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HUMAN PAPILLOMAVIRUS 16 E2 REGULATES HOST CELL PATHWAYS IMPORTANT FOR CANCER PROGRESSION AND TREATMENT SENSITIVITY WHICH MAY CONTRIBUTE TO CANCER OUTCOMES

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A dissertation submitted in partial fulfillment of the requirements for the degree of

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Dedication

This dissertation is dedicated to my grandfather

Mr. Frederick “Seale” Brand

who throughout trials and tribulations has never faltered and served as a role model for the person I want to be. Thank you for teaching me unwavering honesty and dedication and loyalty towards family and friends. The strong work ethic you helped cultivate in me will forever follow me in life in all of my future endeavors.

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Abstract

HUMAN PAPILLOMAVIRUS 16 E2 REGULATES HOST CELL PATHWAYS IMPORTANT FOR CANCER PROGRESSION AND TREATMENT SENSITIVITY WHICH MAY CONTRIBUTE TO CANCER OUTCOMES

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Human papillomaviruses (HPVs) are causative agents in around 5% of all cancers, including cervical and oropharyngeal. A feature of HPV cancers is their better clinical outcome compared with non-HPV anatomical counterparts. In turn, the presence of E2 predicts a better clinical outcome in HPV-positive cancers; the reason(s) for the better outcome of E2-positive patients is not fully understood.

Previously, we demonstrated that HPV16 E2 regulates host gene transcription that is relevant to the HPV16 lifecycle in N/Tert-1 cells. One of the genes repressed by E2 and the entire HPV16 genome in N/Tert-1 cells is TWIST1. In these studies, we demonstrate that TWIST1 RNA levels are reduced in HPV-positive versus HPV-negative head and neck cancer and that E2 and HPV16 downregulate both TWIST1 RNA and protein in our N/Tert-1 model; the HPV16 E6/E7 oncogenes cannot repress TWIST1. E2 represses the TWIST1 promoter in transient assays and is localized to the TWIST1 promoter; E2 also induces repressive epigenetic changes on the TWIST1 promoter. TWIST1 is a master transcriptional regulator of the epithelial to mesenchymal transition (EMT), and a high level of TWIST1 is a prognostic marker indicative of poor cancer outcomes. We demonstrate that TWIST1 target genes are also downregulated in E2-positive N/Tert-1 cells and that E2 promotes a failure in wound healing, a phenotype of reduced EMT.

E2 binds to a variety of host factors that help mediate its function, one of which is tumor suppressor p53. The tumor suppressor p53 primarily functions as a transcription regulatory factor during cellular stress and DNA-damage, leading to cell cycle arrest, senescence, and apoptosis. In HPV positive head and neck cancer, E6 degrades p53 in a cell-cycle specific manner, contributing to oncogenesis yet still allowing significant p53 expression under certain conditions. We found that p53 expression is retained in HPV-positive cell lines and patient derived xenograft models. While we found that TWIST1 repression by E2 is not dependent of the E2-p53 interaction, this interaction is independently important in

sensitizing multiple cell lines to chemotherapy. Moreover, we demonstrated that this E2-p53 interaction is critical for the viral lifecycle and primary keratinocytes immortalized by a mutant deficient in E2-p53 interaction fail to proliferate or complete a viral lifecycle in organotypic raft culture models. We propose that TWIST1 repression, as well as E2 induced chemosensitivity may provide two independent mechanisms for better clinical response of E2-positive HPV tumors.

Chapter 1: Introduction

1.1 Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) are the most common malignancies that arise in the head and neck. These tumors develop in the mucosal epithelia of tissues in the oral cavity such as the tongue, larynx and pharynx as well as nasal tissues (**Fig. 1)** (1). The incidence of new cases of HNSCC are growing worldwide. Oral and laryngeal cancers are classically associated with tobacco and alcohol assumption while those arising in the oropharynx are associated with previous high-risk HPV infection, primarily HPV16 (2, 3).

HNSCC is the sixth most common cancer worldwide was the cause of over 400,000 deaths in 2018 (4). The incidence of HNSCC continues to rise and is estimated to increase by up to 30% by the year 2030 (5). While increasing HNSCC rates in regions such as Southeast Asia are associated with alcohol, tobacco or other carcinogen consumption, in the United States and Europe, HPV infection has been a primary driver of increased oropharyngeal cancer (OPC) rates (6, 7). Moreover, oropharyngeal cancer has a propensity to affect men aged 50 or older and men are three times more likely to develop OPC compared to women (8).

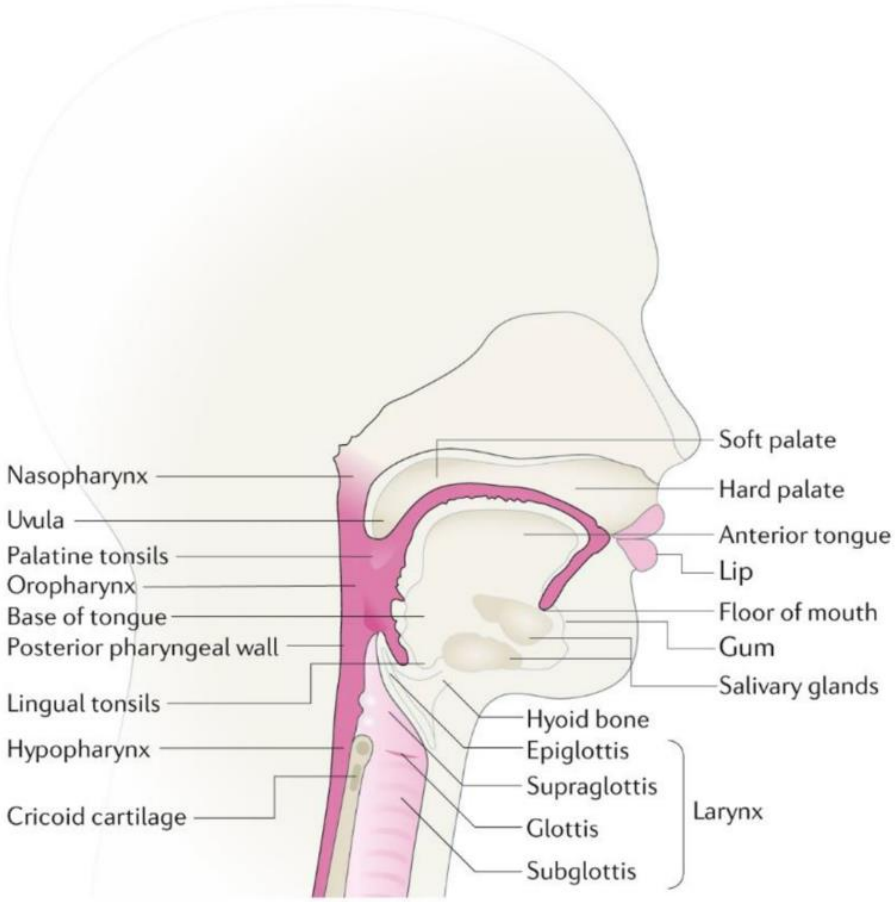


Figure 1. Schematic illustration of primary sites of HNSCC. HNSCC is classified by the site of the arising disease. HNSCC of the Nasopharynx, laryngeal tissues (hypopharynx) or the oral cavity (oropharynx) are most often associated with use of carcinogenic substances such as tobacco and alcohol. Oropharyngeal cancer including that of the base of the tongue and tonsils are most often caused by HPV infection. Image adapted from Johnson *et al. 2020 (1).*

1.2 HNSCC diagnosis and treatment

HNSCC is an adult cancer with a median age of diagnosis of 66 years for HPV-negative and 53 years for HPV-positive tumors (9, 10) The primary presenting symptom is related to mass visualization in oral tumors and functional disturbances such as dysphagia or ear pain with those in the oropharynx or larynx. This leads to later diagnosis and worsening prognosis of those in these more hidden anatomical sites (1). Diagnosis of HNSCC is established by biopsy of the primary tumor and histopathological analysis revealing disorganized cells with irregular keratinization and the appearance of “keratin pearls” (**Fig. 2A**). Interestingly, HPV-positive HNSCC tumors have a more poorly differentiated appearance compared to HPV-negative HNSCC tumors (**Fig. 2B and C**) (11). HPV-positive HNSCCs often have enhanced expression of the cyclin-dependent kinase inhibitor $p16^{INK4A}$, which has become an identifier for HPV-related disease (**Fig. 2C**) (12).

The management of HNSCC involves local surgical resection in patients with no lymph node involvement. In those that do experience nodal metastasis, surgical intervention is followed up with targeted radiation therapy to the head and neck with or without platinum-based chemotherapy such as cisplatin or carboplatin (CRT) (13). Currently the only FDA-approved targeted therapy against HNSCC is the monoclonal antibody epidermal growth factor inhibitor, cetuximab. Interestingly it has been found that cetuximab has lower treatment efficacy in patients with HPV-positive disease, demonstrating that different pathways may be important in HPV-positive versus HPV-negative tumors (14).

In patients with metastatic or recurrent HNSCC, systemic immunotherapy modalities have been developed with limited success. The immune checkpoint inhibitor pembrolizumab is a monoclonal antibody which activates the programmed cell death receptor 1 (PD1). A recent clinical trial has found that pembrolizumab is superior to cetuximab when combined with CRT in patients with HNSCC (15). In the case that a patient is a poor candidate for pembrolizumab, the nucleotide analog 5-fluorouracil or the tubulin targeting agent paclitaxel is added to CRT in advanced stage disease (16). Other clinical trials of combination therapies are ongoing, including the use of Pembrolizumab plus the multiple kinase inhibitor Lenvatinib and are scheduled to be completed in the approaching years (17). Even if remission is achieved, long term quality of life is often poor due to late effects of the standard of care therapy such as continued dysphagia, difficulty speaking (dysphonia) or localized pain, highlighting the need for new therapies and biomarkers for treatment response (1).

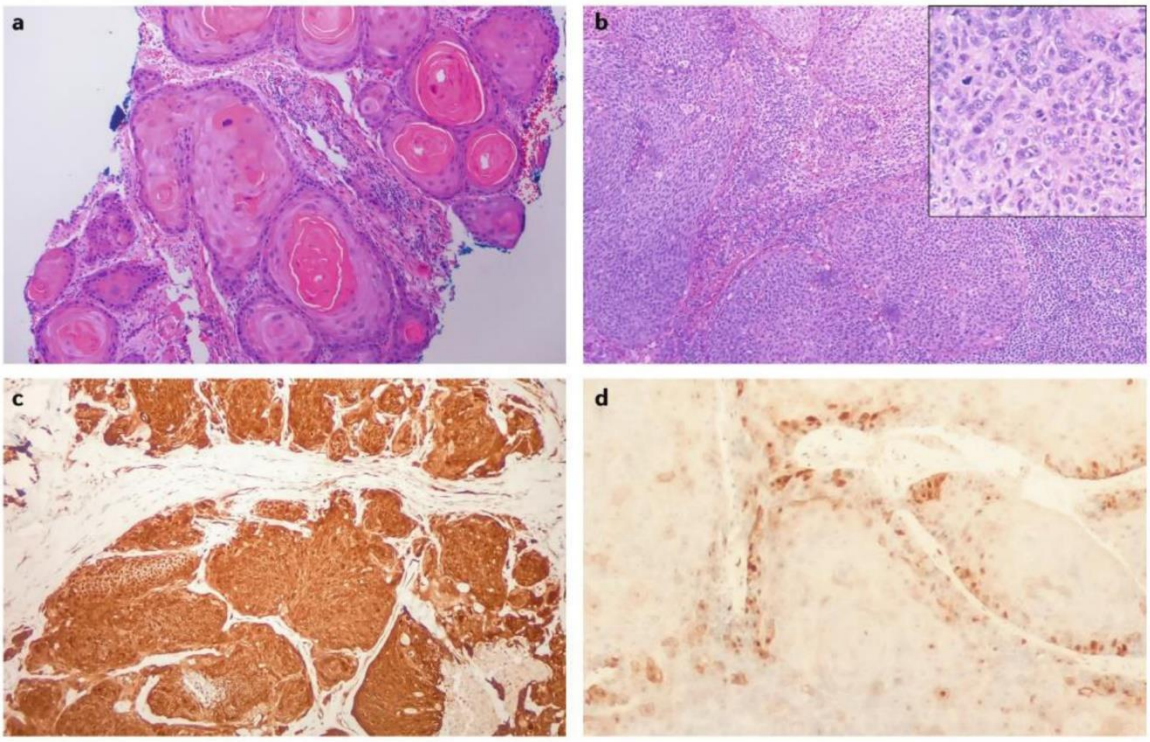


Figure 2. Histopathology sections of several presentations of HNSCC. (**A**) Well-differentiated squamous cell carcinoma deriving from the tongue with notable keratin pearls. (**B**) Poorly-differentiated tumor from the base of the tongue. (**C**) Oropharyngeal tumor with positive IHC staining for $p16^{INK4A}$, which is a clinical correlate with HPV-positive disease. This is compared to a negatively staining sample in **D**. Image adapted from Johnson *et al.* 2020 (1).

1.3 Human papillomaviruses (HPVs)

HPVs are non-enveloped double stranded DNA viruses which possess a tissue tropism for both cutaneous and mucosal epithelia (18). Viruses cause up to 15% of all human cancers and approximately 5% of cancers are caused by HPV (19, 20). The prevalence of HPV associated cancer has reached epidemic proportions in recent years (21). HPVs are broadly classified into high risk and low risk, depending on their carcinogenic propensity. High-risk HPVs, or Alphapapillomaviruses, such as subtypes 16, 18 and 31 are the primary causative agents for most cervical and vulvar cancers in women and oropharyngeal cancers in men **(Fig. 3)** (8). Low-risk HPVs, also referred to as Betapapillomaviruses such as 6 and 11 are cutaneous viruses which cause anogenital warts (18, 22). The high-risk strain HPV16 is the most common of these viral carcinogen in oropharyngeal cancer causing up to 95% of new cases; HPV16 also causes up to 50% of all cervical cancers (23).

HPV-associated HNSCC has distinct epidemiological and molecular characteristics compared to HPV-negative disease (24). Along with a predominance in men and a younger age of diagnosis, HPV-associated HNSCC is associated with fewer and different somatic mutations with oncogenesis being primarily driven by virally expressed oncogenes. Moreover, numerous studies have reported that there exists a difference in patient outcomes. Compared to HPV-negative HNSCC, HPV-positive cancers have improved 5-year disease free survival and overall survival outcomes; the mechanisms mediating these

differences have not been fully elucidated (21, 25-27). These findings have prompted clinical trials focusing on reducing treatment aggressivity In HPV-positive patients. This is referred to as de-escalation therapy, with the goal to mitigate harsh treatment side effects while maintaining favorable treatment response (28-30). However, these efforts have yielded mixed results. Therefore, there exists the need for novel targeted treatments as well as reliable biomarkers for enhanced treatment response in patients with HPV-positive HNSCC.

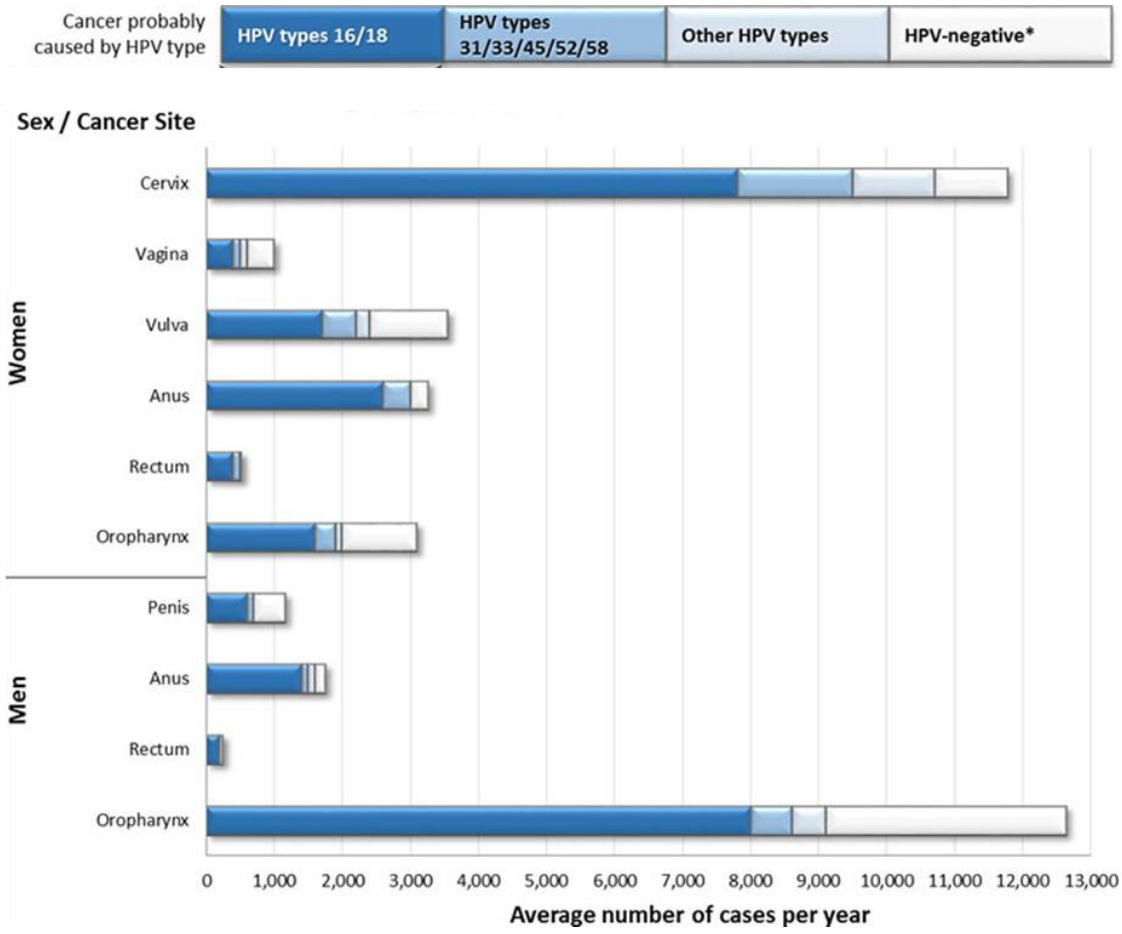


Figure 3. Average number of new cases of HPV positive and negative cancers per year by sex and anatomical site. Data summarizes new cases of HPV associated cancers between 1993 and 2005. HPV types 16 and 18 are the main causative agents in the majority of HPV associated cancers in women. In men, HPV16 is the main causative agent responsible for oropharyngeal cancer. Data adapted from Saraiya *et al. 2015 (8).*

1.4 HPV Genome organization and viral protein function

The HPV DNA genome is 8 kb in length and encodes for nine viral open reading frames, two promoters and a viral long control region (LCR) also known as the upstream regulatory region (URR) which contains the viral origin as well as multiple cis-regulatory sequences for initiating transcription (31, 32) (**Fig. 4)**. The viral proteins are termed either Early (E) or Late (L), depending on time of expression and which of the two viral promoters they are regulated from. Early proteins function primarily as viral and host regulatory factors which assist in the viral lifecycle within the host cell. The late proteins consist of the viral capsid proteins which only function during cellular exit as well during cell entry (33). The main functions of each viral protein are summarized in **Table 1.**

E1 is the only HPV viral helicase. E1 operates in its di-hexameric form to bind to the viral origin during replication and recruit host cellular polymerases to replicate the viral episome (34). Specifically, E1 recruits RPA and DNA polymerases and other host factors to sites of viral replication shortly after its association with E2 which is described below. Moreover, we have reported that E1 can displace Werner Helicase (WRN) from viral replication foci which leads to enhancement of viral replication at the expense of host replication fidelity (35). WRN is critical for the arrest of host replication forks and DNA repair and E1 can destabilize WRN. This leads to enhanced mutational frequency and possibly contributes to both viral integration and carcinogenesis. E1 may also have some

host transcriptional regulatory functions as it appears to regulate several host genes in cellular proliferation and innate immunity (36).

E2 is the primary transcription regulatory factor for HPV and essential for recruiting E1 to initiate replication (37). E2 also has roles in regulating both virus and host transcription and other pathways which will be described in more detail later.

E5 is a hydrophobic membrane protein only encoded by alphapapillomaviruses and is considered a secondary oncoprotein with roles in promoting cellular transformation and cancer progression by activating the epidermal growth factor receptor (EGFR) and also has roles in immune evasion (38).

The E6 oncoprotein acts primarily by inhibiting the function of tumor suppressor p53, both by direct proteasomal degradation with the help of E6-associated protein E3 ubiquitin ligase (E6-AP) as well as transcriptional repression at the p53 promoter (39). This leads to enhanced cellular proliferation and inhibition of apoptosis and senescence in Alphapapillomaviruses. In Betapapillomaviruses, E6 does not degrade p53 but still acts via transcriptional repression (23). E6 has undergone extensive study, revealing other regulatory roles that allow HPV infected cells to escape cell-cycle checkpoint. P53 is acetylated by p300 to promote its stability, leading to enhanced target gene expression (40). E6 can bind to and inhibit p300, leading to reduced acetylation and consequently lower stability synergizing with its E6-AP mediated degradation (41, 42). E6 can also act

independently of its regulation of p53 and block apoptotic signals such as those triggered by tumor necrosis factor as well as directly bind to and inhibit the Fas-associated death domain, a key mediator in the extrinsic apoptotic signaling pathway (43, 44). Conversely, in both high and low-risk HPV, E6 can inhibit the intrinsic apoptosis pathway, by binding to and inducing degradation of the bcl-2 family protein BAK (45, 46).

The E7 oncogene binds to and inactivates the tumor suppressor protein Rb which results in activation of the cell cycle regulatory factor E2F1 leading to reduced growth arrest and senescence. (39). E7 interacts with a variety of other cell cycle regulators such as cyclins A and E and p27, as well as transcription factors such as AP-1 and interferon regulatory factor 1 (IRF-1) contributing to enhancement of cell-cycle dependent viral replication and immune evasion, respectively (47-49). E7 also interacts with the p53 target p21 inhibiting its function and thus synergizing with E6 to assist in cellular transformation (50). E7 has also been shown to activate E2F1 independently of Rb (51).

The viral proteins are expressed as a single long transcript which is then spliced into individual mature messenger RNAs. This splicing gives rise to at least two additional viral proteins with notable functions. E1^E4 is a highly expressed protein in intermediate stages of the viral lifecycle which is controlled by keratinocyte differentiation and is thought to facilitate genome amplification. It also has roles in viral shedding by disrupting keratinocyte intermediate filaments to allow for virus egress from the cell (52). E1^E4 also functions in cell cycle

regulation, promoting G2 arrest to allow for maintenance of sufficient viral genome copies in proliferating cells (53). A recently characterized E8^E2 protein is also the result of alternative transcript splicing. This protein shares the DNA binding motif with full length E2 and may act as a negative regulator of viral transcription to maintain low-level persistent infection (54).

Towards the end of the viral lifecycle, cellular signals initiate transcription at the viral late promoter and the L1 and L2 capsid proteins are expressed. L1 is the primary structural protein which has the ability to self-assemble into the viral icosahedral capsid. The L2 or minor capsid protein has functions in assisting the maturation of the icosahedral capsid as well as assisting in capsid unpackaging upon entry into a newly infected cell (55, 56).

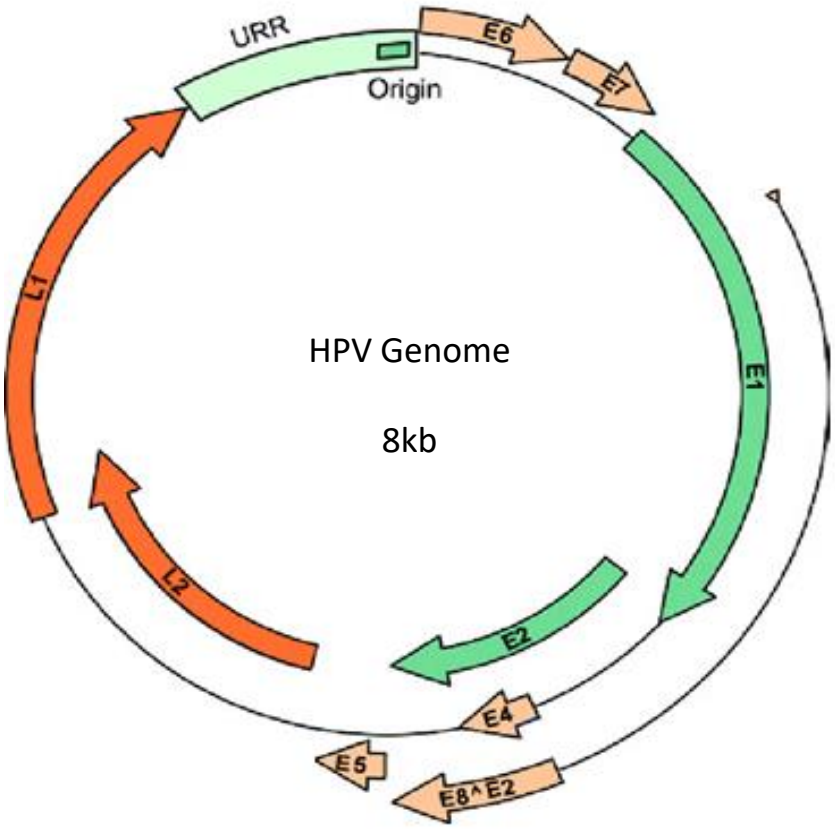


Figure 4. Genome organization of HPV. The 8 kb genome is organized into 7 early genes (E1-E8; there is no E3) and 2 late genes (L1 and L2). The viral upstream regulatory region (URR/LCR) contains the viral origin of replication and the binding sites for E1 and E2. The L1 protein forms the major icosahedral capsid whose assembly and maturation is regulated by L2. The early genes are expressed as a single open reading frame which is then spliced into individual mature transcripts. L1 and L2 are expressed off of an independent late promoter towards the end of the viral lifecycle. Image adapted from Mcbride *et al. 2017* (32)

Table 1. Overview of HPV proteins and their major functions (23)

Viral Protein	Major Functions
E1	Origin binding DNA helicase – requires E2 to initiate viral replication
E2	Homodimeric DNA binding protein – regulates viral transcription, supports DNA replication, segregates viral genomes to daughter cells, regulates host gene expression
E1^E4	Intermediately expressed protein – promotes viral genome amplification, regulates cell cycle by inducing G2 arrest, disrupts keratinocyte intermediate filaments to allow for shedding of mature viral capsids.
E5	Hydrophobic membrane proteins only in Alphapapillomaviruses – disrupt immune detection and acts as minor oncoprotein by activating EGFR
E6	Inhibits and (and degrades in Alphapapillomaviruses) p53 via interaction with the E3 ubiquitin ligase E6-AP – promotes cellular proliferation, inhibition of apoptosis and senescence and leads to cellular transformation
E7	Binds to and disrupts pRB leading to E2F1 activation and cell cycle progression – can directly activate E2F1 promoting cellular proliferation
E8^E2	Contains DNA binding domain of E2 – binds to viral promoter to negatively regulate viral transcription and maintain persistent infection
L1	Major capsid protein – Self assembles into icosahedral capsid containing 360 L1 proteins
L2	Assists in packaging viral genomes in late stages of infection – assists in viral entry into the host cell and nucleus

1.5 HPV viral lifecycle

The mature HPV virion infects the dividing basal cells in cutaneous or mucosal tissues, gaining access to this lower layer of cells via microabrasions or wounds in epithelia (57). The viral capsid attaches itself to heparan sulfate proteoglycans on the basement membrane. The infectious particle is then transferred to a currently unknown receptor(s) on keratinocytes to promote cellular entry (58, 59). The viral capsid is endocytosed and the L2 protein is transferred to the vesicle membrane which conceals the virus from initial immune recognition (60). The HPV genome is then delivered to the nucleus and undergoes initial replication.

During establishment and initial replication, E2 forms homodimers and bind to the viral origin, E1 is then recruited along with host polymerases and the viral episome is rapidly amplified to around 50-100 copies (32). This limited initial replication is thought to be controlled by the negative regulation by the E8^E2 protein described above (61). This initial low viral genome copy number is maintained and replication continues stoichiometrically with the replication of cellular DNA (62). Throughout this maintenance phase, newly replicated episomes are partitioned between dividing daughter cells utilizing E2, providing a direct tether between host chromatin and the viral genome (63). This segregation function of E2 is mediated by several proteins, likely acting in a larger complex including the

BET family member Bromodomain containing 4 protein (BRD4) as well as Topoisomerase-binding protein 1 (TopBP1) (64-66).

As the basal epithelial cells proliferate, the viral oncogenes E5, E6 and E7 work in concert to promote continued proliferation and prevent cell cycle exit. In normal epithelia, as the basal cells divide and grow apically, they terminally differentiate, leading to loss of cellular replicative potential. The viral oncogenes combat this by allowing these post-mitotic differentiating cells to continue proliferating, leading to enhanced epithelial tissue thickening and initiating early stages of pre-cancerous lesions (39, 57, 67) (**Fig. 5)**. Most infections are cleared naturally via an individual's innate and adaptive immune system. However in some patients, HPV is not resolved and this persistent infection can lead to oncogenesis (57).

As the infected keratinocytes continue to differentiate toward the apical surface of the tissue, the viral late promoter located within the E7 gene is activated, triggering expression of the major and minor capsid proteins L1 and L2, respectively. This is partnered with increased levels of E1, E2 and E1^E4 leading to genome amplification to greater than 1000 copies per cell (68). This is referred to as the amplification or vegetative replication stage. Viral episomes are then packaged and shedding occurs by the sloughing off of dead keratinocytes from the surface, or squames (22).

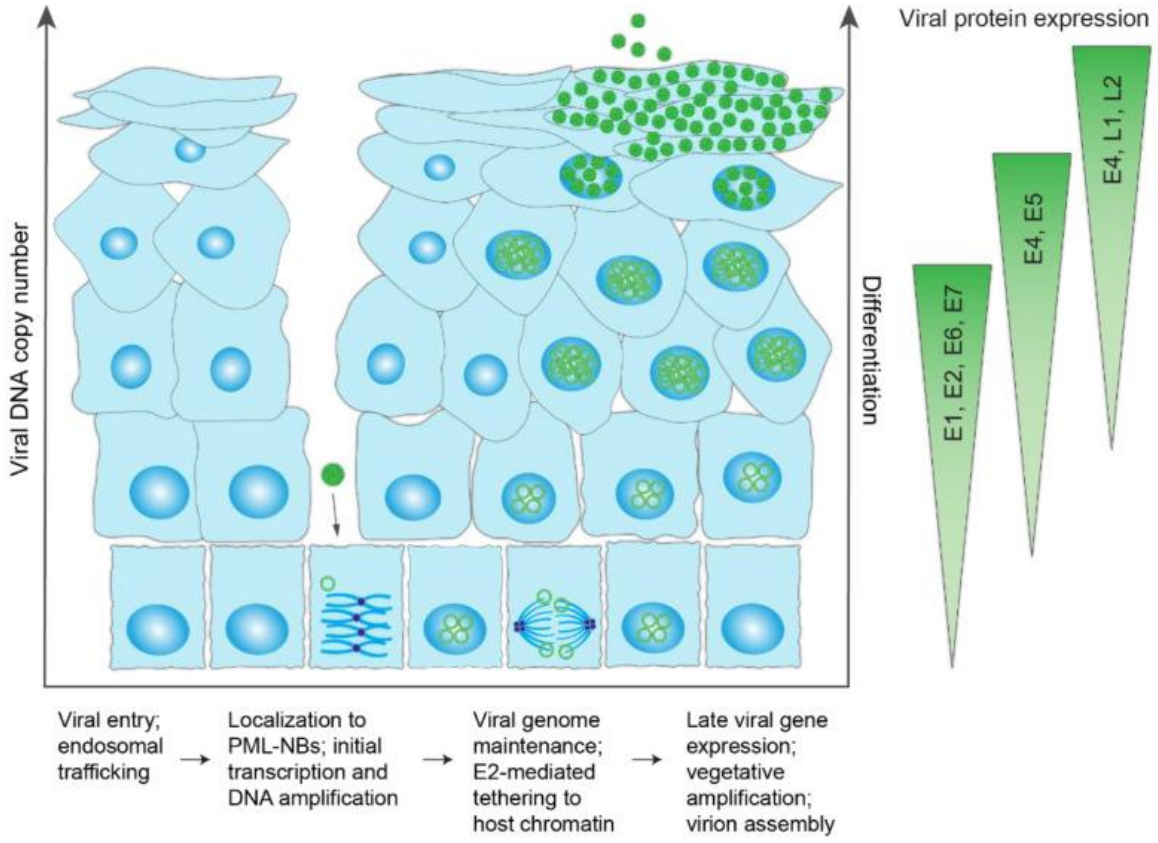


Figure 5. The HPV lifecycle is closely linked to keratinocyte differentiation. Infection is established in the basal epithelial cell layers. Viral particles gain entry to this layer via small microabrasions or wounds in the tissue. Infection is then established by rapid genome amplification to less than 100 viral copies. As the infected keratinocyte differentiates, the oncogenes promote cell cycle progression in post-mitotic cells to promote continued viral replication and maintenance. In the later stages of differentiation, the viral genome is amplified to greater than 1000 copies per cell and new infectious particles are packaged and released in dead keratinocytes or squames. Image adapted from Mcbride *et al. 2021 (57).*

1.6 HPV activation of the DNA-damage response (DDR)

Throughout the viral lifecycle, the virus upregulates cellular DNA repair machinery to assist in its own replication. This is done by activation of Ataxia telangiectasia and Rad3-associated (ATR) and Ataxia telangiectasia, mutated (ATM) proteins, key mediators of the DNA-damage response pathway (DDR) **(Fig. 6)** (69). Both ATR and ATM are serine/threonine kinases. The ATM pathway responds to DNA double-strand breaks initially recognized by the MRN (MRE11-RAD50-NBS1) complex (70). The MRN complex then recruits ATM to sites of damage leading to its autophosphorylation and resulting in phosphorylation in downstream mediators including Checkpoint kinase 2 (CHK2) and phosphorylated Histone 2A (γ-H2AX). ATM also upregulates repair factors such as BRCA1 and BRCA2 which repair these DNA breaks via homologous recombination (HR) (71).

The ATR pathway is activated by cellular replication stress and primarily single-strand DNA nicks at stalled replication forks. Replication protein A (RPA) associates with these single strands and recruits ATR and the binding partner ATRIP. This pathway is facilitated by TopBP1 (72). This results in downstream activation of a series of nucleases which initiate the repair process. While ATM and ATR are activated by different types of genomic stress, they share several common downstream mediators including BRCA1, BRCA2 and others (73).

The activation of the DDR is critical for HPV replication in infected cells (74-76). ATM and ATR activation both contribute to viral replication in different viral

lifecycle stages and the activation of which is induced by several viral proteins. In cells expressing the E6 and E7 alone, or in concert with the rest of the viral genome, ATR and ATM are constitutively active (77). This leads to sequestering of repair complexes important for rapid replication of the viral genome. Moreover, several studies have demonstrated that viral replication itself activates ATM and ATR, most notably by the genomic stress induced by the E1 helicase (78, 79). While in uninfected cells, DDR activation would normally result in pause of the cell cycle, the action of the viral oncogenes on p53, pRB and EGFR allow continued proliferation in the face of this enhanced signaling. This persistent replication in the face of DDR activation is largely responsible for infected cell genomic instability and can result in viral genome integration into host DNA described below (79).

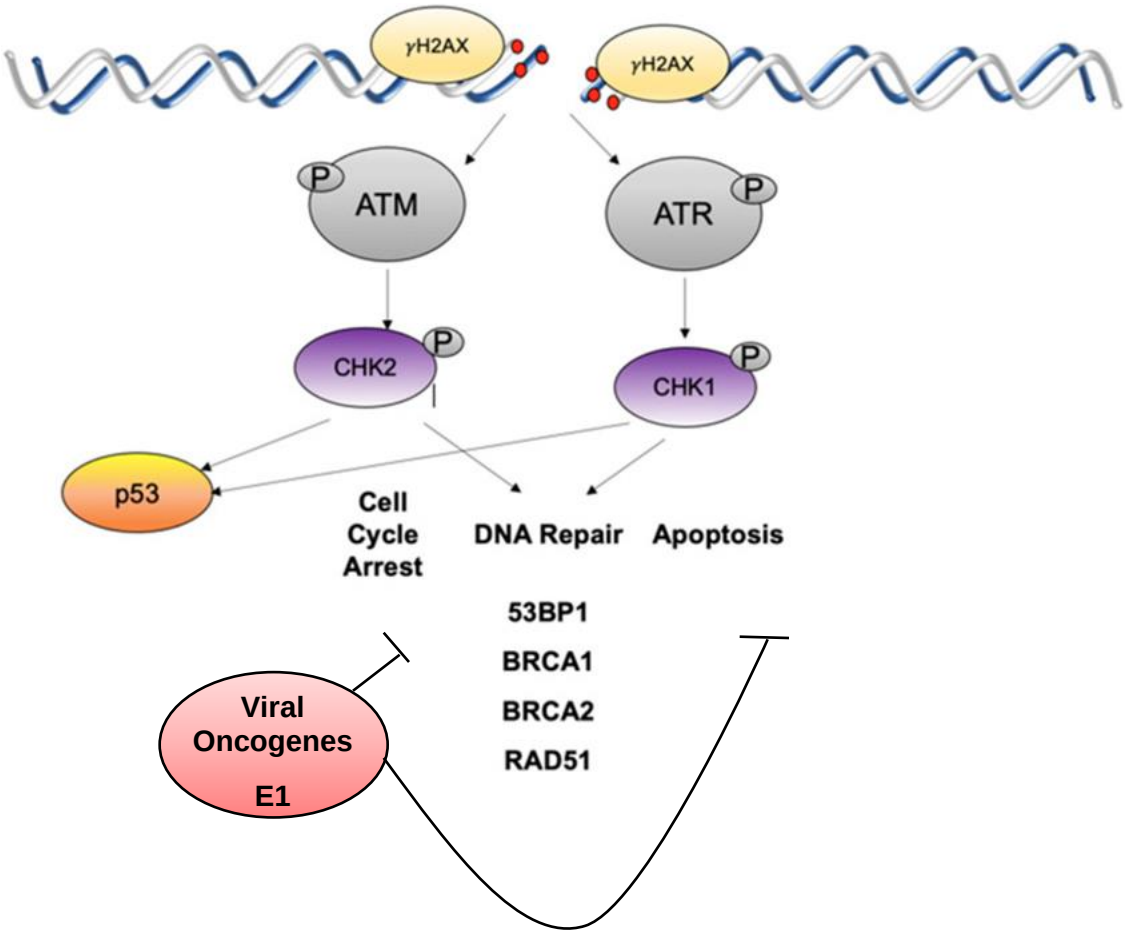


Figure 6. ATR and ATM activation of the DNA Damage response. Several viral proteins including E6, E7 and E1 activate ATR and ATM signaling leading to persistent high DDR levels. The infected cell responds by upregulating repair factors such as BRCA1 and BRCA2 which are sequestered by the virus to promote its own replication. The viral oncogenes act to prevent cell cycle arrest and apoptosis during this process leading to enhanced host genome instability. Figure adapted from Kono *et al.* 2021 (69).

1.7 HPV genome status and integration

Persistent activation of the DDR leads to genomic instability not only for the host genome but that of the virus as well. Like host replication, continued viral replication with elevated genotoxic stress can induce DNA double-strand breaks which provides a substrate for integration into the host genome via low-fidelity repair pathways such as non-homologous end joining (80). This is a common occurrence in cervical cancer caused by high-risk HPVs and is thought to be a key step in cervical cancer progression (81-83).

In the majority of integration events, the viral breakpoint is in the E2 gene resulting in its disruption. This may be due to repetitive DNA sequences which contribute to particular instability (84). Traditionally, it was proposed that because E2 represses viral transcription at the viral LCR, its disruption leads to enhanced E6 and E7 expression leading to enhanced oncogenic potential (85, 86). In agreement with these findings, cervical tumors and cell lines that do retain E2 expression are associated with less aggressive phenotypes (87, 88). While this provides a simple model for carcinogenesis in HPV-positive cervical cancer, in HNSCC, the HPV genome often remains episomal and retains E2 expression (80, 89).

In HNSCC, like cervical cancer, integration is associated with worse outcomes. (90-93). However, for reasons not entirely understood, most HPV positive HNSCCs retain episomal genomes and thus E2 expression. This has permitted

additional studies that have revealed that E2 itself is an independent marker for improved cancer outcomes, independent of E6 and E7 oncogene expression levels (80, 89, 94). These findings have prompted our studies into the potential anti-oncogenic effects of HPV16 E2 in HNSCC.

1.8 The E2 protein

HPV16 E2 is a 43 kDa DNA binding protein and the first viral protein to be expressed in HPV infection (95). E2 has three domains: an approximately 100 amino acid C-terminal DNA-binding domain and a larger ~200 aa N-terminal transactivation domain liked by a flexible hinge domain (**Fig. 7**) (96-98). The DNA binding domain (DBD) recognizes the palindromic sequence $ACCGN_4CGGT$ which is present in close tandem repeats in the viral URR (99). The transactivation domain binds to other viral proteins including E1 which it recruits to the viral origin to initiate replication. This domain also has roles in transcriptional regulation of both the host and virus and viral episome tethering to host chromatin during mitosis. All three domains can bind to a variety of host factors to help facilitate the function of E2; a few of which are described below (64, 99, 100).

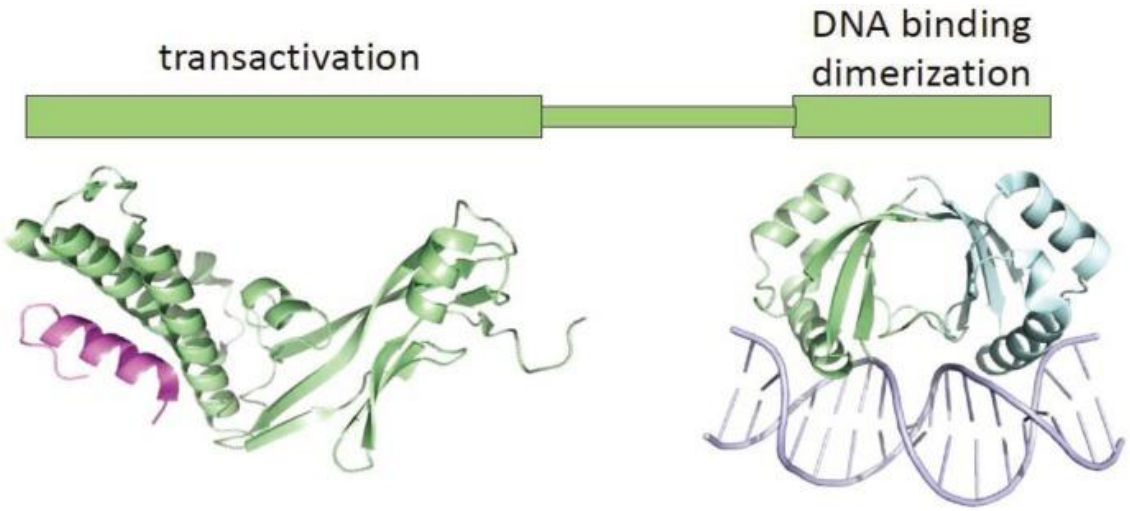


Figure 7. X-ray crystallographic and cartoon schematic images of the TAD and DBD of E2. The DBD recognizes short palindromic sequences with the motif $ACCGN_4CGGT$ which exists repeatedly in the viral URR. Image adapted from Mcbride *et al. 2013* (97, 98, 101).

1.8.1 Functions of E2 and their roles in the HPV16 viral lifecycle

HPV E2 is the primary transcription and replication regulatory factor for the virus. It has additional roles in viral genome regulation, host transcriptional regulation, cell cycle progression, apoptosis and senescence and binds to a wide variety of host proteins to facilitate these functions (**Fig. 8**) (99, 102-105).

1.8.2 Viral replication initiation

E2 is essential for HPV viral replication and is the first detectable viral protein expressed upon initial infection (95). In the host nucleus, E2 homodimerizes and binds to the short palindromic repeats in the URR near the A/T- rich viral origin. The transactivation domain sequesters single E1 molecules which then bind to ATP. ATP binding disrupts the E1-E2 interaction displacing E2 from the origin as well as promoting self-assembly into a di-hexameric E1 complex the origin (106). Afterwards E1 recruits a repertoire of host replication factors including: replication protein A (RPA), topoisomerase 1 and DNA polymerases α and ε (32, 62, 107, 108). In undifferentiated basilar keratinocytes, ATP-dependent replication then proceeds bi-directionally around the 8kb viral episome (109).

1.8.3 Viral episome segregation

During cellular division of the HPV infected host cell, the 8kb viral episome could be unevenly distributed to the daughter nuclei. This disparity could propagate resulting in reduced viral copy number over several generations, leading to

reactivation of innate immune defense and death of the infected cell (63). To ameliorate this, the virus evolved a mechanism to retain and evenly segregate viral genomes to the daughter cells. HPV16 E2 facilitates this process by binding the viral genome via its C-terminal DNA binding domain and tethering it to the host chromosome using its N-terminal domain. This interaction is mediated by TopBP1 which binds to the Serine 23 residue on E2. This interaction can only occur after phosphorylation by Casein Kinase 2, forming a complex with other host factors which allow for proper segregation (65).

1.8.4 Transcription

E2 is the viral transcriptional regulator of HPV (99). E2 binds specifically to sequence motifs in the viral URR to either activate or repress transcription depending on the location of these sites in relation to the gene of interest. When these sites exist in short tandem succession upstream from a heterologous promoter such as the Herpes simplex virus thymidine kinase promoter (TK), the result is robust transcriptional activation and gene product expression (65, 110). Conversely, if the HPV viral LCR is upstream of the same promoter construct, the result is dose dependent repression (111). Expression of exogenous E2 in cervical cancer lines causes a similar repression of the URR resulting in reduced oncogene expression and cell cycle arrest, senescence and apoptosis (112, 113).

E2 has also been shown to regulate host cell transcription (100). E2 can bind to promoter-proximal activator protein 1 (AP-1) sites in the host genome to

transcriptionally activate matrix metalloproteinases (114). Additionally, E2 can bind to and synergize with CCAAT/enhancer binding proteins (C/EBPs) to regulate keratinocyte differentiation (115). E2 by itself and in the context of the full viral genome can induce transcriptional repression of innate immune genes important in the unphosphorylated interferon-stimulated gene factor 3 (U-ISGF3) pathway (116). This pathway is important for cellular response to initial viral exposure as well as DNA damage. Moreover, E2 expression results in repressive DNA methylation via recruitment by DMNT1 at these genes which can be reversed pharmacologically with DMNT1 inhibitors (104, 117).

It has been reported that the splicing of host genes important in cancer and cellular motility is also regulated by E2 suggesting that E2 can regulate host gene expression on multiple levels (100). E2 can also interact with other host factors that themselves regulate host transcription including BRD4, Topoisomerase 1, BRCA1, poly(ADP-ribose) polymerase 1 (PARP1), Sp1 and Mdm2 (118-120). Lastly, we recently found that E2 can transcriptionally repress the epithelial-mesenchymal regulator TWIST1 which has key roles in the cancer progression and treatment sensitivity (94). This will be discussed later and in **Chapter 3.**

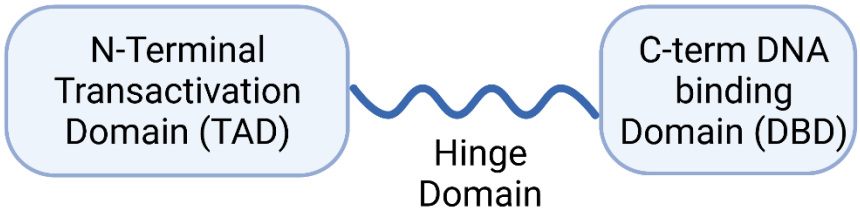
1.8.5 Cell cycle progression, senescence, and apoptosis: The role of p53

In cervical cancer, E2 acts via multiple mechanisms to induce growth arrest and senescence in HPV positive cell lines. As described in above, expression of exogenous E2 in HeLa, SiHa or CaSki cervical cancer cells results in robust binding to the viral LCR causing widespread viral transcriptional repression, and reduction of the E6 and E7 oncogenes expression as well as their function. This causes a restoration of p53 and pRb function and recognition of host cell genomic instability caused by the virus, leading to downstream DDR pathway activation and cell cycle arrest or senescence (85). Consequently, a large proportion of HPV-positive cervical cancer cell lines which are dependent on oncogene function cannot tolerate exogenous E2 expression (99, 110). However, E2 regulation of cell cycle is not limited to its function as a transcriptional repressor.

E2 can also initiate apoptosis in a subset of HPV-negative cell lines by binding the tumor suppressor p53 in Alphapapillomaviruses (121). In this context, the C-terminal DBD of E2 simulates the binding pocket of 53BP2, which closely interacts with p53. This is a direct interaction and has been validated in vitro (121). HPV16 E2 can then directly activate p53 in HeLa cervical cancer cells leading to apoptotic cell death independent of E6 and E7 expression levels. Moreover, specific mutations in E2 that inhibit the DNA binding affinity of the C-terminal domain but retain p53 affinity do not inhibit this function in both HPV-positive and HPV-negative cell lines (122). This suggests that although the DNA and p53 binding share the C-terminal domain, these functions may operate largely

independently of one another. Interestingly, despite the potential genome status and functional differences between cervical and HNSCC cells, few efforts have been made to study the p53-E2 interaction in the context of HNSCC or primary keratinocytes. This work attempts to remedy this lack of knowledge and will be discussed further below and in **Chapter 4.**

Lastly, high-risk E2 has been shown to induce apoptosis in the absence of p53 or any other viral factors by inducing cleavage of caspase-8 a key downstream initiator for the extrinsic apoptosis pathway (123). This is mediated by the N-terminal TAD of E2 and expression of a truncated recombinant TAD is sufficient to activate caspase 8 in Saos-2 osteosarcoma cells. Moreover this function can occur without the need for caspase-8 to interact with its canonical host adapter proteins (124).



Notable host interactors	**BRD4, CASP8, TopBP1**	**SP1**	**BRCA1, PARP, MDM2, TP53**

Figure 8. The many host binding partners of the HPV E2 protein. E2 binds to a large variety of host factors to regulate both the virus and the host. Those mentioned in the text are above. E2 has been shown to have direct interactions with over 40 host proteins, many of which not well described (105) . Image created with Biorender.com.

1.9 The role of p53 in HPV-positive HNSCC

In most cervical cancer cell lines and tumors, the vast majority of p53 is degraded which is mediated by a high level of full-length E6 expression. This direct degradation is initiated by the formation of a larger 40kDa complex with p53 and the E3 ubiquitin ligase E6AP. E6 directs the activity of E6AP to p53, leading to its degradation via the proteasome, promoting genomic instability and cell cycle progression (125-127). This model has led to the dismissal of any importance of the E2-p53 interaction. However, this pathway additionally has a negative feedback mechanism via alternative splicing of E6.

E6 can be alternatively spliced to at least 3 additional shorter transcripts: E6*, E6*I and E6*II (128, 129). E6* and E6*I retain the ability to bind to E6AP, but lose p53 affinity, resulting in a dominant negative inhibition of E6-mediated p53 degradation in a cell cycle specific manner (130, 131). Therefore, instead of global degradation of p53, there appears to be a finelyt tuned regulation of p53 levels in HPV positive cells allowing for enhanced expression during the viral lifecycle. The purpose of cell-cycle specific p53 restoration is not known. While in cervical cancer, these splice variants play a minor role, largely overshadowed by a large amount of full-length E6, we and others have demonstrated that these are actually the dominant variants in HNSCC (92). This once again highlights that there exists key differences between cervical cancer and HNSCC and p53 may be a major contributor. These findings have prompted the studies in **Chapter 4.**

1.10 Epithelial-mesenchymal transition (EMT) and its importance in cancer

Epithelial-mesenchymal transition (EMT) and its reverse process mesenchymal-epithelial transition (MET) are important processes that occur routinely throughout embryogenesis (132, 133). For example, during normal embryo development, undifferentiated stem cells undergo EMT to form the mesoderm which develops into multiple tissue subtypes. This process is known as gastrulation. Later, many mesodermic cells undergo MET to form epithelial organs such as the ovary and kidney (134). These processes modulate pathways important in cellular motility, invasiveness and apoptotic resistance (135, 136). Consequently, EMT plays an important role the process of wound healing, allowing for enhanced migration of keratinocytes to close cutaneous injuries (137). EMT is promoted by a series of master transcriptional regulatory proteins including SNAI1, the zinc finger E-box binding homeobox 1 (ZEB1) and Twist1 (136), which have many overlapping functions (**Fig. 9**). SNAI1 acts to inhibit the expression of Epithelial (E) cadherin molecules and promote loss of both cell-cell adhesion and apical-basilar polarity of epithelial cells (138). The addition of Epidermal Growth Factor (EGF) can directly activate SNAI1 through p21-activated kinase 1 (PAK1). PAK1 phosphorylates SNAI1 promoting its nuclear translocation and transcriptional activity (138). ZEB1 is regulated by multiple pathways including WNT and TGF-β signaling. It also has roles in repressing E-cadherin, resulting in loss of cellular tight junctions and increased motility (139). Twist1 is a basic helix-loop-helix (bHLH) transcription factor activated by the nuclear factor κB (NF- κB)

during mesodermal development in embryonic tissues. It binds directly to the E-box sites on the *E-Cadherin* promoter to also repress its transcription (140). It also has additional roles in inducing the upregulation of lower adhesion N-cadherins as well as upregulation of the mesenchymal cytoskeletal filament vimentin resulting in the heightened motility in mesenchymal cells (141, 142).

EMT has also been found to play an important role in cancer progression, treatment sensitivity and metastasis (140, 143). Cancers that have high expression of EMT markers are associated with poorer survival. Not surprisingly, this is due in part to enhanced stem cell qualities such as motility and loss of adhesion which permits tumor cells in forming secondary metastases in other bodily sites. Twist1 depletion results in reduced metastatic potential of breast cancer cells *in vitro* and *in vivo* (144). Moreover, in hepatocellular carcinoma, Twist1 regulates vimentin expression by depleting micro-RNA's targeting vimentin transcripts enhancing protein translation (141). Additionally, EMT itself is associated with decreased responsiveness to cancer treatment. KPC mice which spontaneously develop pancreatic cancer respond better to treatment with nucleotide analog chemotherapies with transgenic knockout of Twist1 or SNAI1 (145). Additional work has shown the importance of EMT in the treatment of breast cancer. MMTV-*Py*MT mice have tissue specific expression of the Polyomavirus middle T oncoprotein, leading to spontaneous development of mammary adenocarcinoma, which resembles patient breast ductal tumors (146). Fischer *et al.* demonstrated that while disrupting EMT did not result in changes in metastatic burden in these

mice, they elicited enhanced response to cyclophosphamide, a standard of care treatment for advanced stage breast cancer (147).

The relationship between HPV and EMT is a topic of recent interest. Overexpression of HPV16 E6 and E7 oncogenes can induce EMT via STAT3 activation in non-small cell lung cancer cells (148). Moreover expression of the viral oncogenes in cells generated from normal cervical tissue also upregulates Vimentin and decreases E-cadherin expression, but only after treatment with fibroblast growth factors 1 and 2 (FGF1/2) (149). Normal gingival keratinocytes also show markers of EMT after E6 and E7 expression, but only after exogenous overexpression of EGFR, illustrating that the oncogenes alone are insufficient to regulate EMT in keratinocytes (150). Because HPV-positive HNSCC is associated with better treatment outcomes than HPV-negative HNSCC, we postulated that HPV infection may reduce markers of EMT leading to enhanced treatment sensitivity and lower metastatic potential. We also believed that if true, this was likely independent of E6 and E7 and other viral proteins may play a role. These studies are presented in **Chapter 3.**

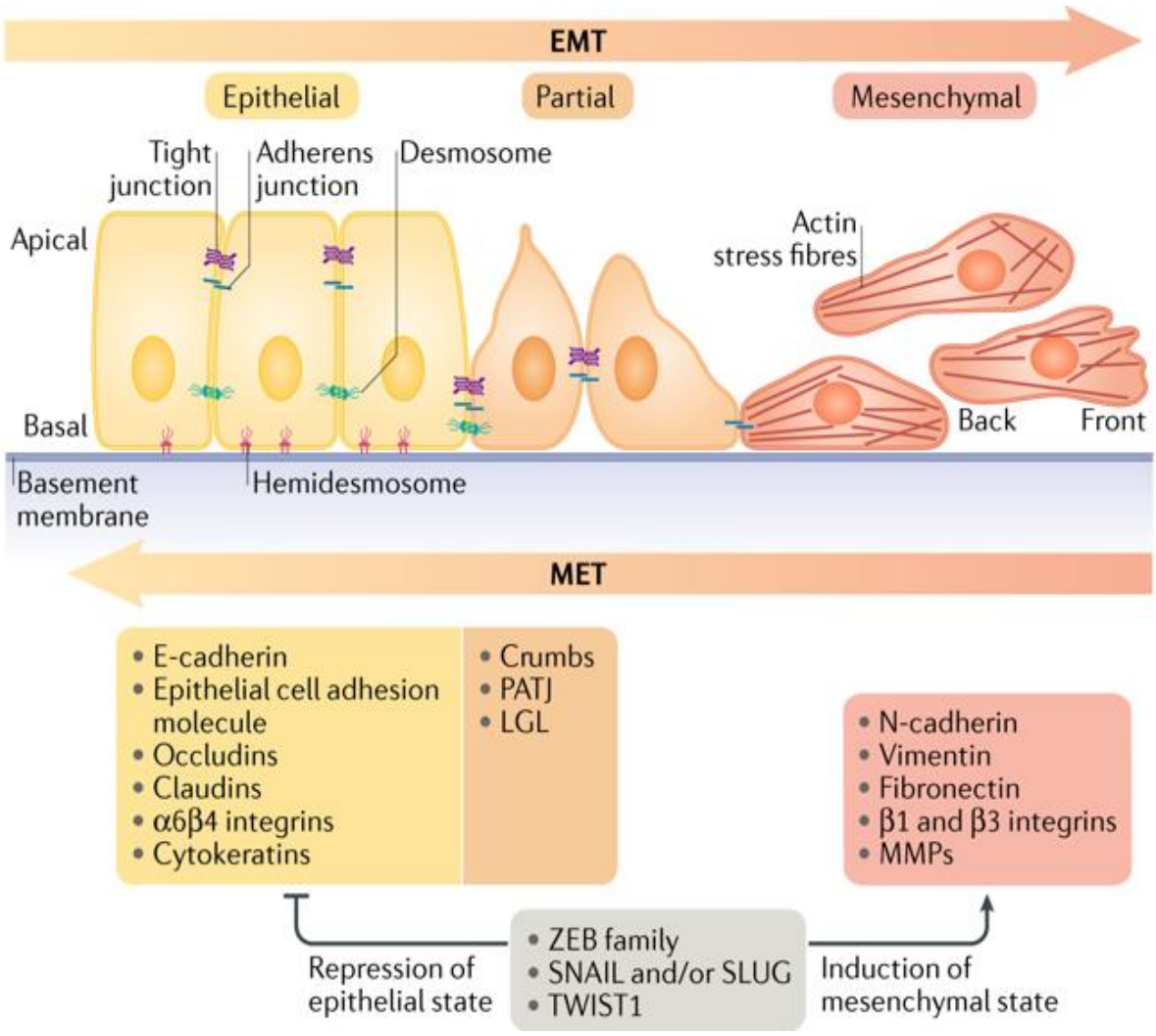


Figure 9. Schematic illustration of EMT and its reverse process MET. EMT is controlled by a small team of master transcription regulators: ZEB, SNAIL/SLUG (SNAI1/2) and TWIST1. These transcription factors repress proteins involved in forming tight junctions between epithelial cells such as E-Cadherin and integrins. Subsequently this occurs with upregulation of N-cadherin, Vimentin, MMPs and other factors leading to loss of basal/apical polarity, decreased cell adhesion and increased motility. Image adapted from Dongre *et al.* 2019 (151)

Chapter 2 – Materials and Methods

2.1 Twist1 Differential expression in TCGA.

Head and neck squamous cell carcinoma (HNSC) Twist1 mRNA expression data were obtained from The Cancer Genome Atlas (TCGA) using the cBio Cancer Genomics Portal (152, 153). These data samples were analyzed for HPV genome status integration HPV16 E2:E7 ratio of mRNA sequencing reads previously described. (3, 93). HNSCC samples without available Twist1 mRNA expression data were omitted. A total 528 samples were identified with HPV status and Twist1 mRNA expression and both variables were correlated and reported using R. Differential gene expression was reported using the $\log_{10}$ V2 SEM method. Statistical analysis was performed utilizing two-way student's t-test with Bonferroni correction for two independent comparisons.

2.2 Cell culture and generation of stable cell lines.

Low-passage N/Tert-1 with stably expressing HPV16, E2 WT, E2(-p53), and E6+E7 were generated as previously described and characterized in previous studies using Lipofectaimine 3000 according to manufacturer's instructions (100, 103, 104, 117, 154). These cells were cultured alongside drug selected empty vector pcDNA™ 3 (Addgene plasmid 2092) and 111µg/mL G418 Sulfate (Genticin) supplementation (Thermo Fisher Scientific). All N/Tert-1 cells were grown in keratinocyte serum-free medium (K-SFM;Invitrogen) supplemented with bovine pituitary extract (Invitrogen), epidermal growth factor (Invitrogen) and 0.3mM

$CaCl_2$. Media was also supplemented with a 1% (vol/vol) penicillin-streptomycin mixture (Thermo Fisher Scientific) containing 4μg/ml hygromycin B (Millipore Sigma). Culture was performed at 37°C in 5% CO_2 and cells were passaged every 3 to 4 days.

The E2(-p53) mutant was created using site directed mutagenesis in the E2 C-terminal DNA-binding domain. Aspartic acid residues 388/344 and tryptophan 341 were all mutated to alanine, disrupting E2's binding affinity to p53. The interaction between E2 and p53 is direct and characterization of this mutant has been previously described (121, 122).

HNSCC primary cancer cell lines UM-SCC-47 and UM-SCC-104 were obtained from Millipore Sigma (Catalog # SCC071 and # SCC072 respectively). UM-SCC-47 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Invitrogen). UM-SCC-104 cells were grown in Eagle's minimum essential medium (EMEM) (Invitrogen) supplemented with 1X nonessential amino acids (NEAA) (Gibco) and 20% (vol/vol) FBS.

Low-passage U2OS osteosarcoma cells with stable expression of empty pcDNA3.0, E2 or E2(-p53) were generated using the calcium phosphate method. U2OS cells were transfected using 1 μg of above plasmids. 48h post transfection, cells were trypsinized and replated in 100mm culture dishes at 1:10 seeding density and selected with 0.75mg/ml G418. N/Tert-1 cells were transfected with 1

μg of previously mentioned plasmids utilizing the Lipofectamine 3000 system according to the manufacturer's instructions. Similarly, transfected cells recovered for 48h and then were transferred to 100mm culture dishes at 1:10 density supplemented with 111 μg/mL G418 Sulfate.

Human foreskin keratinocytes were grown in DermaLife K serum-free media (LifeLine Cell Technology) and immortalized with HPV16 wild-type or mutant genomes as described below (65).

For cell proliferation assays, cells were plated at a density of 1 x 10^5 in $100mm^2$ culture dishes. Cells were then trypsinized, counted and replated again at 1 x 10^5 at indicated time points for up to 40 days. All cells were routinely checked for mycoplasma contamination.

2.3 Immortalization of human foreskin keratinocytes (HFK).

The E2 mutant HPV16 mutant genome (HPV16 E2(-p53), which contained 3 amino acid substitutions in the E2 gene, disrupting its binding affinity to p53) was generated by Genscript (See above) (122, 155, 156). The HPV16 genomes were removed from their parental plasmid using Sph1, the viral genomes were then isolated and then re-circularized using a T4 ligase (NEB) system and transfected into early-passage HFK from two donor backgrounds (Lifeline technology), alongside a G418-resistant plasmid, pcDNA using Lipofectamine 3000. Temporary selection was introduced using media supplemented with 200 ug/mL G418 (Sigma-Aldrich) for 14-days and cells were cultured on a layer of J2 3T3 fibroblast

feeders (NIH), which had been pre-treated with 8 µg/ml mitomycin C (Roche). Throughout the immortalization process, HFK were cultured in Dermalife-K complete media (Lifeline Technology). For studying the effects of p53 depletion in HPV16 immortalized HFKs, 1 µg pMSCV-N-FLAG-HA-GFP, pMSCV-N-FLAG-HA-HPV16E6 or pMSCV-N-FLAG-HA-HPV16E6 ("E6(-p53)) were transfected using a p-Lenti system with psPax2 Gagpol and pmD2.g VSVG Env packaging plasmids. Briefly 293TT cells were plated in 6-well culture dishes at a density of $1x10^5$. The next day, cells were transfected using a Lipofectamine 3000 system and above plasmids according to manufacturer's instructions. 24h later, media was changed to infection target media and the following day, lentivirus laden media was matured with 10 µg/mL polybrene and added to target cells of interest. Target cells were incubated with lentiviruses for 48 hours and stable E6 selection was performed using 5 µg/ml puromycin (Corning). Cells were collected at 24h-post selection (Day 0) and 13 days later to study p53 expression in HPV16 immortalized HFKs (**Chapter 4**).

2.4 SYBR green qRT-PCR.

RNA was isolated using the SV Total RNA isolation system (Promega) following the manufacturer's instructions. 2 µg of RNA was reverse transcribed into cDNA using the high-capacity reverse transcription kit (Applied Biosystems). Resulting cDNA were added to PowerUp SYBR green master mix and relevant primers listed in **Table 2.** (Applied Biosystems) and real-time PCR was performed

using 7500 Fast real-time PCR system. Quantification was expressed as relative quantity over GAPDH using the $2^{-\Delta\Delta CT}$ method.

Table 2. Primers utilized in these studies

Gene/Site of interest	Primer sequence
TWIST1 Forward F	5'-GTCCGCAGTCTTACGAGGAG-3';
TWIST1 Reverse R	5'-GCTTGAGGGTCTGAATCTTGCT-3',
HPV16 E2 F	5'-ATGGAGACTCTTTGCCAACG-3';
HPV16 E2 R	5'-TCATATAGACATAAATCCAG-3',
VIM (Vimentin) F	5'-GACGCCATCAACACCGAGTT-3';
VIM R	5'-CTTTGTCGTTGGTTAGCTGGT-3',
CDH2 (N-Cadherin) F	5'-AGCCAACCTTAACTGAGGAGT-3';
CDH2 R	5'-GGCAAGTTGATTGGAGGGATG-3'.
FLAG-HA Tag F	5'- GACTACAAGGATGACGATG- 3',
FLAG-HA Tag R	5'- GCGTAATCTGGAACATCG -3'.
Twist1 Promoter F	5'-TCAGGCCAATGACACTGCT-3'
Twist1 Promoter R	5'-GACGGTGTGGATGGCCCCGA-3'
HPV16 E6 F	5'- TTGCTTTTCGGGATTTATGC-3'
HPV16 E6 R	5'- CAGGACACAGTGGCTTTTGA-3'
Mitochondrial DNA F	5'-CAGGAGTAGGAGAGAGGGAGGTAAG-3'
Mitochondrial DNA R	5'-TACCCATCATAATCGGAGGCTTTGG -3'
PARP1 F	5'- CGGAGTCTTCGGATAAGCTCT -3'
PARP1 R	5'- TTTCCATCAAACATGGGCGAC -3'
GAPDH F	5'- GGAGCGAGATCCCTCCAAAAT- 3'
GAPDH R	5'- GGCTGTTGTCATACTTCTCATGG- 3'

2.5 Immunoblotting.

Indicated cells were trypsinized, washed with 1X PBS and resuspended in 2x pellet volume protein lysis buffer (0.5% Nonidet P-40, 50mM Tris [pH 7.8], 150 mM NaCl) supplemented with protease inhibitor (Roche Molecular Biochemicals) and phosphatase inhibitor cocktail (Sigma). Cell-buffer suspension was incubated for 30 min on ice and afterwards centrifuged for 20 min at 184,000 rcf at 4°C. Protein concentration was determined using the Bio-Rad protein estimation assay according to manufacturer's instructions. 25µg protein samples were heated at 70°C in equal volume 2x Laemmli sample buffer for 10 minutes (Bio-Rad). Samples were run down a Novex 4-12% Tris-glycine gel (Invitrogen) and transferred onto a nitrocellulose membrane (Bio-Rad) at 30V overnight using the wet blot method. Membranes were blocked with Odyssey (PBS) blocking buffer (diluted 1:1 with 1X PBS) at room temperature for 1-hour and probed with indicated 1° antibody diluted in Odyssey blocking buffer. Membranes were then washed with PBS supplemented with 0.1% Tween (PBS-Tween) and probed with the indicated Odyssey secondary antibody (goat anti-mouse IRdye 800CW or goat anti-rabbit IRdye 680CW) (Licor) diluted in Odyssey blocking buffer at 1:10,000. Membranes were washed thrice with PBS-Tween and an additional wash with 1X PBS. Infrared imaging of the blot was performed using the Odyssey CLx Li-Cor imaging system. Immunoblots were quantified using ImageJ utilizing GAPDH as internal loading control. In **Figure 15B,** the patient derived xenograft samples were a gift from Dr. Devraj Basu at the University of Pennsylvania. Their patient demographics and

characteristics have been previously described. (3, 157). Samples 1-8 are identified in the following order: OCTT52, LST60, LST87, OCTT102, LNT20, OPTT61, OPBT75.

The following primary antibodies were used for immunoblotting: HPV16 E2 (TVG 261) at 1:1000 dilution from Abcam; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724), p53 at 1:1000 (Santa Cruz sc-47698), pRB at 1:1000 (Cell Signaling 9313S), Twist1 at 1:300 (Proteintech 25465-1-AP), phospho-CHK1 (Ser345) at 1:1000 (Cell Signaling 2341S), phospho-CHK2 (Thr68) at 1:1000 (Cell Signaling 2661S), CHK-1 at 1:1000 (Cell Signaling 2360), CHK-2 at 1:1000 (Abcam ab47443), Hemagglutinin tag at 1:1000 (Abcam ab236632), Parp1 at 1:1000 (sc-8007).

2.6 Immunoprecipitation.

Cell protein lysate was prepared as described above. 400 μg lysate was incubated with Primary antibody of interest at a concentration of 1 μg antibody/100 μg lysate. Anti-HA tag or Anti-FLAG tag antibodies were utilized as a negative control when appropriate. Antibody-lysate solution was rotated at 4°C overnight and the following day, 50 μL of prewashed protein A-Sepharose beads (Sigma) were added followed by 4 hours of additional incubation. Samples were washed 4 times with 500 μL Lysis buffer and the precipitated bead complexes were then boiled with 40 μL 4X Laemmli loading buffer (Bio-Rad). Samples were centrifuged at 1000g for 1 minute and then loaded onto a Novex 4-12% Tris-glycine gel

(Invitrogen) followed by transfer to a nitrocellulose membrane and further processed and imaged as described above.

2.7 Transient transfection and analysis of Luciferase reporter plasmids.

N/Tert-1 cells were plated at a density of 1 x 10^6 in 100mm^2 culture dishes. The following Day, these cells were transiently transfected with the plasmids indicated below using the Lipofectamine 3000 system. Transactivation activity of E2 was measured via transient transfection of 1 μg E2 or E2(-p53) along with 10 ng, 100 ng, or 1000 ng of a pTK6E2-Luciferase reporting system. This plasmid has 6 E2 binding sites located upstream from the HSV-1 TK promoter which regulates transcription of luciferase. For LCR (URR) repression function of E2, we used an HPV16LCR-Luciferase plasmid which contains an SV40 promoter. Additional E2 binding to the LCR enhancer regions represses transcription of Luciferase in this system. For Twist1 transcriptional repression, E2 was transfected with 1 μg of a pTWIST1-Luciferase plasmid. This plasmid contains the human Twist1 promoter which was predicted to contain two E2 recognition sites to regulate its transcription (158, 159). Cells were then harvested for luciferase activity or used for Chromatin Immunoprecipitation described below. For luciferase activity: 24h post transfection, N/Tert-1 cells were washed twice with 1X PBS and lysed with 550 μL 1X Luciferase reporter lysis buffer (Promega) via scraping. Protein concentration was estimated with the Bio-Rad protein estimation assay to normalize for raw protein concentrations. Relative fluorescence units were measured using a BioTek

Synergy H1 hybrid reader. RFU's were normalized to protein concentration and reported as normalized luciferase activity.

2.8 HPV16 replication assay.

$5x10^5$ U2OS cells were plated in 100 mm^2 culture dishes. The following day, HPV16 pOri (The viral origin of replication), HPV16 E1, HPV16 E2 and HPV16 E2(-p53) plasmids were transfected at indicated concentrations utilizing the calcium phosphate method as described above (160). 72 hours later, DNA was extracted using the Hirt method and isolated using phenol-chloroform extraction. The low molecular weight DNA pellet was washed with 70% EtOH, dried and resuspended in 150 μL H20. 42 μL of sample DNA was digested using DpnI (New England Biolabs) overnight at 37°C to remove isolate replicated viral DNA. Sample was digested with ExoIII for 1h and heat inactivated at 65°C for 90minutes. Replication was analyzed by real-time PCR as described above using a pORI standard curve for quantification.

2.9 Decitabine treatment.

N/Tert-1 cells were plated at a density of 1.5 X 10^5 in 6-well culture dishes (60-mm^2/well). The next day, cells were treated with 1 μM decitabine (Abcam, ab120842) or 1 μM DMSO control for 72 hours as previously described (104). Afterwards, the cells were trypsinized, harvested and processed for qRT-PCR as described above.

2.10 Chromatin Immunoprecipitation (ChIP).

Depending on the study, N/Tert-1 cells were seeded and either transiently transfected with pTK-6E2 or HPV16-LCR plasmids in 100mm^2 culture dishes as described above or directly plated at a density 2 X 10^6 in 150mm^2 plates. The following day, the cells were crosslinked and fixed using a 1% solution of formaldehyde followed by quenching by 2M glycine. The cells were harvested via scraping and processed for chromatin as previously described (64). Chromatin was sheered by passage through a 25-gauge syringe followed by sonication. Purified chromatin concentration was determined with a NanoDrop spectrophotometer. Approximately 100 µg of chromatin was used per antibody experiment. Chromatin and 1 µg antibody were loaded onto Magnetic A/G protein A beads (Pierce). The following day, beads were washed and precipitated chromatin DNA was processed using the Phenol-Chloroform method for qPCR analysis utilizing relevant primers (**Table 2.**). The following antibodies were used for ChIP: 2µl sheep anti-HPV16 E2 (amino acids 1 to 201) prepared and purified by Dundee Cell Products, United Kingdom; 2µg rabbit anti-Histone H3K9me$_2$ (Abcam, ab1220). Anti-HA antibody was used for negative control.

2.11 Wound healing assay.

2.5 x 10^5 N/Tert-1 cells were plated at the center of each well in 6-well culture dishes (60-mm^2/well). The cells were left to grow to confluency for approximately 48 hours. Afterwards the monolayer was scratched using a 1000µL

pipette tip, creating an ~1mm wound. Wounds were imaged at 0, 12 and 20-hour intervals post scratch (Zeiss, Axiovert 200 M microscope). Multiple images were taken randomly along the wound and measurements were taken from leading cell edge. Wound was measured using ImageJ software and % wound healed was calculated as a ratio of the 0h timepoint or immediately post scratch.

2.12 Southern blotting of HPV16 immortalized HFKs.

Total cellular DNA was extracted via proteinase K – SDS digestions followed by the phenol-chloroform method. 5 µg of total cellular DNA was digested using SphI (to linearize the HPV16 genome) or HindIII (which cannot cut the HPV16 genome). All digestions were supplemented with DpnI to digest all input DNA. Restriction enzymes were purchased from NEB and utilized according to manufacturer's recommendations. Digested DNA was loaded onto an 0.8% agarose gel and underwent electrophoresis. Afterwards, DNA was transferred to a nitrocellulose membrane and probed with radiolabeled (32-P) anti HPV16 sequence probe as previously described (117). Radiolabeled membranes were then exposed to film for 1 – 24 hours. Films were then imaged by an overnight-exposed phosphor screen using a GE Typhoon 9410 imager.

2.13 Exonuclease-V assay.

20 ng genomic DNA was treated with either with exonuclase V (ExoV) (RecBCD, NEB), or untreated for 1 hour at 37°C followed by heat inactivation at 95°C. Because ExoV only digests linear double-stranded DNA, ExoV can digest

integrated HPV16 DNA but not circular episomal genomes. This allows for qPCR analysis of integration status of HPV16 (161). 2 ng of digested or undigested DNA was quantified by real-time qPCR using an Applied Biosystems 7500 FAST thermocycler with SYBR Green PCR Mastermix utilizing indicated primers as described above. The primers utilized are described in **Table 2.**

2.14 Cell viability assay.

1 x 10^4 cells were plated in opaque 96-well cell culture dishes. The following day, cells were treated with an increasing gradient of cisplatin dissolved in DMF or DMF only control. Cells were then incubated for 48 hours. Afterwards, cell viability was assessed using CellTiter-Glo Luminescence Cell Viability Assay (Promega) according to manufacturer's instructions. Luminescence was analyzed using a BioTek Synergy H1 hybrid reader. Percent viability was normalized to DMF treated control cells.

2.15 Clonogenic survival assay.

1x10^4 Cells were seeded in 24-well plates (1cm^2/well) with 1 mL media. The next day, media was replaced with media containing increasing concentrations of cisplatin dissolved in DMF. Cells were incubated for 5-hours with cisplatin or DMF drug-free control; afterwards, cells were washed twice with 1X PBS, trypsinized and counted. 100-200 of the treated cells were replated in 6-well plates and allowed to form colonies for 7-14 days depending on cell line. 6-well dishes were then washed twice with 1X PBS, fixed with 100% MeOH and stained using Crystal

violet. Colonies were counted by a blinded observer. Surviving fraction was calculated using plating efficiency of DMF treated cells. For colony size calculation, entire plates were scanned using an Odyssey CLx Li-Cor imaging system and mean colony size was calculated using ImageJ.

2.16 Cisplatin induced senescence staining.

$7.5X10^5$ Cells were seeded in 6-well dishes ($60mm^2$/well) and treated with 2 μM cisplatin (CDDP) or DMF for 5 hours. Afterwards, cells were washed with 1X PBS and allowed to proliferate for 4 days. Cells were then fixed and stained for senescence using a β-galactosidase staining kit according to manufacturer's instructions (Cell Signaling, Cat# 9860). Six randomly selected 10X fields were counted per replicate/cell line for positively stained cells by a blinded observer. Average number of positively stained cells per field were then calculated and quantified over three independent replicates +/- SE.

2.17 Single-cell gel electrophoresis (Comet) Assay.

$1x10^4$ cells were plated in 24-well dishes with 1 mL media one day prior to harvest. The next day, cells were trypsinized and resuspended in a mixture 0.5% w/v Low molecular weight agarose (Lonza, cat. No. #50101) and PBS at a ratio of 10:1. Suspension was immediately pipetted onto Trivegen CometSlides™ (Cat. No. 4250-004-03) and allowed to dry for 30 min at 4°C. Slides underwent lysis for 90 min at 4°C in the dark (Lysis buffer: 10mM Tris, 100mM EDTA, 2.5M NaCl, 1% TritonX100, 10% DMSO titrated to pH 10.0). Afterwards, slides were placed in

Alkaline buffer for 25 min at 4°C in the dark (Alkaline buffer: 1mM EDTA, 200mM NaOH, pH >13.0). Slides were transferred to an agarose gel electrophoresis box filled with additional alkaline buffer. Electrophoresis was performed at 25V for 20min at room temperature in the dark. Slides were then washed 2x in ddH20 for 5 min at RT and then placed in neutralization buffer for 20 min at RT in dark (Neutralization buffer: 400mM Tris-HCl titrated to pH 7.5). Neutralized slides were then left to dry at 37°C in the dark. Dried slides were stained with DAPI (1:10,000 in dd H2) for 15 min at RT then washed 2x with dd H20 for 5 min. Stained and rinsed slides were left to dry overnight. Slides were imaged using the Keyence imaging system at 20x with >5 images taken per replicate. Quantization of olive tail moments (OTM) was achieved using the CASPLab Comet Assay imaging software by Lu, Y. et al (2017).

2.18 Organotypic raft culture.

Human foreskin keratinocytes were differentiated via organotypic raft culture as previously described (104, 117, 162). Briefly, cells were seeded onto type 1 collagen matrices containing J2 3T3 fibroblast feeder cells. Cells were cultured to confluency atop these collagen plugs, lifted onto wire grids and cultured in dishes at the air-liquid interface. Media was replaced on alternating days. Following 14-days of culture, rafted samples were fixed with formaldehyde (4% v/v) and embedded in paraffin. Multiple 4 µm sections were cut from each sample. Sections were stained with hematoxylin and eosin (H&E) and others prepared for immunofluorescent staining via HIER. Fixed sections were antigen retrieved in

citrate buffer and probed with the following antibodies for immunofluorescent anaylsis: phospho-yH2AX 1/500 (Cell Signaling Technology; 9718), Involucrin 1/1000 (Abcam; ab27495), Keratin 10 1/1000 (SigmaAldrich; SAB4501656), and HPV16 E2. (monoclonal B9)(163) Cellular DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Santa Cruz sc-3598). Microscopy was performed using the Keyence imaging system. Fixing and embedding services in support of the research project were generated by the VCU Massey Cancer Mouse Model Shared Resource, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30 CA016059.

2.19 Statistical Analyses.

All quantitated data is represented as mean ± standard error and calculated from three independent experiments except when indicated. Significance was determined using two-tailed student's *t* test. Bonferroni correction for significance was utilized when indicated.

Chapter 3 – Human Papillomavirus 16 E2 repression of TWIST1 transcription is a potential mediator of HPV16 cancer outcomes

3.1 Purpose of this study

HPV-positive cancer respond better to treatment and have better patient outcomes compared to HPV-negative counterparts (164). Previous work by the Morgan lab and affiliates has revealed that in HNSCC, much of this enhanced benefit may be specific to tumors that retain an episomal status and E2 expression. Integrated tumors have similar outcomes compared to HPV-negative tumors (80, 90, 91, 93). This led us to propose that E2 itself may play an active role in HPV-positive HNSCC. Previous work had identified differentially expressed genes in N/Tert-1 cells with either E2 expression alone or in the context of the HPV16 viral lifecycle. In this RNA-sequencing data, it was discovered that TWIST1, a master regulator of EMT has reduced mRNA expression in cells containing E2 or the full HPV16 genome (104). High TWIST1 expression and elevated markers of EMT are associated with more aggressive cancers (141, 151, 165, 166).

3.2 Hypothesis

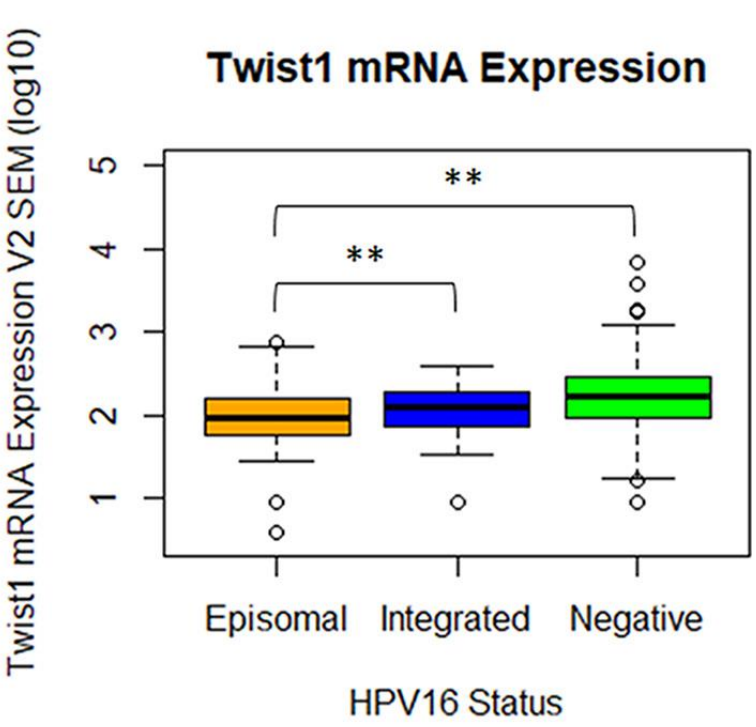
Because E2 has already been demonstrated to directly regulate host genes important in immune response and cancer progression, we hypothesized that E2 may also downregulate *TWIST1* expression, contributing to better treatment outcomes of episomal, E2 expressing tumors.

3.3 Results

3.3.1 TWIST1 is downregulated in episomal HPV-positive HNSCC.

Our previous RNA-seq analysis of N/Tert-1+E2 and N/Tert-1+HPV16 indicated a high statistically significant overlap in E2 regulated genes and those regulated by the entire virus including TWIST1 (104). Analysis of patient tumor samples in The Cancer Genome Atlas (TCGA) revealed a significant downregulation of TWIST1 in HPV16-positive versus negative HNSCCs (92, 93). We first stratified HPV-positive patient tumors as episomal versus integrated, utilizing an E2:E7 expression ratio. Because integration most often occurs in the E2 gene, integrated tumors would have a low E2:E7 ratio while episomal tumors would have a ratio near 1.0. These samples and their HPV genome status were previously identified (92). TWIST1 mRNA expression data were obtained from 528 tumor samples in the Firehose Legacy database using the cBio Cancer Genomic Portal (152, 153). Samples were then grouped as episomal, integrated, or HPV-negative (**Fig 10A.)**. We found that TWIST1 mRNA is expressed at statistically significant lower levels in episomal tumors compared to both integrated and HPV-negative tumors. There was no statistically significant difference between Integrated and HPV-negative samples. This overlapped closely with our previous RNA-seq studies in N/Tert-1 cells expressing E2 or HPV16 compared to vector control cells (**Fig. 10B**).

A



B

N/Tert-1 mRNA Sequencing V2 SEM		
	Relative Expression compared to N/Tert-1	P-value
N/Tert-1 + HPV16	0.464	8.3×10^{-5}
N/Tert-1 + 16E2	0.586	1.4×10^{-4}

Figure 10. TWIST1 mRNA is downregulated by HPV16 E2. (**A**) 528 HNSCC tumors were previously evaluated for HPV viral genome status using the E2:E7 ratio of mRNA sequencing reads. 466 tumors were HPV-negative, 40 were episomal and 17 were integrated. Firehose Legacy TWIST1 mRNA expression data were obtained using cBio Portal. TWIST1 mRNA was then compared to HPV status using student's t test in R. Vertical axis is in $\log_{10}$. (**B**) Previously published RNA-sequencing results in N/Tert-1 cells from Evans *et al*. TWIST1 mRNA expression in N/Tert-1 + E2 and N/Tert-1 + HPV16 were compared to cells with empty pcDNA3.0 vector control. **P<0.001 using Bonferroni correction when appropriate.

3.3.2 TWIST1 mRNA expression is lower in N/Tert-1 cells with E2 alone and HPV16 but not with expression of the E6 and E7 oncogenes.

We validated TWIST1 expression in our N/Tert-1 cells expressing either E2, E6+E7 or the HPV16 genome using RT-qPCR. Several studies have demonstrated that E6 and E7 may play a role in regulating markers of EMT under certain conditions. However, in all of these studies, additional stimulatory factors were required to synergize with E6 and E7 such as treatment with epidermal growth factor or transgenic upregulation of additional EMT pathways (148, 149). We found that in N/Tert-1 cells, E6 and E7 expression was insufficient to change TWIST1 mRNA expression while E2 and HPV16 were (**Fig 11A**). This suggests that in the context of TWIST1, E2 may be playing the primary role in its regulation and not the viral oncogenes.

We then looked at Twist1 protein levels (*TWIST1* is the gene name that encodes for the protein, Twist1). In N/Tert-1 cells, the mRNA downregulation is reflected at the protein level (**Fig. 11B**). This is in direct contrast with our previous studies of HPV16 regulation of innate immune genes where E2, E6 and E7 can all downregulate members of the U-ISGF complex (104, 117). There is clear downregulation of Twist1 protein in N/Tert-1 cells with E2 and HPV16 but not E6 + E7 (compare lanes 2 and 3 to lane 4). These experiments were repeated and quantified in **Fig. 11C**. Vimentin and N-Cadherin are cellular markers of EMT regulated by Twist1 and associated with reduced cellular adhesion and enhanced motility (133, 134, 142, 144). To look at the effects of Twist1 downregulation on

these downstream effectors, we studied their mRNA expression using RT-qPCR (**Fig. 11D).** In agreement with Twist1 expression levels, N/Tert-1 cells expressing E2 and HPV16 but not E6/E7 had lower expression of these markers, indicating that the illustrated Twist1 downregulation results in functional downregulation of EMT related pathways. These results reveal that, to the best of our knowledge, *TWIST1* is the first identified host gene that is likely exclusively regulated by E2 in the HPV16 viral life cycle.

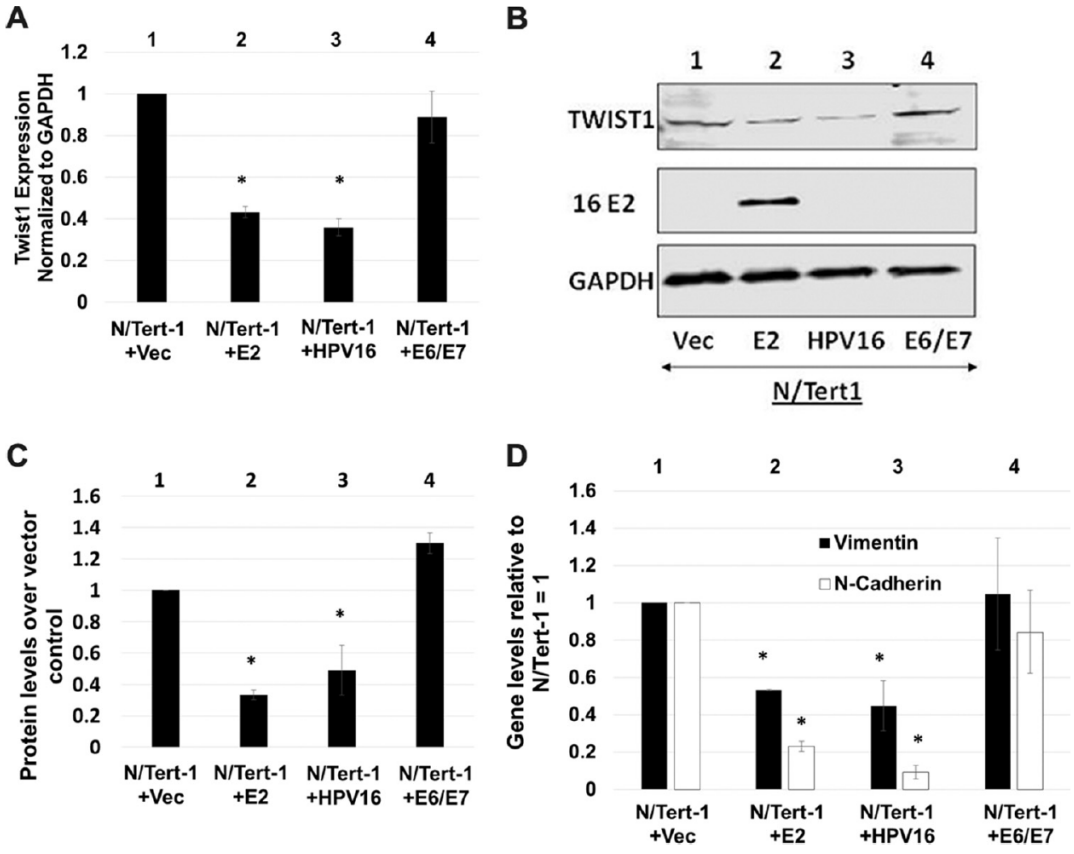


Figure 11. Twist1 is transcriptionally repressed by E2 and the HPV16 genome in N/Tert-1 cells. (**A**) RT- qPCR of N/Tert-1 cells with E2, HPV16, E6/E7, or empty pcDNA3.0 vector. Results are expressed as fold change over vector control cells. (**B**) Twist1 western blot was carried out on extracts from the cells in **A** with GAPDH as internal loading control**.** (**C**) Western blots were quantified and Twist1 protein expression levels were calculated relative to vector using ImageJ. (**D**) Twist1 downregulation leads to reduction in Twist1 target genes, CDH2 and VIM, which are N-Cadherin and Vimentin, respectively. cDNA from **A** was analyzed using primers against CDH2 and VIM. Results are expressed as fold-change over the vector control N/Tert-1 cells. All data represent the mean of at least 3 independent experiments ± SEM, *P<0.05

3.3.3 Twist1 repression is not due to DNA methylation.

We have previously reported that E2 regulates genes related to innate immunity via recruitment of DNA methyltransferase 1 (DMNT1) to the promoter, resulting in DNA-base methylation and transcriptional repression (104). This repression was robustly reversed with the treatment of the DMNT1 inhibitor decitabine (5-aza-cytidine). To test whether TWIST1 is similarly regulated by E2, we treated our N/Tert-1 cells with E2 or the HPV16 genome with 1 μM decitabine for 72 hours, and harvested cells for mRNA expression (**Fig. 12)**. As a positive control, we first tested the efficacy of decitabine treatment by looking at the U-ISGF innate immune genes IFIT1 and MX1 which are robustly repressed by expression of E2 or the HPV16 genome. As previously reported, there was a large increase in expression in all cell lines treated with decitabine and a greater increase in cells expressing E2 and HPV16 (**Fig. 12A**). Next, the same samples were tested for TWIST1 mRNA. Interestingly, while decitabine was able to restore MX1 and IFIT1 expression, it appeared to have no effect on TWIST1 indicating that E2 recruitment of DMNT1 does not play a role in its repression of TWIST1 (**Fig 12B**). This suggests an alternative mechanism for E2 mediated repression of TWIST1.

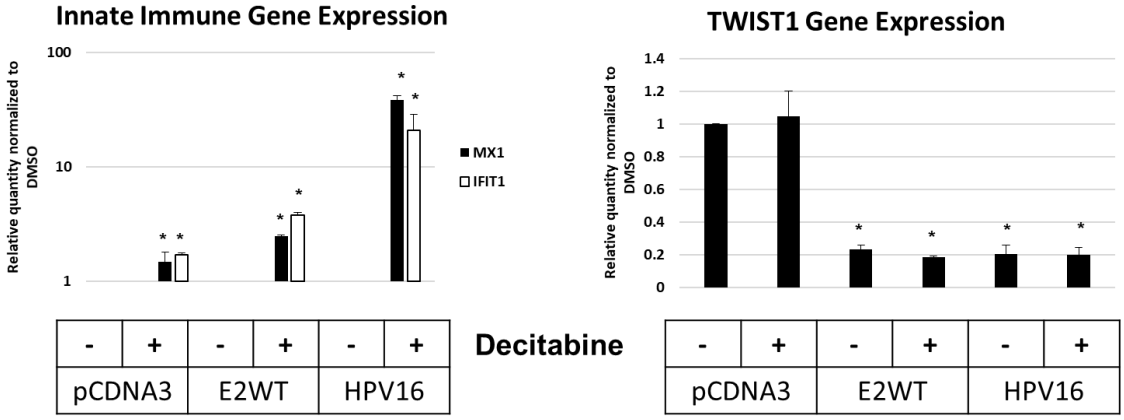


Figure 12. TWIST1 transcriptional repression by E2 is not mediated by DMNT1. (**A**). N/Tert-1 cells were treated with 1 μM decitabine (5-aza-cytidine) for 72 hours and were processed for RT-qPCR. As expected, decitabine treatment was able to derepress innate immune genes MX1 and IFIT1. (**B**) The same samples in **A** were analyzed for TWIST1 expression. Unlike the innate immunity genes, decitabine does not derepress TWIST1. Results are expressed as fold change from that observed in untreated vector control cells. Data in panels A and B represent the mean ± SEM of at least two independent experiments. *P<0.05.

3.3.4 E2 binds to the TWIST1 promoter region, directly repressing transcription

We next investigated whether E2 could directly repress *TWIST1* at the promoter level. To do this, we utilized a luciferase reporting construct containing the *TWIST1* promoter upstream to the luciferase gene (pTWIST1-Luc). This construct was co-transfected with E2 into parental N/Tert-1 cells to ascertain whether E2 could directly modulate TWIST1 transactivation at the promoter (**Fig. 13A**). The presence of E2 resulted in a ~10-fold reduction in Luciferase activity. We verified sufficient E2 transfection efficiency utilizing a pTK6E2-Luc construct that was transfected side-by-side (**Fig. 13B**).

E2 binds to DNA using short palindromic sequence $ACCGN_4CGGT$ in close succession. This sequence is prevalent in the HPV LCR and is used to regulate viral transactivation. Examination of the *TWIST1* promoter region yielded a potential candidate region which E2 may bind to. We generated primers to amplify this region of promoter DNA and performed chromatin immunoprecipitation on this region of the *TWIST1* promoter using a monoclonal antibody against E2 (**Fig. 13C**). Remarkably, we found a significant increase in signal in N/Tert-1 cells with E2 and the HPV16 genome compared to that of the vector control, demonstrating that E2 does indeed bind to the *TWIST1* promoter, likely at the site of interest. We then investigated whether repressive histone epigenetic markers played a role in TWIST1 repression. $H3K9me^2$ is a repressive marker involved in TWIST1 regulation during normal osteogenesis and plays a role in a variety of cancers (167,

168). We found that N/Tert-1 + E2 and N/Tert-1 + HPV16 had elevated levels of this repression marker at the site of interest in the *TWIST1* promoter (**Fig. 13D**). These results indicate that E2 interacts with the *TWIST1* promoter directly, leading to epigenetic modification and resulting reduced transcription. This is the first time that E2 has been illustrated to act as an epigenetic modulator in this manner.

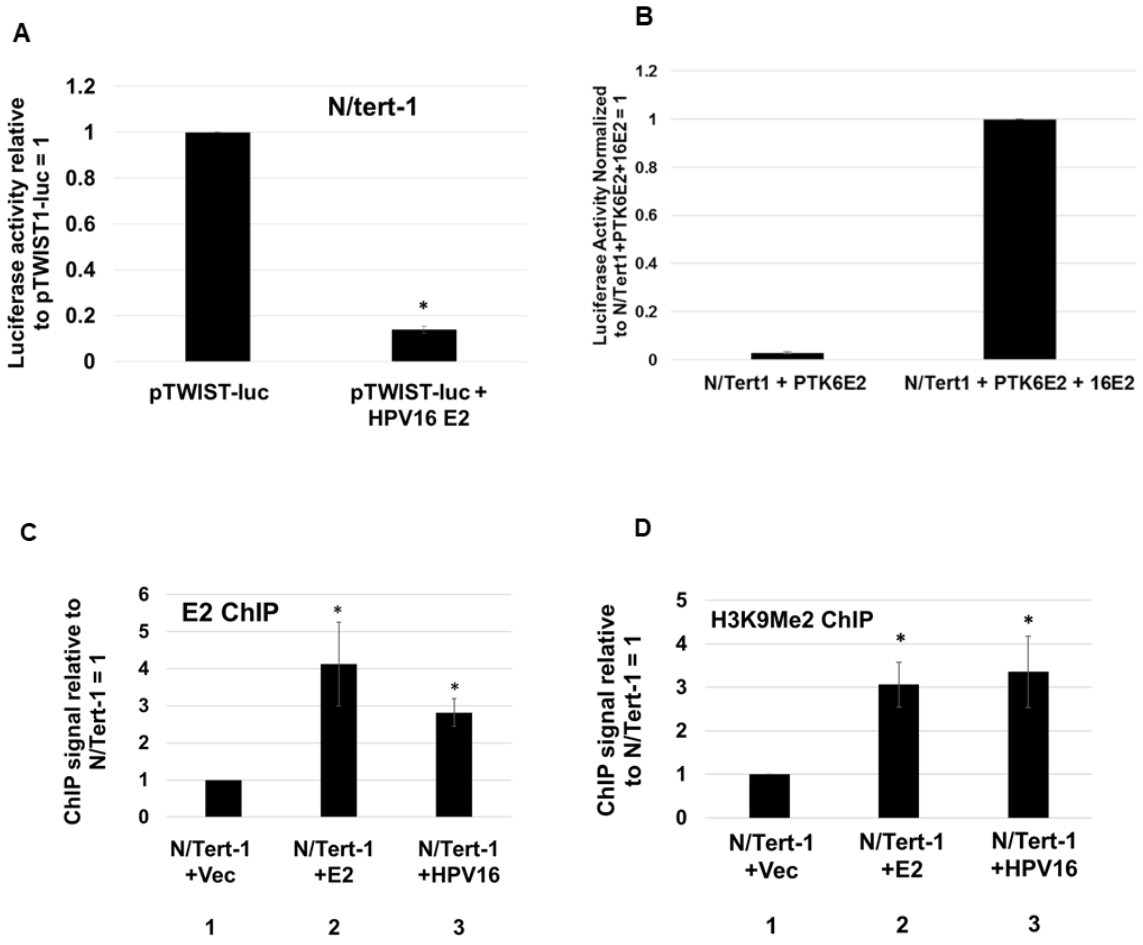


Figure 13. E2 directly binds to the *TWIST1* promoter, actively repressing transcription. (**A**) N/Tert-1 cells were transfected with 1 μg pTWIST1-Luc alone or with 1 μg HPV16 E2. 48h later, a luciferase-based reporter assay was used to monitor levels of *TWIST1* promoter transactivation. Data were obtained as relative fluorescence units (RFU) which were then normalized to total protein concentration. Activity was normalized to pTWIST1-Luc transfection without E2. (**B**) Cells were transfected with pTK6E2-Luc which E2 activates. This demonstrates that E2 is not cytotoxic to cells upon transient transfection as there is robust transactivation by E2. (**C**) Chromatin immunoprecipitation of E2 onto the *TWIST1* promoter in N/Tert-1 cells stably expressing E2, the HPV16 genome or empty vector. (**D**) E2 binding at the *TWIST1* promoter leads to enrichment of the repressive histone marker H3K9me[2]. In both **C** and **D,** there was significant increase in ChIP signal in E2 and HPV16 expressing N/Tert-1 cells compared to vector control. Results were normalized to input DNA and expressed as fold change over vector control. Data represents the mean of at least 3 independent experiments ± SEM. *P<0.05.

3.3.5 E2 expression reduces wound healing capacity in N/Tert-1 cells.

EMT is a fluid and reversible spectrum of phenotypes that range from epithelial to mesenchymal and not a defined status (151). E2 mediated repression of TWIST1 along with repression of genes important for cellular adhesion and motility suggest that there may be an appreciable phenotype in E2-positive N/Tert-1 cells. During wound healing, there is a significant EMT transition of wounded epithelial cells which promotes enhanced motility and wound closure (169). Our group previously reported that U2OS osteosarcoma cells expressing E2 have deficient wound healing capacity in monolayer cell culture (100). The repression of TWIST1 could mediate this phenotype. Therefore, we repeated these wound healing assays in our N/Tert-1 cells (**Fig. 14)**. We plated N/Tert-1 and allowed them to grow to confluency only at the center of the culture dishes. Then we made "scratches" in the cell monolayer to monitor wound healing. 20 hours following the scratch, the wound is almost completely healed in vector control cells as well as cells expressing the E6/E7 oncogenes (**Fig. 14A**) top-right and bottom-right panels). However, in cells expressing E2 or the HPV16 genome, these wounds remained largely unresolved and are quantified in **Fig. 14B**. These results correlate well with Twist1 protein expression in all of these cell lines (**Fig 11**) and suggest once again that E2 and its repression of TWIST1 plays a major role in EMT phenotypes in HPV positive cell lines.

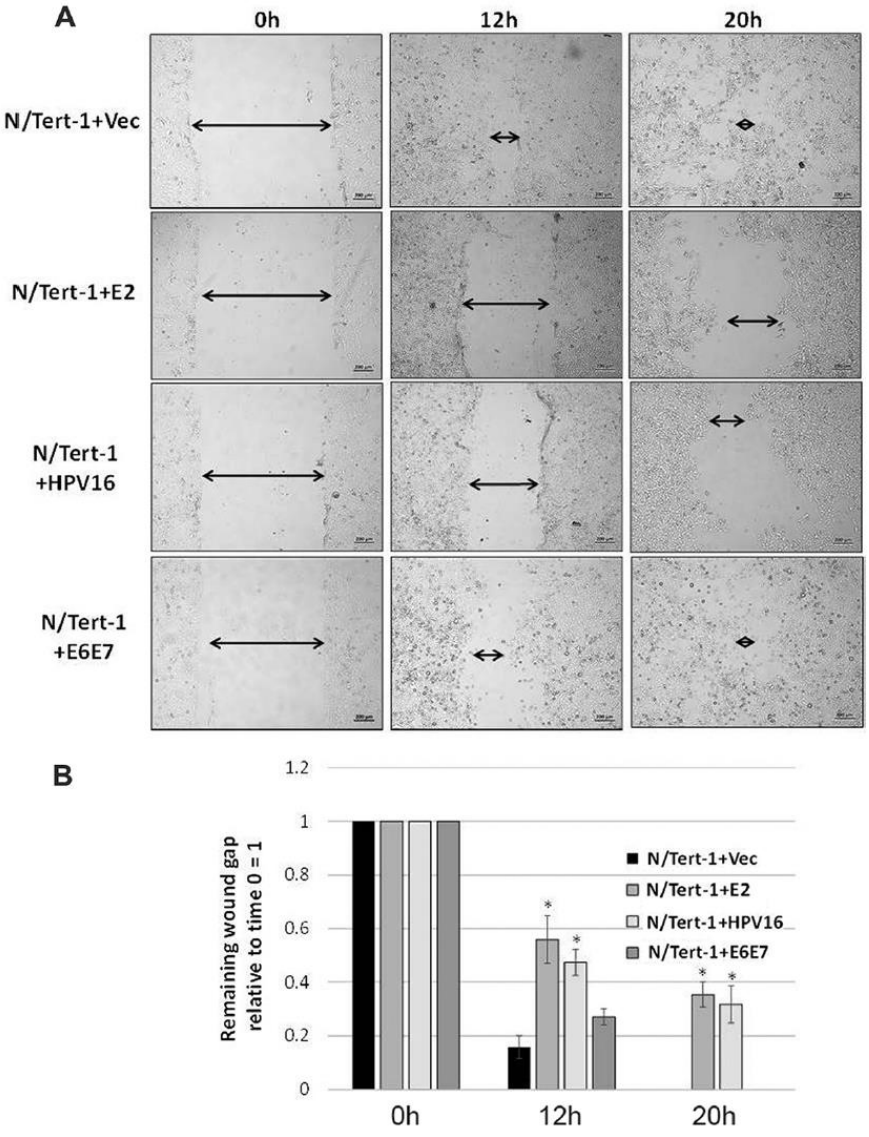


Figure 14. E2 expressing N/Tert-1 cells exhibit deficient wound healing capacity. (**A**) N/Tert-1 cells were plated and allowed to grow to near confluence at the center of 6-well culture dishes. The next day, an ~1-mm scratch was made in the cell monolayer using a pipette tip. The initial wound gap was measured at time of insult and the same field was imaged at 0, 12 and 20h. Arrows are added for clarity. (**B)** Multiple images were taken across the wound at all time points and the remaining wound gap was calculated relative to 0h in ImageJ. Results are expressed as fold change over 0h for each cell line tested. Data represent the mean ± SEM for at least three independent experiments. *P<0.05.

3.3.6 TWIST1 inversely correlates with E2 levels in HPV16-positive HNSCC lines

On average, there is less expression of TWIST1 in HPV-positive HNSCC compared to both integrated and negative HNSCC (**Fig. 10**). Our results suggest that E2 expression likely plays an active role on TWIST1 expression which may be differentially expressed depending on viral genome status. To briefly probe this, we utilized two HNSCC lines that are both HPV16 positive: SCC104 which contains episomal genomes and retains E2 expression, and SCC47 which possesses integrated viral genomes and no E2. We first confirmed the presence of E2 mRNA via RT-qPCR (**Fig. 15A**). As expected, compared to E2 expressing SCC104, there is virtually no E2 mRNA in SCC47 as the viral integration site disrupted the E2 gene (170). Analysis of TWIST1 mRNA in these two lines indicated that E2 expression correlated with a significant decrease in TWIST1 expression (**Fig. 15B**). Moreover, this difference was reflected on the protein level and SCC104 cells had significant lower levels of Twist1 protein compared to SCC47 cells (**Fig. 15C**). These immunoblots were repeated and quantitated (**Fig. 15D**). While this is only a correlation in two HNSCC cell lines, it indicates that the TWIST1 expression levels *in vitro* mirror expression patterns in TCGA as illustrated in **Fig. 10.** These findings support our model of E2 mediated TWIST1 regulation in HNSCC.

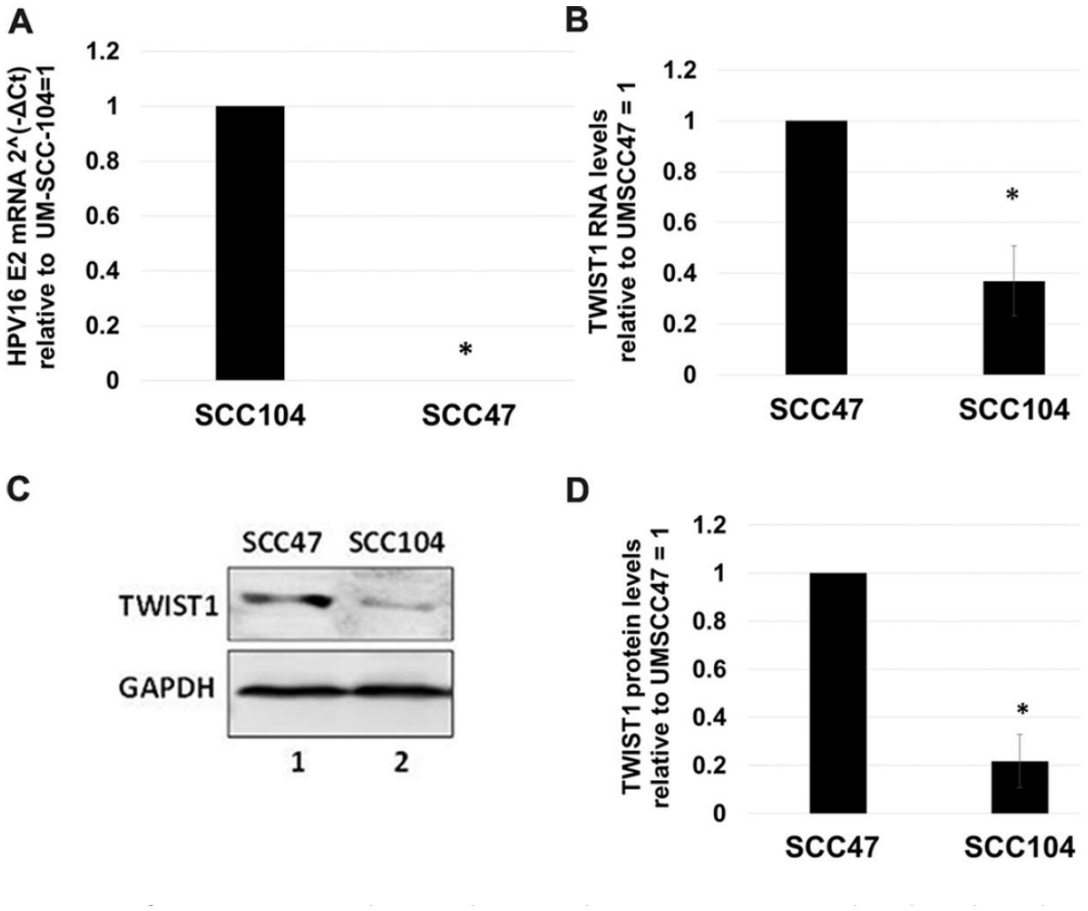


Figure 15. Twist1 expression inversely correlates with E2 expression in HPV16 HNSCC primary cancer cells. (**A**) E2 mRNA expression in SCC47 and SCC104. SCC47 is integrated at the E2 gene disrupting its expression while SCC104 is a purely episomal and retains E2. (**B**). TWIST1 mRNA expression is decreased in the E2 positive SCC104 cell lines compared to the E2 negative SCC47. (**C**) The decreased transcription of TWIST1 is translated to decreased protein expression. (**D**) The immunoblot in **C** was repeated several times and the expression was calculated relative to GAPDH as internal loading control. Results are expressed relative to SCC47 protein expression. Data represent the mean ± SEM of at least three independent experiments. *P<0.05.

3.4 Future directions and discussion for Chapter 3

The HPV E2 protein plays a multitude of critical roles during the viral lifecycle. However, regulation of host gene expression by E2 and its importance for the virus, host and oncogenesis are poorly understood. Our recent work has demonstrated that E2 can regulate host gene transcription relevant for the viral lifecycle (104). In that report, we observed an overlap in function between E2 and E6/E7 in the repression of innate immune genes. Here, we demonstrate for the first time that E2 can bind to a host gene (*TWIST1*) at its promoter leading to its regulation via recruitment of repressive histone modifications and gross modulation of an important pathway in cancer progression (EMT).

TWIST1 repression by E2 may be a determinant of HPV cancer outcomes. Amplification of TWIST1 is associated with poorer overall survival in HNSCC while E2 expression is associated with improved outcomes (171, 172). While these data do grant some insight, the mechanisms of TWIST1 regulation by E2 is yet to be fully elucidated. The transcription factor SP1 activates the TWIST1 promoter and is required for its expression at basal levels (173). E2 has been shown to displace SP1 from the HPV viral URR, leading to viral transcriptional repression (174). E2 may act similarly on TWIST1. Moreover, our previous studies in U2OS cells reported that E2 expression does inhibit cellular wound healing capacity and an E2 mutant deficient in BRD4 binding cannot. However, RNA-seq in these cells did not reveal TWIST1 as a differentially regulated factor in U2OS cells and markers of EMT were largely unchanged (100). This may be due to the mesenchymal

nature of U2OS cells and which are reported to have elevated markers of EMT and may not be repressed to the same level by E2 as in keratinocytes (175). It would be interesting to evaluate the BRD4 binding mutant of E2 in keratinocytes to see if wound healing capacity and additional markers of EMT are de-repressed.

As to why E2 represses TWIST1, there is little known on TWIST1 in the context of keratinocyte differentiation. It is known, however, that HPV regulates epithelial cell differentiation to promote an environment conducive to viral replication (176). E2 and TWIST1 both have roles in the regulation of NF-κB, which may be important for the viral lifecycle (177, 178). TWIST1 has also been shown to downregulate CCAAT/Enhancer binding protein alpha (C/EBPα) (179). C/EBPα is a basic leucine zipper transcription factor which has critical roles in the differentiation of myeloid lineage cells during hematopoiesis (180). C/EBPα also regulates involucrin expression during keratinocyte differentiation and E2 has been shown to directly bind to C/EBPα regulating its transactivation function (115). Therefore, downregulation of TWIST1 by E2 may result higher C/EBPα expression, providing a substrate for E2 regulation of keratinocyte differentiation. Due to the important roles that TWIST1 plays in EMT, cancer progression and chemotherapeutic response, additional studies of TWIST1 regulation by HPV16 is necessary.

Chapter 4 – HPV16 E2 interacts with tumor suppressor p53 to regulate cellular response to DNA damage, a critical function in the HPV16 viral lifecycle

4.1 Purpose of this study

Our previous results indicated that E2 transcriptionally represses TWIST1 leading to decreased markers of EMT and a less aggressive phenotype in N/Tert-1 cells (94). High TWIST1 expression and elevated markers of EMT are associated with reduced response to cancer treatment (141, 145, 147, 151, 169, 172, 181). E2 expression is also independently associated with improved clinical outcomes in HPV-positive HNSCC (91, 171). E2 has direct interactions with a variety of cellular host factors that assists in regulation of the virus and host including p53 (105). P53 is one of the most important cellular factors in recognition of DNA damage, leading to cellular senescence or apoptosis. Moreover, p53-mediated responses are the cornerstone of chemoradiation therapy (CRT), the standard of care in the treatment of HNSCC (182-184). Therefore, we proposed that E2 may help modulate tumor treatment responses and its interaction with p53 may play a role.

4.2 Hypothesis

Because TWIST1 expression and EMT are inversely correlated with treatment response in a variety of tumors and TWIST1 is downregulated by E2,

we believed that E2 may play an active positive role in the sensitivity of cancer cells to chemotherapeutic agents.

4.3 Results

4.3.1 E2 sensitizes multiple cell lines to cisplatin in a p53 dependent manner

Because E2 reduces EMT and is independently associated with favorable treatment outcomes, we wished to determine if E2 can itself grant any differential susceptibility to CRT agents. Cisplatin is a key component of CRT and a standard of care treatment in stage 4 disseminated head and neck squamous cell carcinoma (14, 28, 29, 185-187). Using our established N/Tert-1 cell lines that express E2, we treated cells with an increasing concentration of cisplatin for 5 hours. Afterwards, cells were plated for clonogenic survival and allowed to grow colonies for 7-10 days (**Fig. 16A**). Remarkably, N/Tert-1 cells expressing E2 alone were significantly sensitive to cisplatin compared to vector control cells and had an ~2-fold decrease in EC_{50} compared to the vector control cells (**Fig. 16B**). Additionally, we found that this sensitivity profile was reproducible in U2OS cells expressing E2 (**Fig. 16C and D)**. E2 binds to a wide array of host factors to help mediate its function. In order to determine if any host factor mediates this enhanced sensitivity, we screened against several known binding mutants of E2 (Data not shown). Surprisingly, we found that a previously described E2-p53 binding mutant was deficient in this ability to promote cisplatin sensitivity (**Fig. 16A and C, lower panels**).

We predicted that the observed reduced clonogenic survival in E2 WT could be due to enhanced senescence. To test this, we treated E2WT and E2-p53 mutant N/Tert-1 cells with 2 μM cisplatin for 5 hours similar to **Fig 16.** Afterwards, cells were allowed to recover for 5 days and then stained for β-galactosidase as a marker for senescent cells (**Fig. 17A**). Cisplatin induced senescence in all treated cells compared to mock control. However, we noted that there was a paradoxical decrease in positively stained N/Tert-1 + E2WT compared to the E2-p53 and vector cells following cisplatin treatment. (**Fig. 17A and B**). This was surprising to us as it reveals that while the E2 WT cells did not senesce following cisplatin treatment, they were unable to form colonies over the longer term (7-10 days). Moreover, cisplatin acts mainly non-enzymatically by directly forming DNA crosslinks leading to single strand and double strand breaks (188, 189). Our findings suggest that E2 WT cells do not recognize DNA-damage induced by cisplatin and continue to grow in spite of genomic stress. This intrigued us as while this interaction between E2 and p53 has been relatively well characterized structurally, its mechanistic function was largely unknown. Specifically, p53 binds to E2 directly *in vitro* and this activity may trigger apoptosis in cervical cancer cells (121). However, the nature of this interaction in the context of other HPV-positive cancers was relatively understudied and prompted us to look more closely at p53 in the context of E2 positive HNSCC lines.

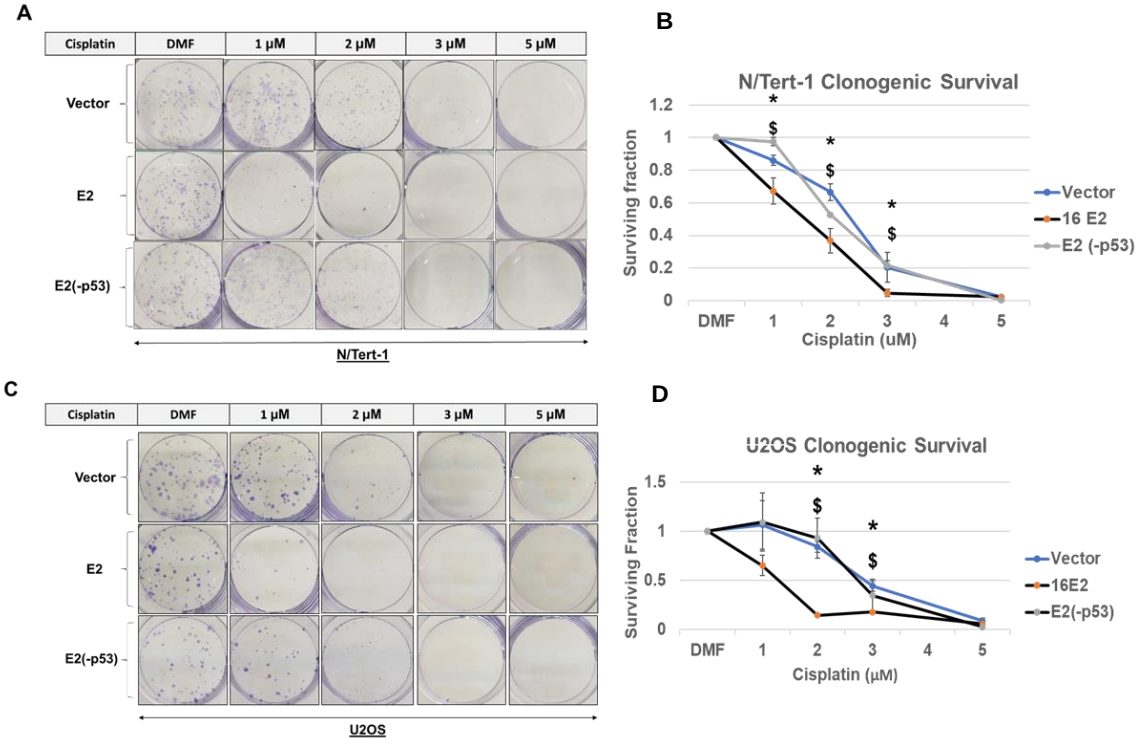


Figure 16. E2 sensitizes multiple cell lines to cisplatin in a p53 dependent manner. (**A**) Clonogenic survival assay of N/Tert-1 cells expressing wild-type E2, a P53 binding mutant of E2 (E2(-p53)) or vector control. Cells were treated with short burst of cisplatin for 5 hours. Afterwards, 200 cells were replated and allowed to form colonies for 7-10 days. (**B**) Clonogenic survival assay was repeated several times and quantitated as surviving fraction taking into account the plating efficiency of each cell line. No significant plating efficiency difference was noted. N/Tert-1 cells with E2 WT cells but not E2(-p53) or vector had a significant decrease in the clonogenic survival following cisplatin treatment. (**C**) The experiment in **A** and **B** were repeated in U2OS cells which have wild-type p53. (**D**) quantitation of multiple experiments yielded similar results to that of N/Tert-1 cells. Results are expressed relative to SCC47 protein expression. Data represent the mean ± SEM of at least three independent experiments. *P<0.05.

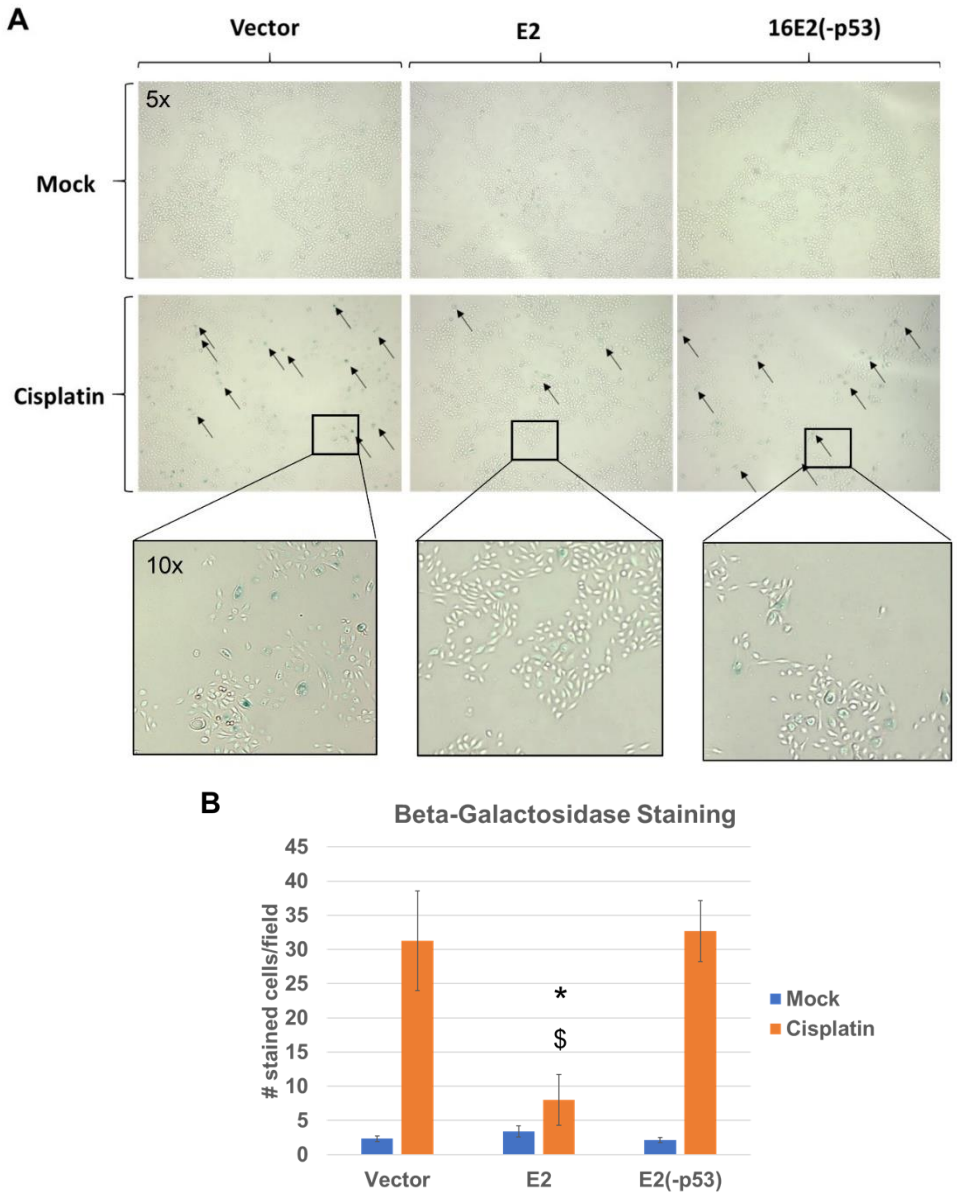


Figure 17. Cells expressing wild-type E2 exhibit reduced senescence despite lower clonogenic survival. β-galactosidase staining of cisplatin treated N/Tert-1 expressing wild-type E2 or E2(-p53) mutant **(A)**. The wild-type E2 expressing N/Tert-1 cells have decreased number of positively staining cells compared to both mutant and vector controls. Images taken at 5x and 10x. Arrows added at 5x for clarity **(B).** Six randomly selected fields were counted per replicate per cell line by a blinded observer and quantitated. Data is portrayed as mean ± SEM. Bonferroni correction utilized when applicable. * $p<0.025$ for Vector vs E2. $ $p<0.025$ for E2 vs E2(-p53).

4.3.2 Tumor Suppressor p53 is expressed in HPV16 immortalized keratinocytes and patient derived xenografts.

Previous reports have demonstrated that alternative splice variants (E6*) are the dominant E6 isoforms in HPV-positive HNSCC, which prevent E6-E6AP-p53 complex formation and inhibit p53 degradation (92, 128-131, 190). In order to determine whether sufficient p53 is expressed to serve as a substrate for E2, we first confirmed the presence of p53 in a series of HPV16 positive cell lines (**Fig. 18**). Expression of HPV16 in N/Tert-1 cells results in a modest reduction in p53 compared to near complete abrogation by expression of the E6/ E7 oncogenes (**Fig. 18A**, compare lanes 2 and 4 to lane 1). Moreover, human tonsil cells immortalized by HPV16 have comparable p53 expression compared to N/Tert-1 vector control cells (compare lane 1 to 3). To further investigate these findings, we studied two independent donors of human foreskin keratinocytes (HFK) immortalized with HPV16. In both donor lines, p53 levels were reduced much less compared to HFK immortalized by E6/E7 overexpression (Lanes 5-8). To determine whether this expression is affected by tumor microenvironment, we surveyed p53 expression in 8 patient derived xenografts (PDXs) from oropharyngeal and oral cavity carcinomas (four HPV16 positive and four negative) (3, 157). All HPV16 positive PDX samples and 3 out of 4 HPV negative retained detectable p53 expression illustrating that there appears to be no clear association between HPV status and p53 expression (**Fig. 18B)**.

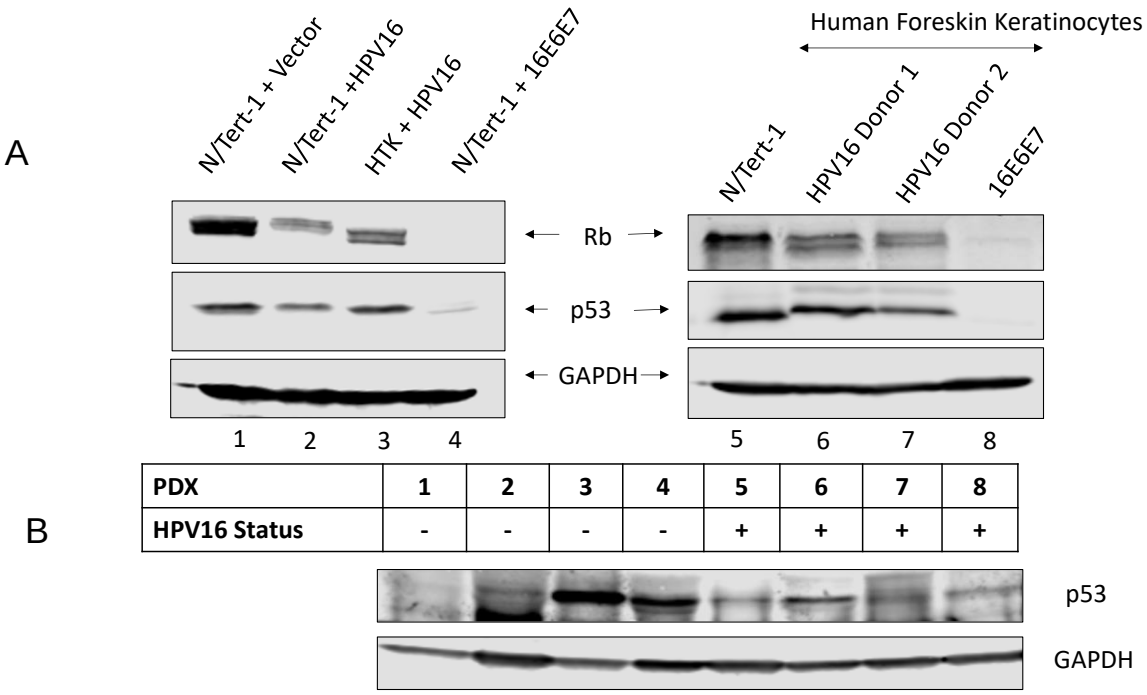


Figure 18. p53 expression is retained in HPV16 immortalized cell lines and PDX models of HNSCC. (**A**) Immunoblot of p53 and pRb in N/Tert-1 cells stably expressing HPV16 (lane 2), E6/E7 (lane 4) or empty vector (lane 1). Rb is the canonical target of the E7 oncogene and overexpression of E7 results in reduction in pRb expression (191). Two independent HFK lines were immortalized with HPV16 (lanes 6 and 7) or E6/E7 (lane 8) as described in **Chapter 2** (**B**) Immunoblot analysis of p53 expression in 4 HPV-negative and 4 HPV-positive patient derived xenografts (3, 157). GAPDH used as internal loading control.

4.3.3 Deficient DNA binding is not the cause of reduced cisplatin sensitivity of the E2(-p53) mutant.

E2 binds to p53 by imitating 53BP2 in its C-terminal DNA-binding domain (DBD). The E2(-p53) mutant was then generated utilizing site specific mutagenesis of residues predicted to be important for E2's interaction with p53 (**See Chapter 2**) (121, 122). Because these mutations are in the DBD, it can be claimed that any phenotype observed with this mutant are due to deficient DNA-binding and not an interaction with p53. To disprove this, we performed functional analysis on this mutant in N/Tert-1 and U2OS cells (**Fig. 19**). We first confirmed that E2 does indeed bind to p53 in N/Tert-1 cells while E2(-p53) does not by immunoprecipitation (**Fig 19A and B**). To study the E2 transcriptional activity, we utilized two-Luciferase reporter systems described in **Chapter 2**. E2 binds to the HPV16 LCR (URR) leading to reduction of viral gene transcription in a dose dependent manner (65). Moreover, when multiple E2 sites are upstream from a gene, E2 exhibits dose-dependent transactivation. As expected, we found that E2 WT and E2(-p53) both repress transcription from the viral promoter in a dose dependent manner leading to reduced luciferase activity (**Fig. 19C**). Next, we transfected the pTK6E2-Luc construct into N/Tert-1 cells and repeated our experiment. While, both E2 WT and E2(-p53) were able to increase luciferase signal over background, we did observe a statistically significant decrease in the E2(-p53) mutant ability to activate transcription of the pTK6E2-luc construct (**Fig. 19D**). To look at DNA binding more directly, we repeated our transfection of

HPV16LCR-Luc in E2 WT or E2(-p53) N/Tert-1 cells and performed chromatin immunoprecipitation. We found a clear and similar increase in normalized ChIP signal over background in both cell lines, indicating that the E2(-p53) can still robustly bind to sites of viral DNA (**Fig. 19E**).

To test the replicative function of E2(-p53), we transfected the viral origin (pORI) and the HPV16 E1 viral helicase into U2OS cells stably expressing E2 WT or the mutant (N/Tert-1 cells do not support this assay). It is well established that E2 and E1 are both required and sufficient to replicate the pOri plasmid (64, 154, 160, 192). After confirming deficient binding to p53 in the mutant using immunoprecipitation (**Fig. 19F**), we found that both the wild-type and mutant E2 retain similar ability to initiate replication (**Fig. 19G**).

We originally proposed that because E2 can transcriptionally repress TWIST1 leading to less aggressive cellular phenotypes (**Chapter 3**), E2 may be more sensitive to CRT agents such as cisplatin. While we found this to be the case and sensitivity was due to the interaction with p53 (**Fig. 16**), we were astonished to find that the expression of E2(-p53) still resulted in TWIST1 mRNA downregulation (**Fig. 20A**). This suggests that while E2(-p53) can bind to sites in the viral promoter, it also bind to the TWIST1 promoter, regulating host transcription. To test this, we performed ChIP to the *TWIST1* promoter similar to **Fig. 13** in **Chapter 3.** As expected, E2 WT and E2(-p53) both bind strongly to the TWIST1 promoter at similar levels, leading to downregulation of TWIST1 mRNA (**Fig. 20B**). These results indicate that overall, E2(-p53) retain most functions of

E2-WT and can still retain both viral and host DNA affinity and function, suggesting that the aberrant growth phenotypes observed with this mutant are likely exclusive to abrogation of p53 binding.

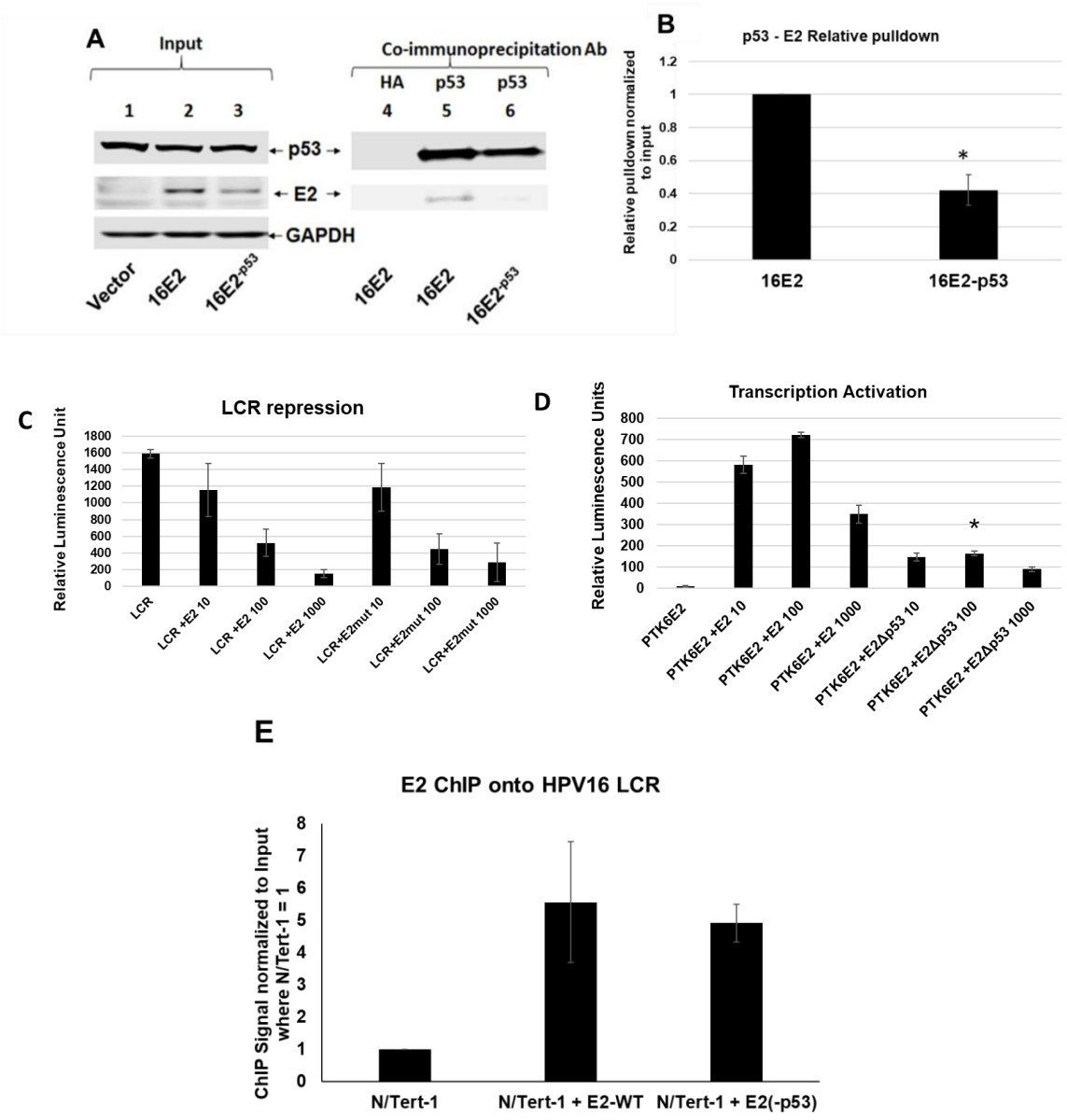
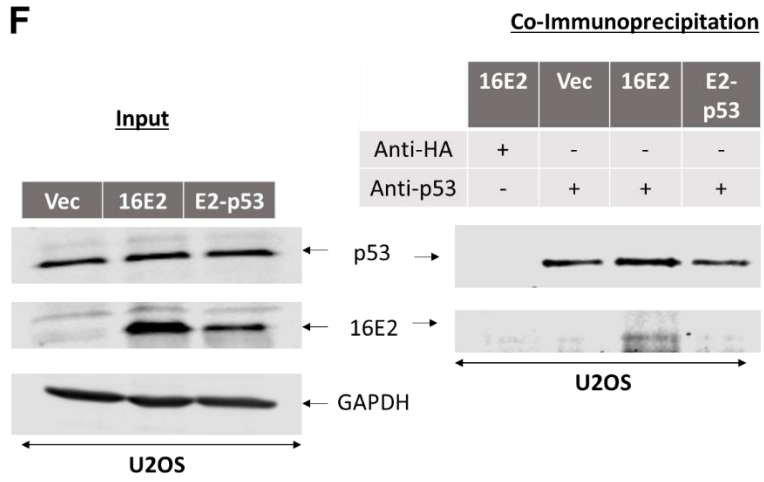


Figure 19. E2(-p53) retains most functions of E2 WT and can bind to both viral and host DNA. (**A**) Immunoprecipitation in N/Tert-1 cells utilizing p53 pulldown of E2. Wild-type E2 can bind to p53 while the E2(-p53) mutant is deficient in binding. (**B**) Because there is decreased expression of E2(-p53) on the input blot in **A,** immunoprecipitation was repeated and quantified. (**C**) Transient LCR repression assay of E2 and E2(-p53). 1 μg of the HPV16LCR-Luc reporter was transfected into N/Tert-1 cells with increasing amounts of E2. Both E2 and E2(-p53) repress transcription from the viral LCR in a dose dependent manner.



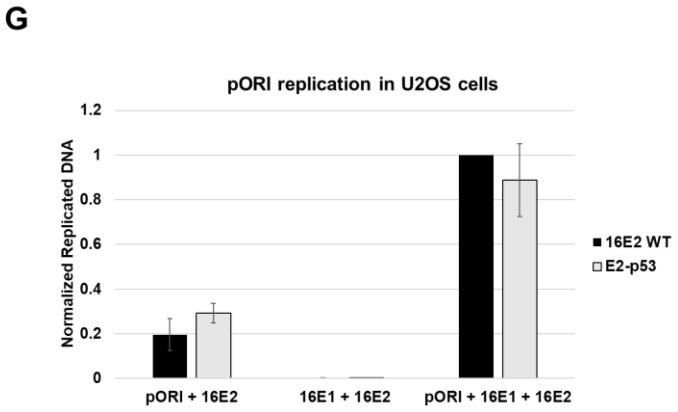


Figure 19 cont. (**D**) Transient transactivation assay of E2. Similar to **C**, 1 µg of pTK6E2-Luc reporter plasmid was transfected along with a gradient of E2. While both E2 and the mutant were able to activate transcription in this assay, there was a statistically significant decrease in E2(-p53) transactivation potential. (**E**) Chromatin immunoprecipitation of E2 onto the HPV16 LCR. Both E2 and the mutant have retained ability to bind to the viral promoter, illustrating that E2(-p53) can still bind to viral DNA. (**F**) p53- E2 Immunoprecipitation of U2OS cells expressing E2 or E2(-p53) confirming deficient binding function of E2(-p53) in these cells. (**G**) Transient replication assay in U2OS cells with stable E2 expression. 1 µg HPV16 E1 was transfected along with 100 ng pORI. Input (unreplicated) DNA was then digested with DPN1 and the resulting replicated DNA was quantified using RT-qPCR. E2(-p53) retains replicative function similar to E2 WT. Quantification of two independent experiments was performed in **B** and **E.** The remaining results are portrayed as the mean ± SEM of at least 3 independent experiments.

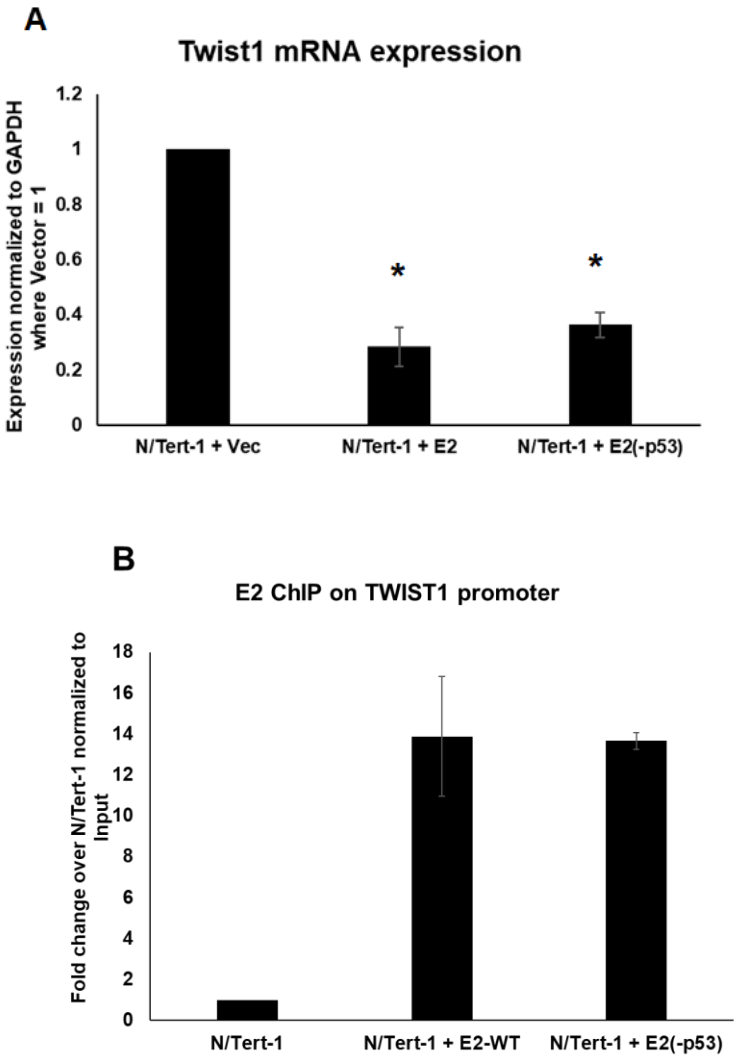


Figure 20. E2(-p53) can bind to the *TWIST1* promoter leading to decreased TWIST1 mRNA expression. (**A**) RT-qPCR of N/Tert-1 cells stably expressing wild-type E2 or E2(-p53). (**B**) E2 Chromatin immunoprecipitation on the cells from **A** using primers designed to amplify DNA sequences in the *TWIST1* promoter. Both wild-type E2 and E2(-p53) bind strongly to the promoter element at similar levels. Despite the difference in cisplatin sensitivity, E2(-p53) can still transcriptionally downregulate TWIST1, suggesting that E2 regulation of EMT is likely not the cause of cisplatin sensitization. The data in **A** represents the mean ± SEM of three independent experiments. The data in **B** is the mean of two biological replicates ± SEM. *P<0.05. ChIP in **Figs. 18 and 19** performed by Drs. Apurva Tadimari-Prabhakar and Claire James.

4.3.4 N/Tert-1 cells expressing wild-type E2 have enhanced cellular viability in the short-term when treated with cisplatin.

We have previously reported that expression of E2 in N/Tert-1 cells does not alter cellular proliferation rate (65, 104, 117). This is important as cisplatin preferentially acts on cells with rapid proliferation, one of the hallmarks in its use as an anticancer agent (189). Our data suggest that E2 positive cells may not be responding to DNA damage by cisplatin resulting in reduced senescence but leading to long term reduced clonogenic survival. Consequently, we believed that continuous treatment of cisplatin may not have a large effect on the cellular proliferation and enhanced cellular viability of E2 expressing N/Tert-1 cells in the short term. To test this, we treated N/Tert-1 cells with E2, the mutant or empty vector continuously for 48 hours and afterwards measured cellular viability using CellTiter Glo© (**Fig. 21**). In accordance with our hypothesis, N/Tert-1 cells with E2 are able to continue to proliferate despite treatment with cisplatin over 48 hours while those with the E2(-p53) mutant and vector control were not. This result once again suggests that there is a lack of response to DNA-damage following cisplatin treatment in E2 positive cells which initially promotes cell cycle progression, but may result in accumulation of unrepaired DNA-damage and resulting genomic catastrophe over the long term (**Fig. 16**).

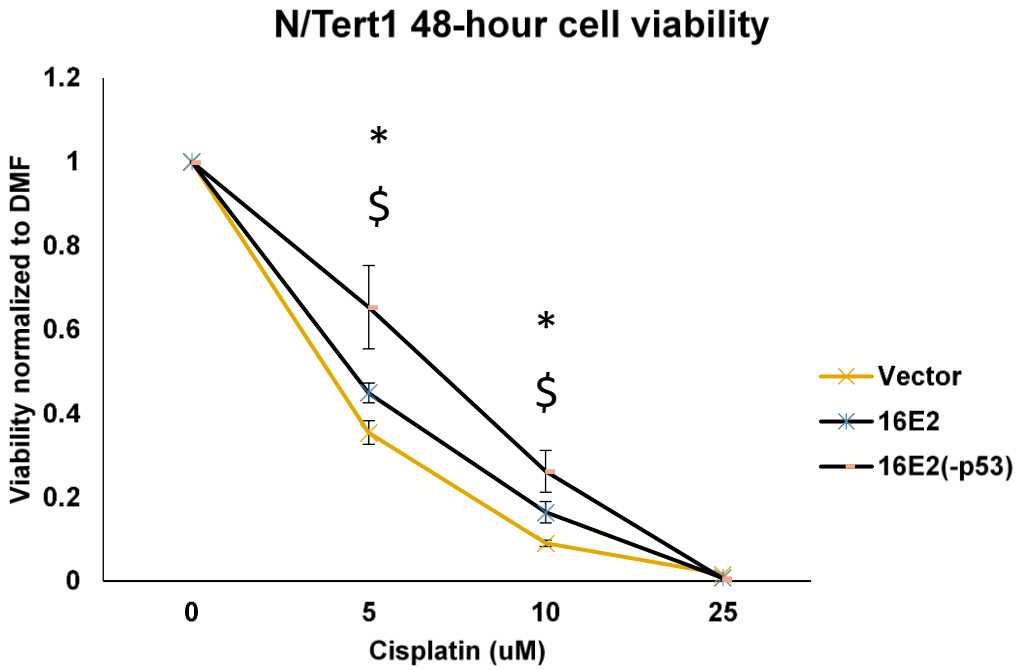


Figure 21. N/Tert-1 cells expressing wild-type E2 have enhanced cellular viability with continuous treatment of cisplatin. $1x10^4$ N/Tert-1 cells with E2 WT, E2(-p53) and vector control were plated in 96 well plates. The following day, indicated concentrations of cisplatin were added to wells and cells were left to grow for 48 hours. Following the treatment period, CellTiter Glo© was added to each well and luminescence was read using a BioTek Synergy H1 hybrid reader. Luminescensce readings were normalized as percent viability over DMF only treatment. Data represents the mean ± SEM of at least six independent experiments. *P<0.05 for 16E2 vs Vector, $P<0.05 for 16E2 vs 16E2(-p53).

4.3.5 The interaction between E2 and p53 reduces recognition and repair of cisplatin induced DNA damage resulting in unresolved DNA fragmentation in N/Tert-1 cells.

The data in **Figs. 16, 17 and 21** suggest that E2 may inhibit cellular response to DNA damage and these cells continue to proliferate in spite of this damage. Following DNA-damage, cells will rapidly begin to recognize and repair the genomic insult by upregulating repair factors such as homologous recombination (HR) and non-homologous end joining (75, 184, 189, 193, 194). Previous studies indicate that these repair pathways are inhibited in high-risk HPV infection (77, 195, 196). To look at the state of DNA more directly after cisplatin treatment, we subjected our E2 expressing N/Tert-1 cells to single-cell gel electrophoresis (Comet) assay on N/Tert-1 cells treated with 2 μM cisplatin as well as 4 days post treatment (**Fig. 22A**). Immediately following treatment (Day 0), cisplatin treatment induced significant DNA fragmentation in all treated cells as illustrated by long, intensely stained comet tails and elevated olive-tail moments (OTMs) (**middle panels**). Following a 4-day recovery period, cells able to repair this damage resolved these tails. While the vector and E2-p53 cells were able to repair cisplatin induced DNA fragmentation, E2 WT cells could not, resulting in persistent comet tails and elevated OTMs (**Right panels**). Moreover, it was found that E2 WT expressing cells had an enhanced background level of fragmentation as mock treated E2 WT had a significant increase in OTM counts compared to vector or E2-p53 cells (**Fig 22A left panels and 22B**). These data reveal that E2

alone is sufficient to modify cellular response to DNA-damage and prevents DNA repair both at baseline and following genomic insult and this E2 function is mediated by interaction with p53.

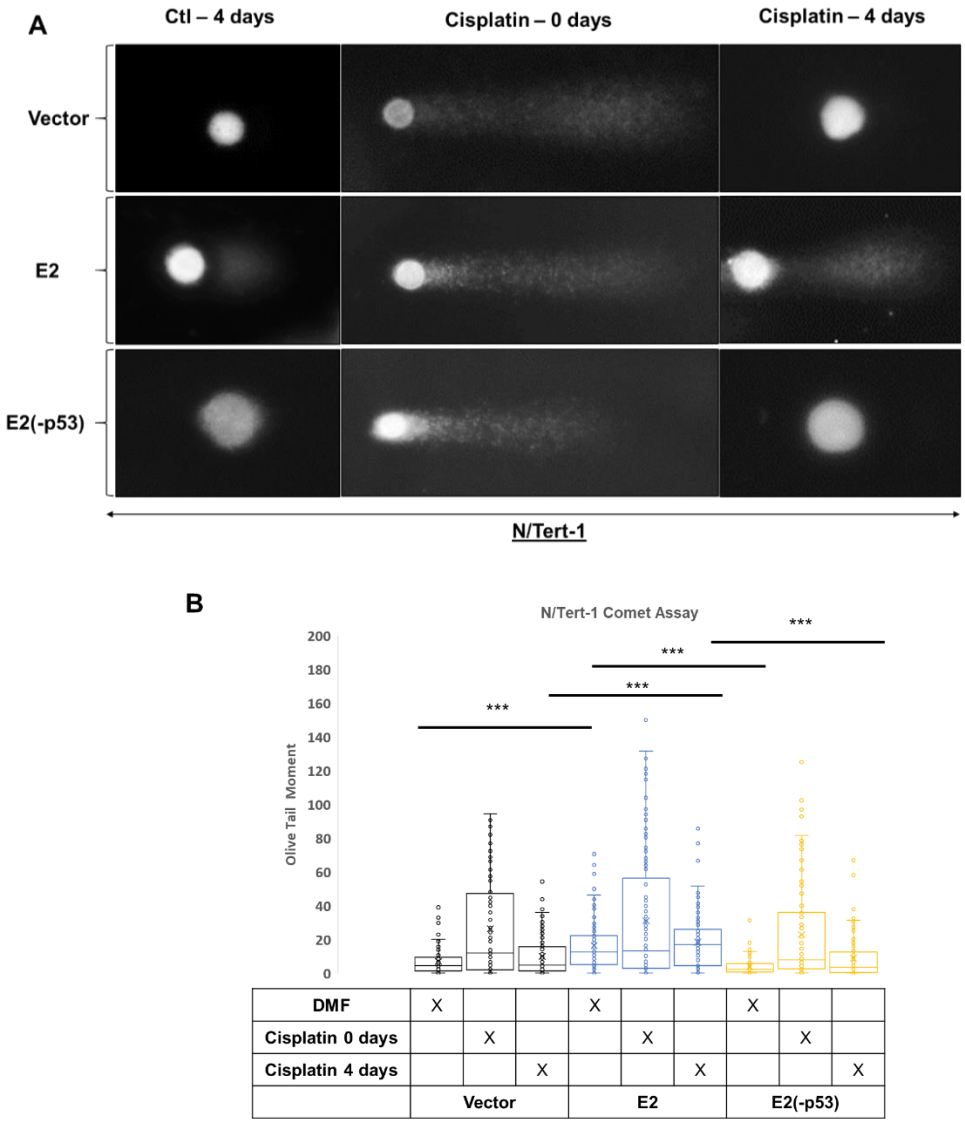


Figure 22. HPV16 E2 binds to p53 preventing recognition and repair of damaged DNA. Single-Cell Gel Electrophoresis (Comet) assay of cisplatin treated N/Tert1 expressing wild-type 16E2 or 16E2^{-p53} mutant **(A)**. The 16E2 N/Tert1 cells exhibit enhanced DNA fragmentation 4-days following cisplatin treatment compared to both 16E2^{-p53} mutant and vector controls **(A and B).** N/Tert1 cells were treated for 5 hours with indicated doses of cisplatin. Cells were then washed with 1XPBS and left to recover for 4 days. Comet assay protocol was adapted from Olive *et al., 2004*. Briefly, following treatment, cells were trypsinized and embedded in 0.5% low molecular weight agarose. The agarose-cell resuspension was placed onto comet assay slides (R&D Systems®, Catalog # 4250-050-03). The plated cells were then lysed and underwent electrophoresis. DNA was stained using DAPI. Olive-tail moment was measured in at least 40 cells per cell-line, per replicate using CASPlab (197). *** $p<0.001$. Analyses were performed using students t-test and α was adjusted for using the Bonferroni approach where $\alpha_{adjusted} = \alpha/n$ where n is the number of comparisons.

4.3.6 p53 is critical for the growth of HPV16 immortalized keratinocytes

E2 is only one of a myriad of viral proteins that are expressed by HPV16 throughout the viral lifecycle. Several other proteins have been shown to modulate cellular response to DDR and modify repair pathways including E6 and E7 (195, 196, 198). In order to look at the role of the interaction between E2 and p53 in the context of the full viral genome, we pivoted our studies to human foreskin keratinocytes immortalized by HPV16 (HFK+HPV16). We immortalized two independent donors of neonatal foreskin cells with HPV16 or the E6/E7 oncogenes using methods previously described (**Chapter 2**) (65).

To determine whether reduction of p53 compromises the growth of HPV16 immortalized cells we introduced stable expression of full length E6 (using a retroviral delivery of the E6 gene which does not allow alternative splicing) into N/Tert-1 (foreskin keratinocytes immortalized by telomerase) and HFK+HPV16 cells. **Fig. 23A** demonstrates that the expression of E6 in N/Tert-1 cells results in significantly increased cellular proliferation as has been described (199). However, introduction of E6 into HFK+HPV16 resulted in an attenuation of cell proliferation (**Fig. 23B**). Because E6 possesses several mechanisms for regulating cellular proliferation independent from p53 degradation, we attempted to isolate these other mechanisms by expressing an E6 mutant unable to initiate degradation of p53 but retains all other known functions (200). The 8S9A10T mutant (Designated as E6Δp53 in these studies for clarity) is deficient in p53 binding but can still immortalize cells and activate telomerase as efficiently as wild-type E6 without any

notable cellular crises. This mutant did not have a deleterious effect on cell growth indicating that it is E6 targeting of p53 attenuates cellular proliferation. Additionally, we found that these proliferation rates inversely correlated with senescence levels (**Fig. 23C and D**). In the HFK+HPV16+E6 cells, we noticed that over time the cells began proliferating once again. To determine whether the recovered cells had a restoration of p53 protein levels we carried out western blots of HFK+HPV16+E6 cells at different stages following E6 introduction (**Fig. 23E**). Lane 4 demonstrates that initially there a reduction in p53 protein levels in these cells immediately following selection compared with control cells (compare lane 4 with lane 3). However, following 13 days of culturing (when we noticed proliferation begin to restore to that of the control cells) we observe restoration of p53 protein expression (compare lane 7 with lane 4). These results suggest that reduction of p53 protein may lead to growth attenuation and enhanced senescence of HFK+HPV16 cells. They also suggest that there is a selective pressure for higher levels of p53 and perhaps less E6 in HFK+HPV16+E6 cells. We monitored the exogenous E6 RNA levels (**Fig 23F**). There is a clear reduction in the E6 RNA expressed from the exogenous vector between days 0 and 13 correlating with the restoration of p53 protein expression and cellular proliferation. While we did not notice an appreciable change in E6 protein levels on western blot, we found that there was a significant increase of E6Δp53 expression compared to wild-type E6 at both time points (**Fig. 23G**), indicating that expression of E6Δp53 may be more permissible than wild-

type E6, supporting our theory that some p53 expression is still required for normal proliferation of HFK+HPV16 cells.

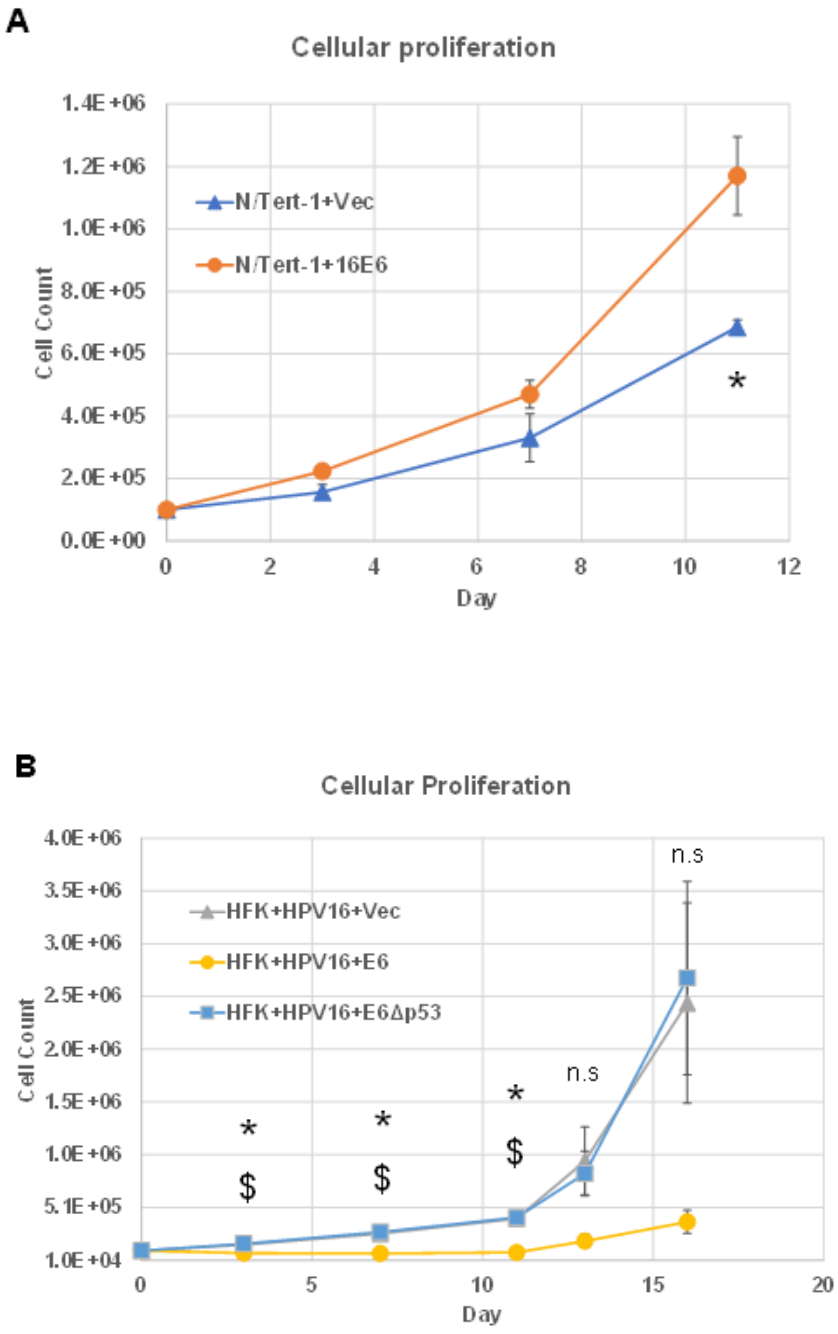


Figure 23. p53 knockdown in via introduction of full length HPV16 E6 reduces cellular proliferation in HPV16 immortalized foreskin keratinocytes. (**A**) 11-day proliferation assay of N/Tert-1 cells expressing exogenous HPV16 E6 compared to empty vector. (**B**) 13-day proliferation assay of human foreskin keratinocytes immortalized by HPV16 and stably expressing exogenous full length E6, mutant E6 that does not bind and degrade p53 (E6Δp53) or GFP control vector.

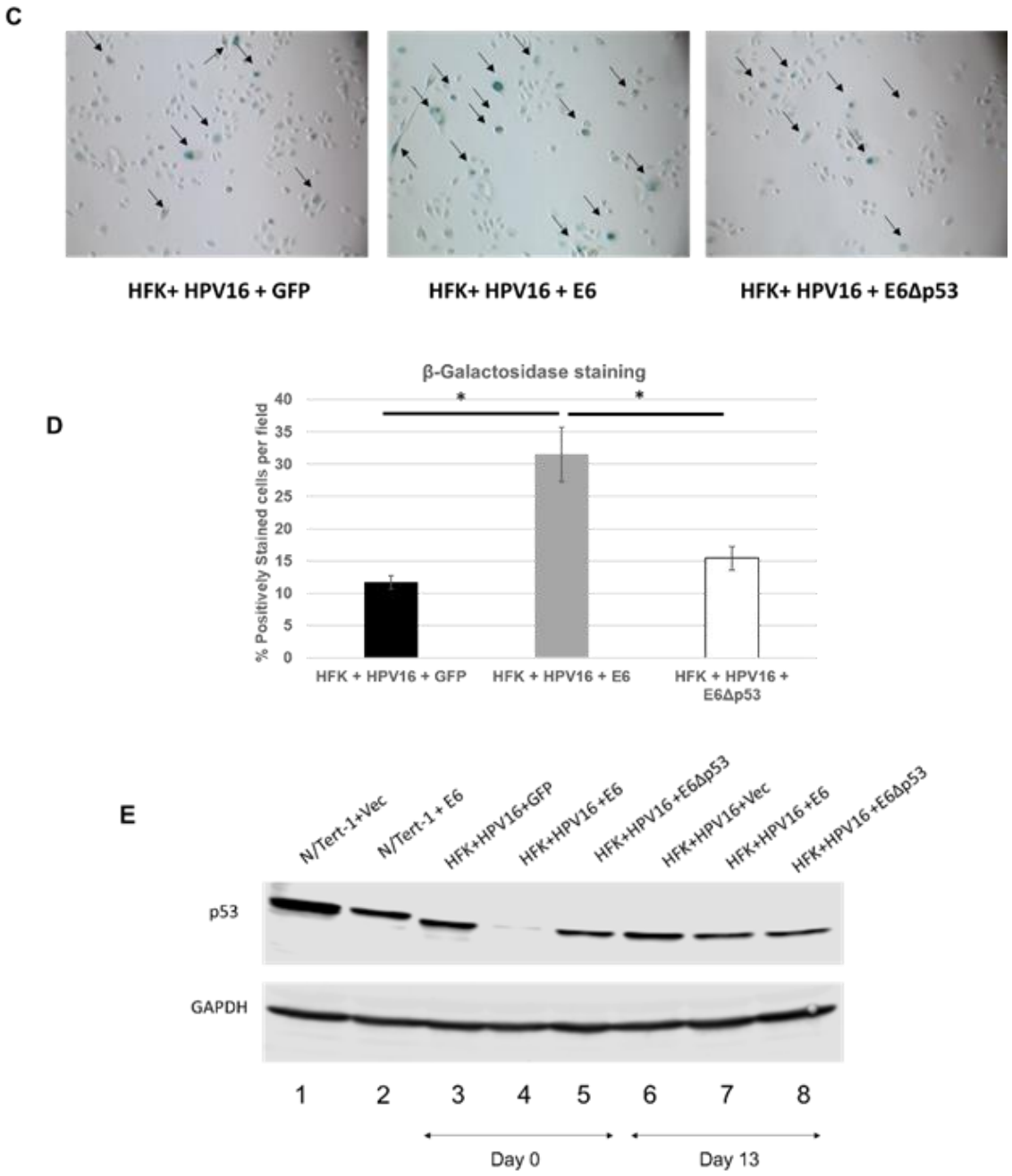


Figure 23 cont. (**C**) Senescence staining of cells in B at day 11. Arrows indicate positively staining cells. (**D**) Quantification of senescence staining in **C**. **(E)** Immunoblot analysis of p53 expression following transfection of E6 plasmids (day 0) and after growth rate recovery of HFK+HPV16+E6 (day 13). GAPDH was used as internal loading control.

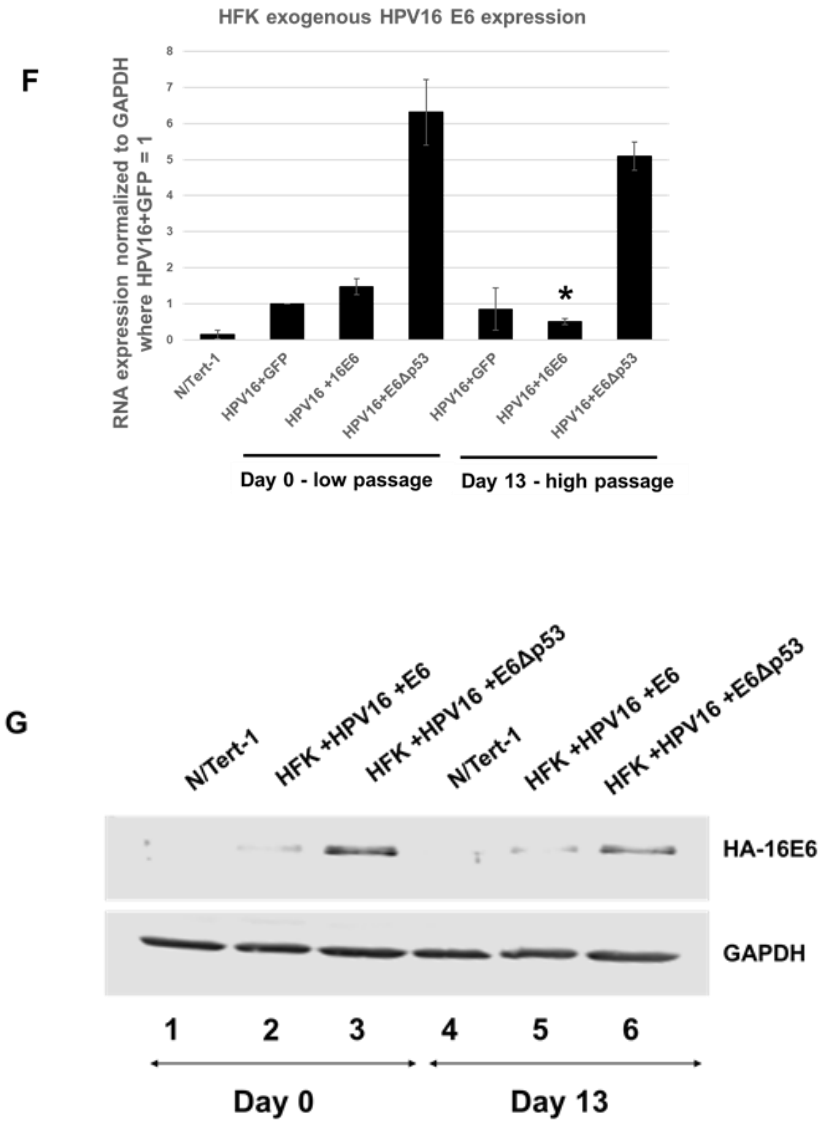


Figure 23 cont. (**F**) RT-qPCR analysis of exogenous GFP, E6 and E6Δp53 expression at day 0 and day 13 using primers against FLAG-HA tag. Bonferroni correction utilized when applicable. (**G**) Western blotting of the indicated extracts using FLAG antibody (the exogenous E6 is double tagged with HA and FLAG). HFK+GFP samples were omitted due to signal oversaturation and bleed over into neighboring wells. The quantified data represent the mean ± SEM for at least 3 independent experiments. *,$P<0.05

4.3.7 HFKs immortalized by HPV16 with deficient E2-p53 interaction elicit attenuated cellular proliferation with a disrupted viral lifecycle.

Since we confirmed that the E2(-p53) mutant retained all critical E2 functions (**Fig 19 and 20)**, we performed similar site-directed mutagenesis which abrogate E2-p53 interaction to entire HPV16 genome (HPV16(-p53)). We introduced the wild-type and these mutant HPV16 genomes into 2 independent primary human foreskin cell populations. We have recently published the utility of these lines to investigate the role of the E2-TopBP1 interaction in the viral life cycle (65). Both the wild-type and mutant genomes efficiently immortalized both HFK donor cells. We carried out Southern blotting on *SphI* cut DNA (a single cutter for the HPV16 genome) (**Fig. 24A**). To further characterize the status of the genomes in these cells we used TV exonuclease assays (this assay is based on the fact that episomal HPV16 genomes are circular and resistant to exonuclease digestion) (161, 201). This assay demonstrated that the viral DNA in the immortalized donor cell lines retained a predominantly episomal status, irrespective of whether the viral genomes were wild-type or HPV16(-p53) (**Fig. 24B**).

Next, we investigated the expression of markers relevant to HPV infection in HFKs. **Fig. 24C** demonstrates that p53 levels are similarly reduced in HFK+HPV16 and HFK+HPV16(-p53) cells when compared with N/Tert-1 cells (compare lanes 2-5 with lane 1). For comparison, cells immortalized with an E6/E7 expression vector had almost no p53 expression (lane 6), likely due to the inability of the E6 to be spliced to E6* variants with this expression vector. To further

characterize these cell lines, we investigated whether the DNA-damage response is active as HPV infections activate both the ATR and ATM pathways. We investigated the phosphorylation status of CHK1 and CHK2 as surrogate markers for activation of these DNA damage response kinases (**Fig. 24D**). Compared with N/Tert-1 cells there is an overall increase of CHK1 and CHK2 levels in cells immortalized with HFK+HPV16, HFK+HPV16(-p53) or E6/E7 expression. CHK1 and CHK2 phosphorylation is also elevated in the presence of all of the HPV16 positive cells when compared with N/Tert-1 cells. It is important to note that E6 and E7 immortalization of HFK induced phosphorylation of CHK1 but not CHK2 when compared with the entire genome (Lane 6). This is likely due to the ATM pathway being largely activated by viral replication rather than by the viral oncogenes E6 and E7 which we have previously reported (78). Overall, these results suggest that markers of HPV16 infection are activated in HFK cells immortalized with HPV16 irrespective of the ability of p53 to bind E2. Even though the HFK+HPV16(-p53) cells had markers indicative of HPV16 immortalization, we noticed an aberrant growth phenotype in both foreskin donor cells (**Fig. 25A**). There was an initial enhanced proliferation of the HFK+HPV16(-p53) cells when compared with HFK+HPV16. However, around the 3-4 week mark, the HFK+HPV16(-p53) cells began to slow their growth and eventually stopped proliferating. To determine the mechanism of the attenuation of cell growth we investigated senescence in N/Tert-1, HFK+HPV16 and HFK+HPV16(-p53) cells by staining for beta-galactosidase

(**Fig. 25B**). There was a significantly increased number of senescent cells with the p53 mutant cells and this was quantitated (**Fig. 25C**).

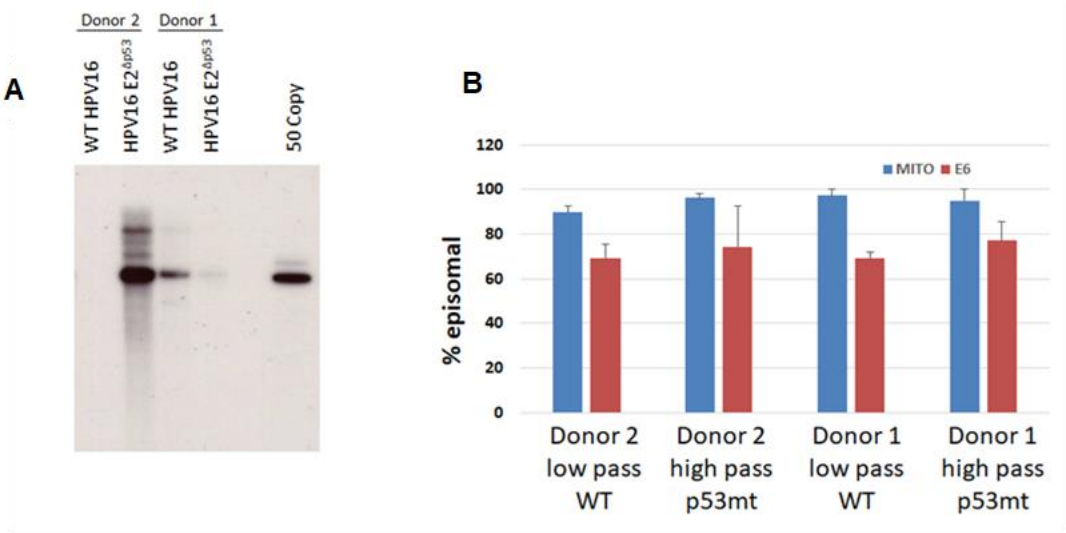


Figure 24. Generation and characterization of HPV16-p53 immortalized human foreskin keratinocytes (HFKs). (**A**) Southern blot of SphI digested DNA (cuts the HPV16 genome once) from the indicated immortalized human foreskin keratinocytes. An over exposure of this blot indicated a band in Donor 2 wild-type cells that migrated around 7.5kbp, indicating a part of the genome may have been lost during immortalization. PCR demonstrates that viral DNA is in these cells, and they are immortalized. With Donor 1 there is less DNA with the mutant genome than the wild-type, the opposite of Donor 2. Therefore, the mutation did not trend towards influencing the levels of DNA in the immortalized HFK. (**B**). To determine whether the viral DNA was episomal, we carried out TV exonuclease digestion. We looked at GAPDH in this assay and called the ΔCt for GAPDH 100% degradation, then we estimated the resistance of both mitochondrial (mito) DNA and HPV16 (E6) to degradation. In all cases the HPV16 DNA is predominantly episomal. As an example, if the ΔCt for GAPDH was 10 following exonuclease treatment, and the ΔCt for mito and E6 1, then they were estimated as 90% episomal DNA (mitochondria have circular genomes that are resistant to the exonuclease). Low pass: pass 7 or less. High pass:12 or greater. This demonstrates that, even following prolonged culture, there is no shift towards integration of the HPV16 genomes. The results shown are the mean ± SEM from duplicate or triplicate experiments. Southern blot and TV assay performed by Dr. Claire James and Raymonde Otoa.

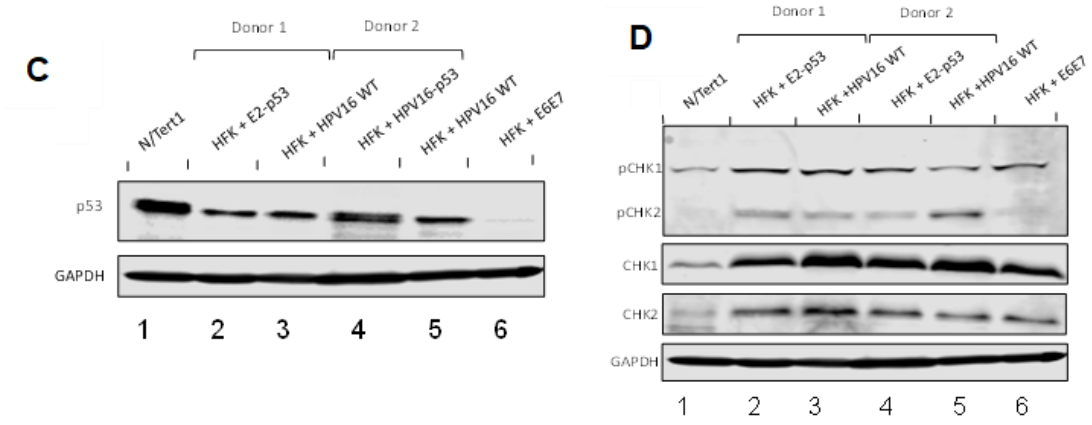


Figure 24 cont. (**C**). p53 protein expression in two independent HFK donors immortalized by wild-type HPV16 (Lanes 3 and 5) and HPV-p53 (Lanes 2 and 4). N/Tert-1 and HFK immortalized by E6 and E7 are provided for reference (lanes 1 and 6 respectively). (**D**) Activation of the ATR and ATM DNA-damage pathways in immortalized HFKs. ATR and ATM activation by HPV16 leads to phosphorylation of Checkpoint kinases 1 and 2 respectively and serve as markers for HPV infection and replication.

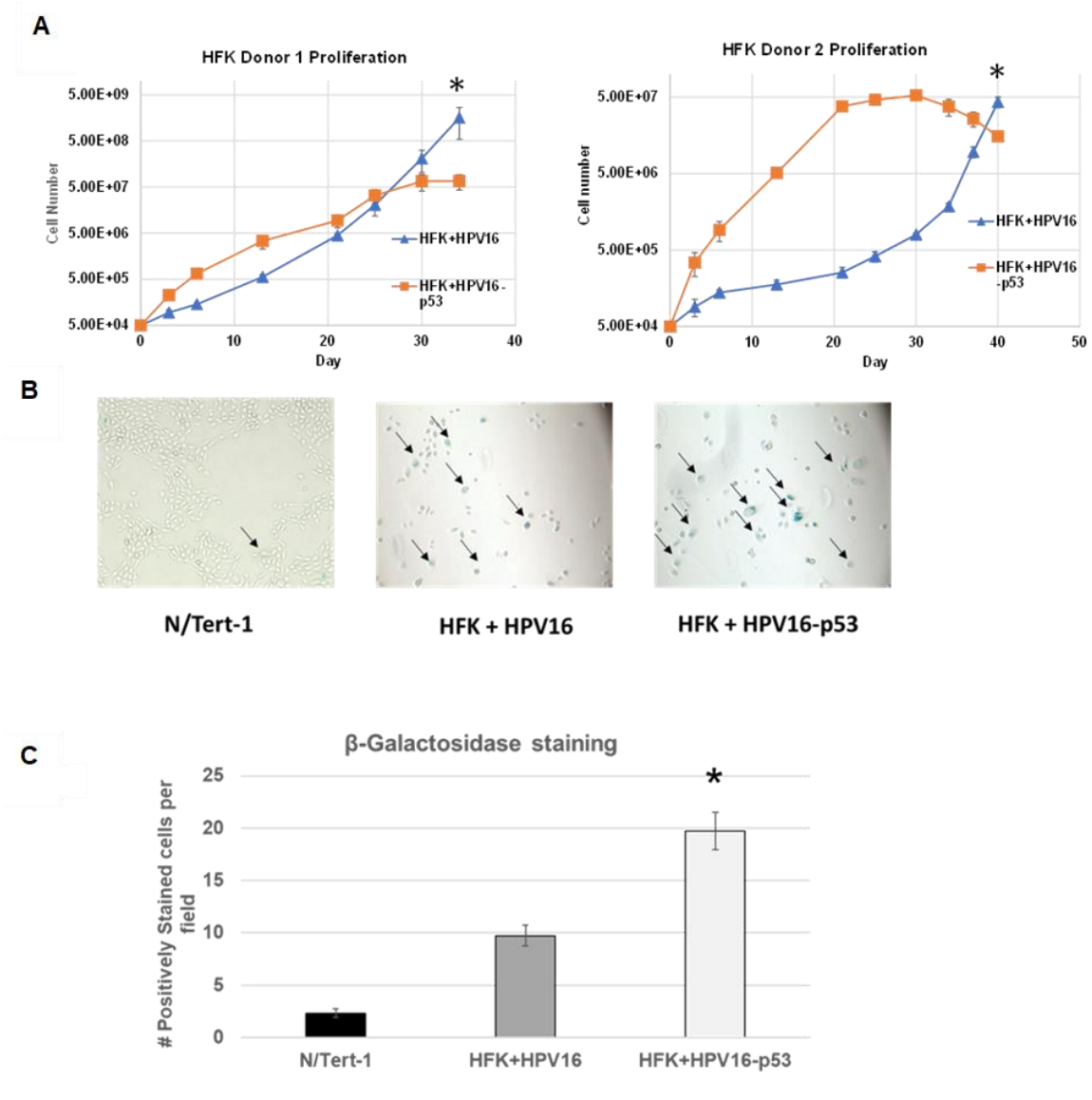


Figure 25. HFKs immortalized by HPV16-p53 exhibit aberrant growth phenotype and elevated levels of senescence (**A**) Extended growth curve on HFK's immortalized by wild-type HPV16 and HPV16-p53. Cells were grown over a period of 34-40 days depending on HFK donor cell line. In general, donor 1 proliferated quicker than donor 2 regardless of HPV genome status. (**B**). β-galactosidase staining as a marker of senescence for proliferating HFK+HPV16 and HFK+HPV16-p53 cells compared to N/Tert-1 cells. Images taken at 10X. Five random fields were imaged per replicate per cell line. Representative image presented with positively stained cells marked by arrows. (**C**) Quantification of β-galactosidase staining. Average number of positively stained cells per high power field were calculated by a blinded observer +/- SEM. *P<0.05.

4.3.8 The interaction between E2 and p53 plays a completely different role in the context of the full HPV16 genome and is critical for a normal HPV16 lifecycle

Senescence can be induced by increased DNA damage, particularly double strand breaks (DSB) (183, 202). Because CHK1 and CHK2 pathway activation was not noticeably different between HFK+HPV16 and HFK+HPV16(-p53), we decided to look at DSBs more directly using our single-cell gel electrophoresis (COMET assays). In **Fig. 22,** we found that expression of E2 in N/Tert-1 cells was sufficient to reduce DNA repair leading to unresolved damage following cisplatin treatment, a characteristic that the E2(-p53) mutant did not have. Because HPV16 activates the DDR by itself, we looked at DNA fragmentation in our HFK cells without cisplatin treatment. As expected, the expression of wild-type or mutant HPV16 genomes in HFKs led to increased formation of DSBs as indicated by OTM counts when compared to HPV-negative N/Tert-1 cells (77) (**Fig. 26A**). However, in a reversal from our findings in N/Tert-1 cells, the mutant HFKs consistently exhibited larger OTM values compared to HFK+HPV16 (**Fig. 26A and B**). This was surprising to us and indicated that the interaction between E2 and p53 plays additional roles in the regulation of DNA damage recognition and repair when the entire viral genome is expressed compared to E2 expression alone.

We demonstrated that p53 knockdown via exogenous expression of full-length E6 attenuates the growth of HFK+HPV16 wild-type cells (**Fig. 23**). We rationalized that expression of E6 should not alter the growth of HFK+HPV16(-p53)

cells. Stable expression of exogenous full length E6 or the E6Δp53 mutant had no additional effect on the growth of low passage HFK+HPV16(-p53) cells, illustrating that the drastic differences in proliferation are potentially due to the E2-p53 interaction (**Fig. 26C**).

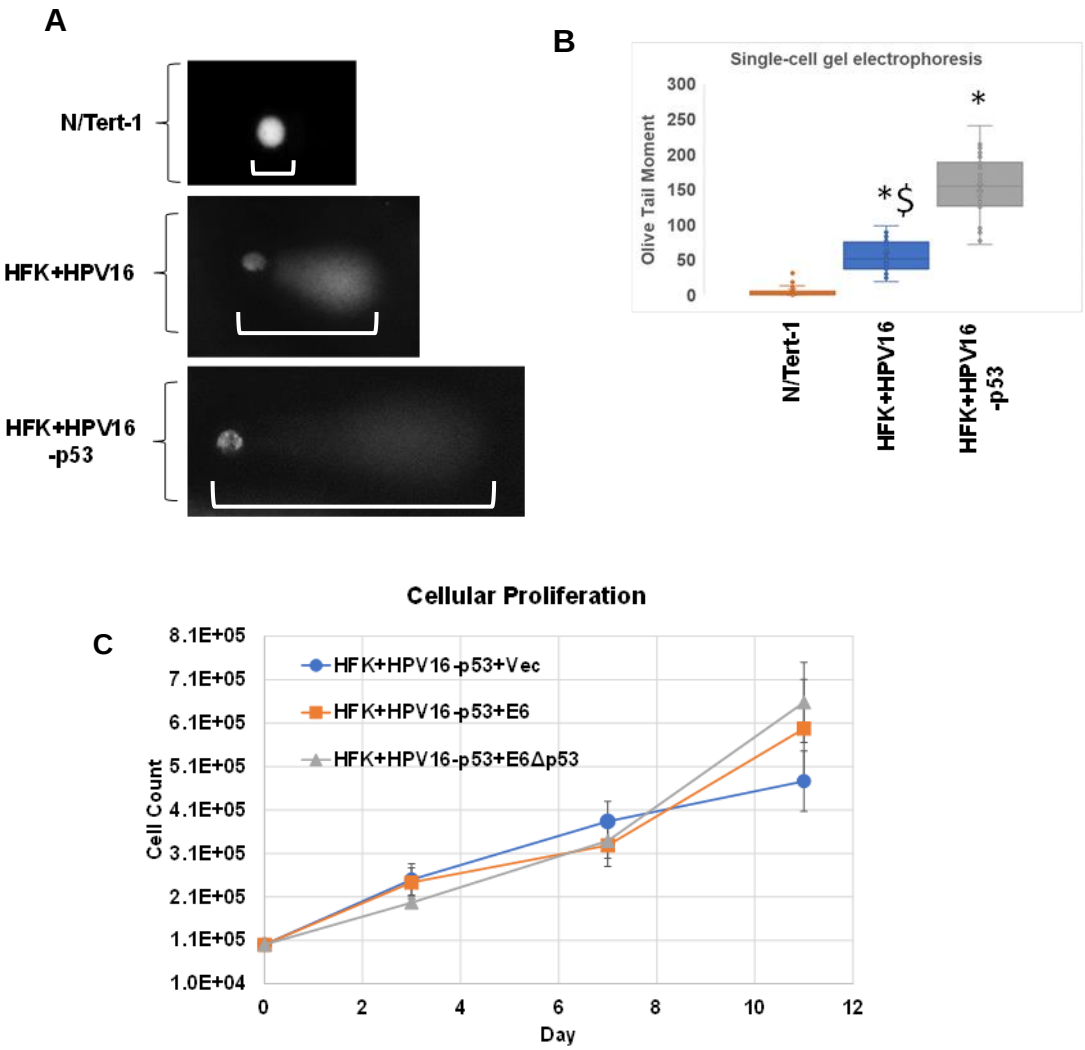


Figure 26. HFKs immortalized by HPV16(-p53) have enhanced levels of DNA fragmentation and are resistant to changes in growth rate with p53 depletion by E6 (**A**) Single-cell gel electrophoresis (COMET) Assay. Cells were grown in 24-well plate for 24 hours then trypsinized, washed, resuspended in 0.5% low molecular weight agarose, and subjected to single cell gel electrophoresis. DNA was stained with DAPI. Five randomly selected fields were imaged at 20x per replicate per cell line. Representative comets are presented with white bars highlighting comet tails. (**B**) The olive-tail moments (OTMs) of all non-overlapping comets in each high-power field were were quantified using CaspLab COMET assay software. Average OTM +/- SEM. *p<0.05 for HFK+HPV16 vs HFK+HPV16-p53. $p<0.05 for HFK+HPV16 vs N/Tert-1. Bonferoni correction used where applicable. (**C**). Eleven-day growth curve on low passage HFK+HPV16-p53 stably expressing exogenous E6, E6Δp53 or GFP control.

4.3.9 HFKs immortalized by HPV16(-p53) have an aberrant life cycle in three-dimensional tissue models.

One of the benefits in using primary keratinocytes for our studies is the ability to subject them to organotypic raft culturing. This allows for a more translational model compared to that of traditional 2-dimensional cell culture. By layering keratinocytes on a layer of collagen, simulating a basal membrane, keratinocytes undergo apical differentiation, leading to cell cycle exit and expressing elevated markers of terminally differentiated epithelia (117, 203). This is a useful tool in studying HPV as the virus' lifecycle is tightly regulated by the differentiation status of the keratinocyte (204). In order to determine the consequences of disrupting the interaction between E2 and p53 in the viral lifecycle more completely, we utilized this technique in our HFKs.

The wild-type HPV and mutant HPV HFK lines in two independent donors were placed on collagen plugs at early passage when the HFK+HPV16(-p53) cells retained proliferative capacity. Due to the large difference in growth rates between the wild-type and mutant cells, the original plating was performed with both $1x10^6$ and $2x10^6$ cells to ensure production of a confluent monolayer on the collagen matrices prior to lifting to the liquid-air interface for differentiation. **Fig. 27** demonstrates an aberrant differentiation process with the HFK+HPV16(-p53) cells when compared with HFK+HPV16 cells at both seeding densities leading to a catastrophic failure of these cells to form normal organotypic tissue. It is important to note that when we utilized our original protocol at the lower cell density ($1x10^6$)

there was a failure to form a monolayer prior to induction of differentiation (as evidenced by gaps between keratinocyte cell clusters on the collagen plug). Using a seeding density of $2x10^6$ eliminated the formation of gaps but did not improve the proliferation. A representative of two independent donors is shown, despite a small difference in basal proliferation between the donors, both donors had identical phenotypes. **Fig. 27B** quantitates the results from two independent rafts from two independent donors; the mutant genomes have dramatically lower raft area when compared with wild-type genomes. To investigate whether differentiation has occurred in these cells we stained with Involucrin and Keratin 10 (**Fig. 27C**). The mutant genome cells stained positive for both differentiation markers demonstrating that, even though raft growth is markedly attenuated, differentiation occurs rapidly and without a normal marker gradient as seen in the wild-type HFKs. We also stained for viral replication using the DNA-damage marker γ-H2AX. Recently we reported that an E2 mutant that failed to interact with TopBP1 results in degradation of E2 during organotypic rafting; this degradation would block viral replication and indeed these cells had no γ-H2AX staining (65). This demonstrates that the γ-H2AX staining indicates the occurrence of viral replication. Fig. 7D demonstrates that there is abundant nuclear γ-H2AX staining throughout HFK+HPV16 cells, indicating replication is occurring however there is a significant reduction in replication in HFK+HPV16(-p53) cells (**Fig. 27C**). These results intrigued us as we have previously reported other mutations in E2 leading to more subtle changes in raft phenotypes (65). This is the first E2 mutant that we

have identified leading to gross failure of raft formation and differentiation, potentially indicating a particular importance of E2 interaction with p53 in the HPV16 viral lifecycle.

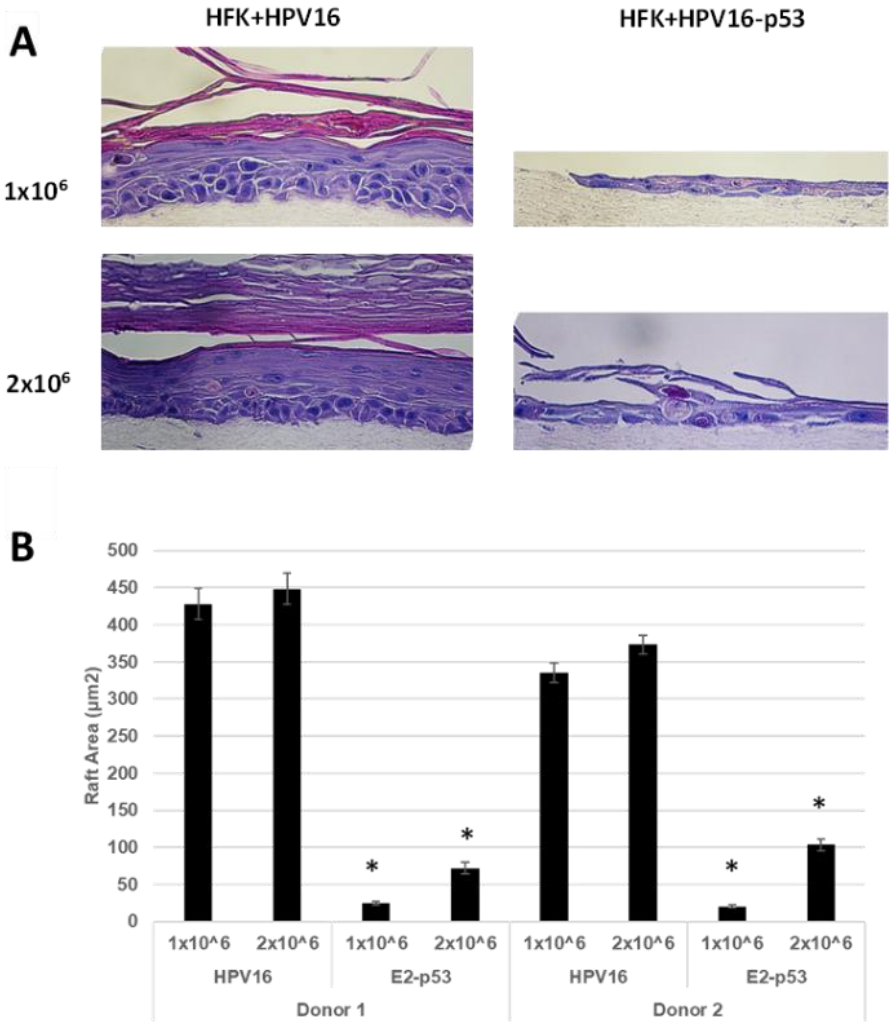


Figure 27. Organotypically rafted HFKs immortalized by HPV16(-p53) exhibit aberrant life cycle with dysregulated differentiation, lower markers of viral replication and overall reduced raft proliferation. (**A**) Organotypic raft cultures and H&E staining of samples from figure 5. HFKs were seeded onto collagen matrices at densities of $1x10^6$ (upper panels) and $2x10^6$ (lower panels). (**B**). The experiment in A was repeated in a second independent HFK donor and average raft areas were calculated for each donor using a Keyence imaging system.

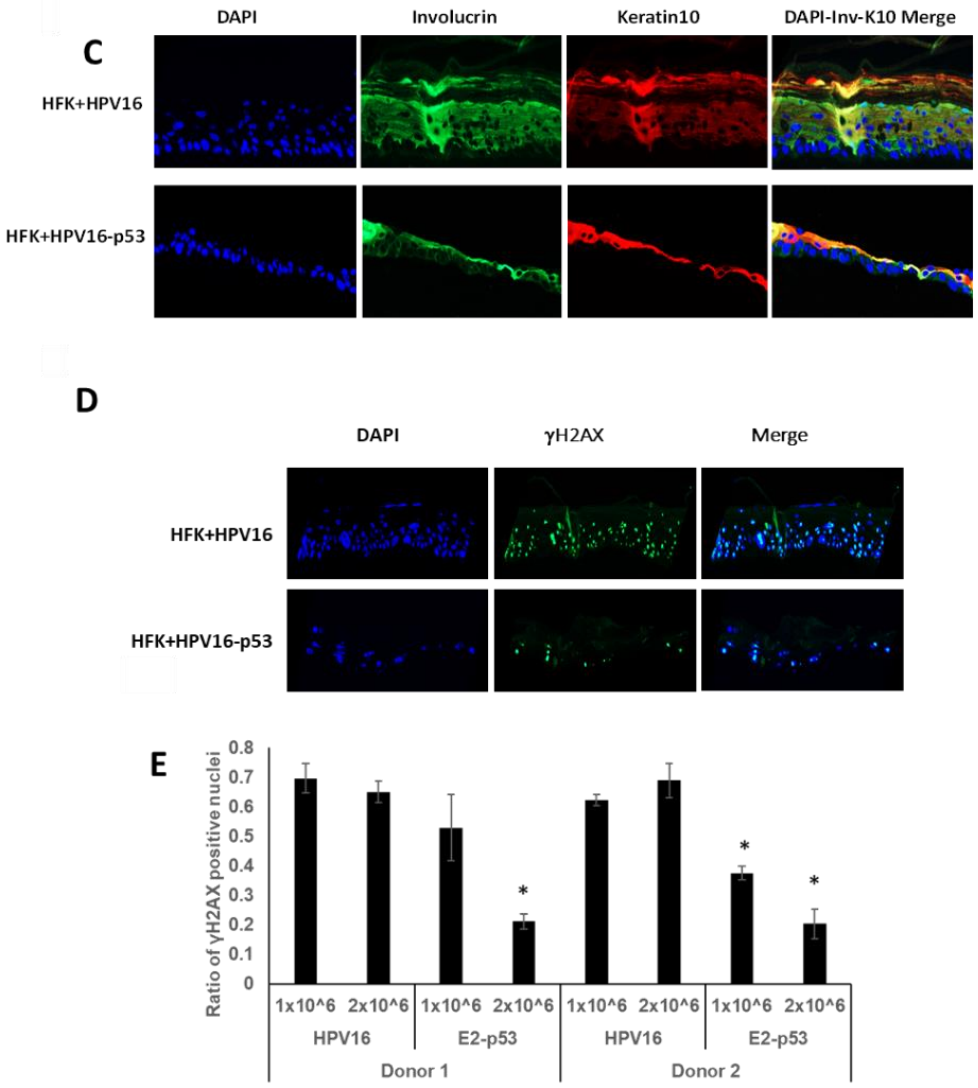


Figure 27 cont. (**C**) HFK rafts stained using indicated antibodies as markers of keratinocyte differentiation. (**D**) DNA damage and viral replication marker γ-H2AX was stained for in HPV16 and HPV16-p53 HFK rafts which is a marker for viral replication. (**E**) γ-H2AX staining was repeated in a second HFK donor and quantified using a Keyence imaging system. Data represents mean ± SEM for at least 2 independent experiments per donor per cell line. *P<0.05. Organotypic rafting, staining and quantification performed by Dr. Claire James.

4.4 Future directions and discussion for chapter 4

Our data reveals a novel importance in the interaction between E2 and p53 in regard to chemosensitivity and the HPV16 viral lifecycle. It was interesting that depending on the context, E2 had varied roles in the regulation of DNA damage either when alone, or with expression of the full viral genome. By itself, wild-type E2 is sufficient to induce DNA fragmentation at baseline as well as after treatment with cisplatin while E2(-p53) was not. Several studies have elucidated a positive association between E2 expression with both reduce cancer aggressiveness and favorable treatment outcomes (80, 90, 92, 93).

There are currently no targeted therapies for HPV positive head and neck cancers. Current treatment regimens for local disease involve surgical resection and targeted radiation therapy (14, 19, 26, 29, 187). In advanced metastatic or otherwise non-resectable disease, systemic platinum based chemotherapy such as cisplatin or carboplatin are routinely added to care (28, 186, 187, 205). It has been known that HPV positivity is associated with longer disease-free progression and enhanced overall survival of patients with head and neck cancer, the mechanisms of which have not been elucidated. While the current standard of care treatments often cure these patients, they contribute to late effects such as paralysis, dysphagia and dysphonia which lead to reduced overall patient quality of life (26, 189). There therefore exists a need to elucidate selective markers for differential treatment response in patients with HPV positive cancers. Our data

suggest that the expression of E2 may be a novel biomarker for treatment response.

The purpose of the interaction between p53 and E2 is still not entirely clear. Following genomic insult, p53 is primed to halt cellular proliferation and initiate senescence if the damage is unable to be repaired by processes including HR (193, 202, 206-208). Interestingly, it is known that HPV hijacks host HR machinery to assist in its own viral replication. Additionally, the virus activates the DNA-damage response pathway via multiple mechanisms potentially to help upregulate these repair factors (77, 79, 162, 195, 196). It is entirely possible that E2 plays a role in this viral function, inhibiting recognition and repair of host DNA damage to sequester repair factors for viral replication. In our attempt to elucidate the mechanism of E2-p53 action, we found a potential candidate. Poly (ADP-ribose) polymerase 1 (PARP1) is an enzyme important in signaling sites of DNA damage leading to recruitment of repair complexes (209). PARP1 is activated by DNA breaks and cleaves nicotinamide adenine dinucleotide (NAD^+), generating ADP-ribose which is added as multimers to acceptor proteins such as PARP-1 itself, histones and other repair proteins (210). The ADP-ribose chains serve as a scaffold and recruit proteins important in the repair of DNA. We found that in N/Tert-1 cells, PARP1 protein is significantly downregulated by wild-type E2 but not E2(-p53) and was not restored by treatment with cisplatin (**Fig. 28A and B**). Moreover, in lanes 3 and 4, PARP1 appears to be modified as it ran slightly larger on western blot compared to E2(-p53) or vector control cells indicating that its

regulation may be primarily post-translational. Due to its critical role in genome maintenance, the downregulation of PARP1 could be at least partially responsible for the reduced recognition and repair of DNA damage by E2.

Additionally, the interaction between other host DNA factors such as TopBP1, or BRD4 may play an important role. TopBP1 is also recruited to sites of DNA damage and is a key downstream mediator of the ataxia telangiectasia and Rad3-related (ATR) leg of the DNA damage response (DDR) (71, 211). Additionally, TopBP1 is also found at HPV replication foci in infected cells, illustrating this crosstalk between host cell DDR and viral replication (64, 79). Because the virally infected cell must continue to proliferate in the presence of an activated DDR, E2 may act by dampening the cells response to damage, ultimately leading to a state of sensitivity to any additional DNA damage such as that evoked by radiation or platinum agents and PARP1 may play a role.

As for the results we observe expression of the full genome in HFKs, it is not surprising that there are some key differences compared to our E2-only N/Tert-1 cells. Nonetheless, we found that wild-type HPV16 immortalized HFKs were more sensitive to cisplatin compared to those with HPV16(-p53), forming fewer and smaller colonies over 14 days (**Fig. 29**), similarly to our data in N/Tert-1 cells (**Fig. 16**). This illustrates that even with the large disparity in Comet assay results in both systems, E2 still appears to confer some p53 dependent chemosensitivity in cells expressing the full viral genome.

As for the difference in Comet OTMs, the DDR is activated by several HPV proteins and including the E1 helicase, the E7 oncogene and viral replication (79). Any significant changes in expression of these could cause runaway DNA damage accumulation. E2 is responsible for transcriptional activation of the viral LCR. However, a recently described viral protein, E8^E2 has also been shown to downregulate viral transcription to maintain low level infection during the maintenance phase of the viral life-cycle (54). E8^E2 shares the C-terminal DNA binding domain and hinge with full length E2 and therefore would also be affected by the site directed mutagenesis of the HPV16(-p53) mutant genome. This could lead to changes in viral gene expression as well as E1/E2 dependent replication via association with NCoR/SMRT family of co-repressor complexes (212). In characterization of our HPV16 immortalized HFKs, we found that cells expressing HPV16(-p53) had an increase in E7, leading to reduced pRb expression. This was reproducible in both donors (**Fig. 30**). Therefore, it is possible that the elevated levels of DNA damage seen in HPV16(-p53) cells may be due to, in part, by elevated E7 expression. Additionally, while the E2(-p53) mutant appeared to have similar replicative function compared to wild-type E2 in our replication assays in **Fig. 19,** we observed significant reduction in viral replication in our organotypically rafted HFK lines **(Fig. 27)**. It is entirely possible that a similarly modified E8^E2 protein could be contributing to changes in viral replication in the context of the full viral genome. There have been no studies on whether E8^E2 interacts with p53

via its shared DBD therefore, the role of E8^E2 in our HPV16(-p53) mutant is an important topic for future study.

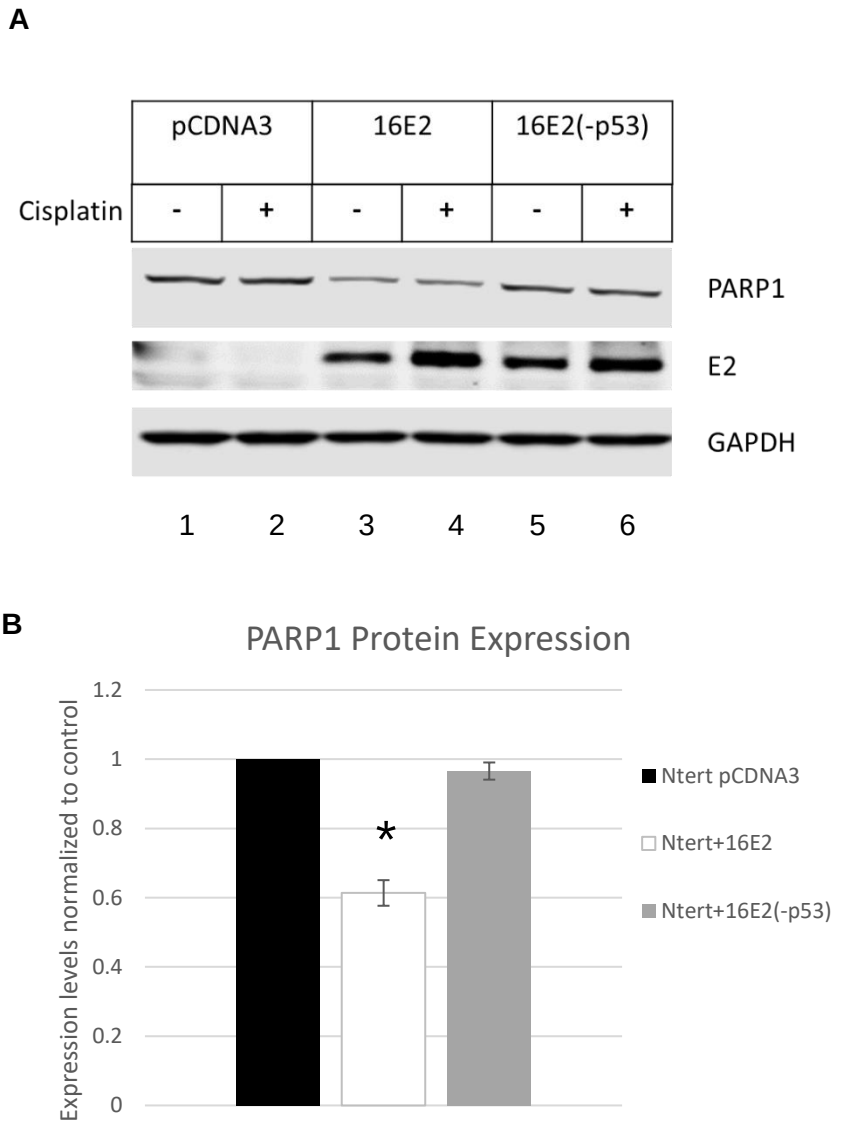


Figure 28. PARP1 is downregulated in N/Tert-1 cells expressing wild-type E2 but not E2(-p53). This regulation is resistant to cisplatin treatment. (**A**) N/Tert-1 cells were treated with 25 μM cisplatin or DMF continuously for 24 hours. Afterwards, cells were harvested for western blot analysis of PARP1 and E2. E2 is stabilized by a wide variety of insults (compare lanes 4 to 3) and is an area of current study. (**B**) PARP1 protein quantitation of several experiments in untreated N/Tert-1 cells. Data represents the mean ± SEM for at least 3 independent experiments. *P<0.05.

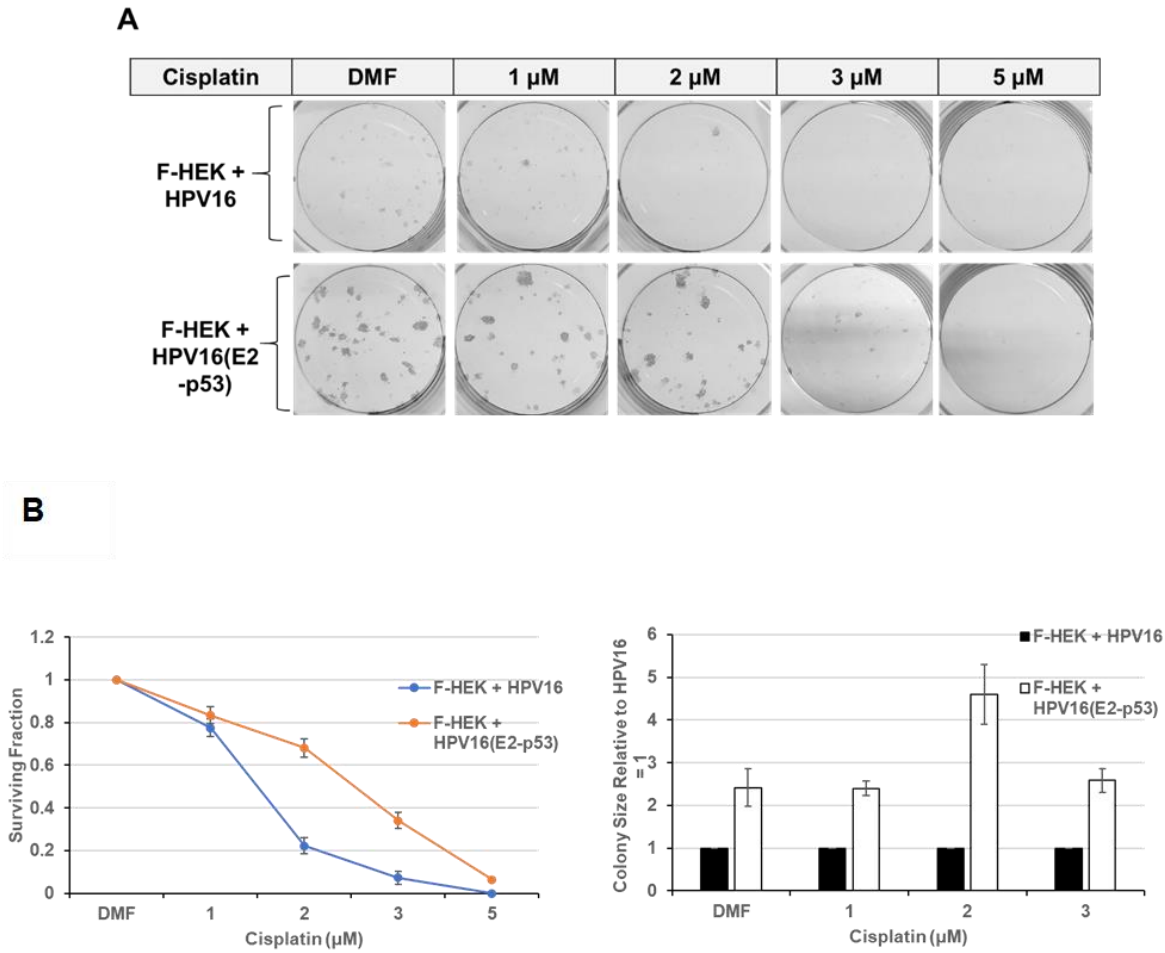


Figure 29. HFK+HPV16 cells are more sensitive to cisplatin compared to HFK+HPV16(-p53) cells. (**A**) Low passage HFKs were treated with increasing levels of cisplatin for 5 hours. Afterwards, 200 cells were replated to 6-well culture dishes and allowed to grow colonies for 12-days. Colonies were stained using crystal violet and counted. A representative replicate is shown. (**B**) Surviving fraction curve of cisplatin treated HFKs taking into account plating efficiency of each cell line. (**C**) Plates were scanned using a Licor imaging system and colony size was calculated in ImageJ. Data represents the mean ± SEM of at least 3 independent experiments. *P<0.05.

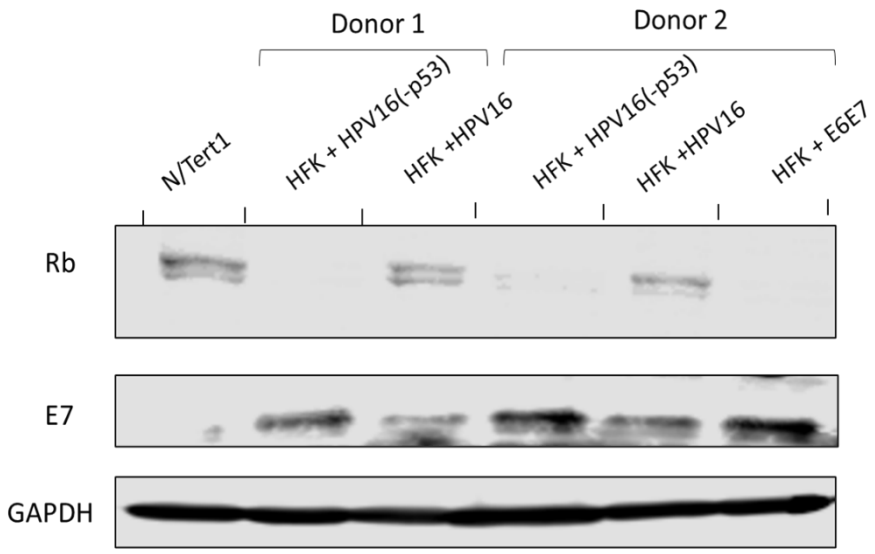


Figure 30. HFK+HPV16(-p53) have elevated levels of E7 and lower Rb compared to HFK+HPV16 cells. This elevated E7 level was found in both donors immortalized by HPV(-p53). Immunoblot analysis of E7 and its molecular target Rb. HFK+E6/E7 used as positive control for E7 and Rb degradation. GAPDH used as internal loading control.

Chapter 5 – Conclusion

In HPV positive HNSCC, tumors often retain episomal viral genomes, and normal expression of all viral genes, including E2 (80, 90, 91). This is in direct contrast with cervical cancer where E2 serves as the most common integration site, leading to its deletion (99). The novel functions of HPV16 E2 in this study provide insight into the differential regulation by HPV in HNSCC and may elucidate the mechanisms behind the improved cancer outcomes in episomal HPV-positive HNSCC.

In **Chapter 3**, we demonstrated that E2 directly binds to the TWIST1 promoter, leading to the addition of repressive histone markers, and resulting in its downregulation. This led to a less aggressive cellular phenotype and reduced wound healing capacity. Tumor cells with reduced markers of EMT have lower metastatic potential and enhanced chemosensitivity in a variety of cancers (141, 145, 147, 213). We found that TWIST1 expression was inversely correlated with episomal status of HPV-positive HNSCC and therefore probable E2 expression in TCGA. Therefore, E2 transcriptional regulation of the host may play an important role in the progression of HPV-positive HNSCC.

The relationship between chemosensitivity and EMT prompted us to look at E2 and cisplatin treatment in **Chapter 4.** We found that E2 is sufficient to sensitize normal keratinocytes to DNA damage via an interaction with p53. Moreover, this interaction has important roles in the cellular response to DNA damage. However,

the E2-p53 interaction and chemosensitivity appear to be unrelated and independent of E2 regulation of TWIST1, highlighting multiple potential roles of E2 in the promotion of less aggressive cancers.

In **Chapter 4,** we also demonstrated that the E2-p53 interaction is critical for the HPV16 viral lifecycle. The primary dogma for high-risk HPVs is that oncogenesis is, in part, mediated by E6 degradation of p53. This has led to an underappreciation of any additional functions of p53 as well as other viral interactors. The results of this work indicate that there is still much to learn on the role of p53 in the HPV16 viral lifecycle. In HPV-negative HNSCC, p53 is often mutated which contributes to cancer progression. While it is true that there are few loss of function mutations in TCGA datasets for HPV positive HNSCC highlighting the role of E6, this does not provide insight to the whole picture. Analysis of TCGA datasets revealed that additionally, there are very few examples of oncogenic gain of function mutations in p53 in HPV positive HNSCC (92) . Put plainly, there appears to be a selective pressure for wild-type p53 in these tumors. HPV is a small virus with relatively few ORFs. Any and all interactions which evolved between viral and host factors are likely critical for normal virus function and should be studied in their fullest, the E2-p53 interaction serving as a prime example.

The fact that patients with HPV-positive HNSCC have improved outcomes has prompted several de-escalation studies (28-30). By treating these patients less aggressively, there was a hope to maintain survival outcomes whilst reducing the side effects and potential late effects of CRT. While there were promising results

from various Phase II trials, the results larger Phase III trials were inconclusive at best (214). We believe that a potential reason for these inconsistencies was failure to stratify by HPV genome status. We have shown that much of the survival benefit of HPV-positive HNSCC is abrogated by viral integration and potentially, E2 disruption. In this work, we demonstrated that E2 has anticancer properties. Therefore, we believe that episomal status and E2 expression may be playing a large role in the outcomes of these patients. The nature of E2 regulation of the host pathways important in cancer and its potential utility as a treatment response biomarker warrant further study.

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Fodde R, Verrijzer CP. 2017. DOC1-Dependent Recruitment of NURD Reveals Antagonism with SWI/SNF during Epithelial-Mesenchymal Transition in Oral Cancer Cells. Cell Reports **20:**61-75.

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Vita

Christian Trent Fontan was born on October 12, 1993 in Jacksonville, Florida USA. He received his Bachelors of Science in 2016 at the College of William and Mary in Williamsburg, Virginia where he studied Chemistry and Pre-medical studies. He joined the Virginia Commonwealth University M.D./Ph.D. combined degree program the same year. After two years of pre-clinical medical studies, he continued his training as a physician-scientist at the Philips Institute in the VCU school of dentistry under the mentorship of Dr. Iain Morgan.

EDUCATION

- **VIRGINIA COMMONWEALTH UNIVERSITY**

Richmond, Virginia 06/2018 – Present

Doctoral Candidate, Oral Health Research **GPA- 4.00**

Mentor – Dr. Iain Morgan

This dissertation focuses on how the viral HPV16 E2 protein regulates cellular pathways important for cancer progression and treatment outcomes. This study will give new insight into why patients with HPV-positive HNSCC respond better than those with HPV-negative cancer and help elucidate both new pathways for targeted therapy and reliable biomarkers for treatment response.

Medical Doctoral Candidate 06/2016 - Present

- **COLLEGE OF WILLIAM AND MARY**

Williamsburg, Virginia 08/2012 – 05/2016

Doctoral Candidate, Oral Health Research **GPA- 3.75**

Mentor – Dr. Douglas Young

My undergraduate research focused on using site-directed mutagenesis to introduce unnatural amino acids into native proteins to be used for functional conjugation. These studies hoped to provide new ways to functionalize proteins and enzymes and modify their efficiency in unfavorable environments, improving their utility in medicine and industry.

PUBLICATIONS

- **Fontan CT,** James C.D., Bristol, M.L., Prabhakar, A.T., Otoa, R., Karimi, E.,Rajagopalan, P., Basu, D., Morgan, I.M. A critical role for E2-p53 interaction during the HPV16 lifecycle. (Pre-print) *BioRxiv.* 2021.11.01.466861. **DOI**: 10.1101/2021.11.01.466861.
- **Fontan, CT,** Das D, Bristol ML, James CD, Wang X, Lohner H, Atfi A, Morgan IM. Human Papillomavirus 16 (HPV16) E2 Repression of TWIST1 Transcription Is a Potential Mediator of HPV16 Cancer Outcomes. *mSphere*. 2020 Dec 9;5(6):e00981-20. **DOI:** 10.1128/mSphere.00981-20.

- Prabhakar AT, James CD, Das D, Otoa R, Day M, Burgner J, **Fontan CT**, Wang X, Glass SH, Wieland A, Donaldson MM, Bristol ML, Li R, Oliver AW, Pearl LH, Smith BO, Morgan IM. CK2 Phosphorylation of Human Papillomavirus 16 E2 on Serine 23 Promotes Interaction with TopBP1 and Is Critical for E2 Interaction with Mitotic Chromatin and the Viral Life Cycle. mBio. 2021 Oct 26;12(5):e0116321. **DOI**: 10.1128/mBio.01163-21.
- James, C.D., **Fontan, C.T.,** Otoa, R., Das, D., Prabhakar, A.T., Wang, X., Bristol, M.L., Morgan, I.M. Human papillomavirus 16 E6 and E7 synergistically repress innate immune gene transcription. *mSphere.* Jan 2020, 5 (1) e00828-19; **DOI**: 10.1128/mSphere.00828-19
- Bristol, M.L., James, C.D., Wang, X., **Fontan C.T.,** Morgan, I.M. Estrogen attenuates the growth of human papillomavirus positive epithelial cells. *mSphere.* Mar 2020, 5 (2) e0004920; **DOI:** 10.1128/mSphere.00049-20
- Murthy AK, **Fontan CT**, Filippa Trikantzopoulou M, Fitzpatrick TH 4th, Levy JM, Alt JA, Schuman TA. Impact of the COVID-19 pandemic on otolaryngology resident rhinology education. Int Forum Allergy Rhinol. 2021 Dec 24. **DOI**: 10.1002/alr.22956.

ABSTRACTS AND SCIENTIFIC PRESENTATIONS

- **Fontan, C.T.,** James, C.D., Basu, D., Morgan, I.M. (2021) *A critical role for tumor suppressor p53 in the human papillomavirus 16 life-cycle.*

Presentation at the Commonwealth of Virginia Cancer Research Conference. Richmond, VA.

- **Fontan, C.T.,** Otoa, R., James, C.D., Morgan, I.M. (2021) *Human papillomavirus 16 E2 inhibits cellular response and repair of DNA damage following cisplatin treatment.* Presentation at Virginia Commonwealth University *The Dental Investigator Research Day.* Richmond, VA.
- **Fontan, C.T.,** Morgan, I.M. (2020) *Human papillomavirus 16 E2 inhibits cellular response and repair of DNA damage following cisplatin treatment.* Presentation at Virginia Commonwealth University *The Dental Investigator Research Day.* Richmond, VA.
- **Fontan, C.T.**, Morgan, I.M. (2019). *A Novel Role for Human Papillomavirus 16 E2 Protein in the Modulation of DNA Damage-induced Apoptosis.* Poster presentation at Virginia Commonwealth University *The Dental Investigator* Research Day. Richmond, VA.
- **Fontan, C.T.**, Morgan, I.M. (2019). *A Novel Role for Human Papillomavirus 16 E2 Protein in the Modulation of DNA Damage-induced Apoptosis.* Poster presentation at Virginia Commonwealth University M.D./Ph.D. Program Retreat. Richmond, VA.
- **Fontan, C.T.**, Morgan, I.M. (2019). *A Novel Role for Human Papillomavirus 16 E2 Protein in the Modulation of DNA Damage-induced Apoptosis.* Massey Cancer Center Research Retreat. Richmond, VA.

- **Fontan, C.T.**, Morgan, I.M. (2019) *Human Papillomavirus 16 E2 modulation of epithelial-mesenchymal transition and chemosensitivity.* Presentation for the Phillips Institute for Oral Health Research Departmental Research in Progress seminar series, Richmond, VA
- **Evans, M.R.**, Fontan, C.T. Morgan, I.M. (2018) *Human Papillomavirus 16 Modulation of Host Innate Immunity.* Presentation for Virginia Commonwealth University School of Medicine *Petite Rounds* seminar series. Richmond, VA
- **Evans, M.R.**, Fontan, C.T. Morgan, I.M. (2018) *Human Papillomavirus 16 Modulation of Host Innate Immunity.* Presentation for Virginia Commonwealth University M.D./Ph.D. seminar series. Richmond, VA

CONFERENCES ATTENDED

- Commonwealth of Virginia Cancer Research Conference

  Richmond, VA

  11/2021

- Massey Cancer Research Retreat

  Richmond, VA

  06/2019

- Massey Cancer Research Retreat

  Richmond, VA

  06/2018

- Massey Cancer Research Retreat

  Richmond, VA

  06/2017

TEACHING AND MENTORING

- Mentor, American Physician-Scientist Association mentorship program Virginia, 2019
- Mentor, Philips institute for Oral Health Research, Virginia Commonwealth University, Fall 2019
- Tutor, Peer Assisted Learning Program, Virginia Commonwealth University School of Medicine, Fall 2019

ORGANIZATIONS

- Member, American Physician-Scientist Association 2016 - Present
- Member, American Medical Association 2016 - Present
- Member, American Medical Student Association 2016 - Present
- Officer (Secretary), Chemistry Club 2014 – 2016

  The College of William and Mary

EXAMINATIONS

1. **United States Medical College Admittance Test** 08/05/2015

   Score:

   511

2. **United States Medical Licensing Exam: Step 1** 03/09/2018

   Score:

   263

WORK EXPERIENCE

1. **VIRGINIA COMMONWEALTH UNIVERSITY**

   Richmond, Virginia 2018-Present

   Graduate research assistant, Dr, Iain Morgan Lab,

Philips Institute of Oral Health Research, Virginia Commonwealth University

Experienced gain in a variety of techniques in molecular biology, cell biology and cancer research.

Lab Techniques Proficient in:

i. Cell culture

ii. Gel Electrophoresis

iii. Western blot

iv. Southern blot

v. DNA and RNA purification

vi. RNA reverse transcription

vii. siRNA protein knockdown

viii. <u>Assays</u>

- Immunoprecipitation assay
- Replication assay
- Luciferase based Transcription assay
- Single-cell gel electrophoresis (COMET) assay
- Cell viability assays
- Clonogenic survival assays
- Anti-cancer drug treatment
- Chromatin Immunoprecipitation
- Real-time qPCR and primer design

Proficient in the use of the following software:

- Microsoft Word, Excel, Power Point, R- programming language, Zeiss microscopy software. Keyence microscopy software.

2. COLLEGE OF WILLIAM AND MARY

Williamsburg, Virginia

2015 – 2016

Student Researcher: Dr. Douglas Young **Department of Chemistry**

Lab Techniques Proficient in

i. Gel Electrophoresis

ii. Ion Affinity Chromatography

iii. Organic synthesis

iv. Nuclear magnetic resonance spectroscopy

v. Microwave “Click” chemistry

vi. Recombinant protein purification