# Investigating the Interaction of Two Human Adenovirus 5 Proteins: E1A and E4orf3 and The Contribution of E1A C terminus on Human Adenovirus 5 Replicative Cycle.

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*“Traveler, there is no path. The path is made by walking”*

-Antonio Machado

*“Caminante no hay camino. Se hace camino al andar”*

-Antonio Machado

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## Abstract

Human adenovirus 5 (HAdV5) has been established as a model system to study cellular processes such as DNA replication, transcription, splicing and the initiation of malignant transformation by DNA tumour viruses. The HAdV5 early region 1A protein (E1A) is the first protein to be expressed upon viral infection. E1A is responsible for inducing quiescent host cells to re-enter the cell cycle via alteration of cellular gene expression and activation of early viral gene expression, with the ultimate goal of promoting viral replication. E1A carries out its functions by binding to cellular regulatory proteins and altering their activities. While many of the interactions between E1A and cellular proteins have been characterized, interactions of E1A with other viral early proteins have not been examined extensively. Here we report, for the first time, the viral early protein E4orf3 as a novel binding partner of the E1A protein.

E4orf3 is a 15kDa protein that weaves through the nucleus of infected cells self-polymerizing into cable-like tracks. E4orf3 functions by forming multiple protein binding interfaces to capture and disrupt numerous cellular protein complexes including members of the DNA damage complex composed of Mre11, Nbs1 and Rad50 (MRN complex) and the oligomeric proteins Promyelocytic Leukemia (PML), amongst others. E4orf3 has also been found to block activation of p53 responsive genes via the formation of repressive heterochromatin at p53 target promoters. We found that E4orf3 binds directly to E1A via the N terminus of E1A. The presence of E4orf3 enhances E1A mediated transcriptional activity of viral genes thus driving their upregulation. In addition, we found that while the E4orf3 protein contributes to inactivation of p53, it is unlikely to be sufficient to block p53 mediated transcription. Instead, our results suggest that E1A and E4orf3 may be working together to achieve suppression of the p53 response pathway.

The second project in this thesis focuses on the functions of the second exon-encoded region of E1A referred to as the C terminus of E1A. This region, is involved in a variety of processes including the regulation of viral and cellular gene expression, immortalization and transformation. Using various exon 2 deletion mutants we found that the C terminus of E1A makes important contributions to the viral life cycle in terms of kinetics of virus growth, activation of viral and cellular gene expression, efficiency of viral genome replication and deregulation of the cell cycle for the induction of S-phase.

## Keywords

Human adenovirus, Early region 1A, E1A, E4orf3, adenovirus early gene, transactivation effects p53 response pathway, C terminus of E1A, S-phase, viral replicative cycle.

## Co-Authorship Statement

Sections 3.11-3.14 of this thesis were published in Viruses, 9(12), December, 2017. I was involved in performing all of the experiments, with the help of Jasmine Frost, Leandro Crisostomo, Oladunni Olanubi and technical assistance from Megan Mendez.

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## List of Abbreviations

| | |
|---|---|
| **Chromatin Immunoprecipitation** | ChIP |
| **Co-Immunoprecipitation** | Co-IP |
| **Conserved region** | CR |
| **C-terminal binding protein** | CtBP |
| **Cytomegalovirus promoter** | CMV |
| **Deoxycytidine monophosphate** | dCMP |
| **DNA Binding Protein** | DBP |
| **DNA double stranded break repair** | DSBR |
| **Fibroblast cells** | IMR-90, WI-38 |
| **Fibrosarcoma cells** | HT1080 |
| **Glutathione S-transferase** | GST |
| **Human Adenovirus 5** | HAdV5 |
| **Human Bronchial Epithelial cells** | HBE |
| **Immunoglobulin** | Ig |
| **Inverted Terminal Repeats** | ITR |
| **Kilo Dalton** | kDa |
| **Kilobase pairs** | kb |
| **Major late promoter** | MLP |
| **Major histocompatibility complex** | MHC |
| **Mre11, Nbs1 and Rad50 complex** | MRN complex |
| **Messenger RNA** | MRNA |
| **Molecular recognition features** | MoRFs |
| **Multiplicity of Infection** | MOI |
| **Non-homologous End Joining pathway** | NHEJ |
| **Osteosarcoma cells** | U2OS |
| **Phosphate-buffered saline** | PBS |
| **Predictor of natural disordered regions** | PONDR |
| **Promyelocytic Leukemia** | PML |
| **Precursor Terminal Protein** | pTP |
| **Receptor internalization and degradation complex** | RID |
| **Retinoblastoma Protein** | pRB |
| **Residues** | R |
| **Retinoblastoma Protein** | pRb |
| **Small Ubiquiti-like Modifier** | SUMO |
| **Structural Protein IX** | pIX |
| **TATA-binding protein** | TBP |
| **TNF-related apotosis-inducing ligand** | TRAIL |
| **Tris-Buffered Saline Tween** | TBST |
| **Terminal Protein Precursor** | pTP |

| | |
|---|---|
| **Terminal Protein** | TP |
| **Tumour Necrosis Factor** | TNF |
| **Virus associated** | VA |
| **Virus encoded protease** | Pr |

# 1. Introduction

## 1.1 Adenoviruses.

Viruses are obligate intracellular parasites that depend upon the host cell machinery for the production of viral progeny (Leber *et al*., 1996; King *et al*., 2018). The ultimate goal of the virus is to make the cell a more favourable environment for efficient viral replication. As a result, viruses posses various mechanisms by which they are able to manipulate and re-organize host networks resulting in wide-spread cellular changes (Ferrari *et al*., 2009; Gulbahce *et al*., 2012; Pelka *et al*., 2008; Rozenblatt-Rosen *et al*., 2012). Viruses serve as useful tools to elucidate different molecular pathways and cellular processes of the host cell. In particular, human adenoviruses have been utilized extensively as models to study important molecular and cellular mechanisms such as DNA replication, transcription, splicing, and cellular transformation (Hay, 1966; Hoeben *et al*., 2013). Adenoviruses have also been used as gene-transfer vectors and oncolytic agents (Hoeben *et al*., 2013).

Adenoviruses belong to the family Adenoviridae, which is divided into 5 genera: *Mastadenovirus, Aviadenovirus, Atadenovirus, Ichtadenovirus* and *Siadenovirus*. Human adenovirus (HAdV) belongs to the *Mastadenovirus* genera, meaning these viruses can only infect mammals. To date, 57 serotypes of HAdVs have been identified (Berk, 2007; Sarantis *et al*., 2004, Walsh *et al*., 2011). The different serotypes can be divided into seven subgroups (A-G) based on based on biological, physiochemical and genetic properties, such as agglutination, ability to induce oncogenicity in rodents, nucleic acid homology, and genetic organization (Bailey *et al.*, 1994; Wigand *et al*., 1969; Rosen *et al*., 1960). The best characterized adenoviruses belong subgroup C: type 2 (HAdV2) and type 5 (HAdV5). As result, most studies utilize one of these serotypes (Alonso-padilla *et al*., 2016), including the results presented in this thesis, which focus on HAdV5.

HAdV was first isolated from human adenoid tissues in 1953 by Wallace Rowe and his colleagues (Rowe *et al*., 1953). In 1954, HAdV was implicated as the etiological agent for acute respiratory illnesses (Hilleman *et al*., 1954). HAdVs have been found to infect multiple mucosal tissues, including the respiratory, gastrointestinal and genitourinary tracts as well as ocular surfaces (Chu *et al*., 1979; Dingle *et al*., 1968; Wood, 1988). Adenoviral infections are self-limiting in otherwise healthy hosts; however, severe manifestations of the illness can occur in newborns or individuals with a compromised immune system (Lewis *et al*., 2009).

The importance of studying HAdV became evident in 1962 when the oncogenic potential of adenovirus was discovered. Trentin and his group showed that injection of subgroup A HAdV12 induced tumour formations in newborn hamsters (Trentin *et al*., 1962). Rodent cells are semi-acquiescent to adenovirus infection, due to a deficiency in both viral DNA replication and late protein production (Eggerdin *et al*., 1986). As a result, HAdV infections in rodent cells are non-productive, leading to oncogenic transformation and tumour development. Not all subgroups of HAdV are capable of inducing tumours in rodents, nevertheless all HAdV subgroups are capable of transforming rodent cells in *in vitro* (Gallimore *et al*., 2001). Unlike rodent cells, human cells are permissive to viral replication, resulting in host cell lysis and progeny virus release (Eggerding *et al*., 1986). To date, there is no conclusive evidence suggesting that HAdV is capable of inducing malignant transformation in humans (Gallimore, 1972). However, early viral proteins have been found to immortalize human cells (Eggerding *et al*., 1986; Graham *et al*., 1977).

Overall, these findings have established HAdV as a useful model system to further our understanding of the molecular biology of DNA tumour viruses in relation to cellular processes such as replication and transcription, and events such as the initiation of malignant transformation (Kümin *et al*., 2002). In addition, these recent studies have highlighted the complex role of viral

proteins in modifying the intracellular environment through changes in DNA replication, cell cycle control, apoptosis, anti-viral host immune responses and mRNA processing (King *et al*., 2018).

**1.2 Human Adenovirus Structure.**

Adenoviruses are small non-enveloped icosahedral viruses, which are made up of a protein capsid surrounding a core that contains the DNA genome (Baron *et al*., 1996); the protein capsid ranges from 70-90 nm depending on the species (Nicklin *et al.*, 2005). The adenovirus virion is composed of 13 structural proteins: 7 capsid proteins, which together form the outer icosahedral capsid of the virus and 6 core proteins, which connect the capsid and the viral DNA (Mackey *et al*., 1976) (Figure 1.1). Polypeptides II (hexon), III (penton) and IV (knobbed fiber), are the three major constituents of the outer capsid of the virion. The capsid consists of 240 hexon trimers and 12 penton pentamers, which make up the 252 subunits called capsomeres (Van Oostrum *et al*.,1985). The pentons form a base for the fiber, the fibers are responsible for tethering the virus to cell surface receptors in order to induce viral uptake. The minor capsid proteins VI, VIII, IX, and IIIa play a role in stabilizing the capsid structure via interactions with the major capsid proteins (Russell, 2009; Vellinga *et al*., 2005). Protein VI is an essential protein that plays multiple roles in adenovirus infection. During entry, this protein alters the endosomal membrane to allow virus entry into the cytosol. In addition, it has a role in facilitating the transportation of the viral genome from the cytoplasm to the nucleus along the microtubular network (Wodrich *et al*., 2006)

The adenovirus core contains proteins VII, V, IVa2, terminal protein (TP), Mu (μ) and the virus-encoded protease (PR) (San Martin, 2012; Rusell, 2009). Protein VII is the most abundant core protein, it functions as a histone-like basic protein around which viral DNA is wrapped (Daniell *et al*., 1981). Protein V can bind the penton base, and polypeptide VI is responsible for linking the core to the capsid (Matthews *et al*., 1998). IVa2 is involved in activation of late

transcription and encapsidation of the viral DNA (Tyler *et al*., 2007). The terminal protein, binds at the end of viral DNA and enables priming of the DNA strands for viral genome replication (Rekosh *et al*., 1977). Mu plays a role in viral chromosome condensation (Anderson *et al*., 1989). Finally, the viral protease is responsible for activating viral protein precursors by cleaving them in order to produce a mature viral particle (Russell, 2009). The mature virions are then capable of infecting neighbouring cells.

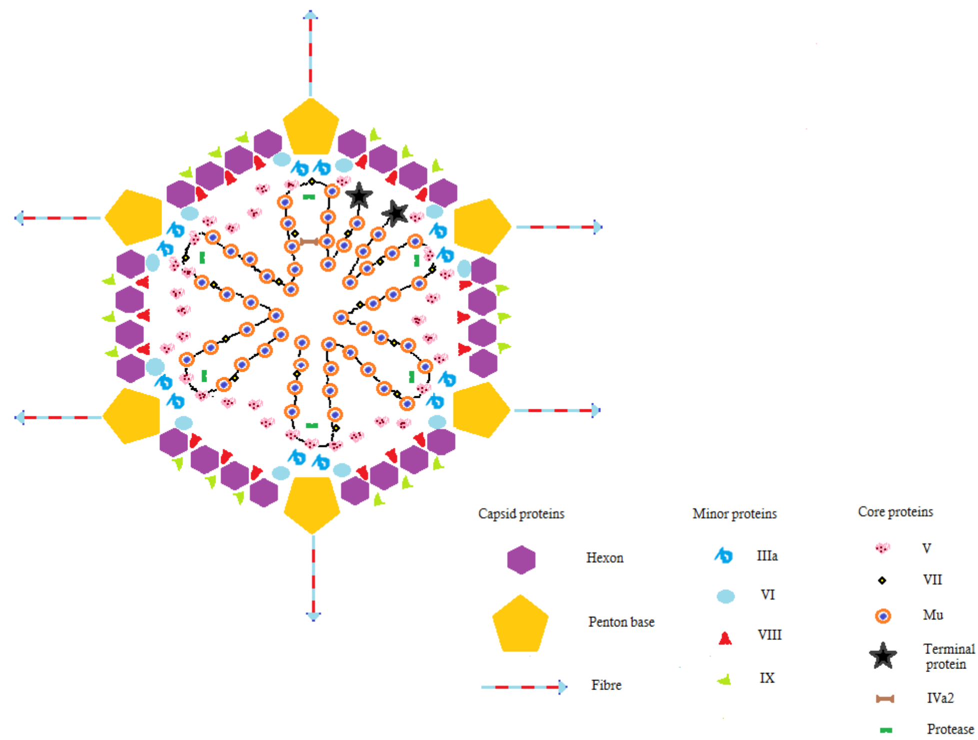



**Figure 1.1. HAdV Morphology**. Adenoviruses have a characteristic icosahedral structure. The capsid is composed of three major proteins, hexon, penton base and a knobbed fiber. There are 4 minor proteins VI, VIII, IX, IIIa and IVa2 which help connect or cement the capsid and viral core. The 6 viral core proteins are also depicted. Illustration used with permission from Brendan Voth.

## 1.3 Human Adenovirus Genome.

HAdV5 has a double-stranded DNA genome of ~35kb (Shayakhmetov *et al*., 2004), the organization of the genome is highly conserved throughout the HAdV family (Hofmayer *et al*., 2009). Viral genes are encoded on both strands of the genome and can be separated into two classes: the early genes which are the first genes transcribed upon viral entry into the host cell and are produced before the onset of viral genome replication, and the late genes which encode the structural proteins that make up the outer viral capsid or help to connect the viral core (Miller *et al*., 2007) (Figure 1.2). All early and late genes are transcribed by the cellular RNA polymerase II (Mishoe *et al*., 1984), while the viral associated RNAs are transcribed by RNA polymerase III (Kidd *et al*., 1995). The early HAdV transcripts are *E1A, E1B, E2A, E2B, E3* and *E4*. These transcripts encode for proteins that are involved in viral transcription and in cellular remodelling mechanisms such as the initiation of S-phase, deregulation of DNA damage response pathways and suppression of the host immune response. Two additional proteins are produced shortly after the onset of viral DNA replication (Binger *et al*., 1984), including the structural protein IX (pIX) and the IVa2 (Lutz *et al*., 1997; Morris *et al*., 2010). In addition, the virus also possesses viral associated RNAs (VA RNAs), VA RNA I and II, which inhibit activation of the interferon response and may influence expression of host genes (O'Malley *et al*., 1986).

The late genes are under the control of one promoter, the major late promoter (MLP) and are transcribed as a single unit that is spliced into five different families of proteins (L1-L5) (Kaufman, 1985; Saha *et al*., 2014). These transcripts encode for structural proteins of the viral capsid and non-structural proteins that are involved in the assembly and packaging of the virion. Both ends of the genome contain 100 bp inverted terminal repeats (ITRs) which serve as the origins of viral genome replication (De Jong *et al*., 2013; Hay, 1985; Saha *et al*., 2014).

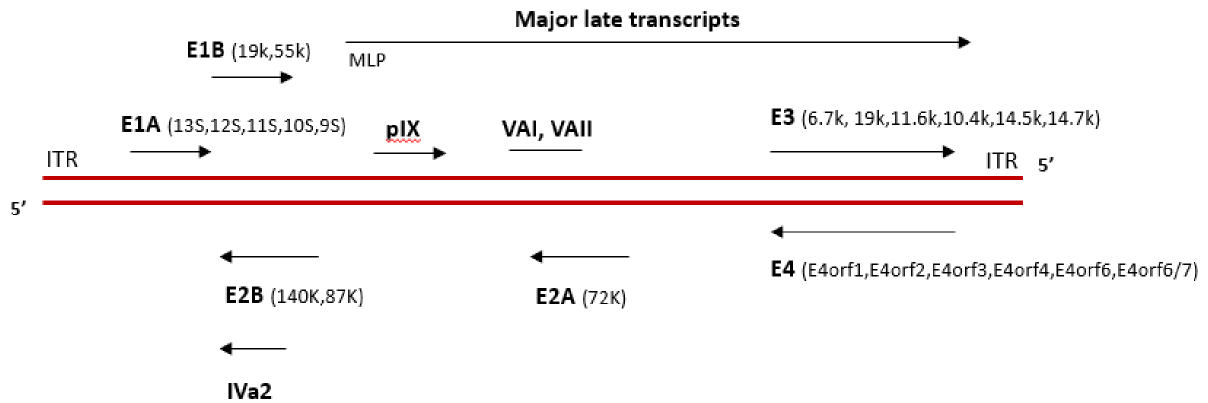



**Figure 1.2 Schematic Representation of HAdV Genome.** Genes are encoded on both strands of the genome and transcription origin sites are found at the 5' ends. Arrows indicate the direction of transcription. Protein names are indicated adjacent or below the arrow of each respective coding mRNA. Adapted from Berk, AJ., 2007 and Saha *et al*., 2014.

## 1.4 Human Adenovirus Replicative Cycle and Host Cell Entry.

Adenovirus infections are usually self-limiting in individuals who are immunocompetent. HAdV is transmitted through direct contact or aerosolized viral particles into the respiratory tract, oropharynx or conjunctiva. Viral transmission can also occur through fecal-oral route into gastrointestinal tracts for non-respiratory HAdV subgroups (Russell, 2009).

HAdV infects quiescent epithelial cells, entry into the cell occurs through receptor-mediated endocytosis. The viral knob of the fiber attaches to the host receptor coxsakie virus and adenovirus receptor (CAR), which is an integral membrane protein that is involved in the formation of tight junctions of epithelial cells (Cohen *et al*., 2001). CAR is the primary receptor for adenovirus subgroups A and C–F (Wu *et al*., 2004). After the virus has bound to the CAR receptor, integrins αvβ3 or αvβ5 bind to the penton base of the virus (Wickham *et al*., 1993). Integrin binding results in fiber shedding from the capsid. In addition, this binding activates signaling pathways that induce endocytosis of the viral particle through clathrin coated pits (Zubieta *et al*, 2005). As the endosome travels toward the nucleus internal acidification occurs, resulting in conformational changes of the capsid, which causes endosomal lysis and escape of virions into the cytoplasm (Medina-Kauwe, 2003). Inside the cytoplasm, the virus is transported along microtubules towards the nucleopore complex (Figure 1.3). The viral capsid is then disintegrated, allowing for the import of the viral DNA into the cell nucleus, after which viral genome replication begins (Fessler *et al*., 1998). Viral progeny are assembled and accumulate within the cell, eventually resulting in cell lysis and release of mature viral progeny.

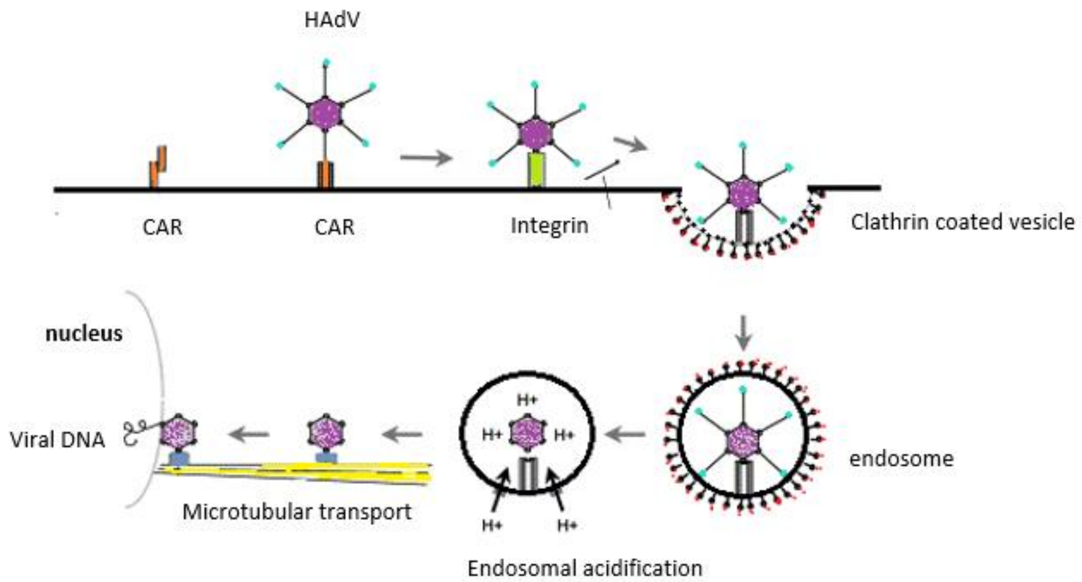



**Figure 1.3 Schematic Representation of HAdV Uptake by the Host Cell.** HAdV knob fiber interacts with CAR. The penton base then binds the integrin αvβ3 or αvβ5. The virus particle is internalized into an endosome, the endosome is acidified as it travels towards the nucleus. The capsid is disassembled and the viral genome enters the nucleus, where viral gene expression occurs. Adapted from Medina-Kauwe, 2003.

**1.5 Transcription and Functions of Early Genes (E1-E4).**

The adenovirus early transcripts are: *E1A, E1B, E2A, E2B, E3* and *E4*, these regions are the first genes transcribed in viral replication and are involved in conditioning of the cell to ensure efficient viral replication (Fessler *et al*., 1998). The functions of viral early proteins are varied and include activation of transcription of other viral genes, induction of the host cell S-phase, initiation of viral DNA replication, and inhibition of anti-viral immune responses (Miller *et al*., 2007).

The first gene expressed after infection is encoded by the E1 region, Early Region 1A, the E1A protein is essential for viral replication as it is responsible for activating the expression of other early genes. In addition, E1A is necessary for initiating cell cycle progression in arrested host cells (Frisch *et al*., 2002; Pelka *et al*. 2008). Due to the multiple and important functions of E1A, it will be discussed in detail in section 1.6.

The E1 region of HAdV also encodes the Early Region 1B (*E1B*) region. The *E1B* region encodes two proteins, E1B 55kDa(k) and E1B 19kDa(k), which inhibit apoptotic responses that are activated in response to S-phase induction by E1A (White *et al*., 1990). The E1B 55k protein targets the p53 tumour suppressor via multiple mechanisms. E1B 55k is able to bind directly to the N terminus of p53 and inhibits p53 mediated transcriptional activation (Blackford, 2009). In addition, E1B 55k functions as a viral small ubiquitin-like modifier (SUMO) ligase and SUMOylates p53 to target it for nuclear export (Muller *et al*., 2008; Pennella *et al*., 2010). E1B 55k is also able to interact with the E4orf6 protein, together they form an E3 ubiquitin ligase complex, which disrupts the functions of the p53 tumor suppressor, the MRN DNA damage repair complex and the DNA ligase IV protein, which is involved in the non-homologous end joining (NHEJ) DNA repair pathway (Blackford, 2009; Querido *et al*., 1997). The E1B 19k protein blocks apoptosis in a p53 independent mechanism by acting as a homologue of the B-cell lymphoma 2

(BCL2) protein. The BCL2 protein inhibits mitochondria mediated apoptosis by binding to BAK and BAX proteins, therefore preventing mitochondrial pore formation and the release of proteins involved in apoptosis, such as cytochrome C (Rao *et al*., 1992; Han *et al*., 1996).

The E2 region plays an important role in viral DNA replication as it encodes the machinery involved in viral genome replication (Hay *et al.*, 1995). The E2 region is divided into a promoter-proximal region (E2A) and distal region (E2B) (Caravokyri *et al*., 1996). E2A encodes for the 72k DNA Binding Protein (DBP), which is essential for elongation during viral DNA replication via binding to the single-stranded template unwinding the double stranded DNA (De Jong *et al*., 2003). In addition, DBP associates with single stranded DNA, which may function to protect the viral genome and prevent the activation of DNA damage pathways.

E2B encodes for the 80k terminal protein precursor (pTP) and a 140k DNA polymerase, which along with DBP and other cellular proteins form a complex involved in viral DNA replication (Parker *et al.*, 1998; Stillman *et al*., 1982). Replication begins when the viral polymerase attaches a dCMP (deoxycytidine monophosphate) residue to the pTP, which allows it to functions as a 5' primer for the initiation of replication (Stunnenberg *et al*., 1988; Parker *et al*., 1998). The viral DNA polymerase, contains 5' to 3' DNA polymerase activity as well as 3' to 5' proofreading exonuclease activity and is the sole DNA polymerase responsible for viral genome replication (Brenkman *et al*., 2002; King *et al*., 1997; Liu *et al*., 2003).

The E3 region is spliced to produce seven proteins: E3-gp19k, 14.7k, 14.5k, 10.4k, 12.5k 6.7k and the adenovirus death protein (ADP/E3-11.6k) (Lichtenstein *et al.*, 2004; Wold *et al.*, 1995). These proteins are involved in blocking the host immune response in order to prevent killing of infected cells by death-inducing cytokines and cytotoxic T cells (Russell, 2009). The E3-gp19k glycoprotein blocks MHC class I antigen receptors from being exported to the surface, thus

blocking killing mediated by cytotoxic T cells (Burgert *et al.*, 1987; Horwitz *et al.*, 2004). The proteins E3-10.4k and 14.5k form the receptor internalization and degradation (RID) complex. This complex blocks the activation of apoptotic pathways by causing endocytosis and lysosomal degradation of receptors such as the tumour necrosis factor (TNF), Fas (CD95) and TNF- related apoptosis inducing ligand (TRAIL) (Tollefson *et al.*, 2001). E3-6.7k works together with RID in downregulating TRAIL and TNF signalling and may block apoptosis independently of other E3 proteins. Furthermore, E3-14.7k functions as an inhibitor of TNF, NF-κB and Caspase 8 mediated apoptosis and blocks TRAIL induced apoptosis (Klingseisen *et al.*, 2012; Lichtenstein *et al.*, 2004). The adenovirus death protein is expressed during the later phases of infection and has apoptotic activity that assists in cell lysis for the release of mature viral progeny (Tollefson *et al.*, 1992). The 12.5k protein appears to not be essential for virus replication, however its function is still unknown (Hawkins *et al.*, 1992).

The E4 region is spliced to produce 7 proteins which are named after their encoding open reading frames (ORFs): E4orf1, E4orf2, E4orf3, E4orf3/4, E4orf4, Eorf6, E4orf6/7. E4orf1 expression has only been detected in HAdV subgroup D viruses 9 and 36 (Tauber *et al.*, 2001; Weiss *et al*., 1997). The E4orf1 protein is responsible for reprogramming the cell to enhance viral replication and to promote mechanisms for cell survival (Frese *et al.*, 2003). E4orf1 also changes host cell metabolism in a MYC-dependent mechanism by promoting glycolysis and increasing the production of nucleotides (Thai *et al*., 2014). E4orf2 is localized to the cytoplasm, but the function of the protein is not known (Dix *et al*., 1995). In addition, the E4orf3/4 protein has not been detected during infection (Tauber *et al*., 2001). The E4orf3 protein forms a polymer in the cell nucleus and plays an important role in the inactivation of several tumor suppressors as well as inhibiting the cellular DNA double stranded break repair (DSBR) (Stracker *et al*., 2002; Sohn *et*

*al.*, 2012). E4orf3 will be discussed in more detail in section 1.8 and 1.9. The E4orf4 protein relocalizes the global cellular phosphatase PP2A, which results in the induction of p53 mediated apoptosis resulting in cell lysis and release of the mature viral progeny (Brestovitsky *et al.*, 2016; Branton *et al.*, 2001). In addition, E4orf4 inhibits the activation of various DNA damage pathways by blocking the phosphorylation of substrates of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, including ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related protein (ATR) (Brestovitsky *et al.*, 2016). As previously mentioned, E4orf6 forms a complex with E1B 55k to ubiquitinate different proteins in order to target them for degradation (Dobner *et al.*, 1996; Querido *et al.*, 2001). Some of the targets of this complex included p53 and the MRN complex. The E4orf6/E1B 55k complex is also involved in RNA processing, transport of late viral mRNAs from the nucleus to the cytoplasm and inhibition of host protein synthesis by preventing cellular mRNA export (Halbert *et al.*, 1985; Yatherajam *et al.*, 2011). Lastly, the E4orf6/7 protein is involved in the activation of E2 transcription by recruiting the E2F transcription factors to the E2 promoter region (Marton *et al.*, 1990; Obert *et al.*, 1994).

**1.6 Adenovirus E1A Structure.**

*E1A* is the first viral gene transcribed upon viral infection and is responsible for activating the transcription of other viral genes, reprogramming host cell gene expression and forcing quiescent cells to enter the cell cycle (Bayley *et al.*,1994; Pelka *et al.*, 2008; Shenk *et al.*, 1991). E1A has been characterized most extensively in HAdV5. Adenovirus E1A is spliced to produce five isoforms (Figure 1.4), the two largest products, 13S and 12S, are expressed at high levels early in infection and encode proteins of 289 and 243 residues (R) respectively. These two isoforms differ only by a 46 amino acid sequence that is present only in the 13S isoform. The primary activator of viral gene expression is the 13S isoform, whereas 12S is generally considered a

repressor (Pelka *et al*., 2009). During the later phases of infection there is a shift to increase the expression of the 11S, 10S and 9S E1A isoforms, which encode proteins of 217, 171, and 55 residues respectively (Radko *et al*., 2015; Stephens and Harlow, 1987; Ulfendahl *et al*., 1987). All isoforms are spliced in the same reading frame except for E1A 9S.

The structure of E1A has not been resolved despite several attempts by multiple groups, which suggests that E1A is a highly disordered protein. In addition, PONDR bioinformatic analysis predicts that E1A is intrinsically disordered (Pelka *et al*., 2008; Romero *et al.*, 2004) (Figure 1.5). However, sequence comparison of the large E1A proteins identified four regions that have conserved sequences, which have been designated as conserved regions (CR) CR1, CR2, CR3 and CR4. The conservation of these sequences suggests their functions are important in adenovirus replication (Kimelman *et al*., 1985). Despite a lack of protein structure, the CR3 region contains four cysteines that are believed to form a zinc-finger domain (Culp *et al*., 1988). In addition, residues 16-28 of the N terminus region of E1A form an amphipathic α-helix (Avvakumov *et al*., 2004; Ferreon *et al*., 2009).

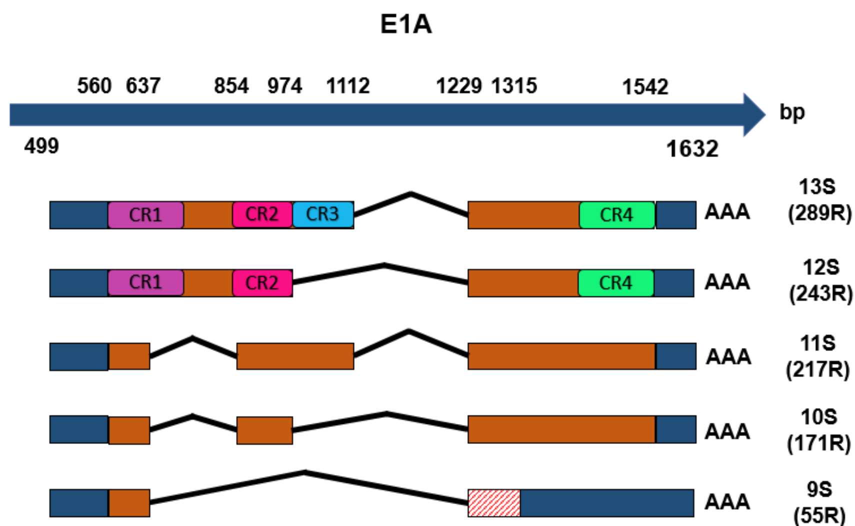



**Figure 1.4 Diagram of E1A Isoforms.** E1A is spliced into 5 different isoforms. Boxes indicate coding regions, bent lines represent introns. Residues for each isoform are listed. All isoforms are translated from the same reading frame, except for the 9S isoform. CR= Conserved Region

.

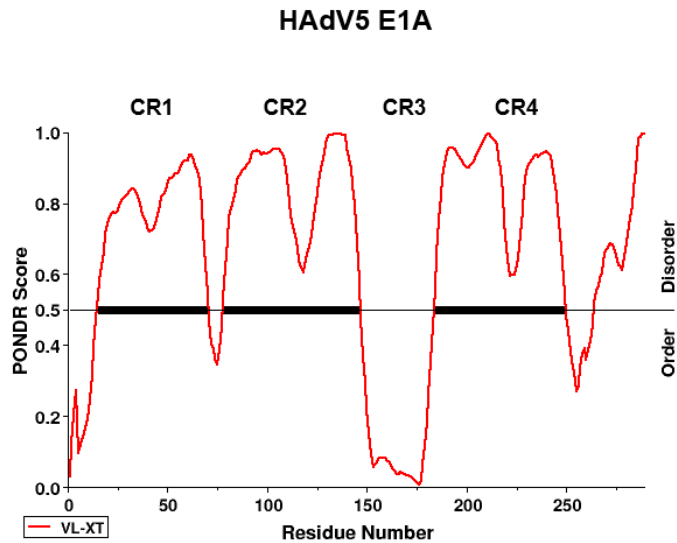



**Figure 1.5 PONDR Graph of Genomic E1A.** Predictor of natural disordered regions (PONDR) is a tool used to predict regions of order or disorder from amino acid sequences. The PONDR score is between 1 and 0, with 1 being the ideal prediction of order and 0 being the ideal prediction of disorder. The threshold is assigned at 0.5, a region is predicted to be disordered if the score higher than 0.5.

### 1.7 E1A Mediated Modulation of Viral and Cellular Transcription.

HAdV E1A is essential for viral infection in host cells. E1A is able to initiate the viral replication cycle by activating the transcription of other early genes as well as by altering transcriptional regulation and gene expression in the host cell (Berk *et al*., 1979; Jones *et al*., 1979). E1A is recruited to more than 17,000 cellular promoters during infection and has been found to alter the transcription of many of these genes (Horwitz *et al*., 2008; Ferrari *et al*., 2008). However, E1A contains no DNA binding capability or any enzymatic activity (Chatterjee *et al*., 1988; Zu *et al,*. 1992; Avvakumov *et al.,* 2002). As a result, E1A relies on protein-protein interactions with cellular factors to remodel the cellular environment (Ferguson *et al*., 1985). E1A is able to bind to multiple cellular proteins through its four conserved regions, as well as short segments called molecular recognition features (MoRFs) (Pelka *et al*., 2008). The disordered nature of the E1A protein allows it to bind to a large repertoire of cellular proteins disrupting a large number of cellular regulatory pathways (Pelka *et al*., 2008).

The regions responsible for transactivation have been mapped to the N terminus and CR3 of E1A (Lillie *et al*., 1989; Martin *et al*., 1990). While the CR2 and CR4 regions are generally thought to be required for induction of S-phase in quiescent cells and induction of oncogenic transformation in rodent cells, respectively (Pelka *et al*., 2008). The N terminus of E1A spans residues 1-41, to date more than 17 different proteins have been found to bind to this region. The N terminus is involved in oncogenic transformation, suppression of cell differentiation, induction of the cell cycle and DNA synthesis (Pelka *et al*., 2008). Moreover, this region of E1A is able to interact with cellular proteins that are involved in signalling networks including Ran GTPase (De Luca *et al*. 2003), the protein kinase Nek9 (Pelka *et al*. 2007, Jung *et al*., 2015) and Protein Kinase A (PKA) (Fax *et al*. 2001). The N terminus works along with the CR1 region to bind cellular proteins or transcription co-factors that are directly involved in the regulation gene expression,

such as p300/CBP. In addition, the CR1 region is essential for the release of E2F factors, which results in the transcription of viral early genes and cellular genes that are involved in the induction of S-phase (Fattaey *et al*., 1993; Ikeda *et al*., 1993)

The CR2 region spans residues 115 to 137 (Avvakumov *et al.*, 2004), four proteins have been found to bind to this region including pRb (Retinoblastoma protein) and related family members (Whyte *et al*., 1998), BS69 (Hateboer *et al.*, 1995), UBC9 (Hateboer *et al.*, 1996), and the S2 subunit of the regulatory complex of the proteasome (Zhang *et al*., 2004). CR2 has multiple functions, including the activation of viral gene expression, cell cycle progression and induction of oncogenic transformation in rodent cells (Gallimore *et al*., 2001; Berk 2005).

The CR3 region spans residues 144 to 191 and is unique to the 13S isoform of E1A. CR3 functions as a strong transcriptional activator that is essential for the expression of early viral genes, it has been shown that even small deletions within the CR3 region negatively affect the transcriptional activity of the E1A protein (Glenn *et al*., 1985; Lillie *et al*. 1986; Moran *et al.* 1986, Pelka *et al*., 2009). CR3 interacts with TATA-binding protein (TBP) and Mediator Complex Subunit 23 (MED23) to form a transcription complex along with RNA polymerase II and transcription co-factors, leading to the transcription of viral early genes. The CR3 region has been found to interact with both transcriptional activators and repressors indicating that CR3 modulates gene transcription in a complex manner (Ablack *et al*., 2010; Pelka *et al*., 2009; Rasti *et al*., 2005).

CR4 or the C terminus of E1A is encoded by the second exon of the gene, which spans residues 240 to 288, this region is conserved between the different isoforms except for 9S which has a unique C terminus due to a frame shift that occurs during splicing (Virtanen *et al*., 1983). The C terminal region of HAdV5 E1A is involved in immortalization of infected cells, oncogenic transformation, tumourigenesis and conversion of the infected cell into an epithelial like cells

(Chinnadurai, 2004; Frisch *et al*., 2002; Mymryk, 1996; Yousef *et al*., 2012). The C terminus region encompasses approximately 40% of E1A, however, only a few proteins have been found to bind to this region including CtBP1/2 (Boyd *et al*., 1993), Qip1 (Kohler *et al*., 2001), FOXK1/2 (Komorek *et al*., 2010), DYRK1A/B (Zhang *et al*., 2001), and HAN11 (Komorek *et al*., 2010). Recently, the Pelka group has identified three additional proteins that bind to the C terminus of E1A: DREF which is localized to PML bodies in the nucleus (Radko *et al*., 2014), Ku70 which is involved in the inhibition of the DNA damage response pathway (Frost *et al.*, 2016) and RuvBL1 which is involved in suppression of type I interferon (Olanubi *et al*., 2017). In addition, we have identified FUBP1 as a novel binding protein of E1A that is involved in the suppression of p53 activity and binds to both the N terminus and CR3 of E1A (Frost *et al*., 2018).

**1.8 Adenovirus E4orf3 Structure.**

E4orf3 is encoded by the open reading frame 3 of the E4 region of HAdV5, the protein product is 15kDa in size. The E4orf3 protein is tightly associated with the nuclear matrix, however it has also been found to accumulate in the cytoplasm (Carvalho *et al*., 1995, Doucas *et al*., 1996; Soria *et al.*, 2010). E4orf3 self-assembles to form a cable-like polymer network within the nucleus. Interestingly, E4orf3 is not a structural homologue of any cellular proteins that form polymers or that function in the pathways that E4orf3 is involved in (Ou *et al*., 2012). The E4orf3 protein first forms a dimer, which then assembles to form a disordered polymer, that has multiple protein binding interfaces (Ou *et al*., 2012). E4orf3 mutants unable to assemble into a polymer do not inactivate tumour suppressor pathways, indicating that the assembly of the E4orf3 is necessary for some of the functions carried out by the E4orf3 protein (Ou *et al*., 2012, Vink *et al*., 2015). E4orf3 has an acidic isoelectric point of 5.1 and the dimer has an electronegative surface potential, which suggests that E4orf3 does not directly bind to DNA. Instead it has been suggested that this

protein acts through intermediate proteins in order to disrupt tumour suppressor pathways and silence target genes (Ou *et al*., 2012).

### 1.9 Adenovirus E4orf3 Function in Host Cell.

The E4orf3 protein is critical for efficient viral DNA replication and is involved in multiple aspects of the viral life cycle, including regulation of viral late mRNA splicing, cytoplasmic mRNA accumulation, and late protein translation (Goodrum *et al*., 1999; Nordqvist *et al*., 1994; Sandler *et al*., 1989; Sheppard *et al*., 2003). Literature has reported that mutant viruses that do not express E4orf3 are deficient in replication (Vink *et al*., 2015). The E4orf3 protein induces drastic changes in nuclear organization and forces the redistribution of different cellular proteins. E4orf3 has been shown to disrupt PML domains within the nucleus (Carvalho *et al.*, 1995; Doucas *et al.*, 1996). PML bodies are involved in multiple cellular functions, including transcriptional regulation, apoptosis, transformation, and response to interferon (Maul, 1998). Upon expression of E4orf3, PML bodies are reorganized from punctate structures into tracks, this function appears to be involved in DNA replication, as these proteins have been found to surround viral replication centers (Ishov *et al*., 1996). In addition, reorganization of PML bodies has been found to be necessary for the inhibition of the interferon antiviral response (Ullman *et al*., 2007). Findings have also reported that multiple cellular factors associated with PML are also rearranged upon expression of E4orf3 (Stracker *et al*., 2002; Stracker *et al*., 2005; Yondola *et al*., 2007). Recently, E4orf3 was found to mediate SUMOylation and proteasomal degradation of PML associated factor TIF-1γ by acting as an E3 ligase for SUMOylation and poly-SUMO chain elongation (Sohn *et al*., 2016). In addition, E4orf3 was discovered to inhibit the activity of another critical tumour suppressor, the p53 protein. E4orf3 induces the colocalization of histone methyltransferases SUV39H1 and SUVH2 at cellular DNA, resulting in the tri-methylation of histone H3 at lysine 9

(H3K9me3) and consequently formation of heterochromatin at p53 responsive promoters, thus preventing the binding of p53 to the promoters of target genes (Soria *et al.*, 2010).

In the absence of the early region E4, the double-stranded DNA genome of HAdV is recognized as broken DNA and is joined into concatemers which are too large to be packaged into the protein capsid (Weiden *et al*.,1994). E4orf3 prevents the concatenation of the viral genome by targeting proteins involved in the double stranded break repair (DSBR) pathway (Boyer *et al*., 1999; Stracker *et al*., 2002), including the DNA-protein kinase (DNA-PK) catalytic subunit and the MRN complex (Evans *et al*., 2005; Stracker *et al*., 2005).

Finally, E4orf3 appears to be involved in transcriptional regulation of several cellular genes. Microarray expression analysis found that expression of E4orf3 leads to changes in expression of over four hundred host cell genes (Vink *et al*., 2015). Overall, the multiple functions of the E4orf3 protein, are essential to allow for maximal genome replication while also inhibiting apoptotic and DNA damage pathways of the host cell (Evans *et al*.,2003).

## Research Objectives and Hypothesis

E1A is essential for HAdV replication in host cells. This protein has been found to bind to multiple host cell factors to alter cellular regulatory networks, in order to ensure successful viral replication. Many of the interactions between E1A and host cell factors have been studied extensively. However, thus far there have not been many reports of E1A interacting with other early viral proteins. Recently, I found that the viral protein E1A co-localizes to the nuclear tracks of the viral protein E4orf3. I am interested in further examining this potential interaction and determining the function that it plays in HAdV infectious cycle. Understanding how E1A is able to communicate and interact with other viral proteins is essential to further our knowledge about the replicative function of HAdV and other DNA tumour viruses in host cells.

Furthermore, the C terminal region of E1A has functions that are essential for the cellular remodelling and alteration of normal function. However, we still lack a complete understanding of how this region contributes to viral replication and the viral life cycle in human cells. Therefore, I am interested in studying the role of C terminal region of E1A in the viral infectious cycle.

The four main objectives of my Masters research are:

1. Characterize the interaction between the viral proteins E1A and E4orf3
2. Examine the effects of E1A and E4orf3 on viral transcriptional activation
3. Explore how the interaction between E1A and E4orf3 affects p53 mediated gene expression
4. Characterize the role of E1A C terminus in the viral replicative cycle

## 2. Material and Methods

### 2.1 Antibodies.

Table 2.1 contains a comprehensive list of all the antibodies used. All primary and secondary antibodies are diluted in Tris-Buffered Saline-Tween20 (TBS-T) containing 5% milk or Tris-Buffered Saline-Tween20 containing 3% bovine serum albumin. For immunofluorescence antibodies are diluted in blocking buffer (1% normal goat serum, 1% bovine serum albumin (BSA), 0.2 % Tween 20 in phosphate-buffered saline (PBS).

### 2.2 Cell cultures.

HT1080 human fibrosarcoma cells, HeLa cervical epithelial cells, IMR-90 and WI-38 primary human lung fibroblast cells, U2OS human osteosarcoma epithelial cells, 293 Human Embryonic Kidney cells and Human Bronchial Epithelial cells (HBE) were grown in Dulbecco's modified Eagle's medium (Hyclone) supplemented with either 10% or 5% fetal bovine serum (Hyclone), 100 units/ml streptomycin and penicillin (Hyclone).

### 2.3. Chromatin Immunoprecipitation (ChIP).

Cells were infected with the indicated viruses at a moi of 30 and harvested 24 hours after the initial infection, cells were cross-linked with 1% formaldehyde, and neutralized with 125mM glycine. Cells were collected, pelleted and re-suspended in ChIP lysis buffer (5mM PIPES pH8, 85 mM KCl, 0.5% NP-40). After lysis, nuclei were pelleted and re-suspended in 400µl ChIP nuclear lysis buffer (500mM TRIS pH 8.1, 10mM EDTA, 1% SDS). Nuclei were sonicated using the Corvaris Focused-UltraSonicator M220 and the supernatant was used for immunoprecipitation. For immunoprecipitation of E1A, the monoclonal M73 and M58 antibodies were used. For immunoprecipitation of p53, anti-p53 mouse monoclonal antibody clone 1C12 was used. Rabbit anti-mouse antibody was used as a negative control IgG. After complexing with Protein-A

Sepharose beads, the beads were washed with: A. Low Salt Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 2mM TRIS pH 8.1, 150mM NaCl). B. High salt wash buffer: (0.1%SDS, 1% Triton, 2mM EDTA, 20mM TRIS pH 8.1, 500mM NaCl). C. LiCl wash buffer (0.25M LiCl, 1% NP-40, 1% Deoxycholate, 1mM EDTA, 20mM TRIS pH 8.1). D. 2X in 1X TRIS-EDTA buffer. Complexes were then eluted. Crosslinking was reversed by adding NaCl to a final concentration of 0.3M and overnight incubation at 65°C. DNA was purified using a PCR purification kit. PCR reactions were carried out for HAdV5 early and major late promoters using SYBR Select Master Mix for CFX (Applied Biosystems) according to manufacturer's directions using 3% of total ChIP DNA as template in a CFX96 Real Time PCR instrument (BioRad). The annealing temperature used was 60°C and 40 cycles were run. Primers for viral promoters can be found in table 2.3.

**2.4. EdU Incorporation Assay.**

WI-38 cells were grown until 100% confluent on Lab Tek II 4- Chamber Slides (Thermo-Fisher). After becoming fully confluent, cells were incubated for another 72 hours to arrest growth. Infections were carried out as described above with a moi of 100 for *dl*309, *dl*311, *dl*1116, *dl*1132, *dl*1133, *dl*1134, *dl*1135, or *dl*1136. One hour prior to fixation, cells were pulsed with EdU for 1 hour as per manufacturer's specifications using the Click-It EdU labeling kit for microscopy (Life Technologies). After labeling with EdU, cells were fixed in 3.7% formaldehyde, stained for EdU using the Click-It kit with Alexa Fluor 488, and labelled for E1A using M58 monoclonal antibody and AlexaFluor-594 conjugated secondary antibody (Jackson Immunoresearch). Cells were visualized using LSM700 laser confocal microscope and analyzed using Zeiss ZEN software package.

**Table 2.1:** Comprehensive list of antibodies used

| Antibody | Details | Use | Dilution factor | Source | Catalogue number |
|---|---|---|---|---|---|
| **Actin** | Mouse Monoclonal | Primary | WB: 1:1000 | Abcam | Ab3280 |
| **Anti-adenovirus type 5** | Rabbit polyclonal | Primary | WB 1:10,000 | Abcam | ab6982 |
| **72K DBP** | Mouse Monoclonal | Primary | WB: 1:400<br>IF: 1:600 | Phil Branton | N/A |
| **E1A (M2)** | Mouse Monoclonal | Primary | WB: 1:400<br>IP: 25 µl | In house | N/A |
| **E1A (M37)** | Mouse Monoclonal | Primary | WB: 1:400<br>IP: 25 µl | In house | N/A |
| **E1A (M58)** | Mouse Monoclonal | Primary | WB: 1:400<br>IP: 25 µl | In house | N/A |
| **E1A (M73)** | Mouse Monoclonal | Primary | WB: 1:400<br>IP: 25 µl | In house | N/A |
| **HA** | Rat Monoclonal | Primary | WB: 1:5000<br>IP: 5 µl | Roche | 11867423001 |
| **ORF3 6A11** | Rat Monoclonal | Primary | WB: 1:100<br>IF: 1:800<br>IP: 20 µl | Thomas Dobner | N/A |
| **p53 1C12** | monoclonal antibody | Primary | IP: 10 µl | Cell Signaling Technology | 2524 |
| **Mouse IgG** | Goat | Secondary | WB: 1:200,000 | Jackson ImmunoResearch | 115-035-003 |
| **Mouse IgG (Alexa fluor 488)** | Goat | Secondary | IF: 1:600 | Jackson ImmunoResearch | 115-545-003 |
| **Rat IgG (Alexa fluor 594)** | Goat | Secondary | IF: 1:600 | Life technologies | A11007 |

**Note:** WB= Western Blot, IF = Immunofluorescence

**2.5. Immunofluorescence.**

HT1080 cells were plated at low density (~40,000 cells per chamber) on chamber slides (Nalgene Nunc), and subsequently infected. 24 hours after infection, cells were treated with cytoskeleton buffer 10 mM piperazine-N,N9-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100], fixed in 4% formaldehyde, blocked in blocking buffer (1% normal goat serum, 1% BSA, 0.2% Tween-20 in PBS) and stained with specific primary antibodies. M73 was used at a 1:100 dilution (hybridoma supernatant), E4orf3 6A11 antibody was used at 1:800 dilution and AlexaFluor 488 and 594 secondary antibodies (Jackson Immunoresearch) were used at a dilution of 1:800. After staining and extensive washing, slides were mounted using Prolong Gold with DAPI (Invitrogen) and imaged using Zeiss LSM700 confocal laser scanning microscope. Images were analyzed using Zeiss ZEN software package.

**2.6 Immunoprecipitation (IP).**

Transfected or infected HT1080 cells were lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.8], 150 mM NaCl) supplemented with a protease inhibitor cocktail (Sigma). One milligram of the cell lysate was used for IP with the monoclonal 6A11 anti-E4orf3 antibody or anti-HA 3F10. E1A was detected using the M73 or M58 monoclonal antibody, while E4orf3 was detected using the rat monoclonal 6A11 antibody or rat monoclonal anti-HA antibody.

**2.7 PCR Primers.**

A comprehensive list of primers can be found in Table 2.2. All primers were purchased from IDT, and annealing temperature of 60˚C was used.

**2.8 Plasmids.**

A comprehensive list of all plasmids used can be found in Table 2.3.

**2.9 Promoter Luciferase Assays.**

HT1080 cells were plated into 6-well plates and grown until 90% confluent. A total of 4µg of plasmid DNA was transfected into each well. 24 hours after transfection, cells were harvested and lysed in 300µl 5x Cell Culture Lysis Buffer (Promega E397A), scraped into labeled eppendorf tubes, which were centrifuged at 13,000 rpm for 10 minutes. After centrifugation, 50µl of lysate was pipetted into white opaque bottom detection plates (ThermoScientific). 50µL of Luciferase Substrate (Promega E151A) was injected by to the 50µL of lysate. Luciferase signal was detected by the SpectraMax ID3 (Molecular Devices). Luciferase activity was measured by fold change differences relative to the reporter only. Western blot was done to verify the presence of E1A in the lysates.

**Table 2.2:** Comprehensive list of primers used

| Primer | Forward Primer (3'-5') | Reverse Primer (5'-3') |
|---|---|---|
| BLM | CACCATGGCTGACACGTTAC | TGTCAATCCCCATTCCAAAT |
| E1A 10S | GATCGAAGAGCCCGAGCA | CCACAGGTCCTCATATAGCAA |
| E1A 13S | TTTTGAACCACCTACCCTTC | CCACAGGTCCTCATATAGCAA |
| E1B | TCAAACGAGTTGGTGCTCATG | CGCGCTGAGTTTGGCTCTAG |
| E2A | GCGGATGAGGCGGCGTATCGAG | GGGGGTGGTTTCGCGCTGCTC |
| E2P | AGAATTCGGTTTCGGTGGGC | AGCAAATACTGCGCGCTGAC |
| E3A | CTCGGAGAGGTTCTCTCGTAGACT | GCCGCCACAAGTGCTTTG |
| E3P | CGCCCTCTGATTTTCAGGTG | CGCGGGACCCCACATGATAT |
| E4orf3 | GAATTCATTCGCTGCTTGAGGCTGAA | CTCGAGTTATTCCAAAAGATTATCCA |
| E4 orf6/7 | CTGCTGCCCGAATGTAACACT | TCCACCTTGCGGTTGCTTAA |
| E4P | GGCTTTCGTTTCTGGGCGTA | TAAACACCTGAAAAACCCTCCTC |
| FIBER | ATGCTTGCGCTCAAAATGGG | TTTTTGAGAGGTGGGCTCAC |
| GADD45 | CAGAAGACCGAAAGGATGGA | ATCTCTGTCGTCGTCCTCGT |
| GADD45AP | GGCGGAAGGTGGTTGGCTGA | AGCTCAGGCCCTGGCGCTCT |
| HEXON | CTTACCCCCAACGAGTTTGA | GGAGTACATGCGGTCCTTGT |
| HGAPDH | GAGTCAACGGATTTGGTCGT | TTGATTTTGGAGGGATCTCG |
| MDM2 | GGTGGGAGTGATCAAAAGGA | GTGGCGTTTTCTTTGTCGTT |
| MDM2P | GGTTGACTCAGCTTTTCCTCTTG | GGAAAATGCATGGTTTAAATAG |
| MLP | CGGGCATGACTTCTGCGCTA | CGCGAATACGTGCAGCTAAA |
| MCM4 | TTGAAGCCATTGATGTGGAA | GGCACTCATCCCCGTAGTAA |

**Table 2.3:** Comprehensive list of plasmids used

| PPB # | Name | Features |
|---|---|---|
| **1** | pCANmyc | Mammalian expression vector with an N terminal myc tag and Neomycin resis marker |
| **14** | pCDNA3-E1A 12S | Mammalian expression vector for 12S isoform of wildtype E1A |
| **27** | GAL-6-Luc | Mammalian two hybrid luciferase reporter plasmid, 6 GAL-4 binding sites |
| **29** | pM | Mammalian two hybrid bait plasmid used to generate a fusion of the GAL4-DI binding domain to a protein of interest. |
| **37** | pM E1A 82T | Mammalian expression vector of HAdV5 residues 1-82 fused to GAL4 DBD |
| **43** | pM E1A CR3 | Mammalian expression vector of HAdV5 CR3 region fused to GAL4 DBD |
| **46** | pCDNA3-E1A 13S | Mammalian expression vector for 13S isoform of wildtype E1A |
| **71** | pCDNA3.1-E1A | Mammalian vector, expressed all E1A isoforms, driven by the CMV promoter pCDNA-3.1 |
| **145** | CMV-HA-E2F1 | Mammalian expression vector HA-tagged E2F1 |
| **146** | CMV-HA-DP1 | Mammalian expression vector HA-tagged DP1 |
| **152** | pGL3-E2F4B-Luc | Mammalian luciferase reporter pGL3 construct with 4 copies of E2F site |
| **154** | pGL3-E3v2-Luc | Mammalian luciferase reporter pGL3 construct with HAdV2 E3 promoter |
| **403** | pCANmyc-E4ORF3 | Mammalian vector with a Neomycin resistant marker, expressing N terminal n tagged E4ORF3 |
| **475** | pCAN-E1A Δ11-13 | Mammalian vector with a Neomycin resistant marker, expressing mutant geno E1A (deletion of residues 11-13) |
| **479** | pCAN-E1A Δ17-19 | Mammalian vector with a Neomycin resistant marker, expressing mutant geno E1A (deletion of residues 17-19) |
| **481** | pCAN-E1A Δ23-25 | Mammalian vector with a Neomycin resistant marker, expressing mutant geno E1A (deletion of residues 23-25) |
| **512** | pCAN-E1A Δ20-22 | Mammalian vector with a Neomycin resistant marker, expressing mutant geno E1A (deletion of residues 20-22) |
| **513** | pCAN-E1A Δ26-28 | Mammalian vector with a Neomycin resistant marker, expressing mutant geno E1A (deletion of residues 26-28) |
| **522** | pCANHA-E4ORF3 | Mammalian vector with a Neomycin resistant marker, expressing N terminal H tagged E4ORF3 |
| **531** | pM E4orf3 | Mammalian expression vector of HAdV5 E4orf3 region fused to GAL4 DBD |

### 2.10 Protein purification and GST pulldown assay.

Glutathione S-transferase fusion of E4orf3 was made by sub-cloning the cDNA into pGEX-6P1 (GE Healthcare Life Sciences) in frame with the N-terminal GST-tag. His-tagged E1A was made by sub-cloning the entire into the pET42 vector (Novagen) in frame with a C terminal 6XHis tag. Proteins were expressed in Escherichia coli strain (*E. coli*) BL21 to express the different proteins.  Liquid culture of the bacteria was grown to an OD600 = 0.85 at 37°C, the culture was then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours at 37°C and centrifuged at 4000 rpm for 10 minutes to obtain a pellet. The bacterial pellet was suspended and lysed in 20ml of NTEN buffer (100mM NaCl, 20mM Tris-Cl pH 8, 1mM EDTA, 1% Triton) supplemented with protease inhibitors and lysozyme. Samples were then sonicated three times for 10 seconds with 30 seconds on ice in between each sonication. The samples were centrifuged at 4000 rpm for 10 minutes to pellet cell debris. 2ml of glutathione s-transferase beads were added to the clarified lysate and incubated together at 4°C for 1 hour. Beads were pelleted and the supernatant was removed. The beads were washed three times with PBS and resuspended with 5ml of PBS and packed into a gravity column. 500µl of fractions were collected through gravity filtration using 50mM Tris-Cl pH 8 and 20mM of reduced glutathione. To verify which fractions contained proteins, 25µl of the samples from each fraction was boiled together with 25µl of 2X sample buffer and DTT (4:1 ratio) at 100°C. The protein samples were resolved on SDS gels and stained with Coomasie blue. To visualize the presence of the protein bands, destain solution (50 volumes of water, 40 volumes of methanol and 10 volumes of acetic acid) was used. Fractions with proteins present were then pooled together and dialysed over night with 1X PBS buffer.

GST-pulldown assay was carried out by incubating purified His-tagged proteins with purified GST-tagged proteins for 1 hour in GST-pull down buffer (50mM HEPES/KOH pH 7.5,

150mM KCl, 1mM EDTA, 10% Glycerol, 0.1% NP-40, 2µg/µl BSA). GST-beads were added and sample was nutated for 1 hour. Beads were then washed with GST-pull down buffer and eluted in SDS sample buffer, sample was boiled and resolved on an SDS–PAGE gel.

**2.11 Real-time gene expression analysis.**

Cells were infected with the indicated viruses. Total RNA was extracted using the TRIzol Reagent (Sigma). 1.25µg of total RNA was used in reverse-transcriptase reaction using SuperScript VILO Reverse Transcriptase (Invitrogen) and random hexanucleotides for priming. The cDNA was subsequently used for real-time expression analysis using the BioRad CFX96 real-time thermocycler. Analysis of data was carried by normalizing CT values of target genes to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA levels. For the C terminus section, real time analysis was done using the Pfaffl method and values were normalized to GAPDH mRNA levels; these were compared to *dl*309 for viral gene analysis and to mock infected samples for cellular genes analysis

**2.12 Transfections.**

Cells for immunoprecipitations were plated in 10cm plates at a density of $2.0 \times 10^6$ cells/plate 24 hours prior to transfection. Transfections were prepared by mixing 1mL of serum-free DMEM, 10µg of total plasmid DNA, and 20µL of linear 1mg/mL solution of polyethylenimine 25kDa reagent from Polysciences (cat# 23966-2). This mixture was vortexed for 10 seconds and incubated at room temperature for 20 min. The complexes were added to the cells and incubated at 37°C. Cells for luciferase assays were plated in 6-well plates at a density of $3.5 \times 10^4$ cells/plate, the total amount of plasmid DNA added was 4µg and 6µL of linear 1mg/mL solution of polyethylenimine.

**2.13 Virus growth assay.**

IMR-90 cells or WI-38, cells were grown to 100% confluence and then contact inhibited cells were incubated for an additional 72 hours, to allow for complete growth arrest. Arrested cells were infected with the indicated viruses in serum-free media. Virus was adsorbed for 1 hour at 37°C, after which cells were bathed in conditioned media and were re-incubated at 37°C. Virus samples were harvested at 48, 72, and 96 hours after infection, all samples were subjected to three cycles of freeze thawing in a dry-ice bath and a room temperature bath, in order to lyse the cells and release the virus. Plaque assays were performed on HEK-293 cells by serial dilution to determine viral titre.

**2.14 Virus Plaque Assays.**

HEK-293 cells were then plated on 6-well plates and grown to 90% confluency, serial dilutions from $10^{-3}$ to $10^{-8}$ of the harvested virus samples were used to infect the HEK-293 cells to quantify the viral growth. Cells were incubated for 1 hour at 37°C, after which the virus was aspirated off. Lastly, the cells were overlayed with a 1:1 ratio of 2X DMEM (Gibco) to 1% Agarose (Invitrogen) and incubated at 37°C with 5% $CO_2$. Three days after overlaying, plaques were counted and used to determine viral titre.

**2.15 Viruses and Viral Infections.**

Cells were infected in serum free media and incubated at 37°C for 1 hour, after which viral infection media was topped off with fresh media. Infected cells were incubated at 37°C until their collection at the respective time points indicated by individual experiments. The viruses used for infections include HAdV5 *dl*309 (wild-type; WT) background, which contains a small deletion in the E3 region, knocking out the E3 14.7k, 14.5k and 10.4k proteins. The series of N terminus and C terminus mutants used were generated in Stanley Bayley's laboratory: *dl*1101(Δ4-25), *dl*1102

(Δ26-35), *dl*1103 (Δ30-49), *dl*1104 (Δ48-60), *dl*311 (Δ203 to 289), *dl*1116 (Δ205 to 221), *dl*1132 (Δ224 to 238), or *dl*1133 (Δ241 to 254), *dl*1134 (Δ255 to 270), *dl*1135 (Δ271 to 284), or *dl*1136 (Δ285 to 289). The mutant virus ΔE4orf3 was generated in Patrick Hearing's laboratory, it contains an 8 bp deletion in the *SSPI* site of E4orf3, preventing full translation of this protein. The ΔE4orf6/ΔE4orf3 virus has the same mutation that block translation of E4orf3, but has also an 8 bp deletion in the second *SSPI* site that terminates the translation of E4orf6 (Huang *et al*., 1989). CMV HA-E4orf3, CMV HA-E4orf3 L103A, CMV HA-E4orf3 N82A and CMV HA-E4orf3 D105/L106 are recombination viruses lacking E1A and most of E1B as well as E4 orf1 to orf3 while possessing CMV-driven HA-E4orf3 wild-type and mutant genes at the left end (Evans *et al*., 2003). The CMV virus was generated by Peter Pelka, it lacks E1A and E1B but is wild type for the rest of the genes, driven under a CMV promoter. The *dl*1520 was made in Berk's laboratory, this virus contains a deletion within the E1B 55k open reading frame from nucleotide 2496 to 3323 and a point mutation at nucleotide 2022 which terminates translation of the protein (Barker *et al*., 1987).

**2.16 Western Blotting.**

Cells were harvested and lysed with NP-40 Lysis Buffer (0.5% NP-40, 100 mM NaCl, 50 mM TRIS pH 7.8, protease inhibitor). Cells in 10cm plates were lysed in 1mL of NP-40 lysis buffer and cells in 6-well plates were lysed in 300µL of lysis buffer per well. Bradford assay was done to equalize the levels of protein. Samples were boiled in a 1:1 ratio of 2X Sample Buffer to 1M DTT. Samples were resolved on a 10% SDS-PAGE self-cast gels or pre-cast Novex Bolt Mini Gels (LifeTechnologies). Gels were run in a BIORAD Mini-PROTEAN Tetra System cell with SDS-PAGE running buffer (25mM Tris, 250mM Glycine pH 8.3, 0.1% SDS) at 200V for 60 minutes. Pre-cast gels were run in Novex Bolt Mini Gel Tank with MES/MOPS SDS Running

Buffer (Life Technologies) at 120V for 50 minutes. Proteins were transferred onto Immobilon-P PVDF membrane (BioRad) in a BioRad Mini-PROTEAN Tetra System cell using transfer buffer (25mM Tris, 250mM Glycine pH 8.3 and 20% MeOH) and were run at 90V for 1 hour at 4°C. The membranes were blocked for 1 hour at room temperature using 5% w/v skim milk powder in TBS-T (10% Tween-20 in TRISbuffered saline pH 7.6). Primary antibody was diluted into
5% w/v skim milk powder in TBS-T and incubated with the membrane overnight at 4°C. The membrane was washed three times, ten minutes per wash with TBS-T. Appropriate secondary antibody with conjugated horseradish peroxidase enzyme was used at a 1:200,000 dilution in 5% w/v milk powder in TBS-T and was incubated with the membrane for 30 minutes. The membrane was washed again three times with TBS-T, ten minutes per wash. Luminata Forte Western HRP Substrate (Millipore) was added to the membrane and incubated for five minutes at room temperature. Amersham Hyperfilm ECL (GE Healthcare Life Sciences) was exposed to the membrane in a dark room. The film was then developed using the automated film developer (Konica Minolta Medical Imaging, Model SRX-101A) in the dark room.

# 3. Results

## 3.1 Relocalization of E1A to E4orf3 Tracks.

Previously, Jung *et al*., identified a link between HAdV E1A with the NimA-related protein kinase 9 (Nek9) and the suppression of the p53-responsive gene *GADD45A*. This study also found that Nek9 colocalizes to the E4orf3 protein tracks and that the suppression of *GADD45A* is partly dependent on the presence of E4orf3 during infection; however, no mechanism was identified. Consequently, it was hypothesized that E4orf3 tracks may play a role in the recruitment of E1A to Nek9-occupied promoters. These findings prompted me to examine if the E1A protein colocalized to E4orf3 protein tracks. To determine this, I did an immunofluorescence assay to examine the localization of these proteins within the nucleus during infection. HT1080 cells were infected with *dl*309 or ΔE4orf3 at an moi of 50. 24 hours after the initial infection (Figure 3.1), the cells were treated with cytoskeleton buffer to remove soluble proteins and phospholipids, the resulting skeletal framework retains the nuclei, dense cytoplasmic filament networks and intercellular junctional complexes (Fey *et al*., 1984). This allows us to have a cleaner view of the tracks formed by the E4orf3 proteins and removes background proteins and non-specific binding. Subsequently, the cells were fixed and stained for E1A and E4orf3. As previously reported, I observed that E4orf3 formed irregular polymers within the nucleus. Interestingly, E1A was found to colocalize to E4orf3 tracks, causing E1A to assume a fibrous or cable-like appearance. The colocalization of E1A to E4orf3 tracks was observed as early as 8 hours after the initial infection. In contrast, cells infected with the ΔE4orf3 virus that lacks E4orf3, show that E1A is distributed evenly throughout the nucleus with some punctuate appearance and does not have a track-like localization.

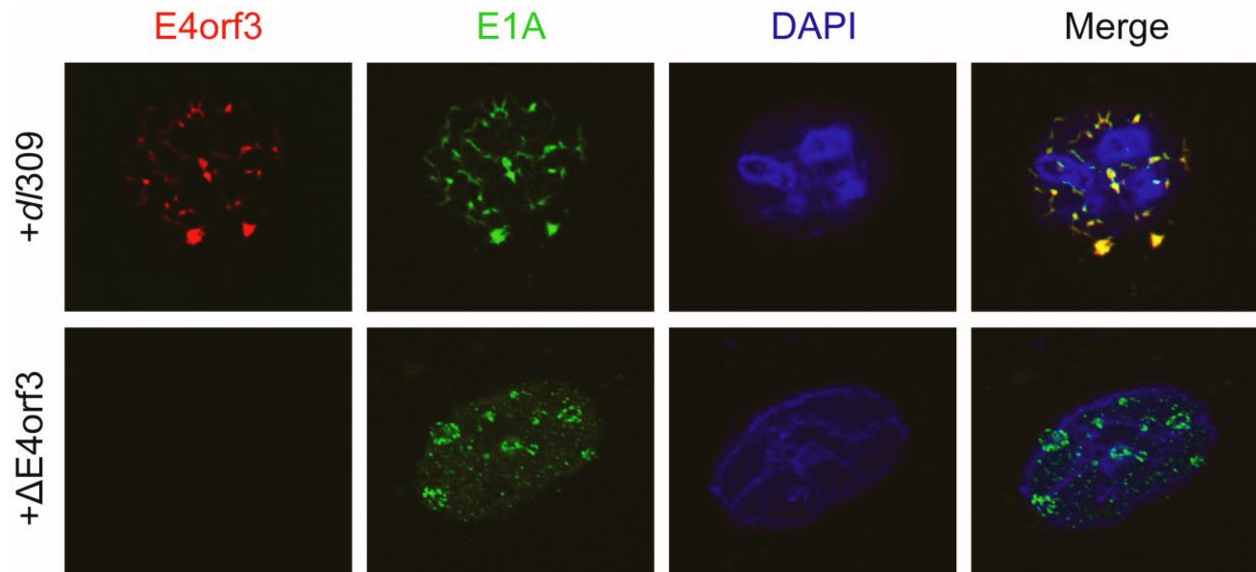



**Figure 3.1. Immunofluorescence Staining Showing E1A Colocalized to E4orf3 Tracks.**

HT1080 cells were plated at low density on chamber slides and subsequently infected with *dl*309 or ΔE4orf3 at an moi of 50. 24 hours after infection, the cells were treated with cytoskeleton buffer 10 mM piperazine-N,N9-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100] for 5 min on ice, then fixed in 4% formaldehyde, blocked in blocking buffer (1% normal goat serum, 1% BSA, 0.2% Tween-20 in PBS) and stained with specific primary antibodies. Anti-E1A M58 and M73 were used at a dilution of 1:100, anti-E4orf3 6A11 antibody was used at 1:800 dilution. AlexaFluor 488 and 594 secondary antibodies were used at a dilution of 1:800. Slides were mounted using Prolong Gold with DAPI and imaged using Zeiss LSM700 confocal laser scanning microscope. Images were analyzed using Zeiss ZEN software package

### 3.2 E1A and E4orf3 Interact Directly During Viral Infection.

Given that E1A and E4orf3 colocalize within the nucleus I wanted to examine if an interaction occurs between the two proteins, to do this, a co-immunoprecipitation assay was carried out. U2OS HA-E4orf3 tet-inducible cells were infected with *dl*309 and induced for E4orf3 expression with doxycycline media for 24 hours. E1A was immunoprecipitated using M73, the samples were run on an SDS-PAGE gel and blotted using 3F10 anti-HA rat antibody. E1A was found to associate with E4orf3 during the course of normal infection (Figure 3.2 A).

A positive co-immunoprecipitation result can not discriminate between a direct and an indirect protein interaction since intermediary factors may mediate the interaction. To determine whether E1A and E4orf3 interact directly, I performed a GST pulldown assay using a GST-E4orf3 fusion vector and an 6xHis-tagged E1A vector. The proteins were bacterially expressed and purified and pull down assays were then carried out. E1A and E4orf3 were found to interact *in vitro*, indicating that this is a direct association (Figure 3.2 B).

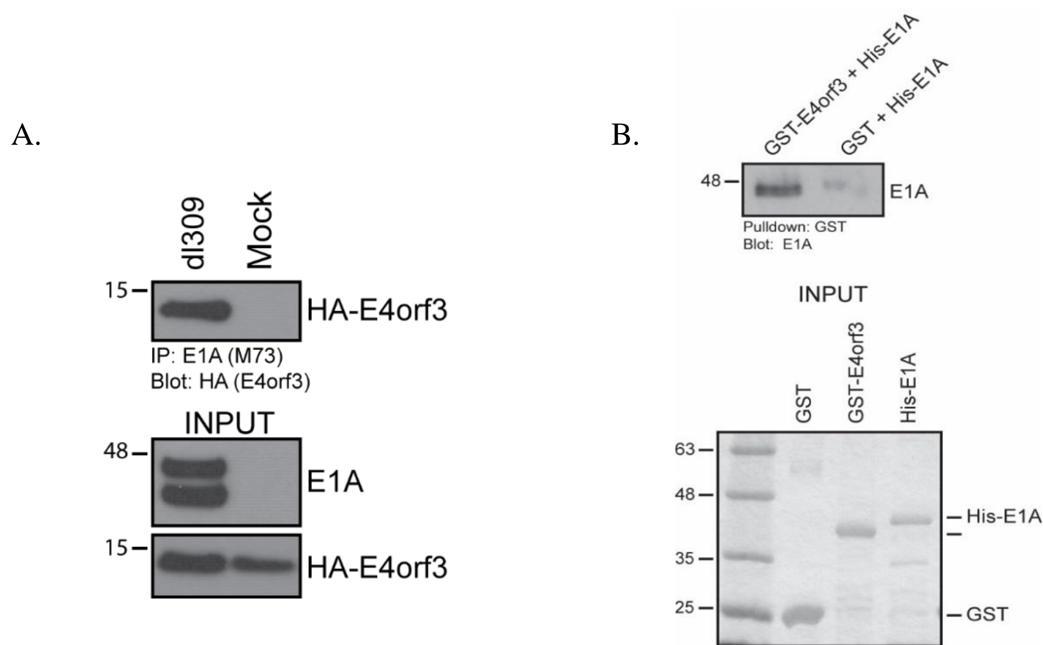



**Figure 3.2 A Direct Interaction Occurs Between E1A and E4orf3 During HAdV Infection.**

A. Co-immunoprecipitation Assay of E1A and E4orf3: U2OS HA-E4orf3 cells were induced for E4orf3 expression with doxycycline media and infected with wild type virus *dl*309 at an moi of 30 for 24 hours. Cells were lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.8], 150 mM NaCl) supplemented with a protease inhibitor cocktail. One milligram of the cell lysate was used for IP with anti-E1A M73. E1A was detected using the M73 and M58 monoclonal antibodies, while E4orf3 was detected using anti-HA rat monoclonal antibody 3F10. n=3

B. GST pulldown of E1A and E4orf3: Glutathione S-transferase fusion of E4orf3 was made by subcloning the cDNA into pGEX-6P1 (GE Healthcare Life Sciences) in frame with the N-terminal GST tag. His-tagged E1A289R was made by subcloning the entire E1A289R cDNA into the pET42 vector in frame with a C terminal 6XHis tag. Proteins were expressed in *E. coli* strain BL21 and purified. GST pulldown was carried out. Input are shown. n=2

### 3.3 E4orf3 Binds to the N Terminus of E1A.

After determining that the interaction between E1A and E4orf3 is direct, I wanted to map the regions of E1A that are required for this association. To do this, I carried out a co-immunoprecipitation assay using doxycycline induced U2OS HA-E4orf3 cells infected with various E1A mutant viruses that contain deletions within the N terminus (*dl*1101, *dl*1102, *dl*1103, *dl*1104) or C terminus (*dl*1133, *dl*1134, *dl*1135, *dl*1136). Protein lysates were immunoprecipitated for E4orf3 using anti-HA 3F10 antibody and blotted for E1A. It was found that all of the E1A C terminus mutants were able to interact with E4orf3. On the other hand, the *dl*1101 (deleted for residues 4-25) N terminus mutant lost all ability to bind the E4orf3 protein. These results indicate that E4orf3 interacts with the N terminus region of E1A, more specifically the region required for this interaction is found within residues 4-25 of E1A (Figure 3.3 A). Using an E1A mutant that is unable to bind to E4orf3 would be useful in determining the functional effects of this interaction. However, the *dl*1101 mutant is also severely deficient for affinity to multiple other proteins (Loewenstein *et al*., 2006). As a result, we decided to find a more specific binding mutant by doing co-immunoprecipitation assay using 13S E1A viruses that contain mutations within residues 3-10 of E1A. The assay showed that all the mutants examined possessed strong binding affinity for E4orf3 (Figure 3.3 B), which indicates that residues 3-10 are most likely not required for the interaction between E1A and E4orf3.

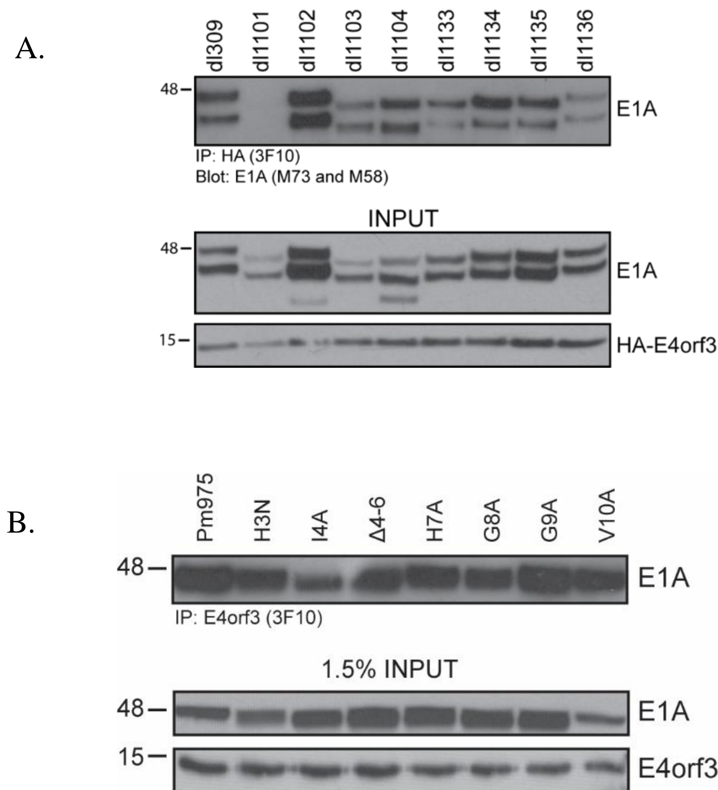



**Figure 3.3 E4orf3 Binds to the N Terminus of E1A.**

A. Co-immunoprecipitation of E4orf3 with E1A N terminus and C terminus mutants: U2OS HA-E4orf3 cells were induced for E4orf3 expression with doxycycline media and infected with the indicated viruses at an moi of 30 for 24 hours. Cells were lysed in NP-40 lysis buffer supplemented with a protease inhibitor cocktail. One milligram of the cell lysate was used for IP with 3F10 anti-HA antibody. E1A was detected using the M73 and M58 monoclonal antibody, while E4orf3 was detected using 3F10. Inputs are shown. n=3

B. Co-immunoprecipitation of E4orf3 with E1A 13S mutants: Assay was carried out the same way as in 3.3 A. Cells were infected with the indicated viruses. Inputs are shown. n=3

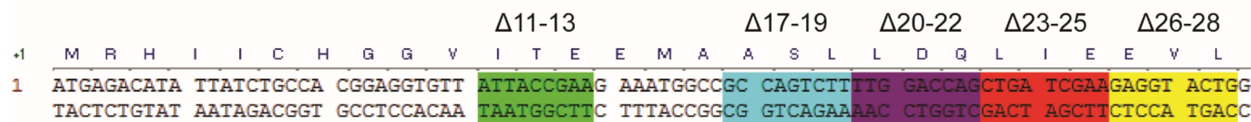
### 3.4 Residues 11-13 of E1A are Necessary for Binding to E4orf3.

Since it was found that mutations of residues 3-10 did not affect the ability of E1A to bind to E4orf3, I decided to generate additional mutants of E1A within residues 11-28: E1A Δ11-13, Δ17-19, Δ20-22, Δ23-25, Δ26-28 (Figure 3.4 A). To do this, I used PCR oligonucleotide primers that contained the E1A deletion within, the amplified PCR product was cloned into pCAN-Myc and transformed into bacteria. The resulting constructs were sequenced and tested for expression in HT1080 cells. Figure 3.4 B shows that all mutants expressed E1A to levels similar to wild type E1A. Next, I did co-immunoprecipitation assays using HT1080 cells that were co-transfected with pCAN HA-E4orf3 and the different E1A mutants. The Δ11-13 E1A deletion mutant was found to lack binding to E4orf3 (Figure 3.5), indicating that these three residues are necessary for the interaction to occur.

### 3.5 Interaction of E4orf3 Polymerization Mutants with E1A.

Previous reports indicate that E4orf3 polymerization is required to sequester and inactivate tumour suppressor proteins. To examine if E4orf3 polymerization is required for binding to E1A I did a co-immunoprecipitation assay using HEK-293 cells infected with ΔE4orf3 as a negative control, CMV-vector virus expressing HA-E4orf3 as a positive control and different HA-E4orf3 mutants: N82A, L103A, D105/L106. The results indicate that mutant virus D105/L106 is unable to bind E4orf3, whereas the N82A and L103A mutants, which are polymerization deficient, were still able to bind E4orf3 (Figure 3.6). These results suggest that polymerization of E4orf3 may not be required for its interaction with E1A.

E1A N-terminus deletion mutants



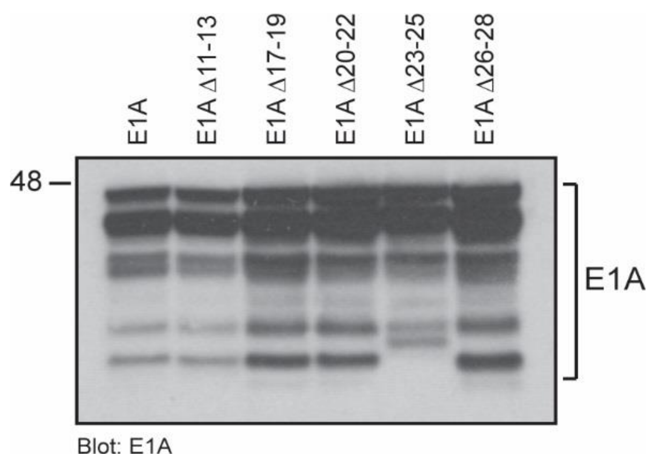




**Figure 3.4 Generation of E1A N Terminus Deletion Mutants.**

E1A mutants were made by PCR amplification using mega-primers containing the deletions within. Genomic E1A was used as a template. PCR products were restricted with XhoI and HindIII and ligated with pCAN-Myc vector. Construct was transformed into Mach-1 *E. coli* and purified using a midiprep kit. Construct was sequenced to verify the deletion. 4µg of each construct was transfected into HT1080s and expression was examined through western blot using M2, M37, M73 and M58 mouse monoclonal antibodies anti-E1A.

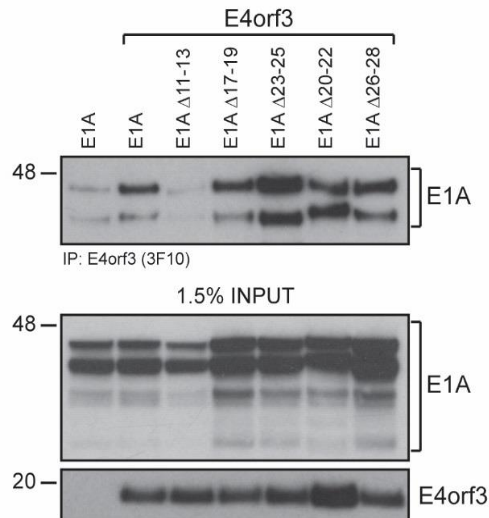



**Figure 3.5 Residues 11-13 of E1A are Important for Binding to E4orf3.**

HT1080 cells were transfected with a pCAN HA-E4orf3 construct and the indicated E1A constructs for 24 hours. Cells were lysed in NP-40 lysis buffer supplemented with a protease inhibitor cocktail. One milligram of the cell lysate was used for IP with 3F10 anti-HA antibody. E1A was detected using the M73 and M58 monoclonal antibody, while E4orf3 was detected using 3F10. Inputs are shown. n=3

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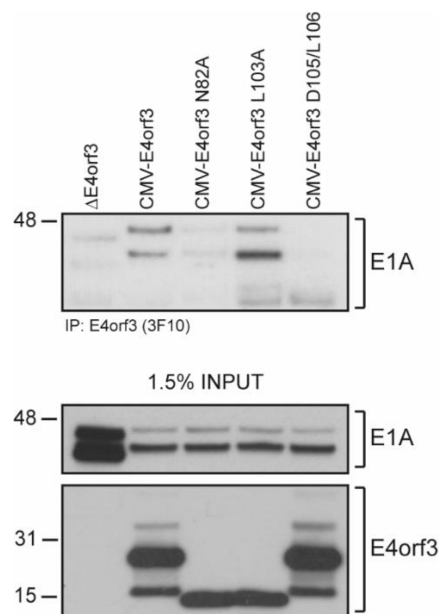



**Figure 3.6 E4orf3 Polymerization Mutants Interact with E1A**

HEK-293 cells were infected with the indicated viruses at an moi of 50 for 24 hours. Cells were lysed in NP-40 lysis buffer supplemented with a protease inhibitor cocktail. One milligram of the cell lysate was used for IP with 3F10 anti-HA antibody. E1A was detected using the M73 and M58 monoclonal antibody, while E4orf3 was detected using 3F10. Inputs are shown. n=3

### 3.6 E1A and E4orf3 Enhance Transactivation of Viral Promoters.

**Suppression of Gal4 Responsive Promoter Transactivation in the Presence of E4orf3.** I wanted to investigate whether the interaction of E1A and E4orf3 resulted in changes to E1A mediated transcriptional activation. To do this, HT1080 cells were co-transfected for 24 hours with a Gal4-responsive luciferase reporter plasmid (pGL2-(UAS)6-Luc), an empty pCAN-Myc construct or pCAN-Myc-E4orf3, Gal4-DBD fused with residues 1-82 of E1A and Gal4-DBD fused to the CR3 regions of E1A and Gal4-DBD alone, the two transactivation domains of E1A. I found that in the presence of E4orf3 the transactivation activity of E1A 1-82 is reduced 2 fold (Figure 3.7 A), while CR3 transcriptional activity was relatively unchanged. To determine if E4orf3 possessed any transactivating activity, I created a fusion of Gal4-DBD to E4orf3. A luciferase assay was then carried out using the pGL2-(UAS)6-Luc reporter with the 12S or 13S isoforms of E1A and GAL4-DBD or Gal4-DBD-E4orf3. We observed a slight reduction in 12S and 13S transcriptional activity with the E4orf3 fusion (Figure 3.7 B), however these changes were not considered to be significant.

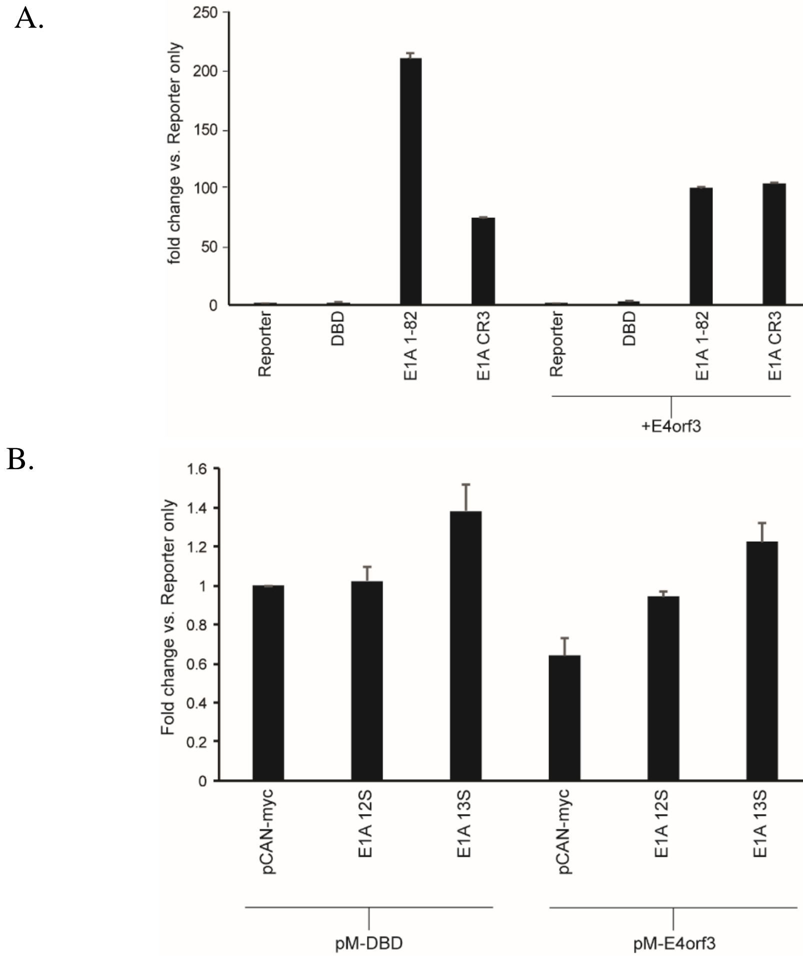



**Figure 3.7 Luciferase Assay of Gal4 Responsive Promoter.**

A. HT1080 cells were transfected with pGL2-(UAS)6-Luc reporter, an empty pCAN-Myc construct or pCAN-Myc-E4orf3, Gal4-DBD fused with residues 1-82 of E1A and Gal4-DBD fused to the CR3 regions of E1A and Gal4-DBD alone. 24 hours after the transfection cells were lysed with culture lysis buffer, 50μl of lysate was mixed with 50μL of luciferase substrate. Luminescence was normalized to protein concentration. n=4

B. HT1080 cells were transfected with pGL2-(UAS)6-Luc reporter, either Gal4-DBD construct or Gal4-DBD-E4orf3 and pCAN-Myc, E1A 12S or E1A 13S as indicated. Assay was done the same way as in figure 3.7 A n=2

**E4orf3 Enhances E1A Transcriptional Activation of the E2F Promoter.** Previous studies have shown that the E2 promoter binding factor (E2F), mediates the transcriptional activation of the viral E2 promoter (Kovesdi *et al*., 1987). Competition experiments indicate that E2F does not bind to other viral promoters such a the E1B, E3, E4, or major late promoter sequences. As a result, the activation of E2F promoter sequences can be uniquely correlated to transcriptional activation of the viral E2 region. In order to examine the effect of E4orf3 and E1A on the transcriptional activity of this promoter a luciferase assay was carried out. HeLa cells were transfected with either an empty pCAN-Myc construct or pCAN-Myc-E4orf3 and E1A (genomic, 12S or 13S). As reported in the literature, E1A was found to activate the transcriptional activity of the E2F promoter (Pelka *et al*., 2011). However, the activation of this promoter is enhanced in the presence of E4orf3 (Figure 3.8), indicating that E1A and E4orf3 may collaborate in the activation of the viral E2 region.

**The E1A 13S Isoform and E4orf3 Enhance Transcriptional Activation of the E3 Promoter.** Previous literature has shown that E1A 13S can strongly activate transcription of a luciferase reporter gene under the control of the adenovirus E3 promoter (Pelka *et al*., 2009). To examine if E4orf3 has an effect on E1A mediated transcriptional activation of the E3 viral region, a luciferase assay was carried out. HT1080 cells were transfected with an empty pCAN-Myc construct or pCAN-Myc-E4orf3, the E3 reporter and E1A (genomic, 12S or 13S). As reported in the literature, the results indicate that E1A alone is able to activate the E3 promoter. Interestingly, the transactivation effect seen is enhanced in the presence of E4orf3, and this effect seems to be more pronounced with the 13S isoform of E1A (Figure 3.9).

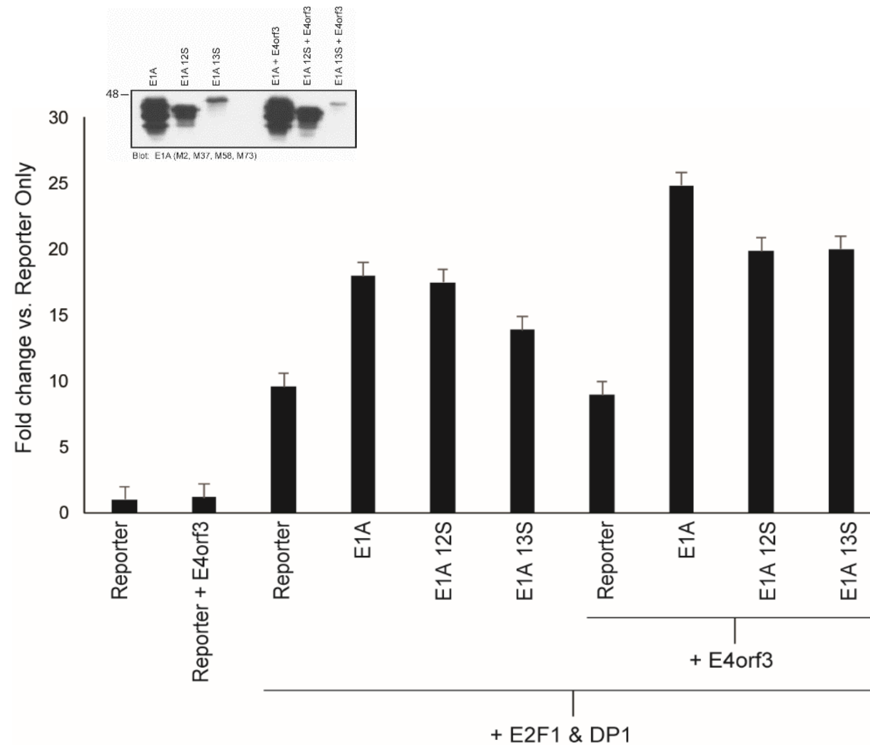



**Figure 3.8 Luciferase Assay of E2F Responsive Reporter.**

HeLa cells were transfected with E2F and DP1 reporter plasmid pGL-E2F reporter (having four synthetic consensus E2F binding sites) together with E2F and DP1, either an empty pCAN-Myc construct or pCAN-Myc-E4orf3 and the indicated E1A constructs. 24 hours after the transfection cells were lysed with culture lysis buffer, 50μl of lysate was mixed with 50μL of luciferase substrate. Luminescence was normalized to protein concentration. n=2

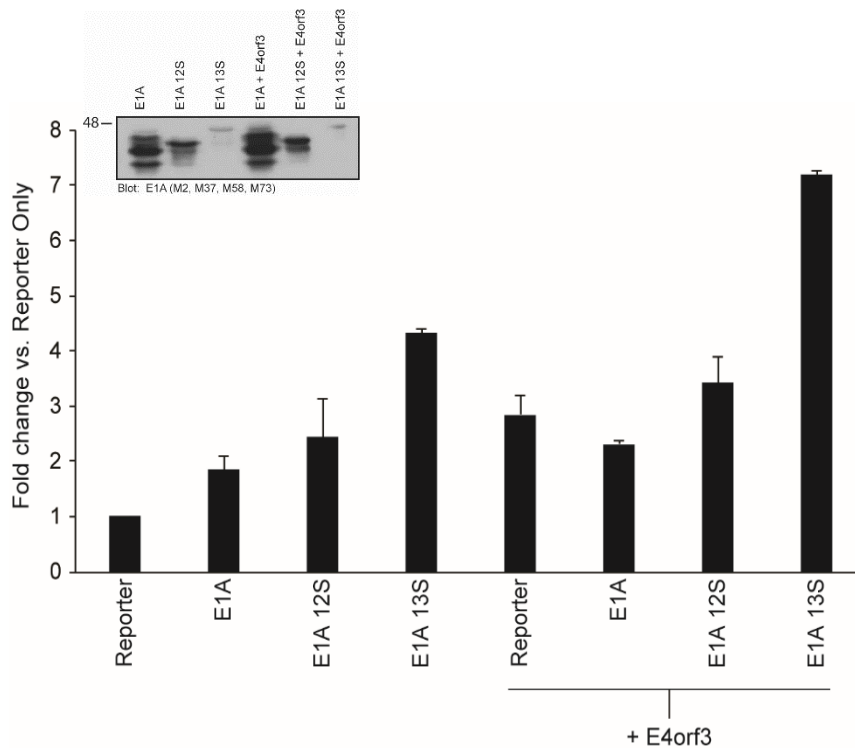



**Figure 3.9 Luciferase Assay of E3 Viral Reporter.**

HT1080 cells were transfected with the E3 reporter plasmid, either an empty pCAN-Myc construct or pCAN-Myc-E4orf3 and the indicated E1A constructs. 24 hours after the transfection cells were lysed with culture lysis buffer, 50µl of lysate was mixed with 50µL of luciferase substrate. Luminescence was normalized to protein concentration. n=4

**E4orf3 Enhances Transcriptional Activation of the E4 Promoter by E1A.** E1A 13S has previously been shown to activate transcription of the E4 viral promoter (Pelka *et al.*, 2009). In order to examine if E4orf3 affects the E1A transactivation of this promoter, a luciferase assay was done using a reporter that has a luciferase gene under the control of the adenovirus E4 promoter region. HT1080 cells were transfected with either an empty pCAN-Myc construct or pCAN-Myc-E4orf3, the E4 reporter and E1A (genomic, 12S and 13S). Transfection of both E1A and E4orf3 results in robust transcriptional activation of the E4 promoter (Figure 3.10 A). In addition, it appears that cells transfected with E1A 13S had increasingly higher levels of transcriptional activation of the E4 reporter when the amount of E4orf3 transfected in cells was increased. However, I do not see the same effect when genomic E1A is transfected with increasing levels of E4orf3 (Figure 3.10 B).

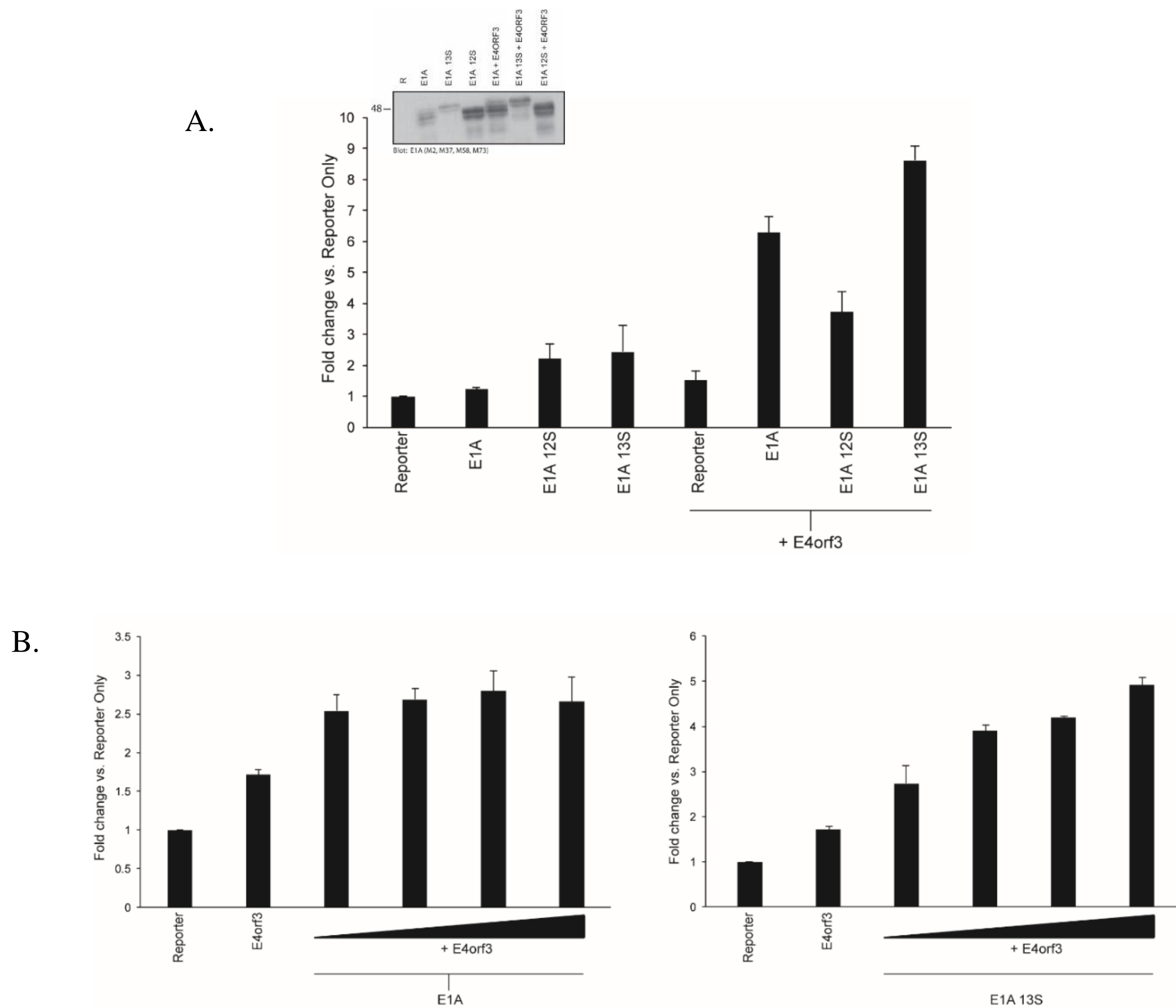



**Figure 3.10 Luciferase Assay of E4 Viral Reporter.**

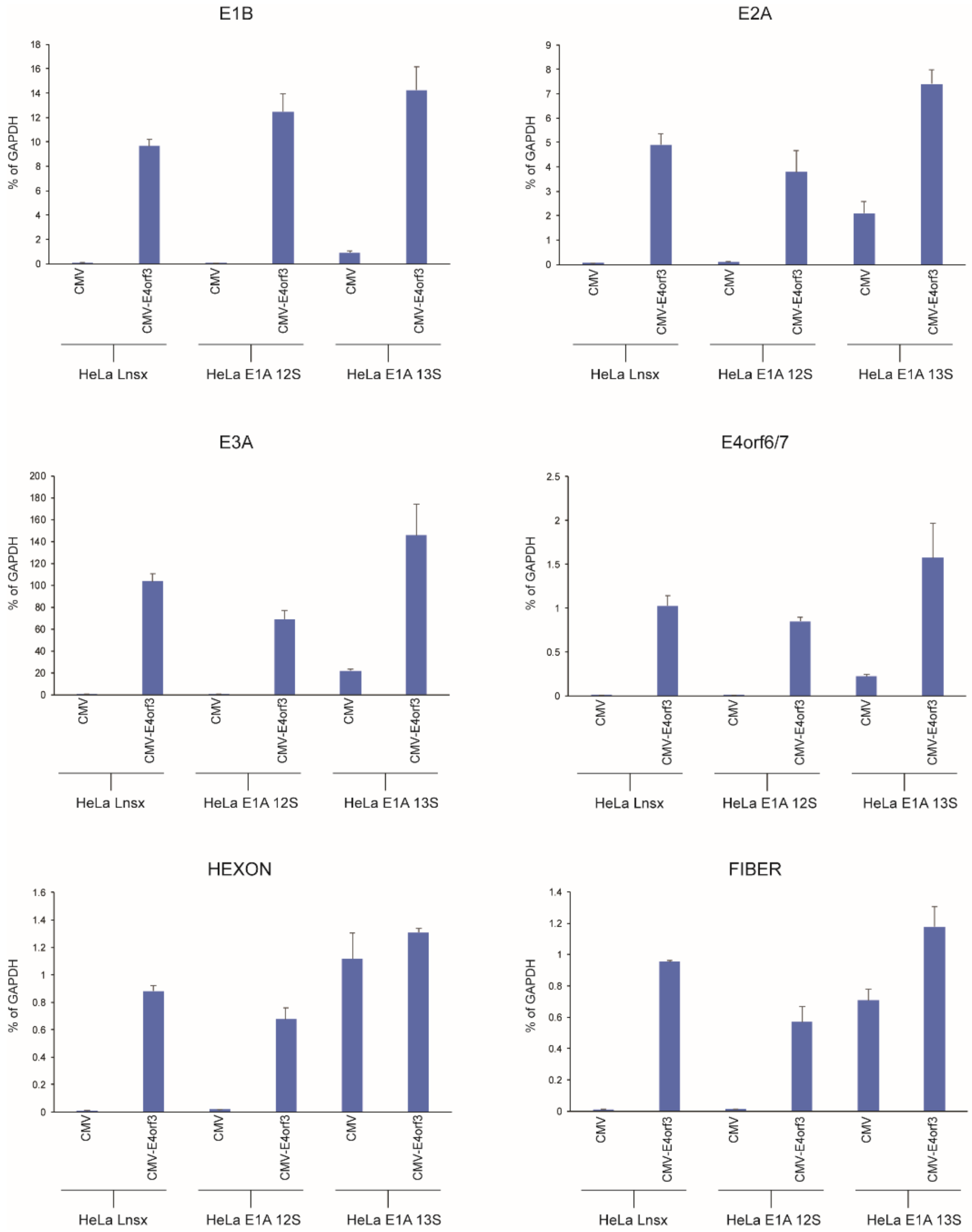
A. HT1080 cells were transfected with the E4 reporter plasmid, either an empty pCAN-Myc construct or pCAN-Myc-E4orf3 and the indicated E1A constructs. 24 hours after the transfection cells were lysed with culture lysis buffer, 50μl of lysate was mixed with 50μL of luciferase substrate. Luminescence was normalized to protein concentration.

B. HT1080 cells were transfected with the E4 reporter plasmid, the indicated E1A constructs and increasing amounts of either an empty pCAN-Myc construct or pCAN-Myc-E4orf3 (0.1µg, 0.5µg, 1µg, 2µg). 24 hours after the transfection cells were lysed with culture lysis buffer, 50μl of lysate was mixed with 50μL of luciferase substrate. Luminescence was normalized to protein concentration.

### 3.7 E1A and E4orf3 Lead to Higher Levels of Viral Gene Expression.

I wanted to examine if the increased transcriptional activation of the viral promoters observed in the luciferase assays, resulted in higher levels of viral gene expression. Three different type of HeLa cells were used: a cell line without E1A (HeLa LNSX), a cell line expressing E1A 12S and a cell line expressing E1A 13S. The cells were infected with a CMV virus or CMV HA-E4orf3 virus. Expression of the viral early genes was examined through qPCR by using primers corresponding to *E2A, E3A*, and *E4orf6/7*. Late viral gene expression was examined by looking at the levels of the structural mRNA encoding genes *hexon* and *fiber*. Cells infected with the CMV HA-E4orf3 virus have higher expression of all viral genes, as compared to cells infected with the CMV virus. Interestingly, I observed that HeLa E1A 13S cells that were infected with CMV HA-E4orf3 have consistently the highest levels of viral gene expression (Figure 3.11 A). However, I do not see the same trend with HeLa LNSX cells infected with the E4orf3 overexpressing virus. The observed changes in gene expression are not due to differences in levels of E1A or E4orf3 expression, as E1A levels in the 12S and 13S cell lines were almost identical and equal levels of E4orf3 expression are observed in the samples infected with CMV HA-E4orf3 (Figure 3.11 B). The overall results indicate that E1A 13S together with E4orf3 increase viral gene expression in a modest but consistent manner.

A.



B.

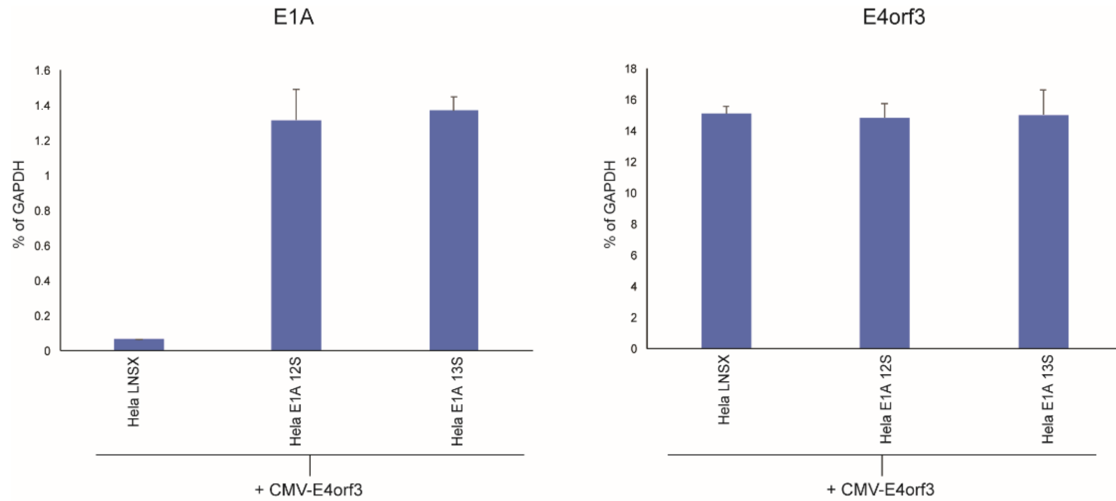



**Figure 3.11 Effects of E1A and E4orf3 on Viral Gene Expression.**

A. 3 different HeLa cell lines were used: HeLa LNSX, HeLa 12S, HeLa 13S. Cells were infected with CMV or CMV HA-E4orf3 as indicated with an moi 30 for 24 hours. Gene expression was analyzed through real time PCR with primers for early viral genes *E1B, E2A, E3A, E4orf6/7* or late genes *hexon and fiber*. Values were plotted as % of GAPDH = CT values of sample over GAPDH value of sample. n=3

B. Assay was done the same way as in 3.11 A, primers for *E1A* and *E4orf3* were used for qPCR. n=3

### 3.8 E4orf3 Affects E1A Enrichment at Viral Promoters.

Since I observed that the E4orf3 together with E1A 13S had a modest effect on virus gene expression, I wanted to determine how the absence of E4orf3 affected the enrichment of E1A at viral promoters during infection. A ChIP assay was done using IMR-90 cells infected with wild type *dl*309 or ΔE4orf3 virus, which does not express E4orf3. The lysates were precipitated with IgG as a negative control, with M73 and M58 antibodies anti-E1A and with 6A11 anti-E4orf3. Real time qPCR was done using primers for the E2 promoter, E3 promoter, E4 promoter and the MLP region. The ChIP analysis showed that samples infected with the wild type virus have enrichment of both E1A and E4orf3 at viral promoters. However, infection with the ΔE4orf3 virus, results in decreased enrichment of E1A at these promoters (Figure 3.12). These results indicate that E4orf3 may play a role in the recruitment or stabilization of E1A or intermediary co-activators at viral promoters.

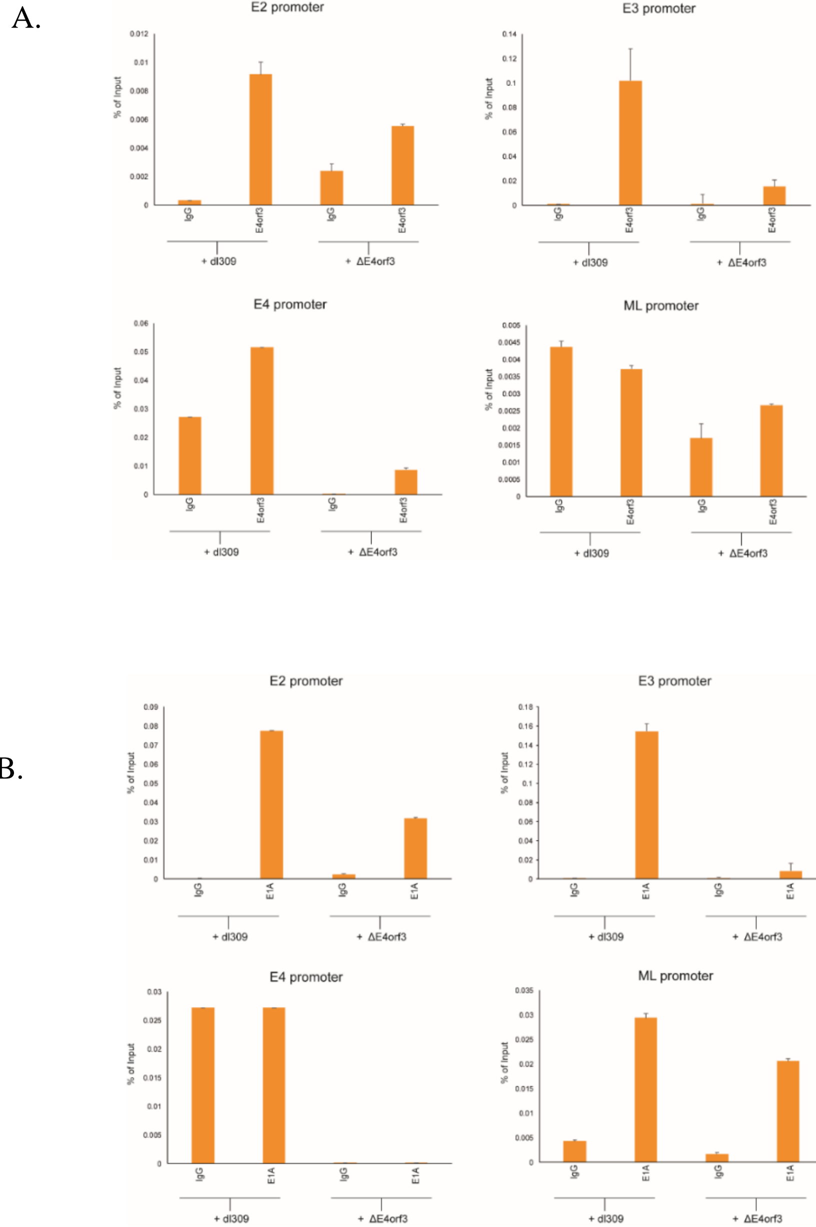



**Figure 3.12 Enrichment of E1A and E4orf3 at Viral Promoters.**

IMR-90 cells were infected with *dl*309 or ΔE4orf3 viruses at a moi of 30 and harvested 24 hours after infection for ChIP-analysis. For immunoprecipitation of E1A, the monoclonal M73 and M58 antibodies were used. For immunoprecipitation of E4orf3 the 6A11 monoclonal rat antibody was used, IgG was used as a negative control. Samples were analyzed by real timer qPCR.

**3.9 Suppression of p53 Transactivation by E1A and E4orf3.**

Previous literature has indicated that DNA tumour viruses are capable of inactivating p53 mediated effects in multiple ways. Adenovirus E4orf3 has been reported to silence p53 activated genes by inducing the formation of SUV39H1/2 H3K9me3 repressive heterochromatin at these promoters (Soria *et al*., 2010). Given that E1A interacts with E4orf3 I wanted to examine the transactivation activity of the p53 promoter when these two genes are transfected together. To do this, a luciferase assay was carried in HT1080 cells transfected with either an empty pCAN-Myc construct or pCAN-Myc-E4orf3, the p53 reporter, p53 expressing plasmid and genomic E1A. The assay indicates that when E1A and E4orf3 are transfected together the transcriptional activity of the p53 promoter is supressed. However, I do not see this suppression when either E1A and E4orf3 are transfected individually (Figure 3.13 A). Next, I wanted to examine if the 12S or 13S isoform of E1A together with E4orf3 result in the same suppression effect. I observed that only the genomic version of E1A is able to decrease the transcriptional activity of the p53 promoter substantially, whereas E1A 13S had minimal suppression effects and 12S did not cause any changes in transcriptional activity of the reporter (Figure 3.13 B). Lastly, we repeated the same luciferase assay using the p53 reporter and the E1A N terminus mutants described in section 3.4. We found that all of the mutants were less efficient at supressing transactivation of the p53 reporter (Figure 3.13 C). Interestingly, some of the E1A mutants that were able to bind E4orf3 had lower levels of p53 reporter suppression. While, the Δ11-13 mutant, which did not bind E4orf3, was more efficient at repressing transcription from this reporter.

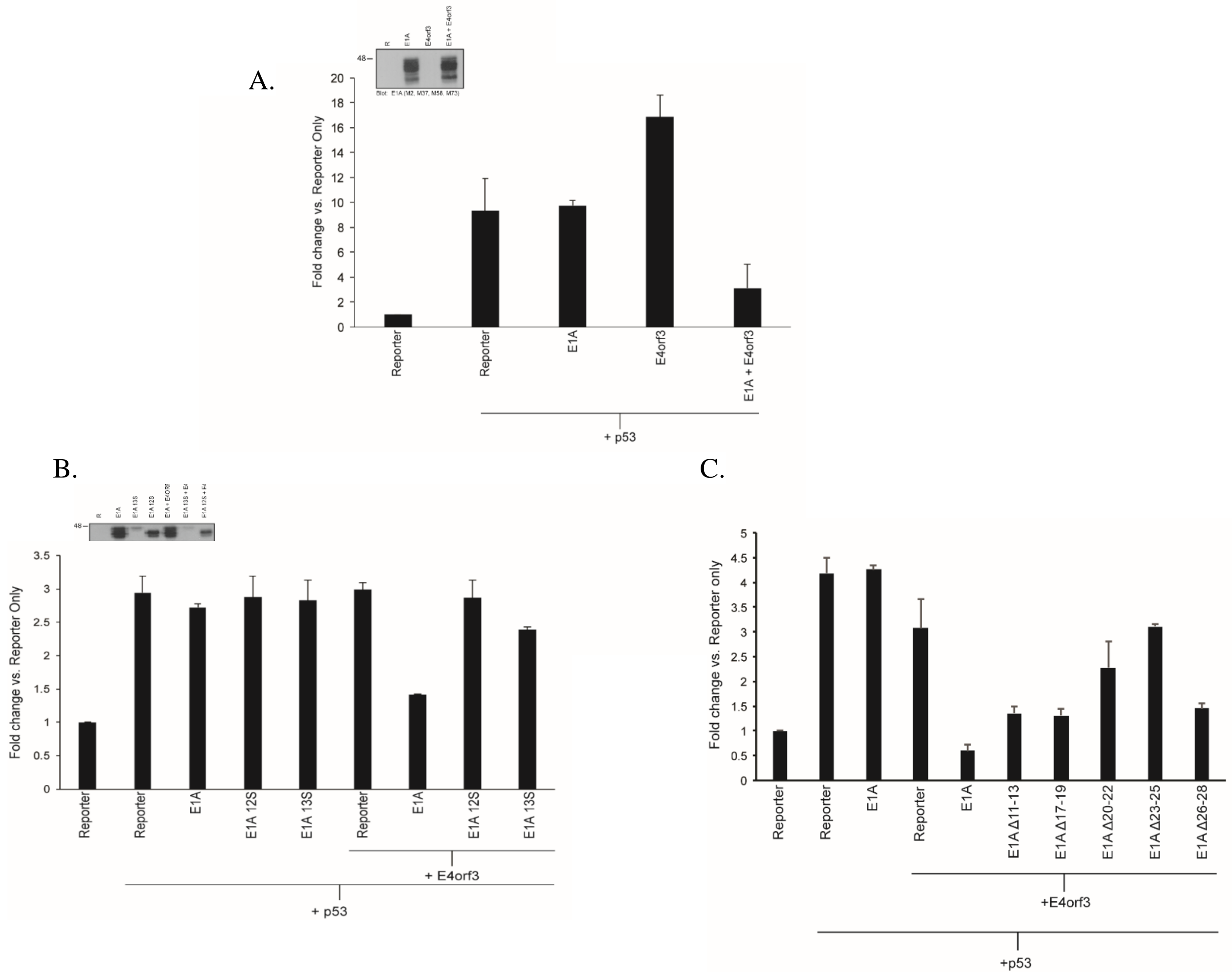



**Figure 3.13 Luciferase Assay of p53 Responsive Reporter.**

A. HT1080 cells were transfected with the p53 reporter, p53 expressing plasmid, E1A and E4orf3 as indicated. 24 hours after the transfection cells were lysed with culture lysis buffer, 50µl of lysate was mixed with 50µL of luciferase substrate. Luminescence was normalized to protein concentration. n=3

B. Assay was carried out the same way as in 3.13A, different E1A constructs were transfected as indicated. n=2

C. Assay was carried out the same way as in 3.13A, different E1A mutant constructs were transfected as indicated. n=3

### 3.10 E4orf3 does not Affect p53 Regulated Gene Expression.

Given that the p53 reporter luciferase assay showed that E1A and E4orf3 suppress p53 promoter activity, I wanted to examine if p53 activated gene expression was affected when E4orf3 is absent during viral infection. To do this, IMR-90 cells were infected for 36 hours with wild type *dl*309 or ΔE4orf3. Gene expression was analyzed through qPCR for p53 responsive genes *GADD45A, MDM2, p21* and *PIG3*. I did not see an increase in the expression of these p53 regulated genes when E4orf3 was absent during infection (Figure 3.14). These results were surprising given that previous literature shows that E4orf3 suppresses the expression of these p53 regulated genes. In order to confirm this observation, I did real time qPCR using 3 different type of HeLa cells: a cell line without E1A (HeLa LNSX), cells expressing E1A 12S and cells expressing E1A 13S, the cells were infected with a CMV virus and CMV HA-E4orf3 virus for 24 hours. I found no significant differences in gene expression between cells that were infected with CMV or CMV HA-E4orf3. In addition, there was no difference is expression of these genes between cells that did not express E1A and the cells that expressed 12S or 13S isoforms of E1A (Figure 3.15). The results of both real time gene expression analysis indicate that expression of p53 regulated genes is not affected by the presence or absence of E4orf3 during the course of viral infection. p53 is required for the activation of p53 regulated genes, however the stabilization of p53 is lower in infection with *dl*309 or ΔE4orf3, as p53 is constantly being degraded by E1B 55k/E4orf6 complex and other mechanisms. Therefore, I decided to examine changes in p53 regulated gene expression using the *dl*1520 virus which does not express the E1B protein and a ΔE4orf6/ΔE4orf3 virus, which has the same phenotype as *dl*1520 in terms in p53 stabilization. Using these viruses ensures that p53 is not degraded during infection, as a result transcriptional changes of p53 target genes should be more noticeable. IMR-90 cells were infected with *dl*1520,

ΔE4orf6/ΔE4orf3 and CMV HA-E4orf3, samples were harvested 16 and 24 hours after the initial infection, gene expression for *GADD45A, MDM2*, *p21* and *PIG3* was subsequently examined through real time qPCR. Interestingly, we found no changes in expression of p53 regulated genes between *dl*1520 and ΔE4orf6/ΔE4orf3, despite p53 stabilization during infection (Figure 5.10).

**3.11 E4orf3 does not Affect the Enrichment of p53 at p53 Regulated Promoters.**

Since I did not observe any changes in p53 expression when E4orf3 was absent during infection, I wanted to examine the levels of p53 enrichment at p53 target promoters. To do this, a ChIP assay was done using HBE cells infected with *dl*309 or ΔE4orf3 for 24 hours, the samples were immunoprecipitated using IgG as a negative control, 1C12 antibody to p53, or M73 and M58 antibodies to E1A. Real time qPCR was done using primers for the promoters of p53 target genes, *GADD45A, MDM2, p21* and *PIG3*. I observed no significant changes in the enrichment of p53 at these promoters in samples that were infected with *dl*309 or samples infected with the ΔE4orf3 virus (Figure 3.16). Interestingly, we see an enrichment of E1A at p53 regulated promoters when the E4orf3 is absent during infection.

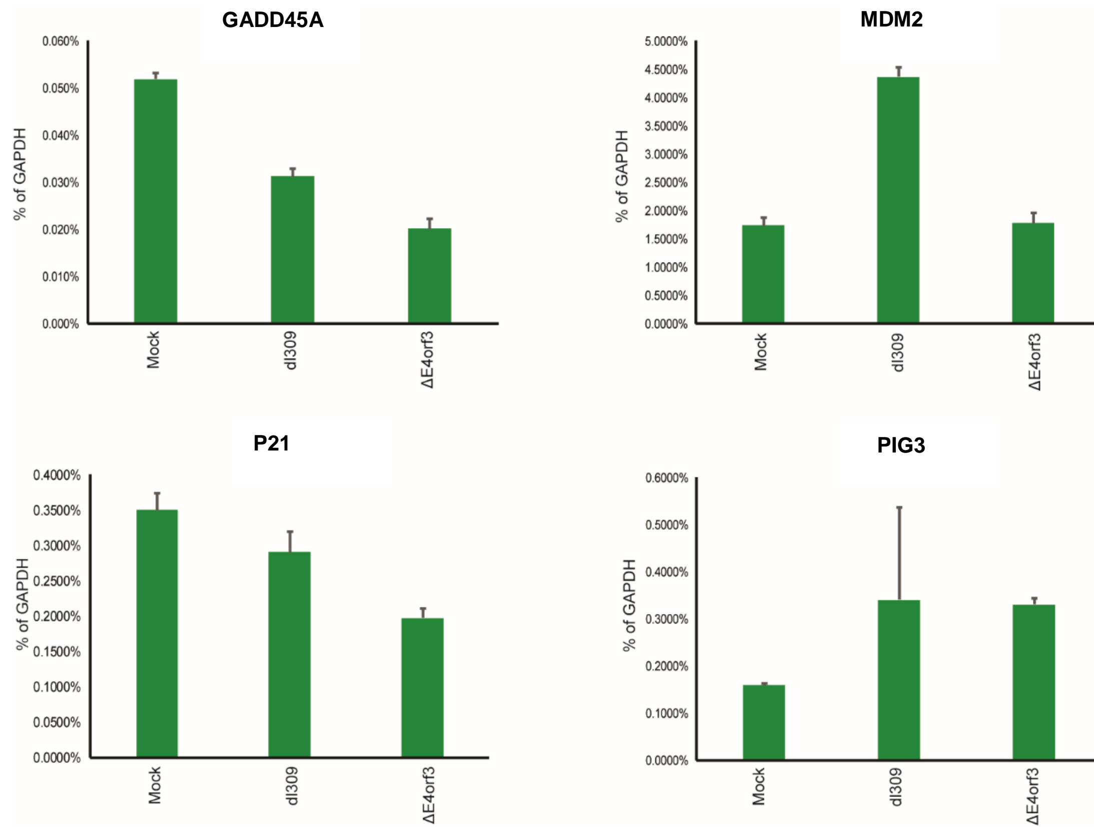



**Figure 3.14 Analysis of p53 Regulated Gene Expression in Fibroblasts.**

IMR-90 cells were infected with either *dl*309 or ΔE4orf3 at an moi of 30 for 36 hours. Gene expression was analyzed through real time qPCR with primers for *GADD45A, MDM2, p21* and *PIG3*. n=3

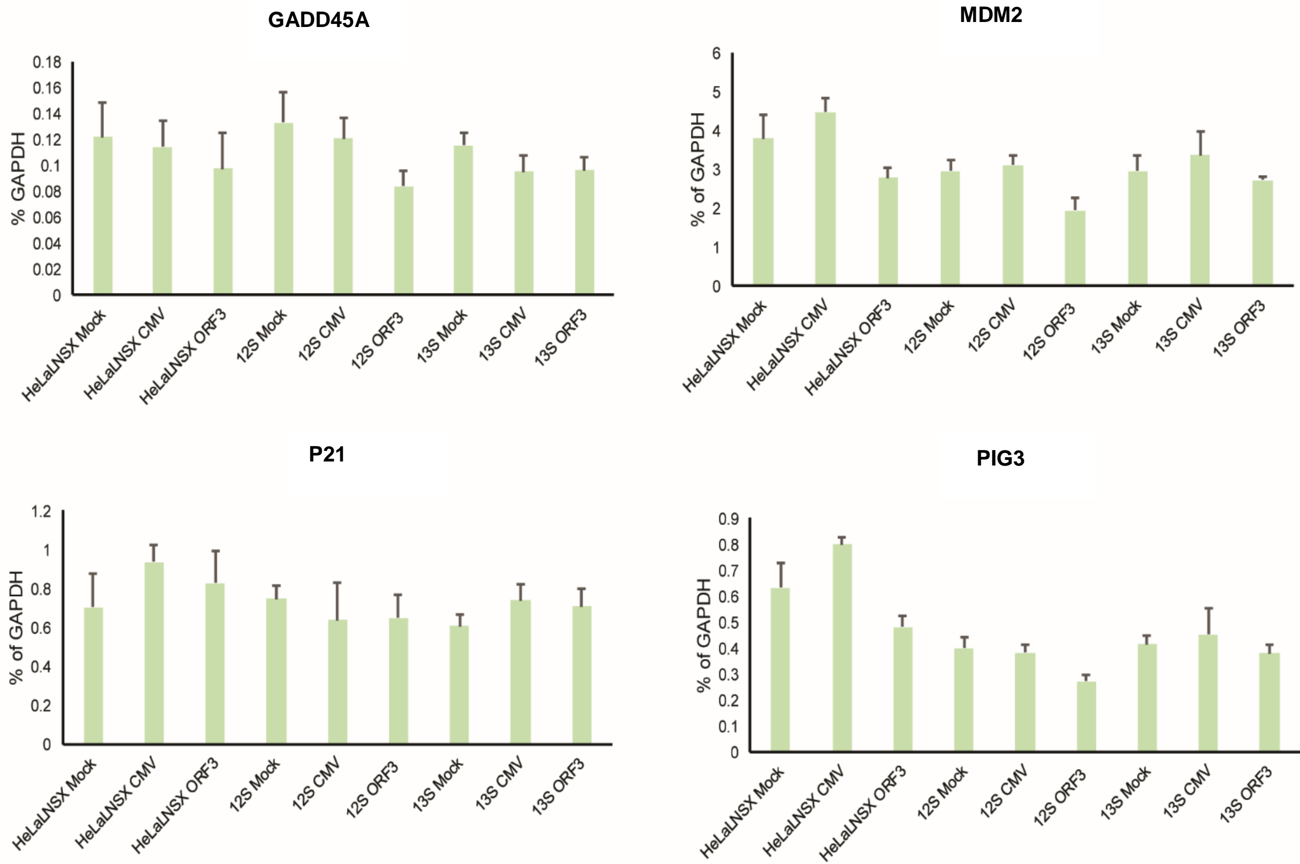



**Figure 3.15 Analysis of p53 Regulated Gene Expression in HeLa Cells.**

3 different HeLa cell lines were used: HeLa LNSX, HeLa 12S, HeLa 13S. Cells were infected with either CMV or CMV HA-E4orf3 at an moi of 30 for 24 hours. Gene expression was analyzed through real time qPCR with primers for *GADD45A, MDM2, p21* and *PIG3.* n=3

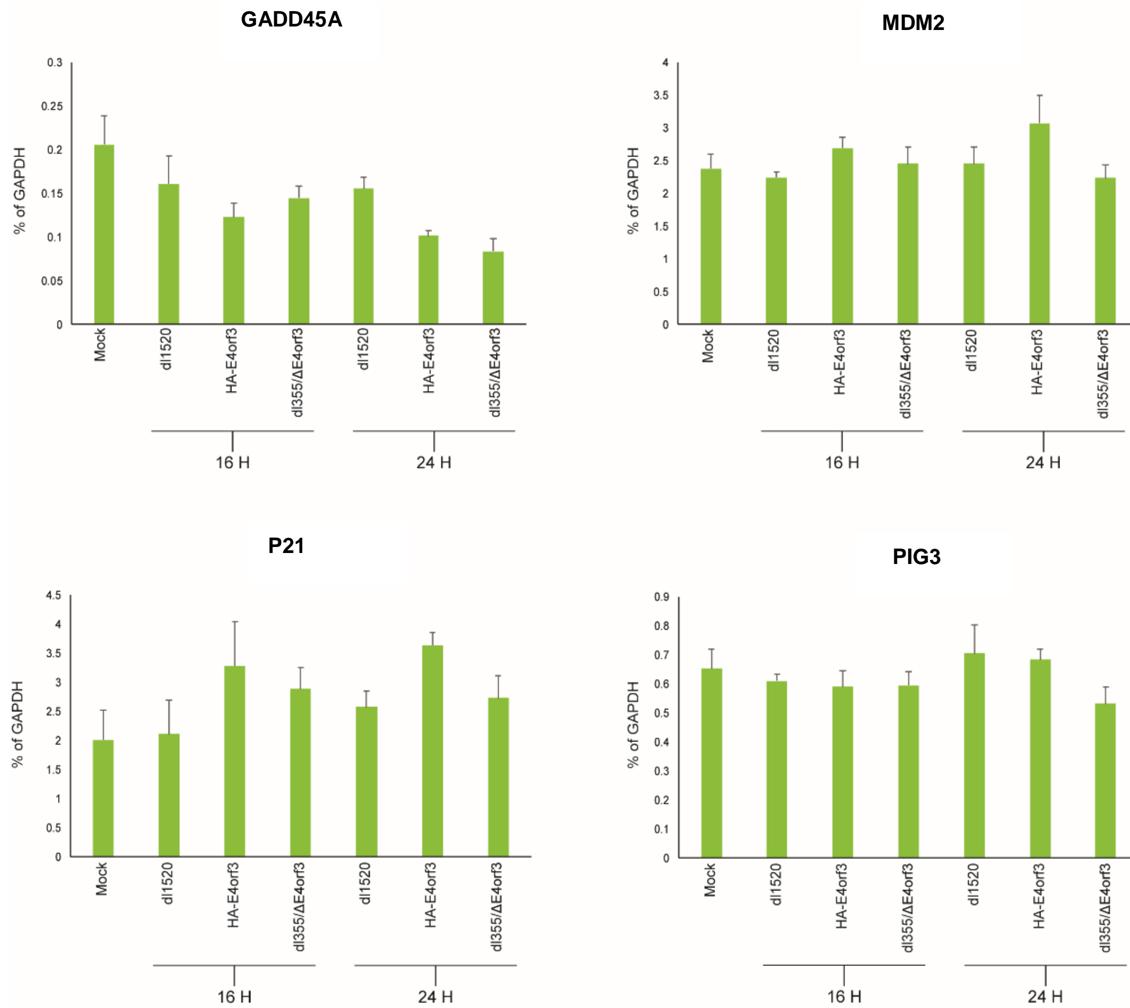



**Figure 3.16 Analysis of p53 Regulated Gene Expression in HT1080 Cells.**

HT1080 cells were infected with either *dl*1520, CMV HA-E4orf3 or ΔE4orf6/ΔE4orf3 (*dl*355/ΔE4orf3) at an moi of 30, samples were collected at 16 or 24 hours after infection. Gene expression was analyzed through real time qPCR with primers for *GADD45A, MDM2, p21* and *PIG3*. n=3

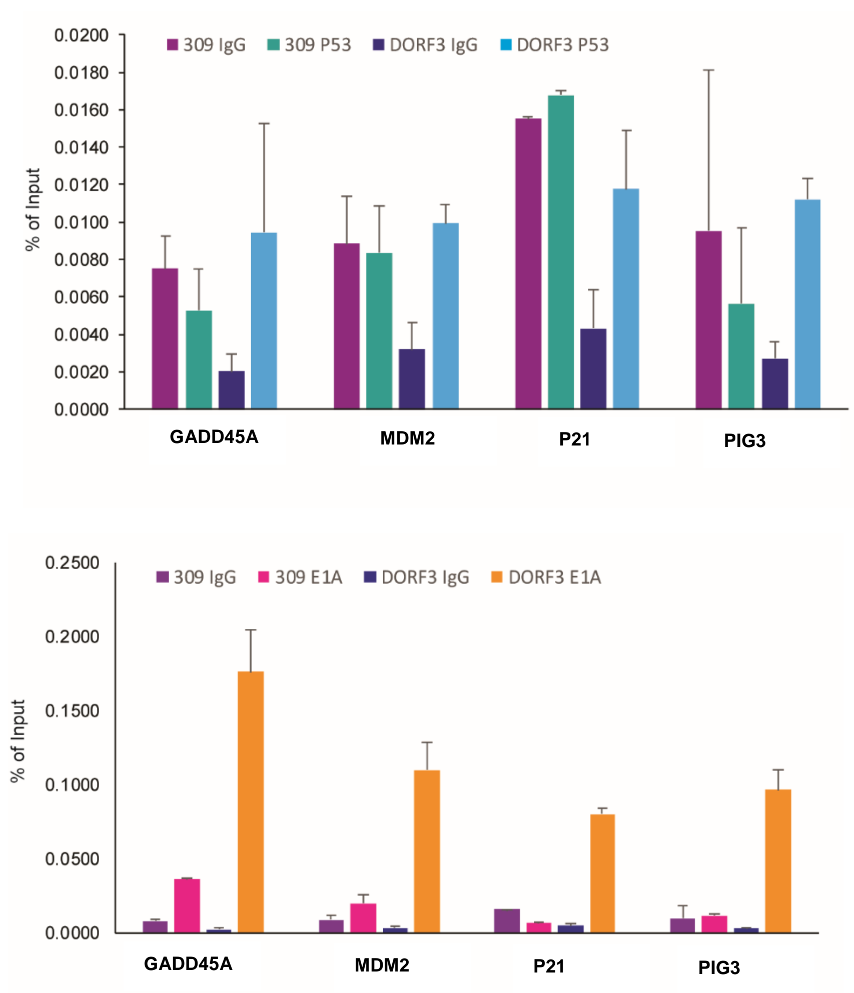



**Figure 3.17 Enrichment of p53 and E1A at p53 Regulated Promoters.**

HBE cells were infected with either *dl*309 or ΔE4orf3 at an moi of 30 and harvested 24 hours after infection for ChIP-analysis. p53 was immunoprecipitated using 1C12 monoclonal antibody, E1A was immunoprecipitated with the monoclonal M73 and M58 antibodies, IgG was used as a negative control. Samples were analyzed by real time qPCR. n=2

**The Contributions of E1A C Terminus on Adenovirus Replicative Cycle.**

Recent studies have examined the interactions of the C terminus of E1A with cellular factors, these interactions have been found to contribute to successful viral replication via the alteration of normal cellular function and the cellular protein network. However, the significance of the C terminus of E1A with regards to viral replication is not well understood. Therefore, we decided to carry out functional analysis using a series of C terminus mutants to examine the influence of the C terminus of E1A on different aspects of the viral replicative cycle.

**3.12 Viral Growth of C terminus E1A Mutants in Arrested Cells.**

To determine how deletions of E1A C terminus affect the ability of HAdV5 to grow we infected arrested WI-38 cells with different E1A mutants that have E1A C terminus deletions: *dl*1116, *dl*1132, *dl*1133, *dl*1134, *dl*1135, *dl*1136 and *dl*311 at an moi of 100. Virus growth was examined at 48, 72 and 96 hours after infection, as no growth was observed at 24 hours (Figure 3.18 B). At 48 hours we found that viruses had most variability in levels on growth, in addition we observed that all mutant E1A viruses had slower growth than *dl*309. However, by 96 hours some viruses had reached similar levels of growth to *dl*309 or had reached equal levels to other mutant viruses. The mutant *dl*311, which lacks most of the exon 2 region of E1A appeared to have very minimal growth during the course of infection. Next, we wanted to examine the morphological changes of cells infected with the different viruses. Arrested WI-38 were infected at an moi of 100 and changes in cellular appearance were observed by microscopy every 24 hours until 120 hours after infection. We did not observe any signs of cytopathic effect (CPE) at 24 hours. However by 72 and 96 hours we were able to see enlargement and detachment of the infected cells (Figure 3.19). Not surprisingly, we observed that cells infected with the *dl*311 mutant showed only minor changes at 120 hours, these results are consistent with the modest growth of this virus.

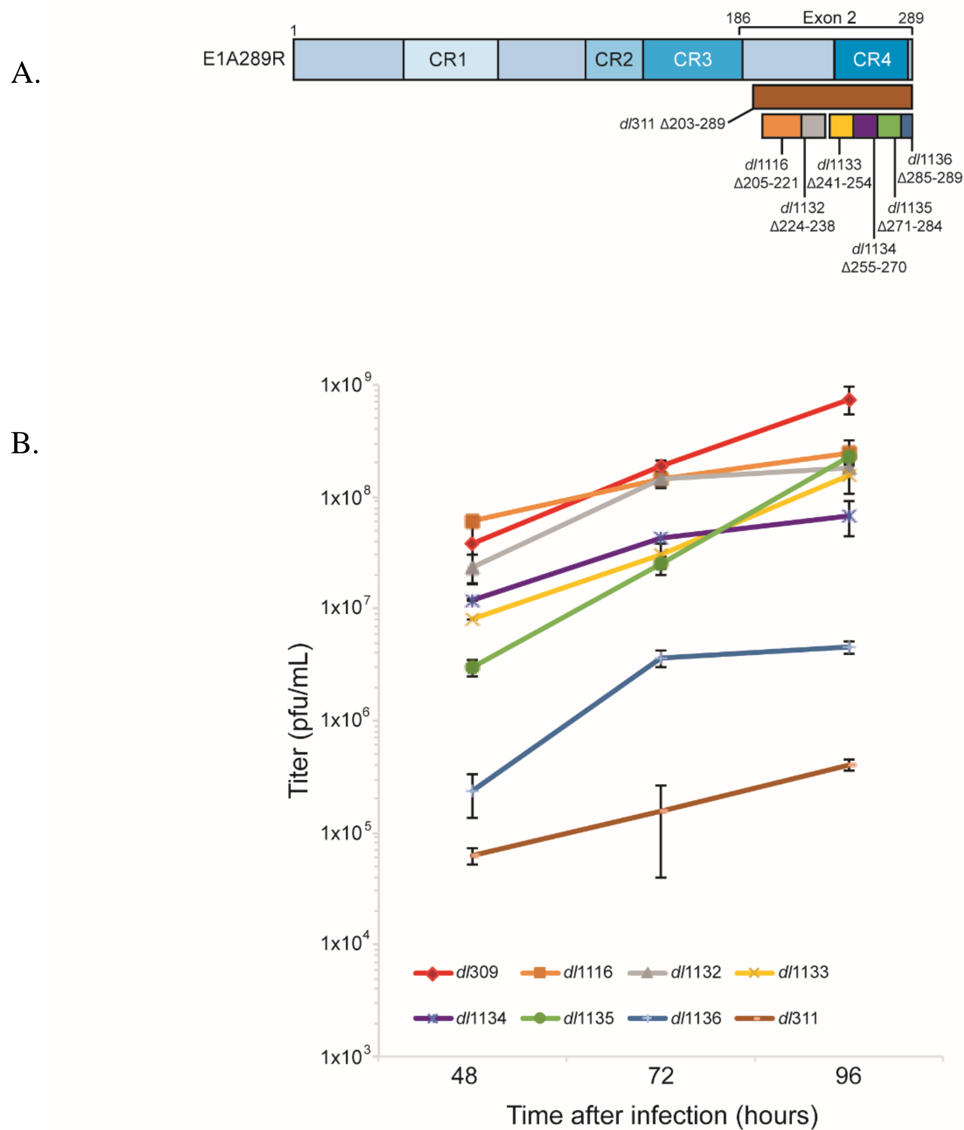



**Figure 3.18 Viral Growth of C Terminus Mutants.**

A. Schematic representation of HAdV5 E1A C terminus deletion mutants.

B. WI-38 cells were infected with the indicated E1A C terminus deletion mutants at an moi of 100 and harvested at 48, 72 and 96 hours. Virus titers were determined by plaque assays using HEK-293 cells. n=3

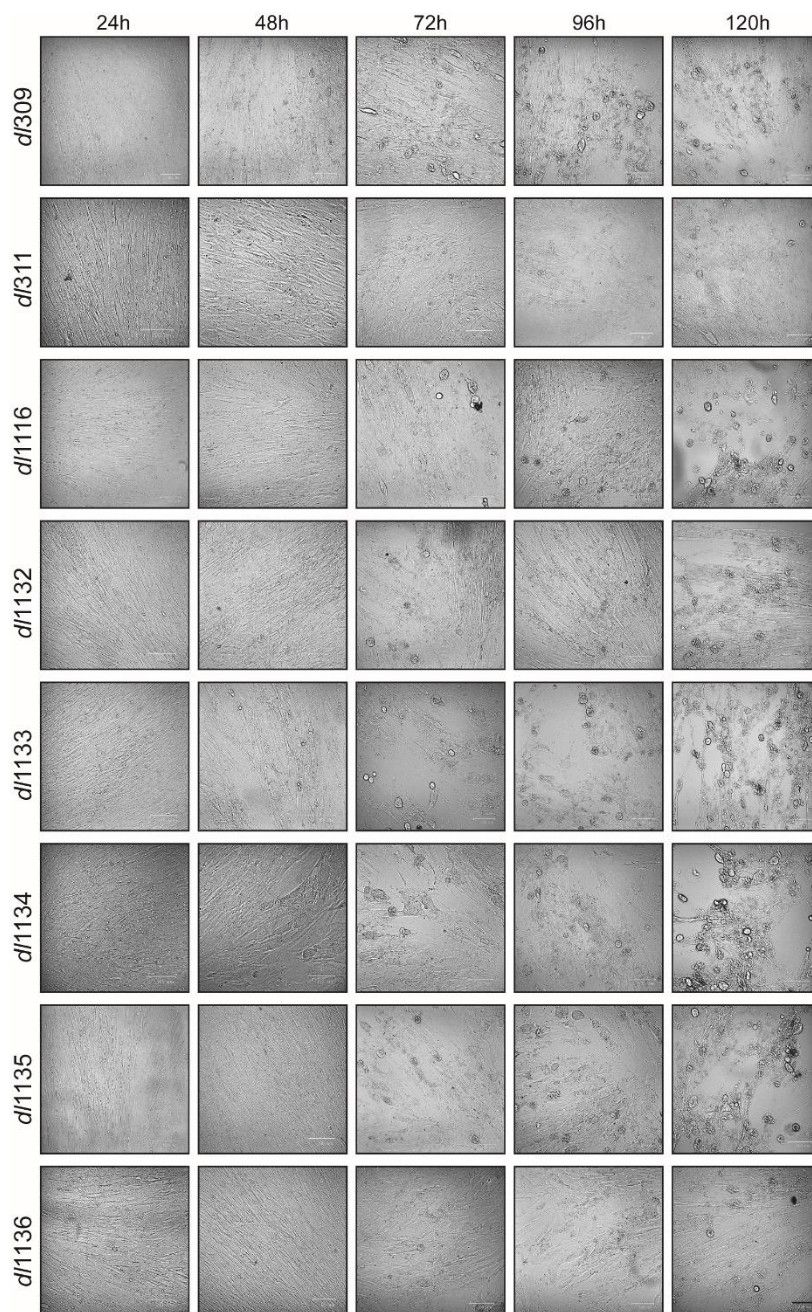



**Figure 3.19 Morphological Changes of Cells Infected with E1A C Terminus Mutants**. Arrested WI-38 cells were infected with the indicated viruses at an moi of 100 and imaged at 24, 48, 72, 96 and 120 hours after the initial infection. Scale bar represents 100 µm. Cells were imaged using a 20× objective lens.

### 3.13 Protein and Gene Expression of E1A C Terminus Mutants.

To determine the effects of E1A C terminus deletions on viral protein expression, infected WI-38 cells were lysed and viral protein levels (E1A, E2 72k DBP and viral late proteins) were examined at 24, 48 and 72 hours through western blot (Figure 3.20). We found that the E1A protein was expressed at high levels 24 hours after the initial infection for all viruses except for the *dl*311 mutant. Levels of E1A increased throughout the infection, as previously reported in literature, we observed a shift in isoform from E1A 12S early in infection to E1A 10S later during infection (Radko *et al*., 2014). We observed DBP expression at 48 hours for the wild type *dl*309 and most of the mutants with the exception of *dl*1133 and *dl*1135. DBP protein increased during infection, as we observed that all mutants showed high levels of this protein by 72 hours after the initial infection. Finally, we found that viral late proteins were readily detectable by 48 and 72 hours after the initial infection for all viruses. Next, we decided to examine the expression levels of viral mRNAs: *E1A-10S, E1A-13S, E1B 55k, E2A, E3A, E4orf6/7* and *hexon* at 16, 24, 48 and 72 hours after the initial infection with the different viruses (Figure 3.21). We observed that the mutants had much lower expression levels of most viral genes compared to *dl*309, however the mutant viruses were approaching *dl*309 expression levels at the later times of infection.

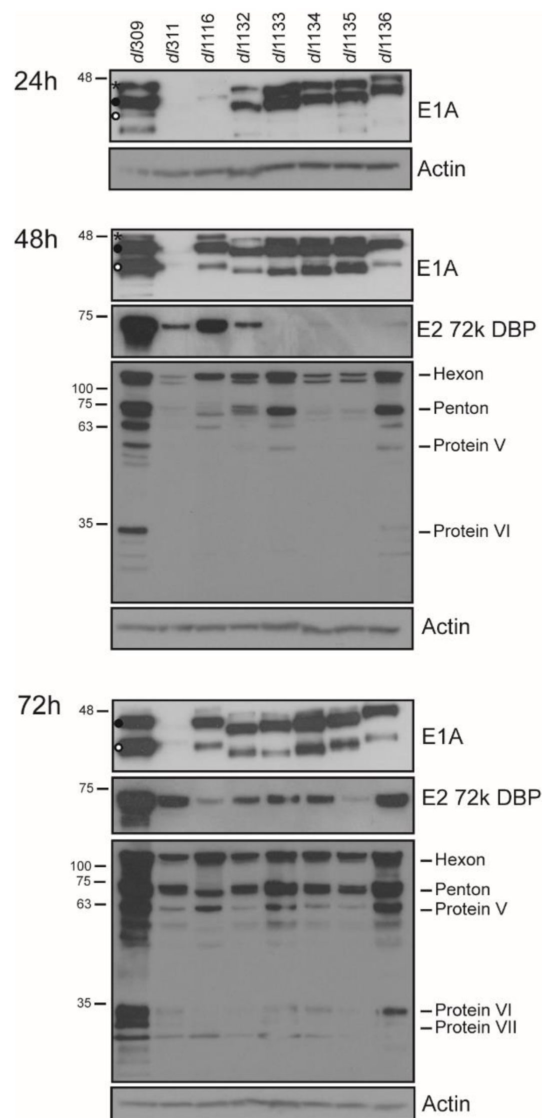



**Figure 3.20 Protein Expression of E1A C Terminus Mutants.**

Arrested WI-38 cells were infected at an moi of 100 and proteins were extracted at 24, 48 and 72 hours. Lysates were run on an SDS polyacrylamide gel and blotted for the indicated proteins. * indicates E1A 13S, • denotes E1A 12S and ° denotes E1A 10S. n=2

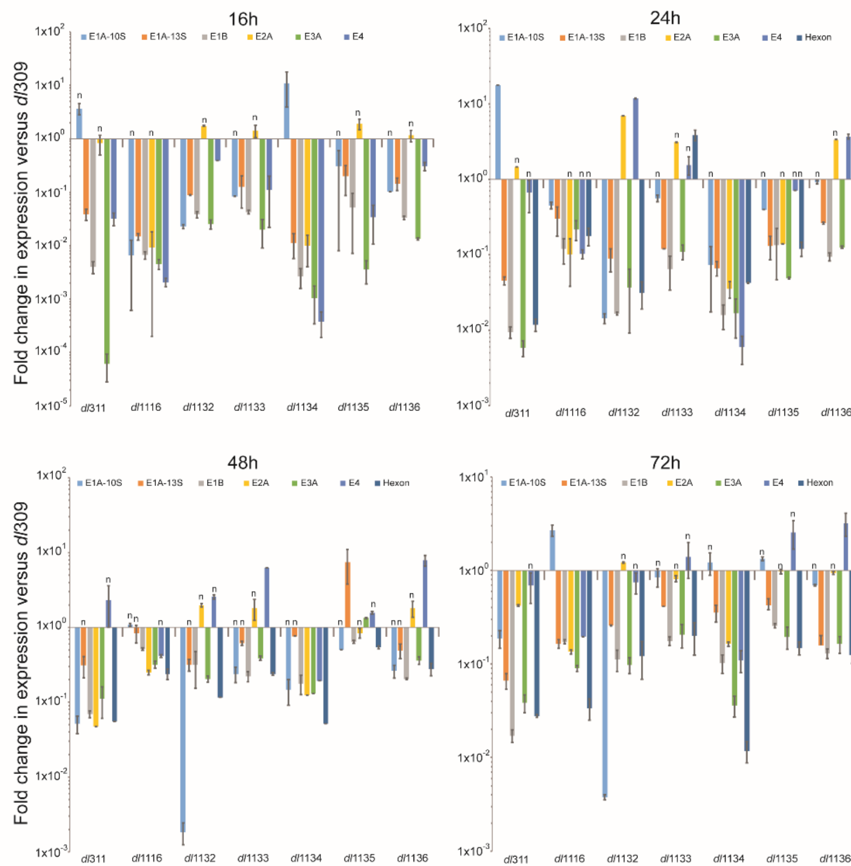



**Figure 3.21 Viral Gene Expression of E1A C Terminus Mutants**.

Arrested WI-38 cells were infected at an moi of 100 with the indicated viruses. Gene expression was analyzed through real time PCR using primers against *E1A 10S, E1A 13S, E1B, E2A, E3A, E4orf6/7* and *hexon*. n=3

### 3.14 Viral Genome Replication of E1A C Terminus Mutants.

HAdV5 E1A is responsible for inducing cell cycle progression of quiescent host cells, leading to DNA synthesis and viral genome replication. We wanted to examine how deletions within the C terminus region of E1A affect the replication of viral genomes. Arrested WI-38 cells were infected with the wild type *dl*309 and the mutant viruses at an moi of 100. Viral genomes were quantified at 24, 48 and 72 hours after the initial infection by real time qPCR using *E1B* primers (Figure 3.22). We did not see any viral genome replication at the 24 hour time point. However, by 48 and 72 hours all viruses had high levels of viral genome replication, with *dl*309 having the highest levels and the mutant viruses lagging behind. Surprisingly, we found that *dl*311 which is growth deficient had similar levels of genome replication to the other mutants.

### 3.15 S-phase Induction of Arrested Fibroblasts Infected with E1A C Terminus Mutants.

Previous literature has shown that E1A is able to upregulate E2F responsive genes involved in cell cycle progression (Chen *et al*., 2007; Shan *et al*., 1994). Therefore, we wanted to examine how the different E1A mutants affected the upregulation of the key genes involved in S-phase induction. Arrested WI-38 cells were infected with the indicated mutant viruses at a moi of 100, samples were collected 16, 24, 48 and 72 hours after the initial infection. Gene expression for *Bloom* (*BLM*), *Proliferating Cell Nuclear Antigen* (*PCNA*) and *Mini-Chromosome Maintenance 4* (*MCM4*) was examined through real time qPCR (Figure 3.23). We observed that *BLM* reached higher levels of expression compared to *PCNA* and *MCM4*. Cells infected with the wild type *dl*309 had higher levels of *BLM* expression at all time points, whereas cells infected with the mutants expressed *BLM* to lower levels, with the *dl*311 mutant being the lowest. The expression of this gene was found to increase consistently from 16 to 48 hours.

We observed that *PCNA* expression was not induced or induced very poorly by all the mutant viruses, cells infected with *dl*309 had the highest expression at 16 hours, however expression levels dropped by 24 hours and remained constant for the rest of the infection. The expression of *MCM4* had a similar trend to *BLM*, with expression increasing from 16 to 48 hours. Cells infected with *dl*309 had the highest expression of *MCM4* overall, except at 48 hours where *dl*1134 shows slightly higher levels of expression than *dl*309.

Lastly, we wanted to determine how the different deletions within the C terminus of E1A affected the ability of the virus to induce S-phase. Arrested WI-38 cells were infected for 24 hours and induction of S-phase/DNA replication was examined through an EdU incorporation assay (Figure 3.24). We found that the wild type virus *dl*309 induced S-phase in over 50% of the cells, whereas most of the mutants induced between 20-40% of the cells. We observed that two mutants *dl*311 and *dl*1132 were able to induce S-phase in less than 20% of the cells.

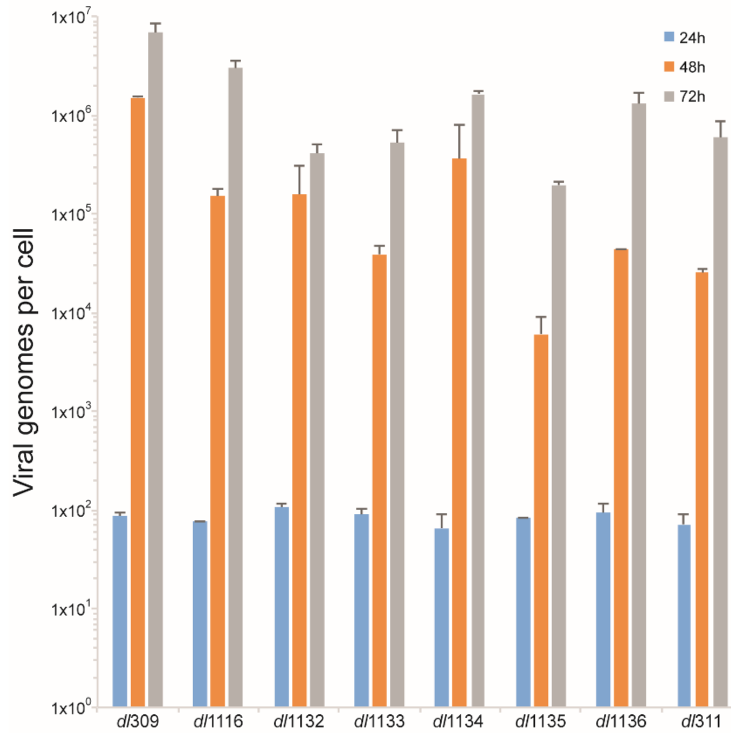



**Figure 3.22 Viral Genome Replication of E1A C Terminus Mutants**.

Arrested WI-38 cells were infected with the indicated mutant viruses at an moi of 100. Samples were collected 24,48 and 72 hours after the initial infection. Viral DNA was extracted and quantified by qPCR using *E1B* primers. Viral genomes are plotted on a per cell basis. n=3

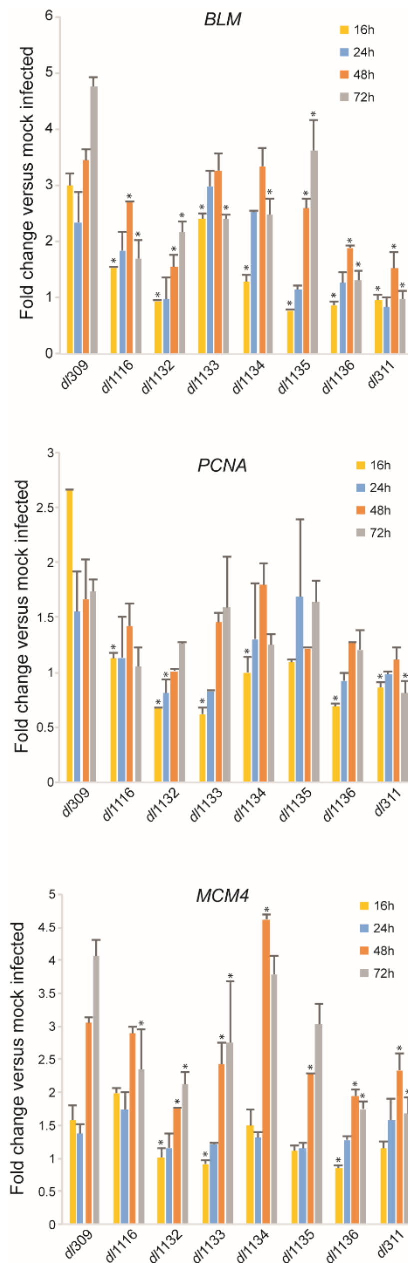



**Figure 3.23 S-phase related Gene Expression of Cells Infected with E1A C Terminus Mutants.**

Arrested WI-38 cells were infected with the indicated mutant viruses at an moi of 100. Samples were collected 16,24,48 and 72 hours after the initial infection. Gene expression was analyzed by qPCR using primers for *BLM, PCNA* and *MCM4*. n=3

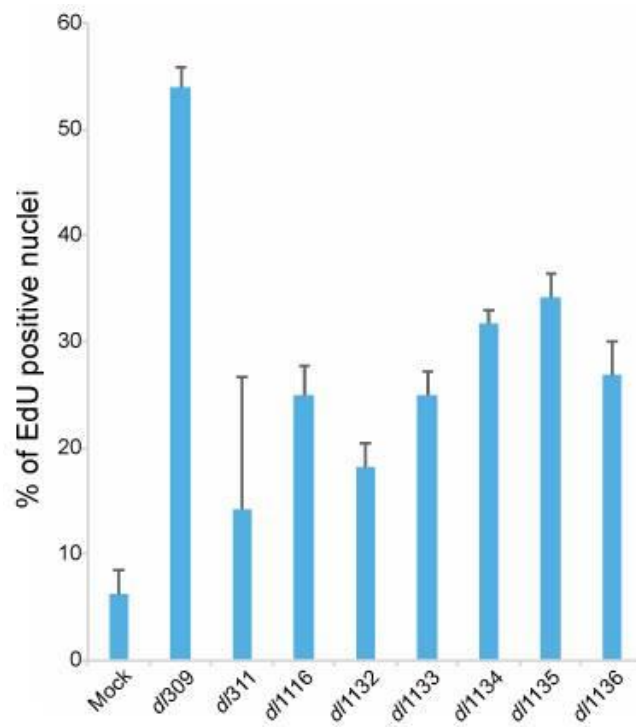



**Figure 3.24 S-phase Induction of Cells Infected with E1A C Terminus Mutants.**

Arrested WI-38 cells were infected with the indicated mutant viruses at an moi of 100. At 24 hours cells were pulsed with EdU for 1 hour and subsequently stained for EdU using the Click-It EdU labeling kit. Cells were stained for E1A using the M58 antibody. n=3

# 4. Discussion

### 4.1 Interaction of E1A and E4orf3.

During infection, E1A targets key cellular proteins involved in multiple processes, in order to remodel the intracellular environment to allow for efficient viral replication. Multiple interactions between E1A and cellular proteins have been studied in detail. However, interactions between HAdV early proteins have not been thoroughly examined.

Previous work has shown that HAdV E1A interacts with the NimA-related kinase Nek9 to supress the activity of p53 regulated gene *GADD45A* (Jung *et al*., 2015). Suppression of *GADD45A* was partly dependent on the E4orf3 protein. It was hypothesized that the nuclear tracks formed by E4orf3 may have a role in enhancing the recruitment of E1A to promoters (Jung *et al*., 2015). Our study has identified, for the first time, an interaction between the adenoviral early protein E1A and early viral protein E4orf3. The initial identification of the possible interaction between E1A and E4orf3 was through immunofluorescence of infected fibrosarcoma cells, where I saw a colocalization of E1A to the tracks formed by E4orf3 in the nucleus (Figure 3.1). The relocalization seen was interesting, as E1A is usually found in a diffuse or punctuate pattern in the nucleus as observed with cells infected with ΔE4orf3 (White *et al.,* 1988). This observation led me to examine if there was an interaction between the two proteins, through a co-immunoprecipitation assay E1A was found to associate with E4orf3 during the course of normal infection (Figure 3.2 A) and GST-pulldown assays confirmed this as a direct interaction (Figure 3.2 B). A series of E1A mutants were used for co-immunoprecipitation assays, the region of E1A involved in the interaction with E4orf3 was mapped to residues 4-25 of the N terminus (Figure 3.3). However, the *dl*1101 mutant (Δ4-25) is severely impaired in many aspects of E1A function, aside from lacking binding affinity to E4orf3, it is also unable to bind the Nek9 kinase involved in p53 gene downregulation as well as the co-regulators p300/CBP and p400 (Flinterman *et al*., 2007;

Jelsma *et al*., 1989). Furthermore, this deletion prevents the *dl*1101 mutant from blocking the activation of interferon stimulated genes or transforming cells with activated Ras (Caporossi *et al*., 1990). Consequently, we cannot use the *dl*1101 virus for studying the functional effects of the interaction between E1A and E4orf3. In order to find the specific residues of E1A required for binding, additional co-immunoprecipitation assays were performed, which revealed that residues 3-10 and 17-28 of E1A are not likely involved in binding to E4orf3. Instead, the E1A deletion mutant Δ11-13 lost binding affinity to E4orf3, indicating this region is necessary for the interaction to occur (Figures 3.3 and 3.5). The N-terminal region of the E1A proteins is contains an α-helix (Avvakumov *et al,* 2004; Ferreon *et al*., 2009), in HAdV5 residues 16 to 28 are critical for helix formation as they have amphipathic characteristics (Pelka *et al*., 2008). Previous functional analysis of the N terminal portion of E1A determined that deletion of residues 11-15 were not required for interaction with p300/CBP and TBP (Loewenstein *et al*., 2006), indicating this mutant is a good candidate for studying the functional effects of the interaction between E1A and E4orf3.

E4orf3 self-assembles into a polymer that acts as a scaffold and forms multiple protein binding interfaces. The formation of this polymer appears to be required for E4orf3 functions since previous studies have found that E4orf3 polymerization mutants are unable to disrupt PML bodies or relocalize NBS1, a key component of the MRN complex (Patsalo *et al*., 2012; Stracker *et al*., 2005). Therefore, we wanted to determine if polymerization is required for E4orf3 binding to E1A. Co-immunoprecipitation assays with E4orf3 mutants N82A, L103A, D105/L106 and E1A, revealed that D105/L106 lacks binding to E1A. However, the polymerization deficient mutants N82A and L103A still interact with E1A, indicating that polymerization may not be required for E4orf3 binding to E1A (Figure 3.6). Residues D105/L106 are not required for E4orf3 self-assembly or disruption of PML, but are essential for relocalization of the MRN complex. It would

be interesting to examine if the ability of E4orf3 to bind to E1A is also connected to disruption of the MRN complex.

### 4.2 Effects of E1A and E4orf3 on Viral Transcriptional Activation.

Adenovirus E1A plays an essential role in activating the transcription of other viral early genes (Berk, 2005). The transactivation regions of E1A have been mapped to its N terminus and CR3 regions. However, the interactions of other E1A regions with different cellular proteins also contribute to transcriptional regulation (Pelka *et al*., 2008). Furthermore, several E4orf3 binding proteins regulate gene expression, linking E4orf3 to transcriptional regulation (Vink *et al*., 2015). In addition, the E4orf3 protein has been found to promote viral gene expression by facilitating the cytoplasmic accumulation of viral transcripts (Sheppard *et al*., 2003). Therefore, I examined how the presence of both E1A and E4orf3 affected the transcriptional activity of different promoters. My initial observations indicated that the transcriptional activity of E1A 1-82 was supressed in the presence of E4orf3, whereas CR3 activity was unchanged. This was not caused by E4orf3 possessing a silencing activity as a GAL4-DBD-E4orf3 fusion had no effect on activity of the luciferase reporter.

The E2F transcription factor is recruited to the E2 early promoter to provide E1A mediated transactivation of E2 genes (Marton *et al*., 1990; Obert *et al*., 1994). Previous literature has shown that isoforms 12S and 13S of E1A use different mechanisms to stimulate E2F-mediated transcriptional activation. Using an E2F responsive promoter, we found that when E4orf3 and E1A are both present we see an increase in the activation of the promoter of approximately five fold with genomic and 13S E1A, and to a lesser extent with the 12S isoform. Examination of the effects on transactivation of the E3 and E4 viral promoters when E1A and E4orf3 were co-transfected showed a modest increase in luciferase activity with E1A13S (Figures 4.3 and 4.4). Taken

together, these results suggest that E4orf3 is a co-activator of E1A mediated transactivation. A similar trend was observed with *in vivo* viral infection and direct examination of viral gene expression, which showed enhanced expression of viral genes in presence of E4orf3, with most genes being upregulated approximately 50% (Figure 4.5). Overall, these results show a modest, yet consistent, enhancing effect of E4orf3 on viral gene expression.

Since I saw an increase in viral gene expression and activation of viral promoters, I wanted to determine how infection in the presence or absence of E4orf3 affected the recruitment of E1A to viral early gene promoters. ChIP indicated that E1A recruitment to viral promoters decreases when cells are infected with a virus lacking expression of the E4orf3 protein. Recent studies from our laboratory have indicated that E1A is not the first gene to be expressed in infection, instead E4orf3 expression begins at approximately the same time (unpublished results). This suggests a model in which E1A works together with E4orf3 to maximize the transcriptional activation of viral genes. The formation of E4orf3 tracks can be seen as early as 8 hours after infection, I hypothesized that E4orf3 may be acting as a scaffold to allow for easier recruitment of host factors that lead to E1A mediated viral gene expression. Previous literature examining the expression of mutant E4orf3 proteins revealed that nuclear track formation is necessary for E4orf3 to induce changes in cellular gene expression (Vink *et al*., 2015). However, E4orf3 polymerization deficient mutants were found to still bind E1A, indicating that a different mechanism is at play. Recent studies, have shown that the E4orf6/E1B 55k complex works together with E1A to enhance the activation of E2F (Dallaire *et al*., 2016). The authors hypothesized that following the E1A mediated activation of early viral gene expression, the E4orf6/E1B 55k complexes function to amplify and sustain the initial effects of E1A in order to maximize viral replication. The HAdV proteins E4orf3 and E4orf6 have a complex inter-relationship and work together to regulate RNA processing, late viral mRNA

nuclear export, the shutoff of host-cell protein synthesis, and neutralization of the host cell DNA damage response during infection (Evans *et al*., 2003; Shepard *et al*., 2003; Tauber *et al*., 2001; Weitzman *et al*., 2005). It is perhaps not surprising, that they share common functions and could be acting synergistically to cooperate with E1A to promote activation of viral gene expression. This avenue has yet to be explored.

### 4.3 The role of E1A and E4orf3 in p53 Regulated Gene Expression.

Previous studies have found that E4orf3 nuclear track formation results in the disruption of the interferon-mediated antiviral response (Ullman *et al*., 2007), inhibition of the DNA damage response (Stracker *et al*., 2002; Sohn *et al.*, 2012), and altered p53-mediated signalling (Soria *et al*., 2010). The tumour suppressor p53 is a key regulator of the cellular responses to stress, as a result HAdV and other DNA virus have evolved multiple avenues of supressing p53 activity. In HAdV5 p53 is targeted for proteasomal degradation by the E1B 55k and E4orf6 proteins which form a complex with p53, this leads to the recruitment of Cullin-containing ubiquitin ligase assembly which causes p53 ubiquitination and subsequent degradation (Querido *et al*., 2001). In addition, the cellular kinase, Nek9, has been found to be involved in E1A mediated suppression of the p53 responsive gene *GADD45A* (Jung *et al*., 2015). Recently, our laboratory has also identified that E1A co-opts the FUBP1 (Far Upstream Element Binding Protein 1) protein for suppression of p53 activity (Frost *et al*., 2018). Finally, the E4orf3 protein has been reported to inhibit p53 function by inducing the formation of repressive heterochomatin H3K9me3 at p53 promoters thus preventing p53 association with p53-responsive promoters (Soria *et al*., 2010). Consequently, I decided to examine if E1A contributes to E4orf3 mediated suppression of p53. Through a luciferase assay using a p53 reporter, we observed that when either E1A or E4orf3 are transfected alone, we do not see a suppression of p53 activity. However, when both proteins are transfected

together we observe approximately a 6 fold decrease in p53 reporter transactivation. In addition, we observed that this suppression only occurs with E4orf3 and genomic E1A, but not with the 12S or the 13S isoforms of the E1A protein. We hypothesized that the smaller isoforms of E1A, which are expressed only by genomic E1A, may be involved in p53 suppression. This avenue has yet to be explored. Interestingly when we tested different E1A deletion mutants, we found that E1A Δ11-13, which is unable to bind E4orf3, was only slightly less efficient at supressing p53 transactivation. While E1A mutants Δ20-22 and Δ23-25 were slightly more efficient with regards to p53 suppression than Δ11-13. It is unclear why this was observed; however, previous analysis of the N terminal portion of E1A have shown that while most residues are involved in binding to one or two proteins, residues 19, 20 and 23 are important for interaction with multiple protein targets. This indicates that the loss of suppression observed may not be specific to p53, but instead could be due to loss of binding to other targets or due to structural defects (Boyd *et al*., 2002; Rasti *et al*., 2005). Surprisingly, analysis of p53-regulater gene expression with *dl*309 or ΔE4orf3 virus showed no significant differences in *GADD45A, MDM2, p21* or *PIG3*. The p53 protein is required for the activation of p53 regulated genes, however in the E1A-wildtype virus *dl*309 or ΔE4orf3 (which also expresses wildtype E1A), p53 is constantly being degraded by E1B 55k/E4orf6 complex and various other mechanisms. Consequently, we decided to use the *dl*1520 virus and the ΔE4orf6/ΔE4orf3 virus. *dl*1520 does not express E1B 55k, therefore p53 is degraded to a lesser extent during infection; while the ΔE4orf6/ΔE4orf3 lacks E4orf6 which acts together with E1B to degrade p53, consequently this virus is virtually identical to *dl*1520 in terms of p53 stabilization. Again, we did not see any difference between a virus with E4orf3 present or absent. This suggests that absence of E4orf3 during infection did not lead to increases in the concentrations of p53-

responsive mRNAs in infected cells. Finally, the results of our ChIP assay showed no difference in p53 enrichment at the promoters of p53 regulated genes.

Overall, we found that the absence of the E4orf3 protein during infection was not sufficient to restore p53 transcriptional activity. These findings contradict previous reports of the p53 suppression mediated by E4orf3 (Soria *et al*., 2010). The discrepancy in our conclusions may result from differences in the cell types used for infection, the earlier study used primary small epithelial airway cells; whereas we used fibroblasts IMR-90, HT1080 and HeLa cells, it could be possible that the basal expression of p53-regulated genes is lower in these cells. Recent studies of E4orf3 function have corroborated our findings, that while the E4orf3 protein contributes to inactivation of p53, it is unlikely to be sufficient to block p53 mediated transcription (DeHart *et al*., 2015; Vink *et al*., 2015). Our findings imply that one or more additional viral proteins contribute to this function, based on the results of the p53 luciferase assay we can suggest that E1A proteins are potentially working together with E4orf3 to block p53-dependent transcription from various promoters. However, the specific mechanism at play is yet to be discovered.

### 4.4 The Contributions of E1A C terminus on Adenovirus Replicative Cycle.

Adenovirus E1A relies on protein interactions with the cellular protein network to reprogram numerous aspects of cell function. Many of these interactions have been thoroughly studied, however functions mediated by the C terminus region of E1A have not been studied as extensively. In HAdV5 E1A, the C terminus region of E1A is encoded by the second exon of the gene which spans residues 187–289 and is conserved in all E1A isoforms, except for the 55 residue protein (Virtanen *et al*., 1983). Most studies of the C terminus of E1A have been in the context of interactions with host cell factor and the effects on cellular processes (Cohen *et al*., 2013; Yousef *et al*., 2012). Therefore, we wanted to examine the functions of the C terminus with regards to

viral replicative cycle. Using a series of viruses which had deletions in the C terminus of E1A we examined the contributions of this region to adenovirus replication, growth and induction of S-phase in arrested lung fibroblasts. We found that viral growth began sometime between 24 and 48 hours after infection for all mutants. Previous reports have also shown that in arrested cells virus release begins after the first 24 hours of the initial infection (Wang *et al*., 2006). Greatest differences in virus growth were observed at 48 hours, all mutant viruses lagging behind the wildtype *dl*309. However, by 96 hours most of the viruses reached similar growth levels, being only half a log lower than the wild type. These results could indicate that the C terminal regions mutated in these viruses may be involved in the initial events of viral replication, making the viruses deficient during the first 72 hours of infection. We observed that the *dl*311 mutants which is deleted for most of the exon 2 region, grew minimally throughout the duration of the assay. This confirms that the C terminal region is essential for efficient viral growth in host cells. Of all deletion mutants, *dl*1136 was the second worst replicating virus. This was unexpected as this mutant expressed most viral proteins to high levels and it was capable of efficient genome replication and S-phase induction. However, we found this mutant to be less efficient at inducing the expression of the S-phase specific genes, these results suggest that even minor transcriptional changes of cell cycle genes result in S-phase induction. Examination of morphological changes of infected cells showed a similar trend, cytopathic effect (CPE) started to become apparent at 48 and 72 hours for cells infected with most viruses, except for *dl*311 and *dl*1136 which showed minor changes at 96 and 120 hours respectively.

HAdV infects terminally differentiated epithelial cells, meaning these cells have exited the cell cycle and the pools of deoxyribonucleotides and DNA replication accessory factors are low (Hofer *et al*., 2012). These conditions, do not allow for efficient replication of viral genomes.

Consequently, HAdV must force quiescent cells to re-enter the cell cycle, the E1A protein plays an essential role in the induction of S-phase. We investigated how deletions in the C terminus of E1A affect viral genome replication in arrested fibroblasts. At 48 hours after infection we observed strong genome replication in all viruses, with all mutants lagging behind *dl*309 which expresses wild type E1A; this trend continued at 72 hours. Interestingly, *dl*311 had high levels of genome replication despite growing very poorly. We observed that there was a correlation between the total number of genomes and levels of the DBP protein. Previous literature has shown that the DBP protein is required for viral DNA replication and the regulation of viral gene expression (Brough *et al*., 1993). This suggests that while cellular factors are important for the initiation of viral gene expression, it is viral factors that dictate the efficiency and the accumulation of viral genomes within the infected host cell. Overall, we found that cells infected with the wild type virus *dl*309 had the highest expression of S-phase specific genes, in addition we observed that induction of these genes was highly variable between the different mutants. Furthermore, analysis of S-phase induction by analysis of DNA replication in infected cells showed that all of the C terminus mutant viruses had deficiencies in S-phase induction compared to the wild type. Interestingly, we found that the results of the EdU incorporation assay correlated with the levels of S-phase specific gene expression observed. These finding are exciting, as previous literature has shown that the CR1-CR2 regions of E1A are the major regions involved in the deregulation of E2F responsive genes including *BLM, PCNA* and *MCM4* that were examined. However, our results suggest that the C terminus of E1A may also be involved in cell cycle regulation and transcriptional control, this would be an interesting avenue to pursue in the future. Finally, we observed that the overall accumulation of viral genomes was not related to S-phase induction, these results correlate with

our previous observations that genome accumulation in the host cell seems to be highly dependent on viral protein levels, and less so on cellular factors.

Overall, we have found that the C terminus of E1A plays an important role in adenovirus replication, growth, gene expression, protein expression, the modulation of cellular S-phase specific genes and induction of the cell cycle in arrested normal diploid lung fibroblasts.

# 5. Summary of findings

## 5.1 Investigating the Interaction of Two Human Adenovirus Proteins E1A and E4orf3.

HAdV has been established as a useful model system to further our understanding of the molecular biology of cellular processes and viral infection. HAdV expresses early proteins which play an essential role in initiating the viral replicative cycle. My thesis project has revealed, for the first time, an interaction between E1A and the early protein E4orf3. We found this to be a direct interaction that occurs during the normal course of HAdV infection. Interestingly, we observed that E1A is localized to E4orf3 tracks within the nucleus. We also found that E4orf3 enhances E1A mediated transcription of viral promoters, this was reflected in increased expression of viral genes. In addition, it was determined that the absence of E4orf3 during infection reduces the levels of E1A enrichment at viral promoters. These results indicate that E1A and E4orf3 are collaborating to activate the expression of viral genes. Finally, our studies of E1A and E4orf3 in regard to p53 function revealed that while E4orf3 may contribute to the repression of p53 regulated genes, it is more likely that E1A and E4orf3 are working together to supress p53 transcriptional activity in order to prevent the activation of apoptotic pathways, thus conditioning the host cell for viral replication.

## 5.2 The Contributions of E1A C Terminus on Adenovirus Replicative Cycle.

The adenovirus E1A protein is responsible for the initiation of the viral replicative cycle after infection. The *E1A* gene is encoded at the left end of the viral genome and consists of two exons. The second exon or C terminus of E1A is involved in a variety of processes, however our understanding of how this region contributes to the viral life cycle is limited. Using small exon 2 deletions mutants we examined how the C terminus of E1A affects viral fitness during infection of primary cells WI-38. Overall, we found that the C terminus region was important for efficient

viral replication as all mutant viruses were deficient in growth compared to *dl*309, which expresses wild-type E1A. In addition, we observed that viral growth correlated with morphological changes observed in infected cells. We found that all mutants had lower levels of viral gene expression and lower levels of viral DNA replication compared to *dl*309. Furthermore, deletions within the C terminus affected the ability of the virus to alter the expression of cellular S-phase specific genes and the overall induction of the cell cycle in arrested fibroblasts. Our studies are the first comprehensive analysis examining how the different regions of E1A C terminus contribute to the viral replicative cycle in arrested human cells.

## 6. References


**Alonso-Padilla J, Papp T, Kaján GL, Benkő M, Havenga M, Lemckert A, Harrach B & Baker AH.** 2016. Development of novel adenoviral vectors to overcome challenges observed with HAdV-5–based constructs. Molecular Therapy 24(1):6-16.

**Anderson CW, Young ME & Flint SJ.** 1989. Characterization of the adenovirus 2 virion protein, mu. Virology 172:506-512.

**Avvakumov N, Kajon AE, Hoeben RC, Mymryk JS.** 2004. Comprehensive sequence analysis of the E1A proteins of human and simian adenoviruses. Virology 329:477–92.

**Avvakumov, N, Sahbegovic M, Zhang Z, Shuen M & Mymryk JS.** 2002. Analysis of DNA binding by the adenovirus type 5 E1A oncoprotein. J Gen Virol 83(Pt 3): 517-24.

**Barker DD & Berk AJ.** 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology 156(1):107-21.

**Baron, S, Fons, M & Albrecht, T.** 1996. Viral Pathogenesis. Med Microbiol. University of Texas

**Bayley, ST & Mymryk, JS.** 1994. Adenovirus E1A proteins and transformation (review). International Journal of Oncology 5, 425–444.

**Bailey A, & Mautner V.** 1994. Phylogenetic relationships among adenovirus serotypes. Virology 205(2):438-52.

**Berk AJ.** 2005.Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. Oncogene 24(52):7673.

**Berk AJ.** 2007. Adenoviridae: The Viruses and Their Replication, p. 2355–2394. In Knipe, DM, Howley, PM (eds.), Fields' Virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

**Berk AJ, Lee F, Harrison T, Williams J & Sharp PA.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell 17, 935–944.

**Binger MH & Flint SJ.** 1984. Accumulation of early and intermediate mRNA species during subgroup C adenovirus productive infections. Virology 136(2):387–403.

**Blackford AN & Grand RJ.** 2009. Adenovirus E1B 55-kilodalton protein: multiple roles in viral infection and cell transformation. Journal of virology, 83(9), pp.4000-4012.

**Boyd JM, Loewenstein PM, Tang Qq, Yu L & Green M.** 2002. Adenovirus E1A N-terminal amino acid sequence requirements for repression of transcription in vitro and in vivo correlate with those required for E1A interference with TBP-TATA complex formation. J. Virol. 76**:**1461-1474.

**Boyd JM, Subramanian T, Schaeper U, La Regina M, Bayley S & Chinnadurai G.** 1993. A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. The EMBO journal 12(2):469-78.

**Boyer J, Rohleder K & Ketner G.** 1999. Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology 263(2):307-12.

**Branton PE & Roopchand DE.** 2001. The role of adenovirus E4orf4 protein in viral replication and cell killing. Oncogene 20, 7855–7865.

**Brenkman AB, Breure EC, Van der Vliet PC**. 2002. Molecular architecture of adenovirus DNA polymerase and location of the protein primer. J Virol 76: 8200–8207

**Brestovitsky, A, Nebenzahl-Sharon, K, Kechker, P, Sharf, R & Kleinberger, T.** 2016. The Adenovirus E4orf4 Protein Provides a Novel Mechanism for Inhibition of the DNA Damage Response. PLoS Pathogens, 12(2), e1005420.

**Brough DE, Droguett G, Horwitz MS & Klessig DF.** 1993. Multiple functions of the adenovirus DNA-binding protein are required for efficient viral DNA synthesis. Virology 196(1):269-81.

**Burgert HG, Maryanski JL & Kvist S.** 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. Proceedings of the National Academy of Sciences of the United States of America 84:1356–60.

**Caporossi D, Bacchetti S.** 1990. Definition of adenovirus type 5 functions involved in the induction of chromosomal aberrations in human cells. Journal of general virology 71(4):801-8.

**Caravokyri C & Leppard KN.** 1996. Human adenovirus type 5 variants with sequence alterations flanking the E2A gene: effects on E2 expression and DNA replication. Virus genes **12:**65-75.

**Carvalho T, Seeler JS, Ohman K, Jordan P, Pettersson U, Akusjärvi G, Carmo-Fonseca M & Dejean A.** 1995. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. The Journal of cell biology 131(1):45-56.

**Chatterjee, PK, Bruner M, Flint SJ & Harter ML.** 1988. DNA-binding properties of an adenovirus 289R E1A protein. Embo J 7(3): 835-41.

**Chen C & Wells AD.** 2007. Comparative analysis of E2F family member oncogenic activity. PLoS One 2:e912.

**Chinnadurai G.** 2004. Modulation of oncogenic transformation by the human adenovirus E1A C-terminal region. In Adenoviruses: Model and Vectors in Virus-Host Interactions Springer, Berlin, Heidelberg.

**Chu W & Pavan-Langston D.** 1979. Ocular surface manifestations of the major viruses Int. Ophthalmol. Clin., 19 (2), pp. 135-167.

**Cohen MJ, Yousef AF, Massimi P, Fonseca GJ, Todorovic B, Pelka P, Turnell AS, Banks L, Mymryk JS.** 2013. Dissection of the C-terminal region of E1A redefines the roles of CtBP and other cellular targets in oncogenic transformation. Journal of virology 87(18):10348-55.

**Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT & Bergelson JM**. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction Proc. Natl. Acad. Sci. USA, 98 pp. 15191-15196

**Culp JS, Webster LC, Friedman DJ, Smith CL, Huang WJ, Wu FY, Rosenberg M & Ricciardi RP.** 1988. The 289-amino acid E1A protein of adenovirus binds zinc in a region that is important for trans-activation. Proceedings of the National Academy of Sciences of the United States of America 85:6450–4.

**Dallaire F, Schreiner S, Blair GE, Dobner T, Branton PE & Blanchette P.** 2016. The human adenovirus type 5 E4orf6/E1B55K E3 ubiquitin ligase complex enhances E1A functional activity. MSphere 1(1):e00015-15.

**Daniell, E, Groff, DE & Fedor, MJ.** 1981. Adenovirus chromatin structure at different stages of infection. Molecular and cellular biology 1, 1094-1105.

**DeHart CJ, Perlman DH & Flint SJ.** 2015. Impact of the adenoviral E4 Orf3 protein on the activity and posttranslational modification of p53. Journal of virology 89(6):3209-20.

**De Jong RN, Meijer LAT & van der Vliet PC.** 2003 .DNA binding properties of the adenovirus DNA replication priming protein pTP. Nucleic Acids Res 31: 3274–3286. pmid:12799455

**De Luca A, Mangiacasale R, Severino A, Malquori L, Baldi A, Palena A, Mileo AM, Lavia P & Paggi MG.** 2003. E1A deregulates the centrosome cycle in a Ran GTPase-dependent manner. Cancer Research 63(6):1430-7.

**Dingle JH & Langmuir AD.** 1968. Epidemiology of acute, respiratory disease in military recruits Am. Rev. Respir. Dis., 97 (6), pp. 1-65

**Dix, I, & Leppard, KN.** 1995. Expression of adenovirus type 5 E4 Orf2 protein during lytic infection. The Journal of General Virology, 76 ( Pt 4)(4), 1051–5.

**Dobner, T, Horikoshi, N, Rubenwolf, S, & Shenk, T.** 1996. Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. Science, 272(5267), 1470–3.

**Doucas V, Ishov AM, Romo A, Juguilon H, Weitzman MD, Evans RM & Maul GG.** 1996. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. Genes & development 10(2):196-207.

**Eggerding FA & Pierce WC.** 1986. Molecular biology of adenovirus type 2 semipermissive infections I. Viral growth and expression of viral replicative functions during restricted adenovirus infection. Virology, 148(1), 97-113

**Evans, JD & Hearing, P.** 2003. Distinct Roles of the Adenovirus E4 ORF3 Protein in Viral DNA Replication and Inhibition of Genome Concatenation. Journal of Virology, 77(9), 5295–5304.

**Evans JD & Hearing P.** 2005. Relocalization of the Mre11-Rad50-Nbs1 complex by the adenovirus E4 ORF3 protein is required for viral replication. Journal of virology 79(10):6207-15.

**Fattaey, AR, Harlow E & Helin K.** 1993. Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. Mol. Cell. Biol. 137267-7277.

**Fax P, Carlson CR, Collas P, Taskén K, Esche H & Brockmann D.** 2001. Binding of PKA-RIIα to the adenovirus E1A12S oncoprotein correlates with its nuclear translocation and an increase in PKA-dependent promoter activity. Virology 285(1):30-41.

**Ferguson B, Krippl B, Andrisani O, Jones N, Westphal H & Rosenberg M.** 1985. E1A 13S and 12S mRNA products made in Escherichia coli both function as nucleus-localized transcription activators but do not directly bind DNA. Molecular and cellular biology 5:2653–61.

**Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ & Kurdistani SK.** 2008. Epigenetic reprogramming by adenovirus e1a. Science 321, 1086–1088.

**Ferreon JC, Martinez-Yamout MA, Dyson HJ, Wright PE.** 2009. Structural basis for subversion of cellular control mechanisms by the adenoviral E1A oncoprotein. Proceedings of the National Academy of Sciences 106(32):13260-5.

**Fessler SP & Young CSH.** 1998. Control of Adenovirus Early Gene Expression during the Late Phase of Infection. Journal of Virology, 72(5), 4049–4056.

**Fey EG, Wan KM & Penman S.** 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. The Journal of Cell Biology 98(6):1973-84.

**Flinterman MB, Mymryk JS, Klanrit P, Yousef AF, Lowe SW, Caldas C, Gäken J, Farzaneh F, Tavassoli M.** 2007. p400 function is required for the adenovirus E1A-mediated suppression of EGFR and tumour cell killing. Oncogene (48):6863.

**Frese KK, Lee SS, Thomas DL, Latorre IJ, Weiss RS, Glaunsinger BA & Javier RT**. 2003. Selective PDZ protein-dependent stimulation of phosphatidylinositol 3-kinase by the adenovirus E4-ORF1 oncoprotein. Oncogene 22:710-721.

**Frisch SM & Mymryk JS.** 2002. Adenovirus-5 E1A: paradox and paradigm. Nat Rev Mol Cell Biol 3(6): 441-52.

**Frost JR, Olanubi O, Cheng SK, Soriano A, Crisostomo L, Lopez A & Pelka P.** 2017. The interaction of adenovirus E1A with the mammalian protein Ku70/XRCC6. Virology 500:11-21.

**Frost JR, Mendez M, Soriano AM, Crisostomo L, Olanubi O, Radko S & Pelka P.** 2018. Adenovirus 5 E1A-mediated suppression of p53 via FUBP1. Journal of virology JVI-00439.

**Gallimore, PH.** 1972. Tumour production in immunosuppressed rats with cells transformed in vitro by adenovirus type 2. The Journal of General Virology, 16(1), 99-102.

**Gallimore PH & Turnell AS.** 2001. Adenovirus E1A: remodelling the host cell, a life or death experience. Oncogene 20: 7824-7835

**Glenn GM & Ricciardi RP.**1985. Adenovirus 5 early region 1A host range mutants hr3, hr4, and hr5 contain point mutations which generate single amino acid substitutions. Journal of virology 56(1):66-74.

**Goodrum FD & Ornelles DA.** 1999. Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. Journal of virology 73(9):7474-88.

**Graham FL, Smiley J, Russell WC & Nairn R.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. The Journal of general virology 36:59–74.

**Gulbahce N, Yan H, Dricot A, Padi M, Byrdsong D, Franchi R, Lee DS, Rozenblatt-Rosen O, Mar JC, Calderwood MA & Baldwin A.** 2012. Viral perturbations of host networks reflect disease etiology. PLoS computational biology 8(6):e1002531.

**Han J, Sabbatini P, Perez D, Rao L, Modha D &White E.** 1996. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and deathpromoting Bax protein. Genes & development **10:**461-477

**Hay RT**. 1966. Adenovirus DNA replication. In DNA replication in eukaryotic cells Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Hay RT, Freeman A, Leith I, Monaghan A & Webster A**. 1995. Molecular interactions during adenovirus DNA replication. In Molecular Repertoire of Adenoviruses II, Current Topics in Microbiology and Immunology Vol. 199, pp. 31 – 48. Springer, Berlin.

**Halbert DN, Cutt JR & Shenk T.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. Journal of Virology 56, 250–257.

**Hateboer G, Gennissen A, Ramos YF, Kerkhoven RM, Sonntag-Buck V, Stunnenberg HG, Bernards R.** 1995. BS69, a novel adenovirus E1A-associated protein that inhibits E1A transactivation. The EMBO journal 14(13):3159-69.

**Hateboer G, Kerkhoven RM, Shvarts A, Bernards R, Beijersbergen RL.** 1996. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes & development 10(23):2960-70.

**Hawkins LK & Wold WS.** 1992. A 12,500 MW protein is coded by region E3 of adenovirus. Virology 188(2): 486-94.

**Hilleman MR & Werner JR.** 1954. Recovery of a new agent from patients with acute respiratory illness.Proceedings of the Society for Experimental Biology and Medicine 85, 183-188.

**Hoeben RC & Uil TG.** 2013. Adenovirus DNA Replication. Cold Spring Harbor Perspectives in Biology, 5(3), a013003.

**Hofmayer S, Madisch I, Darr S, Rehren F & Heim A.** 2009. Unique sequence features of the Human Adenovirus 31 complete genomic sequence are conserved in clinical isolates. BMC genomics 10(1):557.

**Horwitz MS.** 2004. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. The journal of gene medicine **6 Suppl 1:**S172-183.

**Horwitz GA, Zhang K, McBrian MA, Grunstein M, Kurdistani SK & Berk AJ.** 2008. Adenovirus small e1a alters global patterns of histone modification. Science (New York, N.Y.) 321, 1084–1085.

**Huang MM & Hearing P.** 1989.Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. Journal of virology 63(6):2605-15.

**Ikeda MA & Nevins JR.** 1993. Identification of distinct roles for separate E1A domains in disruption of E2F complexes. Mol. Cell. Biol. 137029-7035.

**Ishov AM, Sotnikov AG, Negorev D, Vladimirova OV, Neff N, Kamitani T, Yeh ET, Strauss JF & Maul GG.** 1999. PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. The Journal of cell biology. 147(2):221-34.

**Jelsma TN, Howe JA, Mymryk JS, Evelegh CM, Cunniff NF, Bayley ST.** 1989. Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. Virology 171(1):120-30.

**Jones N & Shenk T.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell 17, 683–689.

**Jung R, Radko S & Pelka P.** 2016. The dual nature of Nek9 in adenovirus replication. Journal of virology 90(4):1931-43.

**Kaufman RJ.** 1985. Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors. Proceedings of the National Academy of Sciences 82(3):689-93.

**Kidd, AH, Garwicz, D & Oberg, M.** 1995. Human and simian adenoviruses: phylogenetic inferences from analysis of VA RNA genes. Virology 207, 32-45.

**Kimelman, D, Miller JS, Porter D & Roberts BE.** 1985. E1A regions of the human adenoviruses and of the highly oncogenic simian adenovirus 7 are closely related. Journal of Virology 53, 399–409.

**King AJ, Teertstra WR, Van der Vliet PC.** 1997. Dissociation of the protein primer and DNA polymerase after initiation of adenovirus DNA replication. J Biol Chem 272: 24617–24623

**King CR, Zhang A, Tessier TM, Gameiro SF & Mymryk JS.** 2018. Hacking the Cell: Network Intrusion and Exploitation by Adenovirus E1A. mBio 9(3):e00390-18.

**Klingseisen L, Ehrenschwender M, Heigl U, Wajant H, Hehlgans T, Schütze S & Schneider-Brachert W.** 2012. E3-14.7K is recruited to TNF-receptor 1 and blocks TNF cytolysis independent from interaction with optineurin. PloS One 7, e38348.

**KoVESDI IM, Reichel R & Nevins JR.** 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proceedings of the National Academy of Sciences. 84(8):2180-4.

**Komorek J, Kuppuswamy M, Subramanian T, Vijayalingam S, Lomonosova E, Zhao LJ, Mymryk JS, Schmitt K, Chinnadurai G.** 2010. Adenovirus type 5 E1A and E6 proteins of low-risk cutaneous beta-human papillomaviruses suppress cell transformation through interaction with FOXK1/K2 transcription factors. Journal of virology 84(6):2719-31.

**Köhler M, Görlich D, Hartmann E, Franke J.** 2001. Adenoviral E1A protein nuclear import is preferentially mediated by importin α3 in vitro. Virology. 2001 Oct 25;289(2):186-91.

**Kümin D, Hofmann C, Rudolph M, Both GW & Löser P.** 2002. Biology of ovine adenovirus infection of nonpermissive cells. J. Virol., 76, pp. 10882-10893

**Leber SM, Yamagata M & Sanes JR.** 1996. Gene Transfer Using Replication-Defective Retroviral and Adenoviral Vectors In Methods in cell biology Vol. 51, pp. 161-183. Academic Press.

**Lewis, PF.** 2009. A community-based outbreak of severe respiratory illness caused by human adenovirus serotype 14. J Infect Dis.199(10):1427-34.

**Lichtenstein DL, Toth K, Doronin K, Tollefson AE & Wold WS.** 2004. Functions and mechanisms of action of the adenovirus E3 proteins. Int Rev Immunol. 23(1-2):75-111.

**Lillie, JW & Green MR.**1986. An adenovirus E1a protein region required for transformation and transcriptional repression. Cell 46(7): 1043-51.

**Lillie JW & Green MR.** 1989. Transcription activation by the adenovirus E1a protein. Nature. 338(6210):39.

**Liu H, Naismith JH & Hay RT.** 2003. Adenovirus DNA replication. Curr Top Microbiol 272:131-164.

**Loewenstein PM, Arackal S & Green M.** 2006.Mutational and functional analysis of an essential subdomain of the adenovirus E1A N-terminal transcription repression domain. Virology 351(2):312-21.

**Lutz PI, Rosa-Calatrava MA & Kedinger CL.** 1997. The product of the adenovirus intermediate gene IX is a transcriptional activator. Journal of virology 71(7):5102-9.

**Mackey JK, Rigden PM & Green M.** 1976. Do highly oncogenic group A human adenoviruses cause human cancer? Analysis of human tumors for adenovirus 12 transforming DNA sequences. Proceedings of the National Academy of Sciences of the United States of America 73, 4657-4661.

**Matthews DA & Russell WC.** 1998. Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. J. Gen. Virol. 79**:**1671-1675.

**Martin KJ, Lillie JW & Green MR.** 1990. Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets.Nature 346**:**147-152.

**Marton MJ, Baim SB, Ornelles DA & Shenk T.** 1990. The adenovirus E4 17kilodalton protein complexes with the cellular transcription factor E2F, altering its DNA-binding properties and stimulating E1A-independent accumulation of E2 mRNA. Journal of Virology, 64(5), 2345–59.

**Maul GG.** 1998. Nuclear domain 10, the site of DNA virus transcription and replication. Bioessays 20(8):660-7.

**Medina-Kauwe LK**. 2003. Endocytosis of adenovirus and adenovirus capsid proteins Adv. Drug Deliv. Rev, 55 (2003), pp. 1485-1496.

**Miller DL, Myers CL, Rickards B, Coller HA & Flint SJ.** 2007. Adenovirus type 5 exerts genome-wide control over cellular programs governing proliferation, quiescence, and survival. Genome biology 8**:**R58.

**Mishoe H, Brady JN, Lancz G & Salzman NP.** 1984. In vitro transcription initiation by purified RNA polymerase II within the adenovirus 2 major late promoter region. The Journal of biological chemistry 259, 2236-2242.

**Morris SJ, Scott GE & Leppard KN.** 2010. Adenovirus late-phase infection is controlled by a novel L4 promoter. Journal of virology 84(14):7096-104.

**Muller S & Dobner T.** 2008. The adenovirus E1B-55K oncoprotein induces SUMO modification of p53. Cell Cycle 7, 754–758.

**Mymryk JS.** 1996.Tumour suppressive properties of the adenovirus 5 E1A oncogene. Oncogene.1581-9.

**Nicklin SA, Wu E, Nemerow GR & Baker AH.** 2005. The influence of adenovirus Fiber structure and function on vector development for gene therapy. Molecular therapy : the journal of the American Society of Gene Therapy **12:**384-393.

**Obert S, O'Connor RJ, Schmid S & Hearing P.** 1994. The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Molecular and Cellular Biology, 14(2), 1333–46.

**Olanubi O, Frost JR, Radko S & Pelka P.** 2017. Suppression of type I interferon signaling by E1A via RuvBL1/Pontin. Journal of virology 91(8):e02484-16.

**O'Malley RP, Mariano TM, Siekierka J & Mathews MB**. 1986. A mechanism for the control of protein synthesis by adenovirus VA RNAI. Cell, 44:391–400.

**Obert S, O'Connor RJ, Schmid S & Hearing P.** 1994. The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Molecular and cellular biology 14:1333–46.

**Ou HD, Kwiatkowski W, Deerinck TJ, Noske A, Blain KY, Land HS, Soria C, Powers CJ, May AP, Shu X & Tsien RY.** 2012. A structural basis for the assembly and functions of a viral polymer that inactivates multiple tumor suppressors. Cell 151(2):304-19.

**Parker EJ, Botting CH, Webster A & Hay RT.** 1998. Adenovirus DNA polymerase: domain organisation and interaction with preterminal protein. Nucleic Acids Res 26: 1240–1247. pmid:9469832

**Patsalo V, Yondola MA, Luan B, Shoshani I, Kisker C, Green DF, Raleigh DP & Hearing P.** 2012. Biophysical and functional analyses suggest that adenovirus E4-ORF3 protein requires higher-order multimerization to function against promyelocytic leukemia protein nuclear bodies. Journal of Biological Chemistry. 287(27):22573-83.

**Pelka P, Ablack JN, Fonseca GJ, Yousef AF & Mymryk, JS.** 2008. Intrinsic structural disorder in adenovirus E1A: a viral molecular hub linking multiple diverse processes. Journal of virology, 82(15), pp.7252-7263.

**Pelka P, Ablack JN, Torchia J, Turnell AS, Grand RJ & Mymryk JS.** 2009. Transcriptional control by adenovirus E1A conserved region 3 via p300/CBP. Nucleic acids research 37(4):1095-106.

**Pelka P, Miller MS, Cecchini M, Yousef AF, Bowdish DM, Dick F, Whyte P & Mymryk JS.** 2011. Adenovirus E1A directly targets the E2F/DP-1 complex. Journal of virology **85:**8841-8851.

**Pelka P, Scimè A, Mandalfino C, Joch M, Abdulla P & Whyte P.** 2007. Adenovirus E1A proteins direct subcellular redistribution of Nek9, A NimA-related kinase. Journal of cellular physiology. Jul 1;212(1):13-25.

**Pennella MA, Liu Y, Woo JL, Kim CA & Berk AJ**. 2010. Adenovirus E1B 55-kilodalton protein is a p53-SUMO1 E3 ligase that represses p53 and stimulates its nuclear export through interactions with promyelocytic leukemia nuclear bodies. J Virol 84(23), 12210-25.

**Querido E, Marcellus RC, Lai A, Charbonneau R, Teodoro JG, Ketner G & Branton PE.** 1997. Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J. Virol. 71**:**3788-3798.

**Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D & Branton, PE.** 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes & Development, 15(23), 3104–17

**Radko S, Jung R, Olanubi O &Pelka P.** 2015.Effects of adenovirus type 5 E1A isoforms on viral replication in arrested human cells. PloS one 10(10):e0140124.

**Radko S, Koleva M, James KM, Jung R, Mymryk JS & Pelka P.** 2014. Adenovirus E1A targets the DREF nuclear factor to regulate virus gene expression, DNA replication, and growth. Journal of virology 88(22):13469-81.

**Rao L, Debbas M, Sabbatini P, Hockenbery D, Korsmeyer S & White E.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl Acad. Sci. USA 89, 7742 – 7746

**Rasti MR, Grand JS, Mymryk PH, Gallimore & Turnell AS.** 2005. Recruitment of CBP/p300, TATA-binding protein, and S8 to distinct regions at the N terminus of adenovirus E1A. J. Virol. 79**:**5594-5605.

**Rekosh DM, Russell WC, Bellet AJ & Robinson AJ.** 1977 . Identification of a protein linked to the ends of adenovirus DNA. Cell 11, 283-295

**Romero P, Obradovic Z & Dunker AK.** 2004. Natively disordered proteins: functions and predictions. Appl. Bioinformatics 3**:**105-113.

**Rosen, L.** 1960. A hemagglutination-inhibition technique for typing adenoviruses. Am J Hyg 71, 120-128.

**Rowe WP, Huebner RJ, Gilmore LK, Parrott RH &Ward TG.** 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine **84:**570-573.

**Rozenblatt-Rosen O, Deo RC, Padi M, Adelmant G, Calderwood MA, Rolland T, Grace M, Dricot A, Askenazi M, Tavares M & Pevzner SJ.** 2012. Interpreting cancer genomes using systematic host network perturbations by tumour virus proteins. Nature 487(7408):491.

**Russell WC.** 2009. Update on adenovirus and its vectors. The Journal of general virology 90: 1-20

**Saha B, Wong C & Parks RJ**. 2014. The adenovirus genome contributes to the structural stability of the virion. Viruses, 6(9), 3563-83.

**Saha B & Parks RJ.** 2017. Human adenovirus type 5 vectors deleted of early region 1 (E1) undergo limited expression of early replicative E2 proteins and DNA replication in non-permissive cells. PloS one, 12(7), p.e0181012.

**Sandler AB & Ketner GA.** 1989. Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. Journal of virology 63(2):624-30.
**San Martin, C.** (2012). Latest insights on adenovirus structure and assembly. Viruses 4, 847-877

**Sarantis H, Johnson G, Brown M, Petric M & Tellier R.** 2004. Comprehensive detection and serotyping of human adenoviruses by PCR and sequencing. Journal of clinical microbiology **42:**3963-3969.

**Shan B, Lee WH.** 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. 14:8166–8173

**Shayakhmetov DM, Li ZY, Gaggar A, Gharwan H, Ternovoi V, Sandig V & Lieber A.** 2004. Genome size and structure determine efficiency of postinternalization steps and gene transfer of capsid-modified adenovirus vectors in a cell-type-specific manner. J Virol. 78(18):10009-22.

**Shenk T.** 1996. Adenoviridae: The Viruses and Their Replication, p. 979–1016. In Fields, B, Knipe, D, Howley, P. Fundamental Virology, 2nd ed. Lippincott Williams & Wilkins, Philadelphia, P.A

**Shenk T & Flint J.** 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. Advances in cancer research **57:**47-85.

**Shepard RN & Ornelles DA.** 2003. E4orf3 is necessary for enhanced S-phase replication of cell cycle-restricted subgroup C adenoviruses. Journal of virology 77(15):8593-5.

**Smith JG, Wiethoff CM, Stewart PL & Nemerow GR.** 2010. Adenovirus. Current Topics in Microbiology and Immunology, 343, 195–224.

**Sohn SY & Hearing P.** 2012. Adenovirus regulates SUMOylation of Mre11-Rad50Nbs1 components through a paralog-specific mechanism. Journal of Virology 86, 9656–9665.

**Sohn SY & Hearing P.** 2016. The adenovirus E4-ORF3 protein functions as a SUMO E3 ligase for TIF-1γ sumoylation and poly-SUMO chain elongation. Proceedings of the National Academy of Sciences 113(24):6725-30.

**Soria C, Estermann FE, Espantman KC & O'Shea CC.** 2010. Heterochromatin silencing of p53 target genes by a small viral protein. Nature 466(7310):1076.

**Stillman BW, Tamanoi F & Mathews MB.** 1982. Purification of an adenoviruscoded DNA polymerase that is required for initiation of DNA replication. Cell 31:613–23.

**Stephens C & Harlow E.** 1987. Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. EMBO J. 6(7):2027–35.

**Stracker TH, Carson CT & Weitzman MD.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature, 418(6895), 348– 52.

**Stracker TH, Lee DV, Carson CT, Araujo FD, Ornelles DA & Weitzman MD.** 2005. Serotype-specific reorganization of the Mre11 complex by adenoviral E4orf3 proteins. Journal of virology. 79(11):6664-73.

**Stunnenberg HG, Lange H, Philipson L, van Miltenburg RT, van der Vliet PC**. 1988. High expression of functional adenovirus DNA polymerase and precursor terminal protein using recombinant vaccinia virus. Nucleic Acids Res 16: 2431–2444.

**Täuber B & Dobner T., 2001.** Adenovirus early E4 genes in viral oncogenesis. Oncogene, 20(54) p.7847.

**Thai M, Graham NA, Braas D, Nehil M, Komisopoulou E, Kurdistani SK & Christofk HR.** 2014. Adenovirus E4ORF1-induced MYC activation promotes host cell anabolic glucose metabolism and virus replication. Cell Metabolism, 19(4), 694–701.

**Tyler RE, Ewing SG & Imperiale MJ**. 2007. Formation of a multiple protein complex on the adenovirus packaging sequence by the IVa2 protein. Journal of virology 81, 3447-3454

**Tollefson AE, Scaria A, Saha SK & Wold WS.** 1992. The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection. Journal of Virology 66, 3633–3642.

**Tollefson AE, Toth K, Doronin K, Kuppuswamy M, Doronina OA, Lichtenstein DL, Hermiston TW, Smith CA & Wold WS.** 2001. Inhibition of TRAIL-induced apoptosis and forced internalization of TRAIL receptor 1 by adenovirus proteins. Journal of virology 75:8875–87.

**Trentin JJ, Yabe Y & Taylor G.** 1962. The quest for human cancer viruses. Science **137:**835-841.

**Ulfendahl PJ, Linder S, Kreivi JP, Nordqvist K, Sevensson C & Hultberg H.** 1987. A novel adenovirus-2 E1A mRNA encoding a protein with transcription activation properties. EMBO J. 6(7):2037–44.

**Ullman AJ, Reich NC & Hearing P.** 2007. Adenovirus E4 ORF3 protein inhibits the interferon-mediated antiviral response. Journal of virology 81(9):4744-52.

**Van Oostrum J & Burnett RM**. 1985. Molecular composition of the adenovirus type 2 virion. Journal of virology 56, 439-448.

**Vellinga J, Van der Heijdt S & Hoeben RC.** 2005. The adenovirus capsid: major progress in minor proteins. The Journal of general virology **86:**1581-1588.

**Vink EI, Zheng Y, Yeasmin R, Stamminger T, Krug LT & Hearing P.** 2015. Impact of Adenovirus E4-ORF3 Oligomerization and Protein Localization on Cellular Gene Expression. Viruses. 7(5):2428-2449.

**Virtanen A, Pettersson U.** 1983. The molecular structure of the 9S mRNA from early region 1A of adenovirus serotype 2. J. Mol. Biol. 165:496–499.

**Walsh MP, Seto J, Liu EB, Dehghan S, Hudson NR, Lukashev AN, Ivanova O, Chodosh J, Dyer DW, Jones MS & Seto D.** 2011. Computational analysis of two species C human adenoviruses provides evidence of a novel virus. Journal of clinical microbiology 49(10):3482-90.

**Wang ZG, Zhao W, Ramachandra M & Seth P.** 2006. An oncolytic adenovirus expressing soluble transforming growth factor-β type II receptor for targeting breast cancer: in vitro evaluation. Molecular cancer therapeutics. 5(2):367-73.

**Weiss RS, Lee SS, Prasad BV & Javier RT.** 1997. Human adenovirus early region 4 open reading frame 1 genes encode growth-transforming proteins that maybe distantly related to dUTP pyrophosphatase enzymes. Journal of Virology 71, 1857–1870.

**Weiden MD & Ginsberg HS.** 1994. Deletion of the E4 region of the genome produces adenovirus DNA concatemers. Proceedings of the National Academy of Sciences 91(1):153-7.

**Weitzman MD, Ornelles DA.** 2005. Inactivating intracellular antiviral responses during adenovirus infection. Oncogene 24:7686–7696

**Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E.** 1998. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334(6178):124.

**White E, Spector D & Welch W.** 1988. Differential distribution of the adenovirus E1A proteins and colocalization of E1A with the 70-kilodalton cellular heat shock protein in infected cells. Journal of virology, 62(11), pp.4153-4166.

**White E & Cipriani R.** 1990. Role of Adenovirus-E1b Proteins in Transformation - Altered Organization of Intermediate Filaments in Transformed-Cells That Express the 19-Kilodalton Protein. Mol Cell Biol **10:**120-130.

**Wickham TJ, Mathias P, Cheresh DA & Nemerow GR.** 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment
Cell, 73 (1993), pp. 309-319

**Wigand R & Meiser W.** 1969. Neutralization and adenoviruses by component antisera.
(Brief report). Archiv fur die gesamte Virusforschung **27:**112-114.

**Wodrich, H, Cassany A, D'Angelo MA, Guan T, Nemerow G & Gerace L.** 2006. Adenovirus core protein pVII is translocated into the nucleus by multiple import receptor pathways. *J Virol* **80**, 9608–9618.

**Wodrich, H, Guan T, Cingolani G, Von Seggern D, Nemerow G & Gerace L.** 2003. Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. The EMBO journal 22, 6245-6255.

**Wold WS, Tollefson AE & Hermiston TW.** 1995. E3 transcription unit of adenovirus. Current topics in microbiology and immunology 199, Pt 1:237–74.

**Wood DJ.** 1988. Adenovirus gastroenteritis.(Brief report). Med. J. (Clin. Res. Ed.), 296 (6617), pp. 229-23.

**Wu E & Nemerow GR.** 2004. Virus yoga: the role of flexibility in virus host cell recognition. Trends in microbiology **12:**162-169.

**Yatherajam G, Huang W & Flint SJ.** 2011. Export of adenoviral late mRNA from the nucleus requires the Nxf1/Tap export receptor. Journal of Virology 85, 1429– 1438.

**Yondola MA & Hearing P.** 2007. The adenovirus E4 ORF3 protein binds and reorganizes the TRIM family member transcriptional intermediary factor 1 alpha. Journal of virology. 15;81(8):4264-71.

**Yousef AF, Fonseca GJ, Cohen MJ & Mymryk JS.** 2012. The C-terminal region of E1A: A molecular tool for cellular cartography. Biochem. Cell Biol 90:153–163.

**Zhang Z, Smith MM & Mymryk JS.** 2001. Interaction of the E1A oncoprotein with Yak1p, a novel regulator of yeast pseudohyphal differentiation, and related mammalian kinases. Molecular biology of the cell 12(3):699-710.

**Zhang X, Turnell AS, Gorbea C, Mymryk JS, Gallimore PH, Grand RJ.** 2004. The targeting of the proteasomal regulatory subunit S2 by adenovirus E1A causes inhibition of proteasomal activity and increased p53 expression. Journal of Biological Chemistry 279(24):25122-33.

**Zu YL,Takamatsu Y, Zhao MJ, Maekawa T, Handa H & Ishii S.** 1992. Transcriptional regulation by a point mutant of adenovirus-2 E1a product lacking DNA binding activity. J Biol Chem 267(28): 20181-7.

**Zubieta C, Schoehn G, Chroboczek J & Cusack S**. 2005. The structure of the human adenovirus 2 penton. Molecular Cell, 17(1), 121-135.