists genes associated with HPV18 E2 functions)**.

4.5 Wildtype 16E2 regulates host gene expression in epithelial cells

The study was then extended into human keratinocytes (NTERT cells) as they offer a mechanism to study a more realistic disease profile, as we want to identify changes with therapeutic relevance. NTERT are epithelial cells immortalised by telomerase (not transformed). NTERT cells were infected with E2 retroviruses as described in the following paper (White, Sowa et al. 2012).

In NTERT cells 16E2 regulates 115 genes, 48 are up-regulated ≥1.5 fold and 67 are down-regulated ≥1.5 fold (**Table 4.5.1**). **(Supplementary Table S.11 displays full list of genes regulated by 16 E2 wildtype ≥ 1.5-fold in NTERT cells).** 8 genes were selected for further validation of the array, 4 of which successfully validated (**Table 4.5.2**). This is most likely due to the low fold-changes gained from the human exon array.

Using Ingenuity Pathway Analysis (as described in **Methods section 2.2.3.2**), networks involved in infection, inflammation and cancer were all identified in the keratinocyte cells (**Table 4.5.3**). 24 of the down regulated genes are known interferon stimulated genes (ISG). (IFIH1, IFI35, DDX58, OAS2, CD14, IFITM1, IFIT3, OAS1, IL8, MX1, IFIT1, IFI44L, IFI27, IFNK, EGR1, KCNMA1, PTGS1, SERPING1, RSAD2, OAS3, DUSP10, DSC1, CLU and ZFP36).

Therefore, E2 potentially interferes with the host innate immune response to promote survival of the infected cell. 14 interferon genes were selected for further validation using qPCR. 10 out of the 14 genes validated when qPCR analysis was carried out in triplicate for these genes (**Table 4.5.4**).

Table 4.5.1

Up-regulated by 16E2	Fold-change	Down-regulated by 16E2	Fold-change
HAS2	2.5879	CLCA4	-3.78519
NR1D2	2.54635	RSAD2	-3.73351
SULT1E1	2.47792	IFNK	-3.56724
CXCL14	2.35527	IFI27	-2.94607
TNFSF4	2.16465	IFI44L	-2.84101
PAPSS2	2.05552	IFIT1	-2.68203
GLIPR1	2.03416	MX2	-2.61854
ANO1	1.92593	MX1	-2.43196
TREM2	1.90045	ANPEP	-2.37873
NEFL	1.89273	TXNIP	-2.28848
RPL35A	1.86539	PBX1	-2.18485
ITGA4	1.84288	CYP1A1	-2.1258
IGFL1	1.80728	SEMA6D	-2.12395
ANGPTL4	1.7995	IL8	-2.08927
BICC1	1.78966	OAS1	-2.0712
SLC1A4	1.77383	IFIT3	-2.06189
FHL1	1.76745	GPR1	-2.03442
IL7R	1.7665	USP18	-2.02222
PHGDH	1.74329	EPHA4	-2.01517
IRAK2	1.73158	HIST1H3F	-1.98992
MMP1	1.72778	HCP5	-1.93073
SPRR2A	1.72519	ABI3BP	-1.92131
MCOLN3	1.71764	IGFL3	-1.90776
DYNC1I2	1.69744	IFITM1	-1.88955
COL12A1	1.69613	CYP3A5	-1.84336
NR1D1	1.68872	TIMP2	-1.83115
SLC10A6	1.68781	FDXACB1	-1.8294
SCG5	1.65218	DDX60	-1.79865
RFTN1	1.65047	ORAI3	-1.78824
SACS	1.64492	NRCAM	-1.78006
DDAH1	1.62976	APCDD1	-1.76576
PLA2G7	1.62757	CD14	-1.76431
IL1F9	1.62607	EPHB3	-1.75591
RAB3B	1.62468	OAS2	-1.75209
SLC9A7	1.61464	DSC1	-1.71474
PI3	1.59609	PARP9	-1.70928
ANXA6	1.5744	EGR1	-1.69221
CLDN7	1.57251	DLX3	-1.69023
ADAMTS6	1.57225	KCNMA1	-1.68519
NUAK1	1.56993	OAS3	-1.66597
CACHD1	1.55891	SLC47A2	-1.64974
SERPINB1	1.5581	DDX58	-1.6428
GSTM3	1.5552	DUSP10	-1.63187
MICALCL	1.54376	ARNTL	-1.62456
IL24	1.53432	ZNF323	-1.62158
MCOLN2	1.53374	PART1	-1.62011
ZC3HAV1L	1.51812	GCET2	-1.60773
FLI1	1.51029	IFI35	-1.60568
		RPS10P7	-1.59981
		ECM1	-1.59722

Table 4.5.1 16E2 NTERT array gene regulation. Human exon array analysis was used to generate lists of genes regulated by HPV16 E2 in epithelial cells. Pawel Herzyk carried out Partek analysis to sort the raw data into lists of genes that had a fold-change (F.C) of ≥ 1.5 and a p-value of ≤0.05. Numbers of genes up and down-regulated by each are shown.

Table 4.5.2

Gene Name	Fold-change in array	Fold-change validation
NR1D2	2.54	3.82 ± 0.15
ANO1	1.92	-4.48 ± 0.34
IGFL1	1.8	2.26 ± 0.12
SPRR2A	1.72	-1.43 ± 0.02
EPHA4	-2.01	2.75 ± 0.25
SEMA6D	-2.12	40.86 ± 1.84
PBX1	-2.18	-2.48 ± 0.28
TXNIP	-2.28	-10.84 ± 0.71

Table 4.5.2: Validation of genes regulated by HPV16 E2 in epithelial cells. The table displays the fold-change of 4 up and 4 down-regulated genes that were regulated by 16 $E2^{-WT}$ in the human exon array. Validation was done in triplicate, and fold-change calculated relative to POZN vector control. Standard error is shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.12**.

Table 4.5.3

Network	Associated network functions
1	Dermatological Diseases and Conditions, Antimicrobial Response, Inflammatory Response
2	Organismal Injury and Abnormalities, Respiratory Disease, Cellular Growth and Proliferation
3	Cancer, Cellular Development, Cellular Growth and Proliferation
4	Cellular Movement, Embryonic Development, Organ Morphology
5	Gene Expression, Cardiovascular System Development and Function, Organismal Development

Table 4.5.3: Top 5 16E2 NTERT regulated pathways from Ingenuity Pathway analysis. The 115 $E2^{-WT}$ regulated genes (≥ 1.5-fold) were subjected to IPA and 5 networks of genes were identified. The table lists the predicted functions with the IPA generated networks. (**Genes associated with this table are shown in Supplementary Table S.12 and p-values associated with functions can be found in Supplementary Table S.13**).

Table 4.5.4

Gene Name	Fold-change in array	Fold-change validation
IFIH1	-1.57941	-1.6 ± 0.05
IFI35	-1.60568	-2.11 ± 0.15
DDX58	-1.6428	-4.98 ± 0.41
OAS2	-1.75209	-8.58 ± 0.67
CD14	-1.76431	83.22 ± 6.28
IFITM1	-1.88955	-2.01 ± 0.2
IFIT3	-2.06189	-15.62 ± 1.14
OAS1	-2.0712	-4.58 ± 0.41
IL8	-2.08927	-1.49 ± 0.09
MX1	-2.43196	-18.72 ± 2.25
IFIT1	-2.68203	-8.61 ± 0.81
IFI44L	-2.84101	-2.28 ± 0.13
IFI27	-2.94607	1.31 ± 0.14
IFNK	-3.56724	-2.01 ± 0.2

Table 4.5.4: Validation of interferon genes regulated by HPV16 E2 in epithelial cells. The table displays the fold-change of 14 interferon genes that were regulated by 16 $E2^{-WT}$ in the human exon array. Validation was done in triplicate, and fold-change calculated relative to POZN vector. Standard error is shown. Sybr Green qPCR validation is described in **Methods section 2.2.1.2**.

4.6 Summary and discussion

As well as trying to gain a better understanding how the viral protein E2, and E2 mutants, regulates the HPV genome for the continuation of the viral life cycle, the question of how E2 regulates the host genome was addressed. Previous studies of how E2 regulates the host genome to aid progression of the viral life cycle have focused on determining the effects of E2 on specific promoters and biological processes. There is a gap in our understanding of how E2 regulates cellular processes on a global scale. Additionally, these studies have used cell types that do not tolerate E2 expression well, which in turn will be reflected in any results gained. Due to the toxicity of E2 in most cell types, previous microarray studies have relied on over expression of E2, usually through delivering the E2 protein via viruses or plasmid over expression to investigate the regulation of cellular genes (Vosa, Sudakov et al. 2012, Ramirez-Salazar, Centeno et al. 2011). These studies have been carried out transiently as the cells are destined for growth inhibition or cell death, and this method of E2 protein delivery adds additional toxicity to the cell. Therefore, the U20S model offers the first study of host gene regulation by physiologically tolerated levels of E2 proteins; therefore, the target genes identified are not related to E2 induced apoptosis or growth arrest. Our model system has also allowed us to answer questions about how E2 regulates the host genome in conjunction with TopBP1 and Brd4.

E2 is essential for viral genome replication and maintenance and needs to carry out this function without causing any damage to the host. E2 binds to 12bp DNA motifs (ACCGN4CGGT) or E2 binding sites (E2BS) within the virus genome. In HPV16 there are four specific E2 binding sites within the long control region (LCR), to which E2 binds as a dimer (Androphy, Lowy et al. 1987). This interaction is essential for the transcriptional regulation of HPV. E2 is responsible for the transactivation of the early PV promoter through interactions with transcription regulatory factors such as CBP, p/CAF,BRCA1, Brm and Brd4 (Schweiger, You et al. 2006, Lee, Hwang et al. 2002, Lee, Lee et al. 2000, McPhillips, Oliveira et al. 2006, Kim, Lee et al. 2003, Kumar, Naidu et al. 2007).E2 is also known to repress viral transcription by blocking transcription factor binding to the early promoter and recruiting chromatin modifying factors (Tan, Leong et al. 1994, Schweiger, Ottinger et al. 2007, Wu, Lee et al. 2006a, Smith, White et al. 2010).

As previously mentioned, there have been various studies of E2's interaction with the host genome. A study by Vosa *et al* looked at the location and function of these E2BS within the host genome, using both computational methods and ChIP. They show that there are over 3,300 E2BSs in the human reference genome and E2 binds to E2BS within active chromatin regions. Interestingly, they made a link between E2 binding to sequence specific DNA and an interaction between Brd4, are both thought to be important for E2 binding to consensus sites (Vosa, Sudakov et al. 2012).

Jang *et al* show that in C33a cells both E2 and Brd4 are located at transcriptionally active promoters. However, binding of E2 did not correlate to the E2 consensus binding sites within these promoters. The interaction of E2 at active nuclear regions enables the virus to evade transcriptional silencing (Jang, Shen et al. 2014). So these E2 binding sites are not an indicator of where E2 will bind to in the host genome.

Another study in C33a cells shows that E2 regulates cellular gene expression independently of the other viral genes, using a recombinant adenoviral vector to overexpress HPV16 E2. They suggest that the cellular processes that E2 regulates in this microarray experiment would indicate that E2 expression allows for a convenient environment for the replication of HPV (Ramirez-Salazar, Centeno et al. 2011). There is no overlap in their set of up and down regulated genes in C33a cells with our U20S data set. This may be due to cell type differences or their method for expressing E2 in these cells.

It was originally thought that the regulation of cellular genes when E2 protein is expressed in cervical carcinoma derived cells may be due to the repression of the expression of the two viral oncogenes, E6 and E7 (Hamid, Brown et al. 2009, Gammoh, Isaacson et al. 2009) (Lagunas-Martinez, Madrid-Marina et al. 2010)(Thierry 2009). However, the work of Vosa (Vosa, Sudakov et al. 2012), Ramirez (Ramirez-Salazar, Centeno et al. 2011) and the work described in this thesis all show that E2 induces changes in the expression of cellular genes, independently of the regulation of E6 and E7.

$E2^{-WT}$ results from "array 2", 881 genes are regulated by $E2^{-WT}$ ≥1.5 fold, 408 were up-regulated and 473 were down-regulated. E2 is known to suppress the

activity of papillomavirus promoters by binding to low affinity binding sites, leading to the displacement of cellular proteins (Tan, Leong et al. 1994, Oldak, Smola et al. 2004, Stubenrauch, Leigh et al. 1996, Tan, Gloss et al. 1992).

It is reasonable to propose that protein-protein interactions could be more significant for E2 mediated transcriptional regulation of cellular genes. E2 proteins from several papillomavirus types interact with a variety of cellular regulatory transcription factors, including Sp1, C/EBP, CBP/p300 and p53 (Steger, Schnabel et al. 2002, Hadaschik, Hinterkeuser et al. 2003, Massimi, Pim et al. 1999, Kruppel, Muller-Schiffmann et al. 2008, Wang, Naidu et al. 2009). E2 is also known to interact with Brd4 and TopBP1, which previous studies and the work presented in this thesis shows that they are vital for viral transcription and replication. Brd4 is an important transcriptional co-factor for E2 transcription activation (Wu, Lee et al. 2006a, Schweiger, You et al. 2006, McPhillips, Oliveira et al. 2006). Brd4 is a BET family member that binds to acetylated histones with two conserved bromodomains and remains associated with chromosomes during mitosis (Dey, Chitsaz et al. 2003). The transactivation domain of most PV E2 proteins interact with the CTD of BRD4 protein (McPhillips, Oliveira et al. 2006). It still remains unclear whether HPV16 E2 binds to Brd4 on mitotic chromosomes. Brd4 binding can increase E2 protein stability and may play a role in viral replication, (my results in Chapter 3 confirm this role) (Gagnon, Joubert et al. 2009).

TopBP1 has been shown to interact *in vitro* and *in vivo* with E2 and can regulate the ability of E2 to control transcription and replication (Boner, Taylor et al. 2002). TopBP1 has been proposed to play a role as a transcriptional regulator. If TopBP1 is overexpressed it co-activates transcription with the HPV16 E2 protein when E2 is bound to target promoters (Boner, Taylor et al. 2002). TopBP1 has also been implicated as being a transcriptional repressor; as TopBP1 can interact with the chromatin modification complex proteins Brg1 and repress transcriptional and apoptotic function of E2F1 (Liu, Luo et al. 2004). A feedback loop is formed as E2F1 positively regulates the TopBP1 promoter (Yoshida, Inoue 2004).

TopBP1 is proposed to be the mitotic chromatin acceptor for HPV 16 E2, the association of E2 with chromatin may play a key role in mediating genome segregation and DNA replication function of the E2 protein (Donaldson, Boner et al.

2007). An absence of TopBP1 results in a redistribution of HPV16 E2 into an alternative cellular protein complex, resulting in enhanced affinity for chromatin. This does not significantly alter the ability of E2 to either activate of repress transcription. TopBP1 may also be the mitotic chromatin receptor for HPV16 E2 as it was shown to co-localise on chromatin at late stages of mitosis (Donaldson, Boner et al. 2007).

Based on this information, E2's regulation of the human genome may rely on its successful interactions with host proteins, such as TopBP1 and Brd4. The array results ("array 2") highlighting the genes regulated by E2 when it is not in complex with Brd4 ($E2^{-Brd4}$ mutant) show that E2 is now regulating a larger set of genes than it did previously when it was interacting with Brd4. This mutant regulated 2814 genes ≥ 1.5 fold, 1885 up-regulated and 929 down-regulated. So the other point to note is that the nature of E2's regulation of the host genome has switched, there is now a greater up-regulation of genes.

A comparison of the top 50 up/down-regulated genes both regulated by $E2^{-WT}$ and $E2^{-Brd4}$ indicated that Brd4 is not involved in the down-regulation of the majority of the top 50 $E2^{-WT}$ targets. This is an interesting finding as previous reports have suggested that E2 repression of the LCR is Brd4 related (Schweiger, Ottinger et al. 2007). We show that down regulation of highly regulated genes by E2 is largely independent of Brd4. However, many of the $E2^{-WT}$ genes that are up-regulated are not regulated due to failure to bind Brd4.

Perhaps this suggests these proteins are required in a complex for efficient transcriptional regulation of host genes, and if either of those proteins unable to interact with the complex the quality of E2s transcriptional regulation may be compromised or result in a loss of control. The results from Chapter 3 for both of these mutants indicate that this occurs, especially for $E2^{-Brd4}$, which has the most compromised transcription activation (**Figure 3.4**).

Cellular movement came up as one of the main pathways affected by E2 (in "array1"). Some of the genes regulated by E2 involved in cellular movement are SH3PXD2B, FGD4, FAP and ARGIDIB. To test how E2 affects cellular migration I employed wound healing assays to evaluate whether cells expressing E2 vs non-

E2 expressing showed any differences in movement. From this work it was clear that E2 expressing cells fail to heal wounds like non-E2 expressing cells, despite growth rates being similar. Movies following the movement of E2 and non-E2 expressing cells over a 24 hour time period (can be found in supplementary files in publication) clearly showed that E2 expressing cells have defective movement, and struggle to migrate. This provides a phenotype to match the pathways regulated in the microarray.

Morison *et al* demonstrated that in HeLa cervical cancer cells, E2 expressing cells had increased motility. However, this is not a good model to investigate the role of E2 in cellular movement as these cells have a background of HPV, and this increase in movement cannot be solely contributed to viral E2 alone (Morrison, Morreale et al. 2011). In cervical cancer, the HPV genome becomes integrated and results in the loss of E2 expression (Mair, Kubicek et al. 2014)(zur Hausen 2009). As discussed in Chapter 1, the loss of E2 results in the increase of expression of E6 and E7 as their promoters are no longer repressed by E2; this may lead to genomic instability and the transformed phenotype associated with cancer. In transformed cells where E2 is no longer expressed, cell movement may reflect the non-E2 expressing cells condition in the wound healing assays. The faster movement of cells may be more reflective to those in a cancerous state. The loss of E2 control on cellular movement could be mediated by the absence of regulation of gene splicing (as mentioned in publication (Gauson, Windle et al. 2014), or due to altered gene regulation.

When performing this same experiment with the two E2 mutants,it was found that the $E2^{-TopBP1}$ had a similar defect to $E2^{-WT}$, but $E2^{-Brd4}$ expressing cells displayed a partial phenotype. This suggests that TopBP1 is not responsible for E2s movement defect, and Brd4 is perhaps only partially involved. Other proteins that interact with E2 are controlling E2 regulation of genes whose products are involved in cellular movement.

Overall this study suggests that when the E2-TopBP1-Brd4 complex is defective, the interaction of the viral E2 protein with the host genome is altered. An interesting follow up to this experiment would be to see the effect of the E2 mutant that fails to bind both TopBP1 and Brd4; does this lead to a loss of control of the

regulation of host genes? As the viral life cycle is closely linked to the differentiation of host cells, and the regulation of which, is also linked to interactions with host proteins, perhaps not binding TopBP1-Brd4 and the non-specific regulation of the host genome may be related to the quality of viral transcription and replication of the viral genome or transcriptional control of the host genome.

HPV18 E2 also regulated similar pathways to HPV16 E2, although there were similarities between the two high-risk HPV types there were also differences. IPA analysis showed that both of these high-risk HPV types are involved in cellular movement, cellular assembly and organisation and cellular development functions. No movement assays were carried out for HPV18 E2, although, it is expected they would demonstrate a similar phenotype due to the pathways the genes are involved in. These findings complement the functional assay results gathered in Chapter 3, which also show that these two HPV types have differences. This highlights the fact that studies between HPV types are not comparable when looking at processes such as replication.

Genes altered by HPV16 E2 in the U20S cell model were also shared with genes regulated in clinical samples of HPV infected cells (found in 3 separate microarray studies). Genes changed in low grade and high grade cervical squamous intraepithelial lesions compared to a normal cervix were investigated, as well as comparisons of genes changed between high grade cervical squamous intraepithelial lesions, cervical squamous cell carcinomas, and normal cervix.

20 genes out of 74 from “array 1” were found to be regulated in a similar way to at least one of the data sets probed. This suggests that E2 may reprogram the host genome during infection and could leave epigenetic marks to continue E2 regulation of genes, even after E2 expression is lost after integration of the viral genome. It could be speculated that some of the changes of E2 gene regulation of the host could be epigenetically inherited, and despite E2 being lost in transformed cells, E2’s influence on the genome may still be active. Equally, a subset of the genes that were found to be regulated by E2 in the array may not still be regulated upon transformation. Further experiments to determine what happens epigentically with E2’s regulation of the host genome in patient tumour samples is required to elucidate the key changes that are responsible for the progression to cancer. Where does E2 locate in host chromatin? Is this different

in transformed and non-transformed cells? Is this location on host chromatin linked to the interaction with the E2-TopBP1-Brd4 complex? If yes, can we target these proteins to make antiviral therapies?

Chromatin modifications at E2 regulated promoters could be responsible for the alterations in gene expression. A better understanding of these chromatin modifications is required in order to reverse the control that E2 has on the cellular genome. This is important as we have shown E2 manipulates the host genome and this likely contributes to the viral life cycle. Ultimately, reversing the effects of E2 on the genome may affect HPV transformed cells and we could utilise pre-existing therapeutics that target chromatin-modifying enzymes and epigenetic regulators to reverse the effect of E2. Using resources such as TCGA and ENCODE could offer to be useful tools to progress this element of research.

The U20S model is not the most suitable to answer this question. Future work will focus on NTERTs/NOKS and even looking directly at patient samples. The preliminary results in the E2 expressing NTERT cells suggest that E2 is regulating interferon genes to promote viral infection, and evade immune response. Future RNA-seq and ChIP-seq experiments will be carried out in epithelial cells that stably express E2.

Chapter 5 – Conclusions and Future Work

Despite the availability of prophylactic vaccines against HPV16/18/6/11, the battle against HPV related cancers is not over (Schiller, Castellsague et al. 2012).The need to develop novel antiviral therapies still remains. The vaccine only protects against the two most prevalent types of HPV and cannot clear pre-existing HPV infection. It is possible that other high-risk oncogenic HPV types may become more prevalent after the introduction of this vaccine. Small molecule inhibitors that block the interaction of the viral replication factors, E1 and E2, are not effective across all HPV types due to slight differences between E1-E2 interactions in different HPV types (White, Titolo et al. 2003). However, the interaction between TopBP1 is confirmed in BPV1 E2 which suggests that the interaction is conserved across all PV types, which makes this interaction an excellent candidate for viral therapy (Donaldson, Boner et al. 2007). It is likely that the interaction between the viral proteins and that of the host is more conserved across HPV types.

From Chapters 3 and 4 we have established that E2 forms a complex with the host proteins TopBP1 and Brd4, and this complex is essential for the initiation of viral replication, as well as the regulation of the host genome, in both HPV16 and 18. This complex offers a good target for anti-viral therapies for HPV related cancers. However, TopBP1 may prove to be the better pan-type specific target as it is required in both HPV16 and 18 viral replication and transcription, whereas HPV18 E2 does not require Brd4 for replication function. Perhaps HPV18 E2 interacts with a different set of proteins for this regulation, and may explain why the disease outcome is different between these two high-risk types.

Inhibiting the interaction between E2 with TopBP1, or disrupting TopBP1 function, is a promising target for the following reasons:

1. TopBP1 is essential for optimum E2 mediated DNA replication, and has been shown to be required for replication initiation.

2. TopP1 is a candidate mitotic chromatin receptor for HPV16 E2.

3. TopBP1 helps E2 regulate the host genome.

4. There are known enzymes that target TopBP1

TopBP1 is both phosphorylated and acetylated by other host proteins, such as; Akt and SIRT1. These modifications act as a switch to regulate the many functions of TopBP1, and these modifications can be altered using enzymes that target TopBP1. Three Akt inhibitors which are in clinical trial include; perifosine (KRX-0401, Aeterna Zentaris/Keryx), MK- 2206 (Merck), and GSK-2141795 (GlaxoSmithKline)(Pal, Reckamp et al. 2010). There are also a number of SIRT1 inhibitors such as; Suramin (Trapp, Meier et al. 2007), Sirtinol (Grozinger, Chao et al. 2001), Tenovin-1 (Lain, Hollick et al. 2008), Salermide (Lara, Mai et al. 2009), and Cambinol (Heltweg, Gatbonton et al. 2006). Here, how TopBP1 and E2 modifications may benefit the viral life cycle and why these inhibitors could be useful in future studies will be discussed.

Lysine acetylation has been found in a diverse range of organisms, from bacteria to humans which would suggest that the regulatory functions of acetylation are well conserved (Kim, Yang 2011, Soufi, Soares et al. 2012). This would allow the virus a mechanism to alter the host regulation of multiple cellular processes. Acetylation regulates nucleic acid-protein interactions and also protein-protein interactions. Acetylated lysines act as docking sites for proteins that contain acetyllysine recognition domains, such as bromodomains (Zeng, Zhou 2002, Zeng, Zhang et al. 2010). Lysine acetylation is regulated by three types of proteins: bromodomain (BRD) proteins (Filippakopoulos, Knapp 2014, Barneda-Zahonero, Parra 2012), histone acetyltransferases (HATs), histone deacytalses (HDACs) and sirtuins (SIRTs) (Dhalluin, Carlson et al. 1999, Seto, Yoshida 2014, Chen, Fu et al. 2014, Anand, Brown et al. 2013). These proteins all regulate lysine acetylation in different ways. BRD proteins bind to acetylated lysine and therefore act as readers for the state of lysine acetylation. HATs affect lysine acetylation acting as writers. Finally, HDACs and SIRTs remove acetyl groups and are erasers (Anand, Brown et al. 2013).

TopBP1 and Treslin cooperate in the loading of Cdc45 onto replication origins (Kumagai, Shevchenko et al. 2010, Zegerman, Diffley 2007, Zegerman, Diffley 2010). Lui *et al* have shown that the acetylation of TopBP1 regulated by SIRT1 is essential for the TopBP1-Treslin interaction and DNA replication (Liu, Lin et al.

2014), and propose that this offers to be a mechanism to regulate DNA replication under normal and stress conditions. TopBP1 is normally acetylated in S phase, allowing for TopBP1 to bind to Treslin and in turn initiation of replication. However, in unfavourable nutrient deprived conditions, the activity of SIRT1 is up-regulated (Canto, Auwerx 2009, Cohen, Miller et al. 2004, Escande, Chini et al. 2010) and TopBP1 acetylation is decreased, resulting in a decrease of TopBP1-Treslin interaction and the activation of metabolic checkpoint. Treslin is also a target for S phase Cdk2 or possibly E2(Johansson, Graham et al. 2009). Therefore, it is possible that the E2-TopBP1 interaction could also be regulated by phosphorylation and acetylation pathways.

Several proteins are known to be phosphorylated upon entry into S-phase which are involved in DNA replication. Proteins are phosphorylated by kinases such as Cdk2 and this is essential for replication function (Aleem, Berthet et al. 2004). The increase of Cdk2 activity in early S phase is known to promote the loading of proteins onto the viral origin of replication such as Cdc45 for the initiation of replication. Additionally, phosphorylation of the viral E1 protein by Cdk2 is essential for the initiation of viral replication (Ma, Zou et al. 1999).

The work of Johansson *et al* shows that E2 is stabilised in S-phase of the cell cycle and this may be associated with an increase in phosphorylation of E2 protein. Phosphorylation of E2 may be essential for optimum viral DNA replication (Johansson, Graham et al. 2009). In Lorna Macintosh's thesis work (**Figure 28**- accessible though Glasgow Libraries), E2 is shown to have a preferential interaction with TopBP1 in S-phase, phosphorylated E2 and TopBP1 peak in S-phase. The $E2^{-TopBP1}$ mutant does not show this increase in S-phase. The E2 mutant may not be a substrate of Cdk2, and hence is not phosphorylated.

Another kinase which may phosphorylate E2 is Cdc7. Cdc7 is a serine-theronine kinase which is essential for the initiation of S-phase. If Cdc7 is inhibited in cancer cells it prevents the progression into S-phase, accumulating DNA damage, followed by the induction of p53-independent cell death (Yoshizawa-Sugata, Ishii et al. 2005, Im, Lee 2008, Kim, Kakusho et al. 2008, Montagnoli, Tenca et al. 2004). However, if Cdc7 is inhibited in normal cells it does not significantly affect cell viability. Cdc7 inhibitors are non-toxic and causes cell death preferen-

tially in only cancer cells. Many Cdc7 inhibitors, first were identified by at Nerviano Medical Sciences Srl by high throughput screening using biochemical kinase assay (Montagnoli, Valsasina et al. 2008, Vanotti, Amici et al. 2008, Jackson 2008). Due to their low toxicity, Cdc7 inhibitors may be a good tool to reduce E2 phosphorylation, and therefore, blocking E2-TopBP1 interaction, without being toxic to cells.

HPV infection has also been linked to increased Akt activity (Pim, Massimi et al. 2005)(Menges, Baglia et al. 2006). Akt stimulates cell survival signalling pathways and inhibits DNA damage checkpoint response (King, Skeen et al. 2004, Tonic, Yu et al. 2010, Xu, Hegarat et al. 2010). Akt is a protein kinase which is known to phosphorylate TopBP1 at the Ser-1159 residue, which in turn induces oligomerisation of TopBP1 through its 7^{th} and 8^{th} BRCT domains. This phosphorylation event is crucial for TopBP1 to interact with and repress E2F1(Liu, Paik et al. 2006). The repression of E2F1 by TopBP1 is mediated by TopBP1s recruitment of the Brg1-Brm chromatin –remodelling complex (Liu, Luo et al. 2004). Liu *et al* show that oligomerisation effects TopBP1 function in checkpoint activation by preventing its recruitment to chromatin and subsequent binding to ATR upon replicative stress, as well as, it induces the interaction with E2F1. Therefore, they propose that Akt may function as a switch for the function of TopBP1, switching between checkpoint activation to transcriptional regulation (Liu, Graves et al. 2013). Akt inhibitors inhibit TopBP1 oligomerisation and result in its function to switch back to checkpoint activation and no longer promote cell survival.

TopBP1 is also a known mediator for the oncogenic gain-of-function activities of mutant p53 (mutp53) in cancer (Chowdhury, Lin et al. 2014). A drug capable of blocking TopBP1 oligomerisation and p53 binding is Calcein AM. This drug has significant anti-tumour activity in a wide range cultured cancer cells harbouring high levels of TopBP1 (Chowdhury, Lin et al. 2014). The activity of this drug results in the re-activation of E2F1-dependent apoptosis and blockade of mutp53 gain-of-function. Therefore, proving that this drug has potential for targeting TopBP1 for cancer therapy (Chowdhury, Lin et al. 2014).

Brd4 may also be a valuable target for novel antiviral therapies. In the field of cancer research, epigenetic deregulation of transcription is now appreciated to

be as important for carcinogenesis as genetic mutation. Bromodomain-containing proteins are of substantial biological interest, as components of transcription factor complexes and determinants of epigenetic memory (Filippakopoulos, Knapp 2014, Rosner, Hengstschlager 2012, Simo-Riudalbas, Esteller 2014). The bromodomain and extraterminal domain (BET) family of proteins are known to play an essential role in regulating transcription by RNA polymerase II (Pol II) (Dey, Ellenberg et al. 2000). Bromodomain proteins are chromatin "readers"; they recruit chromatin-regulating enzymes, including "writers" and "erasers" of histone modification, to target promoters and regulate gene expression (Rosner, Hengstschlager 2012).

Brd4 is recognised as a critical mediator of normal and disease functions through its interactions with both cellular and viral factors (Wu, Chiang 2007a), and is now known to be a promising target for many cancers (acute myeloid leukemia, multiple myeloma, Burkitt's lymphoma, NUT midline carcinoma, colon cancer) because of its fundamental role in transcriptional processes (French, Miyoshi et al. 2003, Crawford, Alsarraj et al. 2008, Zuber, Shi et al. 2011, Mertz, Conery et al. 2011, Rodriguez, Huidobro et al. 2012). Brd4 promotes transcription by recruiting the transcriptional elongation factor, p-TEFb, to promoters to enhance phosphorylation of the C-terminal tail (CTD) of RNA polymerase II promoters, and additionally by directly phosphorylating the RNA polymerase II CTD (Wu, Chiang 2007a, Devaiah, Lewis et al. 2012). Brd4 is also known to remain bound to transcriptional start sites of gene expression during M1/G transition, influencing mitotic progression (Yang, He et al. 2008, Dey, Nishiyama et al. 2009). Hence, Brd4 recruits P-TEFb to mitotic chromosomes resulting in increased expression of growth promoting genes. Brd4 has also been identified as an essential protein for viral transcription in HPV as well as many other viruses (Wu, Chiang 2007a). Brd4 is an essential transcriptional co-activator for all E2 proteins, and is found ubiquitously in all proliferating cells (Houzelstein, Bullock et al. 2002), therefore blocking the interaction of the host protein with viral E2 is a viable target for HPV related cancers.

There are currently a number of clinical trials investigating the targeting of BET family proteins. These include RVX-208, I-BET 762, OTX 015, CPI-0610 and TEN-010. Results from the trial of OTX 015 has shown promising results for the treatment hematologic malignancies (Boi, Gaudio et al. 2015, Ito, Umehara et al.

2011, Chung, Coste et al. 2011, Zeng, Li et al. 2005). Two groups have reported inhibitors of BET bromodomains, JQ1 and I-BET, which have high affinity for all bromodomains of the BET family. Both JQ1 and I-BET engage the bromodomain pocket in a manner that is competitive with acetylated peptide binding, which in turn causes the displacement of all four BET proteins from chromatin in cells upon exposure to these compounds (Ceribelli, Kelly et al. 2014).

Initial studies on cellular model systems of viral infections have demonstrated that bromodomain inhibitors have promising efficacy, but more detailed mechanistic studies are needed to understand the complex mechanisms that control viral replication and regulation of viral latency *in vivo*.

Figure 5.1

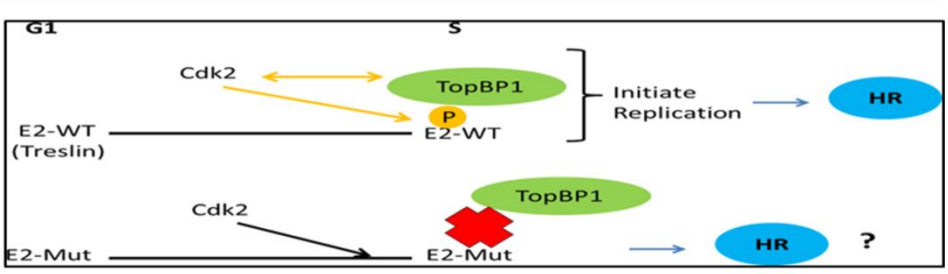


Figure 5.1: E2 is phosphorylated by Cdk2 to facilitate an interaction with TopBP1 to initiate viral replication. Mutant E2$^{-TopBP1}$ is not a substrate of Cdk2 and cannot be phosphorylated. TopBP1 is not able to interact with E2$^{-TopBP1}$ and cannot initiate replication.

Future directions

E2 is phosphorylated by Cdk2 to facilitate an interaction with TopBP1 which is required for initiation of viral replication (**Figure 5.1**). The $E2^{-TopBP1}$ mutant may not be a substrate of Cdk2, and the lack of phosphorylation may prevent the binding of TopBP1 (**Figure 5.1**), hence the defect in replication seen in Chapter 3 (**Figure 3.3 and 3.5**). The E90V mutation in E2 lies between two possible phosphorylation sites (**Figure 1.7**), which may be responsible for a lack of phosphorylation by Cdk2 or Cdc7 kinases, resulting in impaired interaction between E2 and TopBP1. As the $E2^{-TopBP1}$ mutant is not completely null in replication, $E2^{-TopBP1}$ may utilise interactions with other host proteins to carry out viral replication, although not as efficiently. Results from Chapter 3 (**Figure 3.8.1** and **3.8.2**) clearly show that TopBP1 is required for the initiation of replication and for the formation of replication foci. Under normal conditions, HPV activates the ATM-dependent DNA damage response to induce viral genome amplification upon epithelial differentiation. Gillespie *et al* have shown that homologous DNA recombination factors are localised to viral replication foci, and suggest that HPV activates the ATM pathway to recruit these repair factors to foci to aid in HPV replication (Gillespie, Mehta et al. 2012). If replication foci fail to form, proteins involved in homologous DNA recombination may not co-localise together to facilitate replication. In the absence of replication foci, replication can still occur, although, the quality of the replication may be severely compromised. Perhaps the mechanism the virus uses to replicate may also change when E2 fails to bind to TopBP1.Additonally, the results from the U20S microarray show that when E2 fails to bind TopBP1, it regulates the expression of an increased number of genes. Perhaps, these are interactions that E2 uses to compensate for the roles TopBP1 fulfils within the viral life cycle. Future work should focus on making a phosphorylation mutant of E2 to test in viral life cycle models.

It is possible that acetylated TopBP1 may bind to one of Brd4's bromodomains at one side, and to E2 on the other. Brd4 would still free to interact with both the host chromatin and transcription factors, perhaps bringing the E2-TopBP1 complex to open sites of chromatin and the promoters of host genes for their regulation. This is a perfect environment for the virus to conduct viral transcription and replication as it has everything it needs. TopBP1 may also bind to host chro-

matin. Previous work suggests that TopBP1 may be the mitotic chromatin acceptor for HPV16 E2, and could play a role in mediating genome segregation and DNA replication functions of the E2 protein (Donaldson, Boner et al. 2007).

Perhaps Brd4 and TopBP1 chaperone E2 to the viral origin to initiate replication as the result from the ChIP assays in Chapter 3 (**Figure 3.6**) show that E2 does not locate to the origin independently of E1. Alison McBride's group did a ChIP-on Chip experiment to investigate where E2 binds to in the host genome, they found that E2 did not locate to E2 binding sites, but instead there was an overlap between E2 and Brd4 on chromatin (Jang, Kwon et al. 2009a).

Drugs that target Brd4 and TopBP1 could be used in future studies. There are no inhibitors of E2-Brd4 or E2-TopBP1. Inhibiting the function of TopBP1 or Brd4 with FDA approved drugs could affect viral replication.

Inhibitors could be offered to vulnerable groups such as, pregnant women, transplant patients, HIV infected individuals, and women in third world countries. The treatment could potentially be offered in a similar manner to the treatment program currently offered to those with HSV-2 infection, to dampen down the production of infectious virus particles. If one of the discussed inhibitors could be given at sub-toxic doses it offers to be a potential therapeutic.

Supplementary data

S.1: Vec vs $E2^{-WT}$ (array 1)

S.2: $E2^{-WT}$ IPA (array 1)

S.3: Vec vs $E2^{-WT}$ (array 2)

S.4: $E2^{-WT}$ IPA (array 2)

S.5: Vec vs $E2^{-Brd4}$ (array 2)

S.6: $E2^{-Brd4}$ IPA (array 2)

S.7: Vec vs $E2^{-TopBP1}$ (array 1)

S.8: $E2^{-TopBP1}$ IPA (array 1)

S.9: Vec vs 18E2 (array 2)

S.10: 18E2 IPA (array 2)

S.11: NTERT 16E2 (array 3)

S.12: NTERT 16E2 IPA (array 3)

S.13: IPA gene function p-values

All supplementary files can be found on the accompanying disc.

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