Analyzing the Interaction of Human Adenovirus E1A Oncoprotein with the Cellular Proteins Ku70 and FUBP1

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To my parents, Jacqueline and Wesley Frost, who have always encouraged and supported me in everything I do.

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Abstract

Early region 1A proteins (E1A) are the first proteins expressed upon viral infection. E1A is therefore responsible for the remodeling of the intracellular environment to promote viral replication. It is also the major transactivator of viral early gene expression. E1A carries out its functions predominantly by binding to cellular regulatory proteins and altering their activities. The unstructured nature of E1A enables it to bind to a variety of proteins and form new molecular complexes with novel functions. While there are only a handful of known binding partners to the E1A C-terminus, it has recently been shown that Ku70 and Far Upstream Element Binding Protein 1 (FUBP1) are novel E1A binding proteins.

Ku70, a component of the Non-Homologous End Joining and DNA damage response pathway is important for viral growth as depletion leads to decreased viral growth. My studies also show that Ku70 is localized to viral replication centers, Ku70 associates with the viral genome, and is recruited to cellular cell cycle regulated promoters upon infection.

FUBP1, a protein first identified as a regulator of *c-Myc* transcription also has implications for viral proliferation. Upon infection FUBP1 is re-localized to punctate nuclear structure of unclear function and it binds to viral mRNAs.

My results show that these proteins are critical for efficient viral growth and proliferation, with implications for understanding virally-induced carcinogenesis.

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List of Abbreviations

Adenovirus	AdV
Chromatin Immunoprecipitation	ChIP
Co-Immunoprecipitation	Co-IP
Complementary DNA	cDNA
Conserved region	CR
C-terminal binding protein	CtBP
Cyclin Dependent Kinase	CDK
DNA Binding Protein	DBP
Endoplasmic Reticulum	ER
Far Upstream element Binding Protein 1	FUBP1
HAdV	Human Adenovirus
Hepatitis C Virus	HCV
Immunoglobulin	Ig
Internal Ribosomal Entry Site	IRES
Japanese Encephalitis Virus	JEV
K-homology domain	KH
Kilo Dalton	kDa
Kilobase pairs	kb
Non-homologous End Joining pathway	NHEJ
Nuclear Pore Complex	NPC
Nucleophosmin	NPM
Precursor Terminal Protein	pTP
Retinoblastoma Protein	pRB
Terminal Protein	TP
Virus associated	VA
Virus encoded protease	Pr

1. Introduction

1.1. Adenoviruses

Adenovirus (AdV) was first discovered in 1953 by Wallace Rowe and associates. (Rowe *et al.*, 1953) This discovery was due to the search of etiological agents that were the cause of acute respiratory infections. Adenoviruses were first isolated from adenoid tissue, hence the name adenovirus. In 1962, it was discovered that human adenoviruses were capable of causing tumors in rodents, making this adenovirus particularly interesting to study. (Trentin JJ, Yabe Y, 1962) Adenoviruses belong to the family *Adenoviridae*. This family consists of five genera, *Mastadenovirus, Aviadenovirus, Atadenovirus, Ichtadenovius* and *Siadenovius.* Human adenoviruses (HAdV) belong to the *Mastadenovirus* genera. Currently there are over 50 serotypes of human adenoviruses that have been characterized. (Berk, 2007; Sarantis *et al.*, 2004) These can be further divided into seven subgroups (A-G) based on a variety of characteristics such as their ability to agglutinate erythrocytes, antigenic relationship, oncogenicity, and fiber length. (Wigand & Meiser, 1969)

HAdV varies in its infective potential, in rodent cells it is able to cause tumors, while in human cells it cannot. This variance is due to the type of host cell it is infecting, as well as the viral serotype. If the host cell is acquiescent, the virus will reproduce indefinitely until lysis occurs ultimately causing cell death. Conversely, if the cell is semi-acquiescent, the viral infection will not result in lysis or cell death because the cell only permits viral infection at a low efficiency. (Dobner *et al.*, 1996) With human infection, HAdV generally results in an upper respiratory tract infection. This can result in pharyngitis, or pharyngoconjunctival fever. In addition to this,

adenovirus has been shown to cause conjunctivitis and ear infections. (Hall, 2007; Kitamura, 2001; Murtagh *et al.*, 1992) Adenovirus is transmitted either by direct contact or by an individual coming into contact with contaminating viral particles on surfaces. (Sambrook & Russell, 2006) The majority of the population is able to recover from human adenovirus infection with the help of their own immune systems. Those individuals who are immunocompromised, such as transplant or cancer patients, may experience fatal infections. Currently there are no approved anti-adenoviral drug therapies available. (San Martín & Carmen, 2012)

Adenovirus serves as a critical model for the study of how viruses are able to cause transformation in human cells. In addition to this, the HAdV E1A protein has been shown to share similarities to a number of other viral proteins. For example, the Human Papilloma Virus E7 open reading frame has been shown to encode transcriptional activation and transformation functions analogous to E1A. (Phelps *et al.*, 1988) Therefore the study of mechanisms that HAdV participates in is crucial to understanding the cellular remodelling that occurs, and how this contributes to the oncogenicity of these viruses.

1.2. Human Adenovirus Structure and Morphology

Human adenoviruses are a family of small non-enveloped viruses. They are composed of a linear double-stranded DNA genome of approximately 35 kilobase pairs (kb) in size. Adenovirus has an icosahedral, non-enveloped capsid with a protein core. (Baron *et al.*, 1996) Adenovirus capsid is comprised of 240 hexon trimers, this is the main component of the capsid. The hexon protein is situated on the 20 faces of the icosahedral capsid. (Russell, 2009) There are four types of hexon proteins, H1, H2, H3 and H4. (Burnett, 1985) The H1 hexons have been found to associate with the pentons at the 12 apices. The residual hexon proteins are found on the 20 faces

of the icosahedron structure. (Russell, 2009) The capsid of adenovirus also contains the penton proteins.

The penton subunit are a covalent complex of two proteins, the penton and the fiber protein which can be found protruding from the 12 vertices of the capsid. (Russell, 2000) The fiber is comprised of a knob and a rod attached to the penton base. This is the first virus element to interact with a host cell. The fiber knob interacts with the Coxsackie Adenovirus Receptor (CAR) receptor on the cell surface. The CAR receptor is a 46kDa integral membrane protein with an extracellular region consisting of two immunoglobulin (Ig)-like domains that the adenovirus fiber is able to interact with. (San Martín & Carmen, 2012).

Additionally, there are other minor proteins VI, VIII, IX, IIIa and IVa2 that comprise the virion. These proteins were named based on the increasing order of their electrophoretic mobility on an SDS-PAGE gel. (Nicklin *et al.*, 2005) Proteins V, VII, mu (μ) and the terminal protein (TP) become packaged along with the genomic DNA in the viral core. Finally, the virus contains a virus-encoded protease (Pr), which is required for the processing of structural proteins. (Russell, 2000)

1.3. Human Adenovirus Host cell entry and Replication Cycle in Humans

In order for HAdV to infect non-cycling cells, it requires proteins that possess the ability to drive a cell into re-initiating the cell cycle. This requires proteins that are able to interact and manipulate key cellular regulatory networks. The knob of the fiber protein is responsible for binding to the host cell receptor to mediate entry of adenovirus into the host cell. The CAR receptors are used by all HAdVs, however Group B HAdVs are also able to use the CD46 cell receptors. (Wu & Nemerow, 2004) Next, the penton base interacts with integrin's αvβ3 and αvβ5

via a conserved RGD motif. (Russell, 2000; Wu & Nemerow, 2004) This allows the adenovirus to become internalized via clathrin-mediated endocytosis. The endosome then acidifies, and the viral capsid is dissociated causing the escape into the cytoplasm. (Meier & Greber, 2004) The capsid is then ferried to the nuclear pore complex (NPC) through the use of microtubules. Here, the viral genome is injected into the nucleus and primary viral transcription is initiated. (Blumenthal *et al.*, 1986) The viral progeny accumulates within the cell, eventually resulting in the lysis of the host cell, indicating a productive adenoviral infection. (Pombo *et al.*, 1994)

Adenovirus replication in human cells occurs in two distinct phases, the early and late phase. These phases are separated by the initiation of viral DNA replication. The early phase begins promptly at the point of viral adsorption. This entails the entry of the virus into the host cell, and the migration of the viral genome to the nucleus. Then the transcription and translation of the early genes occurs. In turn, early genes will eventually drive transcription and translation of the late genes, which occurs at maximum 20 hours post infection. (Russell, 2009)

1.4. Human Adenovirus Genome

Adenoviruses have a linear double stranded genome that ranges from 26-42 kb. There is a small amount variation between the adenovirus serotypes genomes. The HAdV-5 genome of 35kb has been extensively studied and characterized. (Ghebremedhin & Ghebremedhin, 2014) The genome is split into two replication phases and consists of the early region genes *E1A, E1B, E2A, E2B, E3, E4* and late region genes *L1, L2, L3, L4,* and *L5*. (Figure 1.1) (Miller *et al.*, 2007; Pettersson U, 1986) The early genes are the first genes transcribed upon viral entry into the host cell. The early genes code for proteins involved in viral transcription and replication, the initiation of S-phase as well as genes that are necessary for the suppression of the host immune response to viral infection. (Miller *et al.*, 2007; Pettersson U, 1986) The late genes encode for proteins that

comprise the viral capsid. The late genes share a promoter region resulting in transcription of all five genes occurring at the same point during the viral life cycle. (Brown *et al.*, 1975) The transcripts of HAdV can be alternatively spliced, resulting in multiple mRNA products. For example, the *E1A* gene can be alternatively spliced to give rise to five mRNA products, as shown in Figure 1.2. (Miller *et al.*, 2007; Pettersson U, 1986)

1.5. HAdV Early Genes

Adenovirus early genes are responsible for the replication of the viral genome as well as initiating the late gene replication phase. The early transcripts are, E1A, E2A, E2B, E3 and E4. (Miller *et al.*, 2007; Pettersson U, 1986) The first gene expressed after viral infection is the *E1A* gene. E1A is critical for viral replication as it is responsible for remodelling the cellular environment, initiating S-phase induction and activating the transcription of the remaining viral early genes. (Subramanian *et al.*, 2006) This will be discussed in section 1.6.

The *E1B* gene encodes for two proteins, a 55kDa and a 19kDa protein. (White & Cipriani, 1990) The E1B proteins play a critical role in preventing apoptosis, as this is a normal response when abnormal cell cycle changes occur like those induced by E1A. The E1B 55kDa protein is able to arrest apoptosis via a variety of mechanisms. For example, E1B can bind directly to the tumor suppressor protein p53 and in turn repress the activation of p53-responsive promoters. (Debbas & White, 1993) The E1B 19kDa protein is a BCL1-like protein. It inhibits mitochondria-mediated apoptosis via the prevention of mitochondrial outer membrane pore formation and the release of apoptogenic proteins from the pores. (Han *et al.*, 1996)

The E2 transcript has been shown to be critical for viral DNA replication. It encodes the viral DNA polymerase as well as the DNA binding protein. The transcript is divided into the E2A region and the E2B region. (Caravokyri & Leppard, 1996) The E2A protein encodes the 72kDa DNA Binding Protein (DBP). This protein is essential for enhancing initiation as well as the elongation process during viral DNA replication. (Russell, 2000) The E2B region has been found to encode for two proteins, a 140kDa DNA polymerase, as well as a terminal protein precursor (pTP). The precursor terminal protein acts as a primer for the initiation of DNA replication. It does so by covalently binding the 5' end of viral DNA, and becomes cleaved to the TP during virion maturation, thus allowing the DNA polymerase to make contact forming a pre-initiation complex (Smart & Stillman, 1982)

The E3 region encodes for proteins that are involved in the evasion of host immune response; E3-gp19K, 14.7K, 14.5K, 10.4K, 6.7K and the adenovirus death protein (ADP/E3-11.6K). (Russell, 2000) The E3-gp19K protein is a glycoprotein found localized in the membrane of the endoplasmic reticulum (ER). Here it binds MHC Class I antigens, preventing them from being detected by cytotoxic T lymphocytes by preventing their export out of the endoplasmic reticulum. (Horwitz, 2004; Smart & Stillman, 1982) The E3-14.5K along with two E3-10.4K form the receptor internalization and degradation complex. This complex along with the E3-14.7K protein block proapoptotic pathways by binding to ligands such as Fas (CD95), tumour necrosis factor and TNF-mediated inflammatory responses. The E3-6.7K also assists the receptor internalization and degradation complex. (Chin & Horwitz, 2006; Krajcsi *et al.*, 1996) The adenovirus death protein is responsible for the release of the fully assembled viral progeny. (Russell, 2000)

Finally, the E4 region encodes for multiple proteins, E4orf1, E4orf2, E4orf3, E4orf6 and E4orf6/7. (Stone *et al.*, 2003) Due to the multitude of proteins this region encodes, this region hosts a wide range of functions. Some of these functions include; promoting DNA replication, activation of the mTOR pathway (E4orf4) and inhibition of p53 induced apoptosis along with the E1B 55kDa protein (E4orf6). (Dobner *et al.*, 1996; Russell, 2000)

1.6. Human Adenovirus Early 1A protein (E1A)

E1A is the first protein expressed upon viral infection, and is the protein responsible for initiating cell cycle and the reprogramming of cellular pathways. E1A accomplishes this by recruiting hub proteins in order to alter cellular pathways. The ability of E1A to bind a variety of proteins is due to its unstructured nature. (Frisch & Mymryk, 2002) Literature has shown that proteins can be structured or unstructured containing disordered regions. (Dyson & Wright, 2005; Pelka *et al.*, 2008) It has also been shown that protein-protein interactions can be facilitated by molecular recognition features (MoRFs). MoRFs can be characterized as having structure fluidity or disordered regions. The E1A protein contains MoRF regions that allow it the ability to adopt multiple confirmations. This is how it is able to bind to such a variety of proteins, truly transforming the intracellular environment. (Han *et al.*, 2004)

There are 5 isoforms of E1A that arise due to differential splicing. The largest two at 289 and 243 residues in HAdV-5 have been the most extensively studied. Importantly, E1A has the ability to immortalize cells and with the assistance of a second oncogene such as the E1B protein or Ras, it is able to fully transform cells in rodents. (Subramanian *et al.*, 2006)

E1A consists of four conserved regions (CR) termed; CR1, CR2, CR3 and CR4. (Arany *et al.*, 1995) CR1 consists of residues 42-72 in the HAdV-5 E1A protein. This region works alongside the N-terminus to bind several transcriptional regulators such as the protein acetyltransferase p300, this association has been shown to be critical for oncogenic transformation by HAdV E1A. (Egan *et al.*, 1988; Wong & Ziff, 1994) It has been shown that the CR1 region is critical for the interaction with the CH3 region of p300. This binding occurs via the MoRF within the residues 66-72 in HAdV-5 E1A. (Egan *et al.*, 1988; Stein *et al.*, 1990) CR1 has also been shown to play a role in releasing E2F proteins that are associated with the retinoblastoma protein (pRb). This is critical for viral replication, as the release of the E2F proteins results in the activation of the early gene. (Ikeda & Nevins, 1993)

CR2 consists of residues 115-137 in HAdV-5 E1A. (Avvakumov *et al.*, 2004) CR2 has been found to be involved in a variety of functions related to virus replication. This includes the activation of viral early gene expression as well as driving the host cell to enter the cell cycle. (Berk, 2005; Gallimore & Turnell, 2001) CR2 was found to be required for the oncogenic transformation of rodent cells in culture along with other functions. (Jelsma *et al.*, 1989; Lillie *et al.*, 1986) Some of the known targets for this region are retinoblastoma protein, BS69, UBC9, p130 and the S2 subunit of the 19S regulatory complex of the proteasome. (Hateboer *et al.*, 1996; Zhang *et al.*, 2004) The retinoblastoma protein was the first protein discovered to interact with E1A. The interaction between E1A and pRB is crucial for E1A to be able to transform rodent cells. (Whyte *et al.*, 1988) E1A was found to interact with hypo-phosphorylated pRB through the LXCXE motif (residues 122-126). (Ikeda & Nevins, 1993; Mittnacht *et al.*, 1994) CR2 has a high affinity for pRb and so the local concentration of pRb relative to E1A is increased allowing the pRb binding motif in CR1 to bind which leads to pRb dissociating from the transcription factors of the E2F family.

E2F is then free to activate the early viral genes. (Felsani *et al.*, 2006) However, it has also been shown that the E1A 289R isoform can also activated E2F-responsive gene expression without the binding of Rb. (Pelka *et al.*, 2009)

The CR3 region spans residues 144-191 in HAdV-5. (Avvakumov *et al.*, 2004) It has three functional domains, a N-terminal C4 zinc finger, a C-terminal promoter targeting region and an acidic region known as auxiliary region 1 (AR1). The C4 zinc finger functions as a powerful transcriptional activation domain. (Webster & Ricciardi, 1991) Although E1A is thought to be relatively unstructured, the presence of the Zn-finger domain strongly suggests that CR3 is a structured domain, and that this is important for its function. (Culp *et al.*, 1988) Importantly, E1A is able to be recruited to viral promoters through an interaction between a promoter targeting domain located at residues 183-188 and cellular sequence specific DNA binding transcription factors. (Pelka *et al.*, 2008)

CR4 is located at residues 240-288. (Avvakumov *et al.*, 2004) This final conserved region has been less extensively studied, and as a result there are fewer known binding partners. CR4 has been shown to be essential for the oncogenic transformation of rodent cells along with E1B but has also been shown to be a suppressor of transformation with activated Ras. (Boyd *et al.*, 1993; Douglas & Quinlan, 1995) To date, only a handful of known binding proteins have been identified, these include, CtBP1/2, Qip1, DREF, FOXK1/2, DYRK1A/B, HAN11 and most recently Ku70 (Cohen *et al.*, 2013; Frost *et al.*, 2017; Radko *et al.*, 2014) The lack of knowledge surrounding this region indicates there is still much to learn about how CR4 contributes to the overall function of the E1A proteins.

1.7. Hub proteins and E1A

One of E1A's primary roles in cellular reprogramming is to initiate the cell cycle in adenovirus infected cells by altering cellular gene expression on a global scale. During this process the activation of other viral genes also occurs, thus enabling viral replication. (Bayley & Mymryk, 1994) In order to efficiently manipulate the cellular environment, E1A is known to target hub proteins. Hub proteins can be defined as those proteins that are involved in a multitude of cellular pathways. Hub proteins are critical for cellular regulatory pathways, as they allow them to be resilient to disruption. (Fraser *et al.*, 2002; Pelka *et al.*, 2008) The redundancy found in these networks is a preventative measure against the disruption of an individual node by random events, such as a mutation. Therefore, pathogens such as viruses tend to target key regulators of these pathways, which are the hub proteins. When a hub protein is disrupted, the results is a disruption of the key functions of the cellular network in which the hub protein was a part of. (Jeong *et al.*, 2001)

Most notably, E1A enables viral replication by targeting hub proteins centralized in the control of cell growth and differentiation. Therefore, E1A subsequently serves as a "hub detector", identifying potential proteins that are at the heart of cellular regulatory networks.

To further reveal the function of E1A, Dr. Pelka has performed affinity purification combined with mass spectrometry (MS) to identify novel binding partners that interact with E1A via its C-terminus. This approach has identified two proteins previously not reported to bind to E1A, these are, the Ku 70 complex and Far Upstream Element Binding Protein 1 (FUBP1). This indicates that both Ku70 and FUBP1 may be hub proteins, due to this recent E1A binding ability discovery.

1.8. Ku70 Protein

The human protein Ku70 was first discovered in patients with autoimmune diseases as it was target of autoantibodies. The name is derived from the first two letters of the patients name from which Ku was organically identified in. (Mimori *et al.*, 1986). Elevated levels of anti-Ku antibodies have been detected in patients with autoimmune disease such as systemic lupus erythematosus, polymyositis. (Reeves, 1992)

Ku70, encoded for the by the *XRCC6* gene is 70kDa in size and often found in a heterodimer state with its homolog Ku80. (Walker *et al.*, 2001). This relatively conserved protein has homologs in a variety of organisms. The homologs found in different organisms have been shown to vary in sequences and yet they all share similar sizes and DNA binding properties. (Barnes & Rio, 1997) Ku70 consists of three domains, a N-terminal alpha helix/beta barrel von Willebrans A (vWA) domain, a central DNA binding core domain that is required for both DNA binding and dimerization, and finally a helical C-terminal domain. (Walker *et al.*, 2001)

One of the better characterized roles of Ku70 is its critical role in the Non-Homologous End Joining pathway (NHEJ). The NHEJ pathway is one of two DNA damages response pathways in mammalian cells. This pathway is responsible for the rejoining of DNA ends without the use of sequence homology. (Jeggo, 1998) The Ku70/80 heterodimer is responsible for first binding to double stranded DNA breaks to initiate the NHEJ pathways. This heterodimer plays an important role in aligning the DNA ends while subsequently protecting them from degradation. The Ku complex then acts as an initial building block for the assembly of repair factors. (Lees-Miller & Meek, 2003)

Ku70 has also been shown to be involved in a multitude of other roles. It has been demonstrated to be involved in the maintenance and regulation of telomeres, playing a role in transcriptional silencing of telomere-proximal genes. Paradoxically, Ku70 also plays a protective

role in telomere maintenance. As telomeres are located at the ends of chromosomes, they can potentially be recognized as double-stranded breaks. Studies in mice models deficient for Ku70 have shown conflicting results regarding the shortening or lengthening of telomeres in this environment. However, human cells that were subsequently depleted of Ku show telomere loss. (Celli *et al.*, 2006) Similar with its role in the NHEJ pathway, Ku70 protects telomere ends from recombination and various degradation events. (Shi *et al.*, 2007)

Studies have also shown that Ku70 plays a role in V(D)J recombination in vertebrates. It is responsible for repairing double stranded breaks (DSBs) that are created during V(D)J recombination, which is crucial for the formation of genetic diversity in the immune system. The recombination of the V, D, and J segments of immunoglobulins allows for the recognition of a variety of antigens, adding to the adaptive immune response. (Gellert, 2002) In addition to having nuclear functions, Ku70 has also been shown to have cytoplasmic activity. To encourage cell survival, Ku70 binds and sequesters Bax, a pro-apoptosis protein away from mitochondria. This inhibits apoptosis, and this has been suggested to be mediated by various mechanisms including protein-protein interactions as well as post-translational modification of the Ku protein. (Gomez *et al.*, 2007)

Ku70 has also been shown to play roles in viral life cycle. SV40 replication relies on both the ATM and ATR DNA damage response pathways. However, Ku70 was previously shown to be recruited to SV40 replication centers following ATM DNA repair inhibition. This finding indicated that the ATM DNA repair pathway influences which DNA repair pathway is used by preventing the recruitment of NHEJ factors such as Ku70. This inhibition is thought to occur in order to prevent concatemerization of the viral genome. (Sowd *et al.*, 2014). Ku70 has also been

shown to be degraded during infection with Hepatitis C virus, in the presence of the Hepatitis C virus, ubiquitination levels of Ku70 were enhanced. (Saitou *et al.*, 2009)

Ku70 has been shown to be active in a diverse array of functions, many of which surround the protection of DNA ends or DNA repair pathways. Ku70 has also been shown to interact with viruses, as well as play a role in the inhibition of apoptosis. Taken together this suggests that Ku70 may in fact be a hub protein, making it a logical target of the HAdV-5 E1A proteins.

1.9. Far Upstream element Binding Protein 1 (FUBP1)

The far upstream element binding protein 1 (FUBP1) was first recognized in 1994 as a transcriptional regulator of c-Myc. It was found that FUBP1 binds to the Far Upstream Element (FUSE) located approximately 1500 base pairs upstream from the c-Myc promoter. This binding only occurs in undifferentiated cells. (Duncan *et al.*, 1994) c-Myc is a critical transcription factor that has been shown to regulate the expression of about 10% of cellular genes. Importantly, it regulates genes involved in cell proliferation, cell differentiation, and apoptosis. C-Myc is often up-regulated in cancers leading to constant cell proliferation and differentiation. (Levens, 2002; de Nigris *et al.*, n.d.) Due to the discovery that FUBP1 was involved in the regulation of the proto oncogene c-Myc, this prompted interest in studies of FUBP1 and its functions within the cell.

Since the initial discovery of FUBP1, it has been shown to be up-regulated in a variety of cancers, including Burkitt's lymphoma, T-cell leukemia and prostate cancer. Interestingly, FUBP1 has also been shown to be up regulated in cancers independently of c-Myc. (Bazar *et al.*, 1995; Davis-Smyth *et al.*, 1996) (Table 1.1) Therefore the study of why FUBP1 interacts with the E1A oncoprotein of HAdV will provide crucial insights into FUBP1s role in viral life cycle and cellular processes that can lead to cancer, especially as FUBP1 is not a well characterized protein. By

identifying the reasons behind E1A targeting of FUBP1 we will gain insight into FUBP1 function in the cell.

The FUPB1 gene encodes for a 644 amino-acid protein that is composed of three defined domains. These include, an amphipathic helix N-terminal domain, a tyrosine-rich C-terminal transactivation domain and a DNA-binding domain. (Duncan *et al.*, 1996) The DNA-binding domain has been shown to contain four K-homology (KH) motifs. These are conserved protein domains responsible for binding RNA. (Valverde *et al.*, 2008) Importantly, it has been determined that the KH3 domain with the KH4 domain are sufficient for the binding to the FUSE element. (Braddock *et al.*, 2002)

Literature shows that FUBP1 has been found to be involved in a number of roles. One of which is the regulation of nucleophosmin (NPM). Nucleophosmin is a regulator of cellular growth and protein synthesis. NPM controls the rate of ribosomal subunit export from the nucleus to the cytoplasm. NPM has also been found to be overexpressed in certain cancers such as colon, prostate and gastric cancers. (Olanich *et al.*, 2011) A study by Olanich et al. showed that FUBP1 is able to bind the 3' UTR of NPM mRNA, subsequently inhibiting the mRNA from being translated.

FUBP1 has also been shown to induce translation of p27Kip1 from an internal ribosomal entry site. p27Kip1 is a cyclin dependent kinase inhibitor that plays a role in controlling cell cycle via regulation of the transition between G1 and S-phase. (Zheng, Yuhuan ., Miskimins, 2012) It was found that FUBP1 binds to the U-rich region of p27 5'UTR to promote recruitment of ribosomes. This is contrasting to the inhibitory effect FUBP1 has on NPM, suggesting that FUBP1 is a multifunction protein. (Zhang & Chen, 2012)

Interestingly, FUBP1 has also been shown to mediate the replication of several retroviruses via RNA binding. It has been shown that Hepatitis C virus (HCV) replication in host cells is dependent on the binding of FUBP1 to the poly(U) tract within the 3'UTR of HCV. This is critical for HCV RNA replication. (Dixit *et al.*, 2015) FUBP1 has also been shown to negatively affect viral replication. It has been shown that Japanese encephalitis virus (JEV) is suppressed by FUBP1, via binding to UTR of JEV. (Chien *et al.*, 2011) In addition to these examples, a recent study on Enterovirus 71 (EV71) has shown that the KH3 and KH4 domains of FUBP1 are responsible for the interaction of the internal ribosomal entry site (IRES) of the 5'UTR of EV71. FUBP1 is able to induce the replication of this hand-foot-mouth disease retrovirus by competing with inhibitory IRES transacting factors. (Huang *et al.*, 2011)

FUBP1 has also been shown to bind to cellular mRNAs. An example of this can be seen with p21, a cyclin-dependent kinase inhibitor. The stability of p21 mRNA has been shown to be influenced by FUBP1 regulation through AU-rich elements (AREs). When FUBP1 is depleted, p21 mRNA levels increased 5-fold. (Rabenhorst *et al.*, 2009)

Taken together, the data on FUBP1 suggest that it may have oncogene-like properties. Notably, the role FUBP1 has been shown to play in cancers, viral replication and RNA regulation suggest that it is a hub protein, and therefore a target for HAdV-5 E1A. Determining the role FUBP1 plays in HAdV life cycle and reasons behind its targeting by E1A will provide further insight to FUBP1's role within cellular networks.

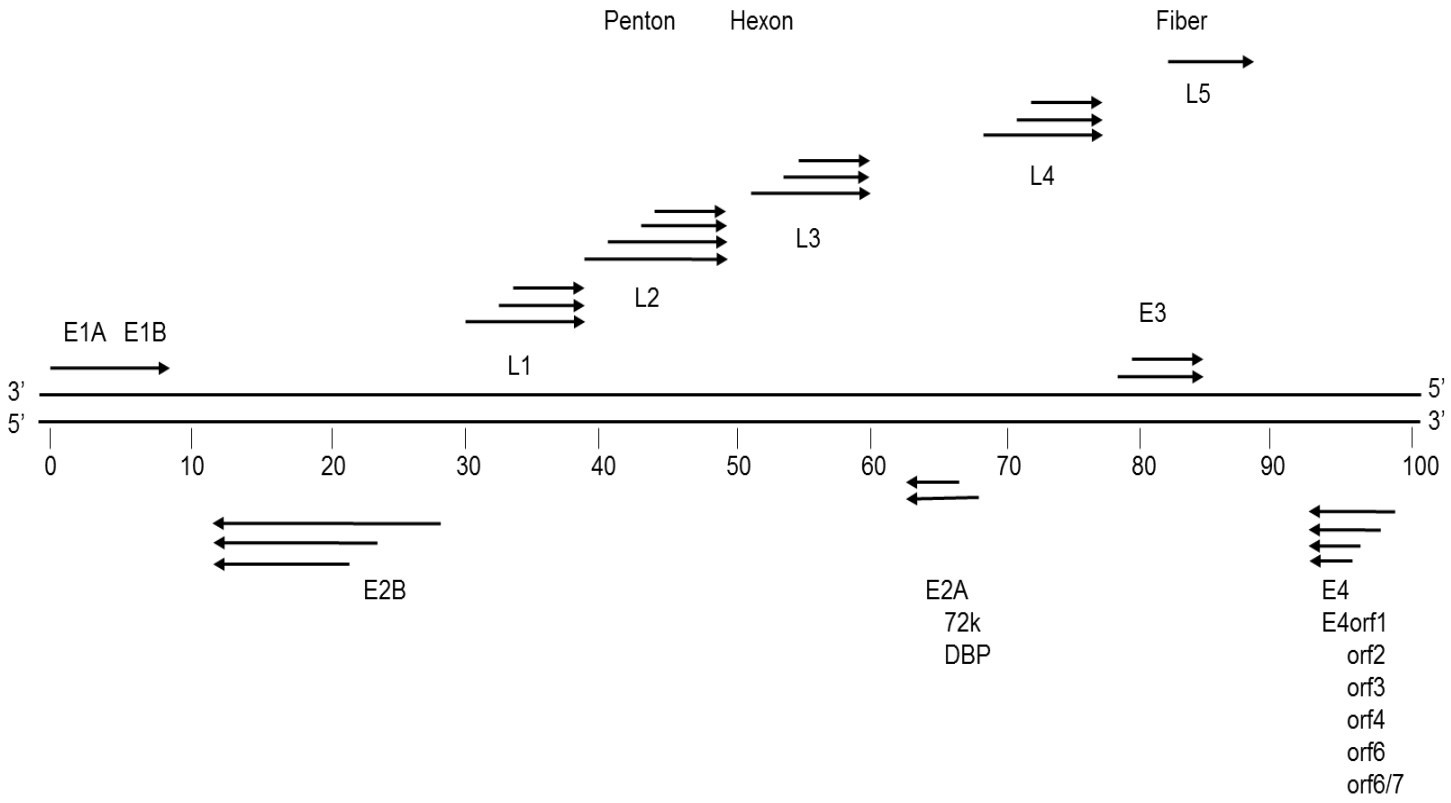


Figure 1.1.: Map of the Human Adenovirus Genome. Gene transcription occurs at both strands, arrows indicate the direction of transcription. Genes are listed above and below the respective strand. Modified from Berk, 2007.

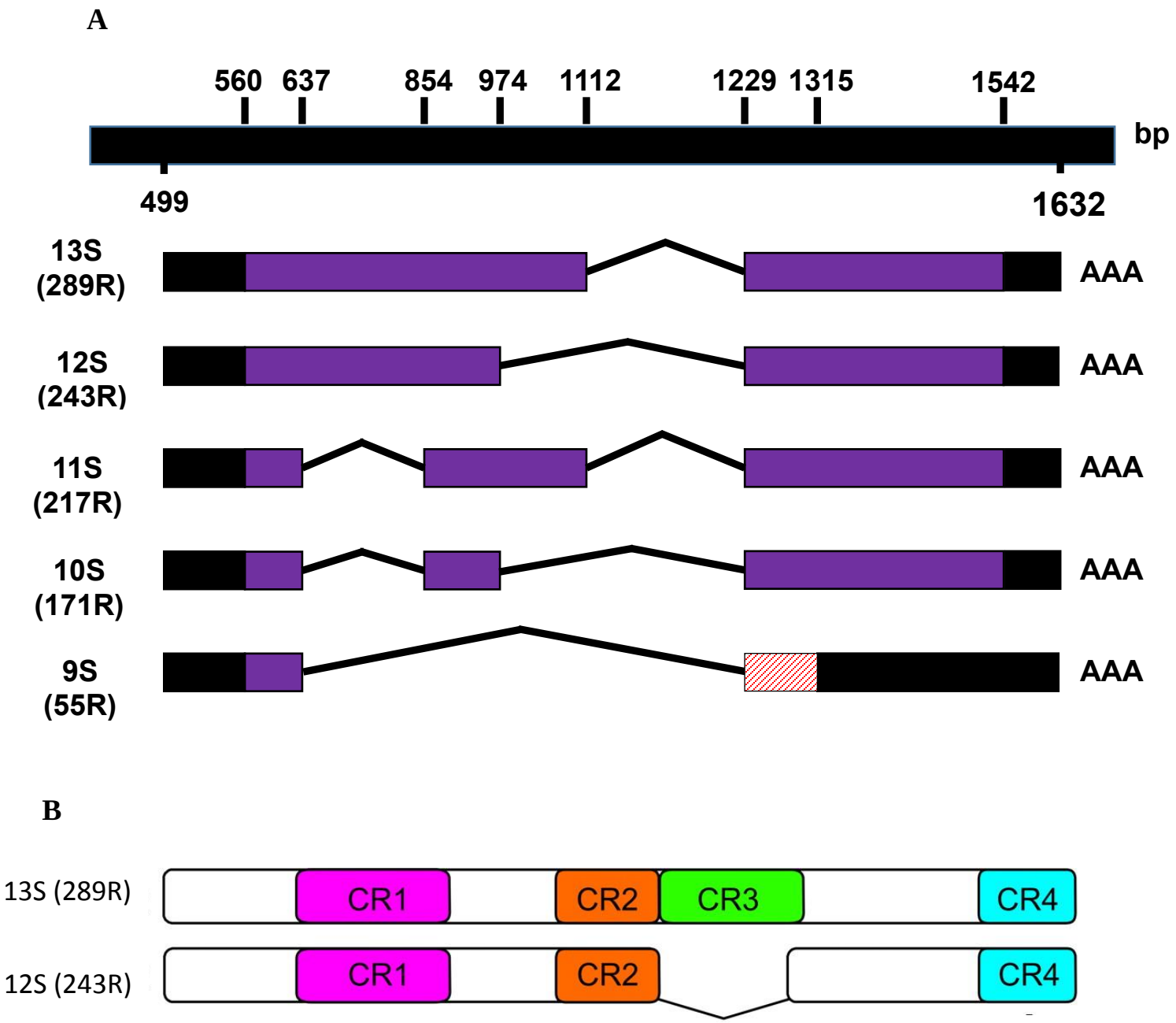


Figure 1.2: E1A Isoforms. A. Splice variants of E1A isoforms. Boxes represent coding regions, lines represent introns. **B**. Diagram depicting the differences in sequence alignment of the two largest isoforms of E1A (13S and 12S). The 12S isoform lacks the CR3 region. Modified from Pelka *et al.*,2008.

Table 1.1: FUBP1 involvement in cancers. List of human malignancies FUBP1 has been shown to be involved in and the downstream targets of this involvement. FIR= FUBP-interacting repressor. Based on Zhang & Chen, 2012.

MALIGNANCY	ALTERATIONS IN FUBP1	DOWNSTREAM TARGET
BLADDER CANCER	FUBP1 increase	N/A
BREAST CANCER	FUBP1 increase	N/A
CLEAR CELL RENAL CANCER	FUBP1 increase	Increase in c-Myc
COLON CANCER	FIR truncation	Increase in c-Myc
LIVER CANCER	FUBP1 increase	Increase Stathmin 1 and Cyclin D2, decrease in p21, p15
NON-SMALL CELL LUNG CANCER	FUBP1 increase	Increase Stathmin 1
OLIGODENDROGLIOMAS	FUBP1 mutation	N/A
PROSTATE CANCER	FUBP1 increase	N/A

Research Objectives and Hypothesis

To further investigate the function of E1A and its C-terminus binding partners, affinity purification combined with mass spectrometry (MS) was used to identify novel binding proteins. This approach has identified two new E1A binding proteins that are; Far Upstream Element Binding Protein 1 (FUBP1) and the Ku 70 protein.

Ku70 participates in pathways related to DNA repair and protection. Determining how E1A is able to utilize Ku70 for its own benefit could give insight on how other viruses are able to manipulate Ku70. This is especially important as Ku70 has been shown to have diverse roles within the cell.

While Ku70 is a well characterized protein, the function of FUBP1 is not as well understood. Therefore, studies with E1A could elucidate the role of FUBP1 in cellular regulatory networks. Moreover, identifying novel binding partners of the E1A C-terminus allows for the further understanding of how E1A is able to reprogram the cell for viral replication.

Therefore my main research objectives were:

1. To examine the interaction between Ku70 or FUBP1 and HAdV-5 E1A
    a. Determine if the interaction is direct or indirect
    b. Identify the region required for binding
2. Elucidate the role of Ku70/FUBP1 in the viral life cycle
    a. Determine the effect of Ku70/FUBP1 on viral growth and replication
3. To investigate the reasons behind E1A targeting of Ku70 and FUBP1
    a. Characterize Ku70 and FUBP1 in regulatory networks

Thus, my hypothesis is that E1A targets Ku70 and FUBP1 in order to modulate their activities in support of viral replication and deregulation of cellular growth.

2. Materials and Methods

2.1. Antibodies

Mouse monoclonal anti-E1A M73 and M58 antibodies were previously described (Harlow *et al.*, 1985) and were grown in-house and used as the hybridoma supernatant. Mouse monoclonal anti-72k DBP antibody was previously described (Reich *et al.*, 1983) and was used at a dilution of 1:400 for western blot. Table 2.1 contains a comprehensive list of all antibodies used.

2.2. Cell and virus culture

IMR-90 primary human lung fibroblast cells (Nichols *et al.*, 1977) (ATCC# CCL-186), HT1080 human fibrosarcoma cells (Rasheed *et al.*, 1974) (ATCC# CCL-121) and Human Embryonic Kidney (HEK) 293 cells (Russell *et al.*, 1977) were cultured with 10% fetal bovine serum (Seradigm), and 1% streptomycin and penicillin (HyClone). Cells were incubated at 37˚C with 5% CO_2. All virus infections were carried out in serum-free media for 1h after which saved complete media was added without removal of the infection media.

2.3. Chromatin Immunoprecipitation

IMR-90 cells were infected with the indicated adenoviruses at a m.o.i. of 10 and harvested 24h after infection for ChIP-analysis 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 via the Corvaris Focused- UltraSonicator M220. Supernatant was used for immunoprecipitation. For immunoprecipitation of E1A, the monoclonal M73 and M58 antibodies were used. For immunoprecipitation of Ku70 the polyclonal anti-Ku70 antibody was used, and for the

immunoprecipitation of FUBP1 the monoclonal anti-FUBP1 antibody was used. Rabbit anti-rat 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 using a 5M NaCl solution. PCR reactions were carried out for HAdV-5 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

IMR-90 cells were grown until 100% confluent on Lab Tek II 4- Chamber Slides (Thermo-Fisher). After becoming fully confluent, cells were incubated for another 72h to arrest growth. Infections were carried out as described above with a m.o.i. of 20 for *dl*309. One hour prior to fixation, cells were pulsed with EdU for 1h 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 M73 monoclonal antibody and AlexaFluor-594 conjugated secondary antibody (Jackson Immunoresearch). Cells were visualized using LSM700 laser confocal microscope and ZEN software suite.

2.5. Immunofluorescence

IMR-90 cells were plated at low density (~40,000 cells per chamber) on chamber slides (Nalgene Nunc), and subsequently infected as described above. Sixteen or twenty-four hours after infection, cells were 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 neat (hybridoma supernatant), E2 DBP antibody was used at 1:100 dilution (hybridoma supernatant), Ku70 antibody or FUBP1 antibody, was used at a dilution of 1:300, and AlexaFluor-488 and 594 secondary antibodies (Jackson Immunoresearch) were used at a dilution of 1:600. 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

Transfected 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 M73 or M58 anti-E1A antibody. E1A was detected using the M73 or M58 monoclonal antibody, while Ku70 or FUBP1 was detected using the rabbit monoclonal antibody or rabbit polyclonal antibody respectively.

2.7. PCR primers

A comprehensive list of primers can be found in Table 2.3. All primers were purchased from IDT, and annealing temperature of 55˚C was used.

2.8. Plasmids

The expression plasmid for pcDNA3.1-E1A was previously described (Pelka *et al.*, 2007) and it expressed all E1A isoforms. The expression plasmid pEGFP-C1-FLAG-Ku70 was purchased from Addgene (plasmid #46957) and was previously described (Britton *et al.*, 2013). A comprehensive list of all plasmids used can be found in Table 2.2.

2.9. Protein purification and GST pulldown assay

Glutathione S-transferase fusions of Ku70 and FUBP1 were made by sub-cloning the cDNA into pGEX-6P1 (GE Healthcare Life Sciences) in frame with the N-terminal GST-tag. His-tagged E1A289R was made by sub-cloning the entire E1A289R cDNA into the pET42 vector (Novagen) in frame with a C-terminal 6XHis tag. Proteins were expressed in Escherichia coli strain BL21 (DE3) and purified on their respective resins according to the manufacturer's specifications. GST-pulldown assay was carried out by incubating His-E1A289R with purified GST-Ku70 or GST-FUBP1 for 1h 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 agitated for 1 h. Beads were washed with GST-pull down buffer, eluted in SDS sample buffer and resolved on a SDS–PAGE.

2.10. RNA Immunoprecipitation

HT1080 cells were infected with *dl*309 at a m.o.i. of 20 for 48h. Cells were collected, pelleted and lysed in 10ml solution (2ml PBS, 2ml Nuclear isolation Buffer (1.28M Sucrose, 40mM Tris-HCl pH 7.5, 20mM $MgCl_2$, 4% Triton X-100), 6ml H_2O). Nuclei were pelleted and re-suspended in 1ml RIP Buffer (150 mM KCl, 25mM Tris pH 7.4, 5mM EDTA, 0.5mM DTT, 0.5% NP-40) with protease inhibitor added. Nuclei were mechanically sheared with a Dounce Homogenizer (15-20 strokes), and nuclear membrane/debris was pelleted by centrifugation. Extracts were pre-cleared

with 40µl of Protein-A Sepharose beads for 1h. Antibodies were added (10µl of FUBP1, 50µl M73 (E1A) and 2µl Rabbit anti-Rat IgG) and agitated for 2h. 20µl of Protein-A Sepharose beads were added and agitated for 1h at 4˚C. Beads were pelleted and washed with RIP buffer 4 times. RNA was extracted using the TRIzol Reagent (Sigma) at the indicated time points according to manufacturer's instructions. Associated RNA was detected using real-time PCR analysis.

2.11. Real-time gene expression analysis

IMR-90 cells were infected with *dl*309 at a m.o.i. of 10. Total RNA was extracted using the TRIzol Reagent (Sigma) as previously described. 1.25 µg of total RNA was used in reverse-transcriptase reaction using SuperScript VILO Reverse Transcriptase (Invitrogen) according to the manufacturer's guidelines using random hexanucleotides for priming. The cDNA was subsequently used for real-time expression analysis using the BioRad CFX96 real-time thermocycler. Analysis of expression data was carried out using the Pfaffl-method (Pfaffl, 2001) and was normalized to GAPDH mRNA levels. These values were compared between siControl and siKu70 or siFUBP1 transfected cells. Total E1A was detected with primers binding within exon 2: TCCGGTCCTTCTAACACACC and GGCGTTTACAGCTCAAGT CC, as previously described (Radko *et al.*, 2015).

2.12. siRNA knockdown

IMR-90 cells were transfected with Ku70 specific Silencer siRNA (Life Technologies s5455 or s5457) or FUBP1 specific Silencer siRNA (Life technologies s16967) using SilentFect reagent (BioRad) according to manufacturer’s specifications using 10nM final siRNA concentration. Silencer Select negative control siRNA #1 (Life Technologies) was used as the negative siRNA control.

2.13. Transfections

Cells were plated in 10 cm plates at a density of 2.0×10^6 cells/plate 24h prior to transfection. Transfections were prepared by mixing 1 mL of serum-free DMEM, 10 μg of total plasmid DNA, and 20 μL of linear 1 mg/mL solution of the polymeric transfection agent polyethylenimine 25 kDa reagent from Polysciences (cat# 23966-2). This was vortexed for 10 seconds and incubated at room temperature for 20 min. The complexes were added to the cells and incubated for 24–48h.

2.14. Viral genome quantification

IMR-90 cells were lysed in lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA and 1% SDS) on ice for 10 minutes. Lysates were sonicated briefly in a Covaris M220 Focused-UltraSonicator to break-up cellular chromatin and subjected to digestion using Proteinase K (NEB) according to manufacturer's specifications. Following digestion, viral DNA was purified using GeneJET PCR Purification Kit (Thermo-Fisher). PCR reactions were carried out using SYBR Select Master Mix for CFX (Applied Biosystems) according to manufacturer's directions using 2% of total purified DNA as template in a CFX96 Real Time PCR instrument (BioRad). Standard curve for absolute quantification was generated by serial dilution of a pXC1 plasmid containing the left end of HAdV-5 genome starting with a concentration of 1.0×10^7 copies per reaction down to 1.0 copy per reaction. The primers used were the same as those used for expression analysis of E1B region; the annealing temperature used was 60°C and 40 cycles were run.

2.15. Virus growth assay

Arrested IMR-90 cells were infected with HAdV-5 *dl*309 (Jones & Shenk, 1979) at a m.o.i. of 10 or as indicated, in serum-free media. Virus was adsorbed for 1h at 37°C under 5% CO_2, after which cells were bathed in conditioned media and were re-incubated at 37°C under 5% CO_2. Virus titers were determined at 48h, 72h, and 96h after infection, and plaque assays were performed on HEK 293 cells by serial dilution.

Table 2.1: Comprehensive list of antibodies used

Antibody	Details	Use	Dilution factor	Source	Catalogue number
72k DNA Binding Protein	Mouse Monoclonal	Primary	WB: 1:400 IF: 1:600	Phil Branton	N/A
9E10	Mouse Monoclonal	Primary	WB: 1:300	In house	N/A
Actin	Mouse Monoclonal	Primary	WB: 1:1000	Abcam	Ab3280
E1A (M58)	Mouse Monoclonal	Primary	WB: 1:400 IF: Neat	In house	N/A
E1A (M73)	Mouse Monoclonal	Primary	WB: 1:400 IF: Neat	In house	N/A
FUBP1	Rabbit Monoclonal	Primary	WB: 1:3000 IF: 1:300 ChIP: 5µl RNAIP: 10 µl	Abcam	Ab181111
GFP	Rabbit Polyclonal	Primary	1:1000	Clontech	632592
HA	Rat Monoclonal	Primary	WB: 1:5000	Roche	11867423001
Ku70	Rabbit Polyclonal	Primary	WB: 1:3000 IF: 1:300 ChIP: 5µl	Pierce	PA5-27538
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
Rabbit IgG	Goat	Secondary	WB: 1:200,000	Jackson ImmunoResearch	111-035-003
Rabbit IgG (Alexa fluor 594)	Goat	Secondary	IF: 1:600	Jackson ImmunoResearch	111-585-003
Rat IgG	Goat	Secondary	WB: 1:200,000	Jackson ImmunoResearch	111-035-003

Note: WB= Western Blot, IF = Immunofluorescence, Primary and Secondary antibodies are diluted and applied to membranes in Tris-Buffered Saline Tween 20 (TBS-T) containing 5% skim milk powder or in immunofluorescence blocking buffer (1% normal goat serum, 1% bovine serum albumin (BSA), 0.2 % Tween 20 in phosphate-buffered saline (PBS)).

Table 2.2: List of plasmids used

PPB #	NAME	PARENT VECTOR	FEATURES
36	pcDNA2-E1A 12S wt	pcDNA3	Expression vector for 12S cDNA wt E1A
45	pEGFP-C2-CR3 (Ad5)	pEGFP-C2	Expresses the CR3 region of HAdv E1A (139-204R)
46	pcDNA3-E1A-AD5-13S	pcDNA3	Expression vector for full length Ad-5 13S E1A
48	pEGFP-X2	pEGFP-C2	Expresses the C-terminal portion of HAdV E1A (187-289R)
49	pEGFP-CR2	pEGFP	Expresses the CR2 region of HAdV E1A (93-139R)
50	pEGFP - 1-82T	pEGFP	Expresses the N-terminal portion of HAdV E1A (1-82R)
71	pcDNA3.1-E1A	pcDNA3.1 Hygro	HAdV-5 genomic E1A
263	pCGNM-HA-FUBP1	pCGNM	Expresses HA-tagged FUBP1
272	pGEX-6P-1	N/A	GST-fusion vector for N-terminal fusion proteins
277	pGEX-6P-1-FUBP1	PGEX-6P-1	Expresses GST-fused FUBP1
421	pEGFP-FLAG-Ku70	pEGFP	Expressed GFP and Flag-tagged Ku70
433	pGEX-Ku70	PGEX-6P-1	Expresses GST-fused Ku70

Table 2.3. Comprehensive list of primers used

PRIMER	FORWARD PRIMER (3'-5')	REVERSE PRIMER (5'-3')
BLM	CACCATGGCTGACACGTTAC	TGTCAATCCCCATTCCAAAT
CCNB1	CGGGAAGTCACTGGAAACAT	AAACATGGCAGTGACACCAA
CDC25C	CCGTGGCCATAGAAAGAGAG	TAGGCCACTTCTGCTCACCT
CDK6	TGGACTCCCAGGAGAAGAAG	GGTGGGAATCCAGGTTTTCT
CDK6p	TAAGCCGTGACATTGACGTG	CCGCTTAATCCTTCCTGGTT
CDKN1A	GGAAGACCATGTGGACCTGT	AAGATGTAGAGCGGGCCTTT
CDKN1Ap	GTGGCTCTGATTGGCTTTCTG	CTGAAAACAGGCAGCCCAAG
E1A	CACGGTTGCAGGTCTTGTCATTAT	GCTCAGGTTCAGACACAGGACTGTA
E1Ap	GTTTTCTCCTCCGAGCCGCT	TTACATCAACTCATTCAGCAAAC
E1B	CGCGCTGAGTTTGGCTCTAG	TCAAACGAGTTGGTGCTCATG
E1Bp	GAATAAGAGGAAGTGAAATCTG	CGGCTCGGAGGAGAAAACTC
E2A	GGGGGTGGTTTCGCGCTGCTCC	GCGGATGAGGCGGCGTATCGAG
E2p	AGCAAATACTGCGCGCTGAC	AGAATTCGGTTTCGGTGGGC
E3A	GCCGCCACAAGTGCTTTG	CTCGGAGAGGTTCTCTCGTAGACT
E3p	CGCGGGACCCCACATGATAT	CGCCCTCTGATTTTCAGGTG
E4orf6/7	CTGCTGCCCGAATGTAACACT	TCCACCTTGCGGTTGCTTAA
E4p	TAAACACCTGAAAAACCCTCCTGCC	GGCTTTCGTTTCTGGGCGTA
Exon 2	GGCGTTTACAGCTCAAGT CC	TCCGGTCCTTCTAACACACC

Hexon	CTTACCCCCAACGAGTTTGA	GGAGTACATGCGGTCCTTGT
hGAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
MCM3	CGGGGTGGAGTCATCCTGGG	CGCAGCTCCACATCGTCC
MCM4	TTGAAGCCATTGATGTGGAA	GGCACTCATCCCCGTAGTAA
MCM4p	CCGAGCGAGGCCTACTTCT	GGACAGTGCCGCTTCTTTCA
MLP	TCGGCCTCCGAACGGTAAGA	AACTTTATGCCTCGCGCGGG
PCNA	GAAGCACCAAACCAGGAGAA	TCACTCCGTCTTTTGCACAG
PCNAp	CTGGCTGCTGCGCGA	CACCACCCGCTTTGTGACT

3. Results

Ku70

3.1 Ku70 binds directly to E1A

Ku70 was one of the novel proteins found by Dr. Pelka, through affinity purification and mass spectrometry to bind to the C-terminus of the E1A protein. To determine whether this interaction was direct or indirect, purified GST-fused Ku70 protein was used to perform a GST-pulldown assay with His-tagged HAdV-5 E1A 289R (13S) protein. The pull down was analyzed with an SDS-PAGE gel and blotted for E1A. (Figure 3.1.1). The results showed that E1A interacts with Ku70.

To determine if the interaction between HAdV-5 E1A and Ku70 occurred during viral infection, HT1080 cells were infected with *pm*975 (expressing only the E1A 289R isoform), or *dl*520 (expressing only the E1A 243R isoform) for 24h. After infection, cells were collected and lysed, and the lysate was used to perform a co-immunoprecipitation using M73 and M58 E1A antibodies. This was subsequently run on an SDS-PAGE gel and blotted for Ku70. (Figure 3.1.2) The results show Ku70 interacts with E1A during infection, and is able to bind both the 289R and 243R isoforms of E1A.

3.2 Ku70 binds in the CR4 region of E1A

In order to map the interaction site of Ku70 to E1A, HT1080 cells were infected with HAdV expressing wild-type E1A (*dl*309) or mutant HAdV's with deletions within E1A for 24h. The mutants chosen were those that mutated the N-terminus, CR3, and C-terminus of E1A due to Ku70's role in transcription (Fell & Schild-Poulter, 2015), as well as the initial identification of this interaction using the C-terminus of E1A. The results indicate that most of the mutants were

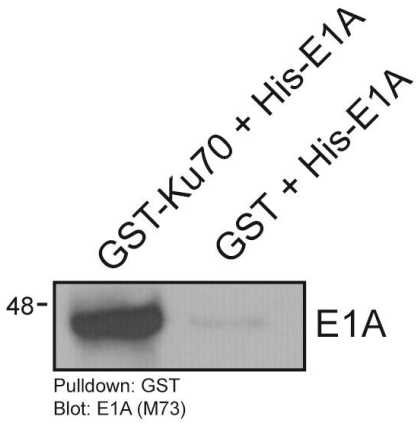
still able to interact with Ku70. However, mutations in the CR4 region of E1A resulted in a complete inability to bind Ku70. The CR4 region spans residues 241-289 in the 289R isoform of E1A (Avvakumov *et al.*, 2004), and is partially deleted by the HAdV mutants *dl*1133-*dl*1136. (Boyd *et al.*, 1993; Jelsma *et al.*, 1988). A reduction in binding can also be observed with the N-terminus mutant *dl*1103, which deletes residues 30-49 in the N-terminus (Figure 3.2). (Jelsma *et al.*, 1988)

3.3 Ku70 is required for efficient adenovirus growth

To determine how Ku70 affects viral growth, IMR-90 cells were treated with control siRNA that does not target any known human mRNA or one that targets Ku70. After Ku70 depletion, the cells were infected with HAdV *dl*309 and virus growth was assessed at 48h, 72h and 96h after infection. There was a reduction of *dl*309 growth by approximately 3-fold in those cells depleted of Ku70 compared to control treated cells (Figure 3.3 A). This suggests that Ku70 is important for maximal virus growth and replication.

To test whether the interaction between Ku70 and E1A was important for viral growth, a virus expressing E1A mutant unable to bind to Ku70 was assayed for growth on IMR-90 cells. The mutant used, *dl*1134, was reduced for growth similarly to what was observed after Ku70 depletion (Figure 3.3 B). This suggests that the effects seen in viral growth are due to the loss of interaction between Ku70 and E1A.

Figure 3.1.1: Ku70 binds directly to E1A. Bacterially purified GST-Ku70 and His- E1A289R were incubated together for h at 4 °C, and pulled down using glutathione beads. Bound proteins were eluted from bead, and then resolved by SDS-PAGE. E1A was detected using M73 monoclonal antibody. Inputs represent a Coomassie-stained gel showing 1 μg of each protein as used in the pull-down assay. (Frost *et al.*, 2017)


GST-Ku70 + His-E1A
GST + His-E1A
48
E1A
Pulldown: GST
Blot: E1A (M73)

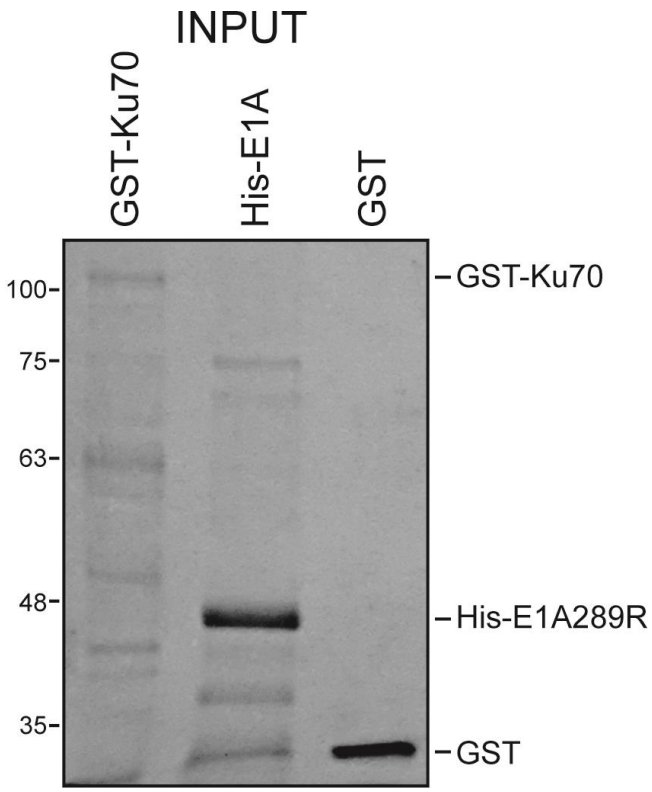

INPUT
GST-Ku70
His-E1A
GST
100
75
63
48
35
GST-Ku70
His-E1A289R
GST

Figure 3.1.2: E1A interacts with Ku70 during viral infection. HT1080 cells were infected with *pm*975 or *dl*520, Twenty-four hours after infection, cells were lysed and immunoprecipitated for E1A using the M73 antibody. Associated protein complexes were eluted from the beads and resolved by SDS-PAGE gel. They were then detected by western blot using anti-Ku70 antibody. Inputs shown. (Frost *et al.*, 2017)

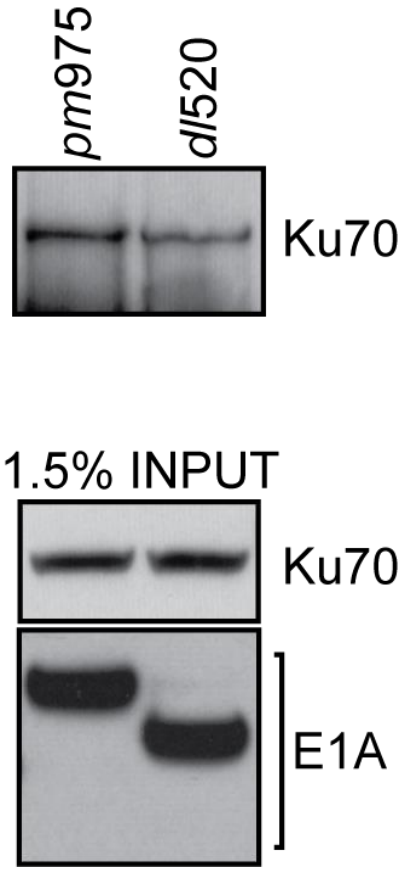
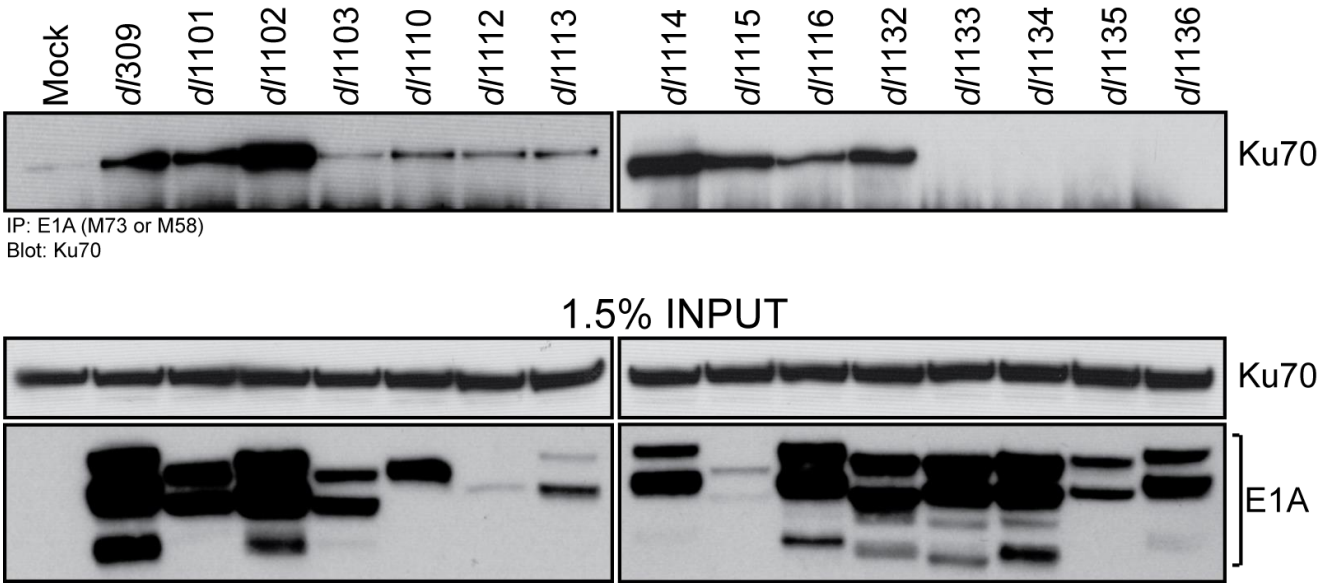

pm975
dl520
Ku70
1.5% INPUT
Ku70
E1A

Figure 3.2: Ku70 binds in the CR4 region of E1A. HT1080 cells were infected with the indicated HAdV-5 mutants or wt virus (*dl*309). Mock represents uninfected cells. Twenty-four hours after infections cells were lysed and immunoprecipitated for E1A using either M73 or M58 antibodies, M58 was used for mutants *dl*1135 and *dl*1136 as these do not have the M73 epitope. Associated protein complexes were then eluted from the beads, resolved by SDS-PAGE and detected by western blot using anti-Ku70 antibody. Inputs are shown.(Frost *et al.*, 2017)


Mock
dl309
dl1101
dl1102
dl1103
dl1110
dl1112
dl1113
dl1114
dl1115
dl1116
dl1132
dl1133
dl1134
dl1135
dl1136
Ku70
IP: E1A (M73 or M58)
Blot: Ku70
1.5% INPUT
Ku70
E1A

3.4 The effect of Ku70 on viral genome replication and gene expression

The reduction in viral growth upon Ku70 depletion indicates that Ku70 plays an important role in viral replication. To determine if Ku70 affects the virus's ability to replicate its genome, viral genomes were quantified from IMR-90 cells depleted of Ku70 and control treated cells. As seen in Figure 3.4.1, no significant change in viral genomes was observed at 24h or 48h after infection. However, at 72h there was consistent reduction in viral genomes observed in cells depleted for Ku70.

To elucidate the effect Ku70 has on HAdV gene expression, quantitative real-time reverse transcriptase PCR (qPCR) was performed on IMR-90 cells treated with Ku70 siRNA and control treated cells at 24h and 48h. While most genes did not show a significant difference between Ku70 depleted cells and control siRNA cells, the viral gene *E4orf6/7* was approximately 3-fold higher in those cells depleted for Ku70. (Figure 3.4.2) Overall these results showed that Ku70 has a marginal effect on HAdV ability to replicate its genome and on viral gene expression.

3.5 Ku70 associates with the HAdV genome

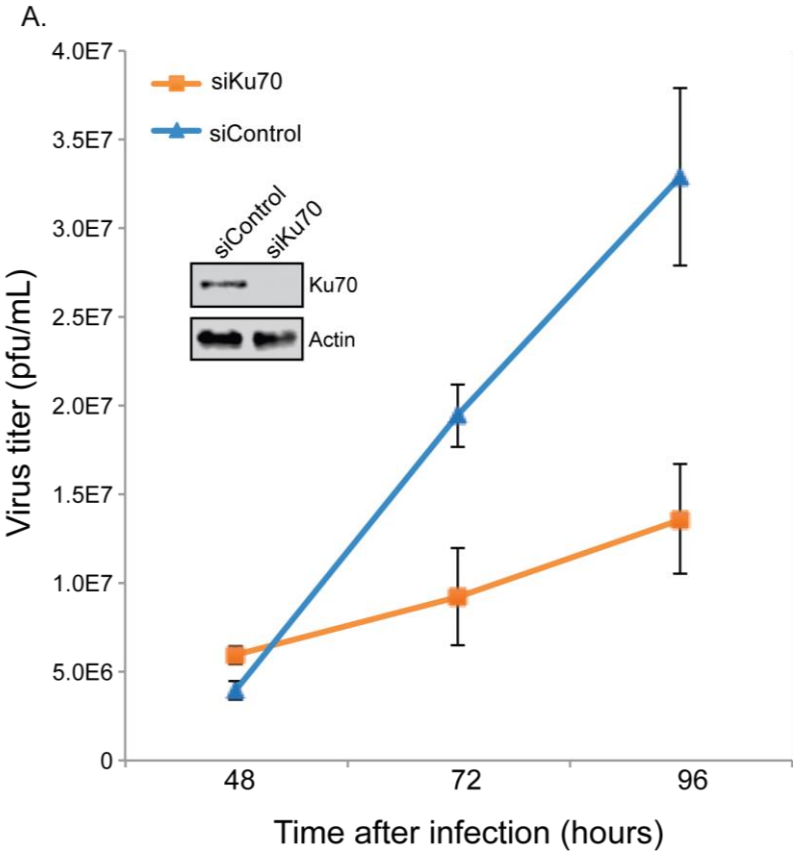
Despite marginal effects of Ku70 on viral gene expression, it was important to determine whether Ku70 was recruited to viral promoters or to the viral genome in general. IMR-90 cells were infected with *dl*309 for 24 hours, and chromatin immunoprecipitation (ChIP) was carried out to examine Ku70s occupancy on viral promoters and within viral protein coding regions. The results showed low levels (approximately 3-fold over IgG control) of Ku70 occupancy at most viral promoters (Figure 3.5 A). In contrast, E1A is found at approximately 40-60 fold enrichment at these promoters (Figure 3.5 B). Unexpectedly, Ku70 was found to be abundant at the coding region of the *hexon* gene in comparison to the levels of Ku70 found at viral promoters (Figure 3.5

A). This trend was also observed at the E1B, E3 and E2A open reading frames. These results show that Ku70 is not enriched significantly at viral promoters, but is recruited to the viral genome more generally.

3.6 Ku70 sub-cellular localization is restructured during adenovirus infection

Since the ChIP results showed that Ku70 is recruited to the viral genome, it was important to determine if Ku70 was recruited to viral replication centers. IMR-90 cells were infected with *dl*309 for 24h, and then fixed and stained for E1A, DBP, Ku70 and DAPI. As seen in Figure 3.6, during late infection, Ku70 is re-localized from being fairly evenly dispersed in uninfected cell nuclei (with a few concentrated spots of protein), to being reorganized in infected cells to more punctate structures that localize with viral replication centers (DBP staining). Ku70 does not seem to localize specifically to E1A early on in infection (16h).

Figure 3.3: Ku70 depletion decreases adenovirus growth. **A.** Ku70-depleted IMR-90 cells were infected with wild-type HAdV-5 *dl*309 at an m.o.i. of 10 for the indicated times, after which cells were harvested and virus released by freeze-thaw. Virus titer was then quantified by plaque assay on 293 cells. Error bars represent standard deviation of 3 biological replicates. **B.** IMR-90 cells were infected with HAdV-5 *dl*309 or *dl*1134 at an m.o.i. of 10 for the indicated times, after which cells were harvested and virus released by freeze-thaw. Virus titer was then quantified by plaque assay on 293 cells. Error bars represent standard deviation of 3 biological replicates. (Frost *et al.*, 2017)


A.
4.0E7
3.5E7
3.0E7
2.5E7
2.0E7
1.5E7
1.0E7
5.0E6
0
Virus titer (pfu/mL)
siKu70
siControl
siControl
siKu70
Ku70
Actin
48
72
96
Time after infection (hours)

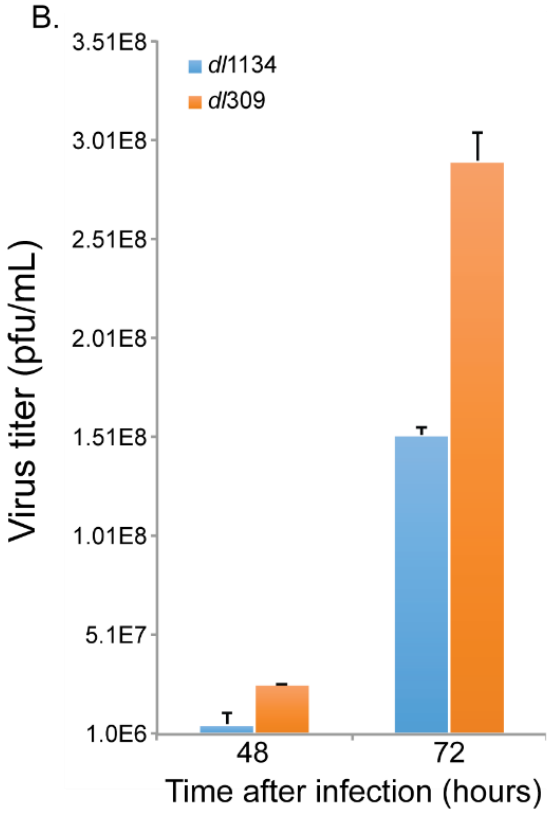

B.
3.51E8
3.01E8
2.51E8
2.01E8
1.51E8
1.01E8
5.1E7
1.0E6
Virus titer (pfu/mL)
dl1134
dl309
48
72
Time after infection (hours)

Figure 3.4.1: The effect of Ku70 on viral genome replication. IMR-90 cells transfected with control siRNA or siRNA targeting Ku70 were subsequently infected with *dl*309 at an m.o.i. of 10 for the indicated times and assayed for viral genomes by qPCR. Error bars represent standard deviation of 4 biological replicates.(Frost *et al.*, 2017)

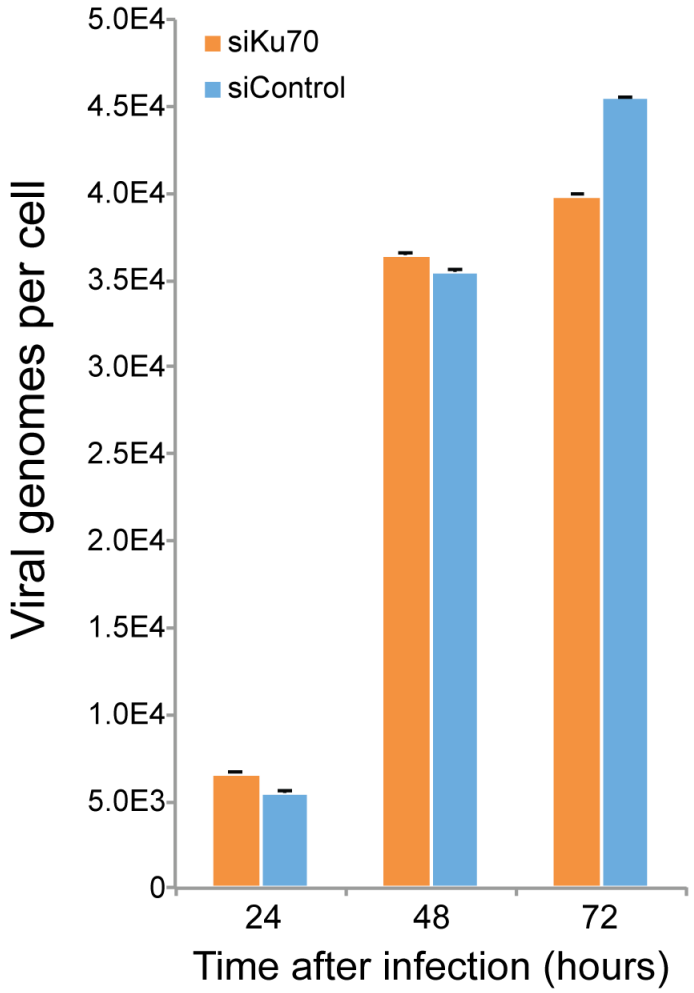

5.0E4
4.5E4
4.0E4
3.5E4
3.0E4
2.5E4
2.0E4
1.5E4
1.0E4
5.0E3
0
siKu70
siControl
Viral genomes per cell
24
48
72
Time after infection (hours)

Figure 3.4.2: The effect of Ku70 on viral gene expression. IMR-90 cells transfected with control siRNA or siRNA targeting Ku70 were infected with *dl*309 at an m.o.i. of 10 for 24h or 48h. Total RNA was extracted using the TRIzol method and viral gene expression was determined using reverse-transcriptase qPCR. GAPDH was used as a normalization control. Gene expression is represented as fold change in expression versus control siRNA transfected cells. Error bars represent standard deviation of 3 biological replicates.(Frost *et al.*, 2017)

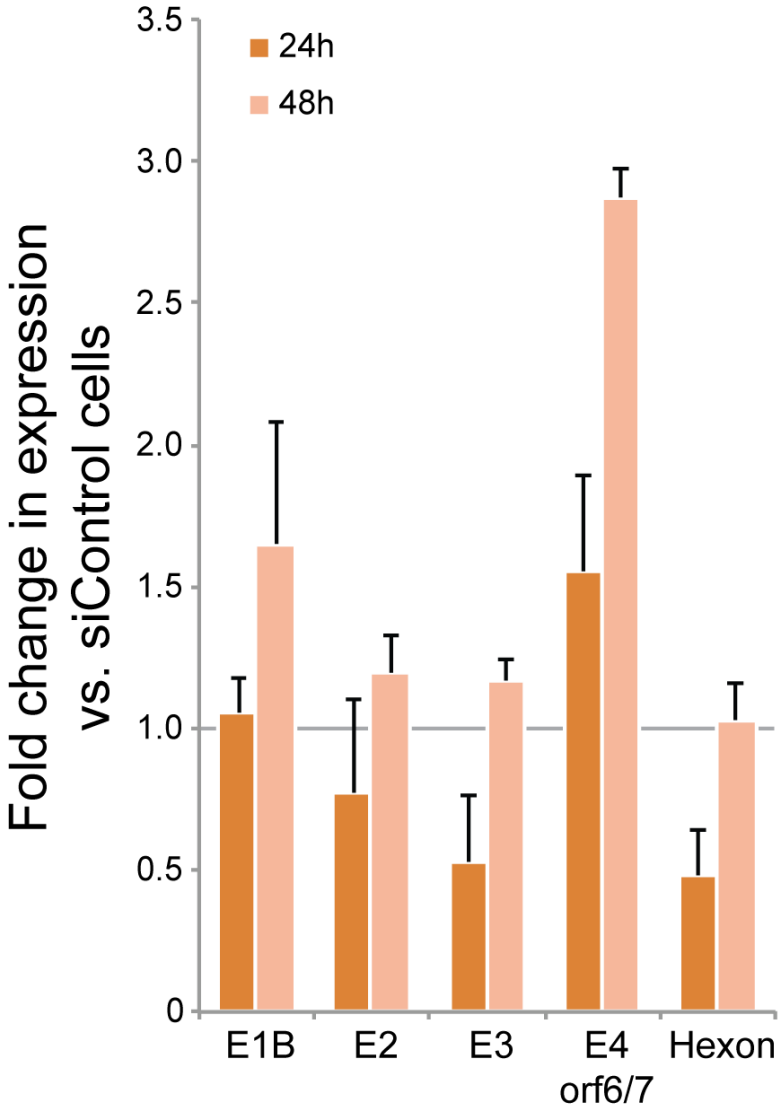
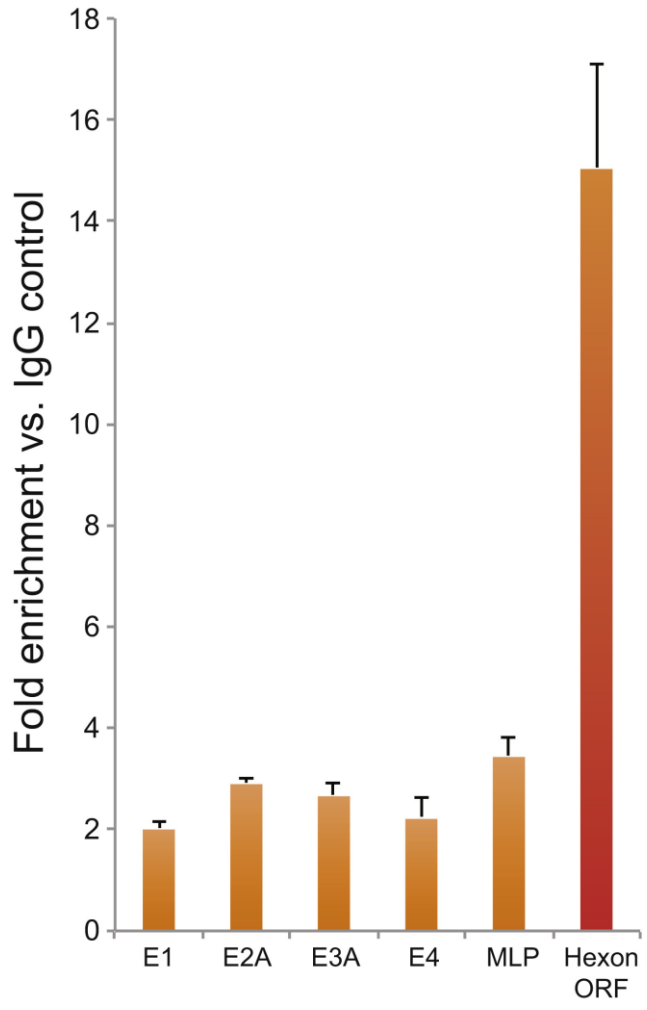

Fold change in expression vs. siControl cells
24h
48h
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
E1B
E2
E3
E4 orf6/7
Hexon

Figure 3.5: Ku70 associates with the viral genome. A. Twenty-four hours after *dl*309 infection at m.o.i. of 10 of IMR-90 cells, the cells were fixed, lysed, and ChIP was performed for Ku70 and IgG negative control. Quantification of promoter or coding region occupancy was performed by qPCR and is represented as fold enrichment versus IgG negative control. Error bars represent standard deviation of 3 biological replicates **B**. Cells treated the same as A were lysed and ChIP was performed for E1A and IgG negative control. Quantification of promoter occupancy was performed by qPCR and is represented as fold enrichment versus IgG negative control. Error bars represent standard deviation of 3 biological replicates. (Frost *et al.*, 2017)

A


Fold enrichment vs. IgG control
18
16
14
12
10
8
6
4
2
0
E1
E2A
E3A
E4
MLP
Hexon ORF

B

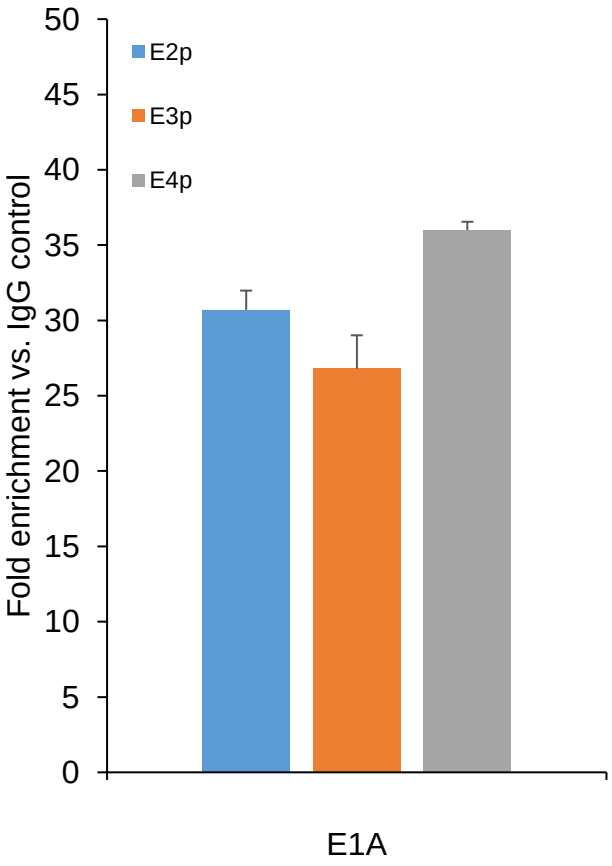
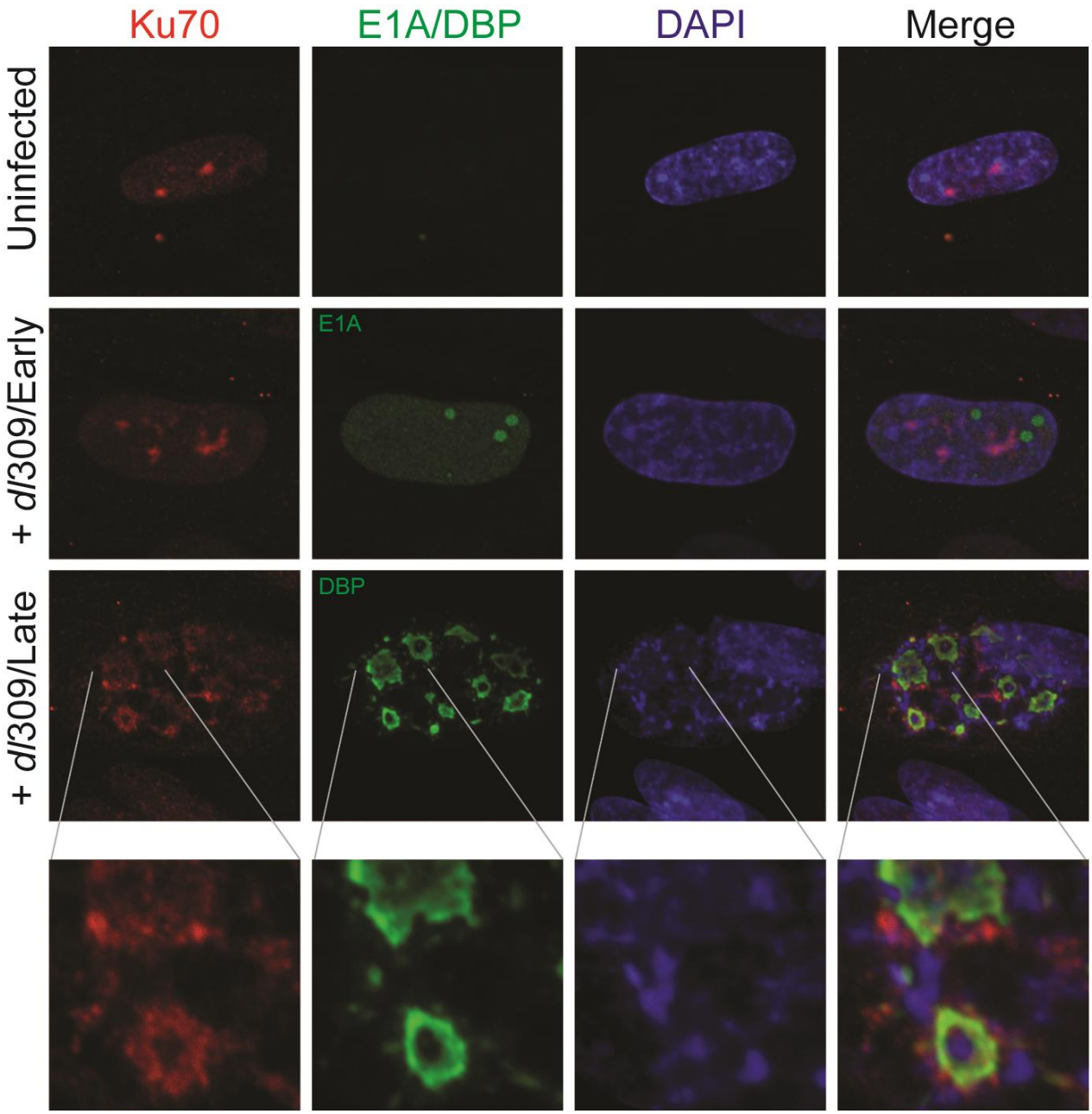

Fold enrichment vs. IgG control
50
45
40
35
30
25
20
15
10
5
0
E2p
E3p
E4p
E1A

Figure 3.6: Ku70 is recruited to viral replication centers. IMR-90 cells grown in chamber slides were infected with *dl*309 for 16h (Early) or 24h (Late), fixed and stained for E1A, DBP, Ku70 and DAPI. Cells were visualized using Zeiss LSM700 laser confocal microscope and analyzed using Zeiss ZEN software and composed using Adobe Illustrator. (Frost *et al.*, 2017)


Ku70
E1A/DBP
DAPI
Merge
Uninfected
+ dl309/Early
+ dl309/Late
E1A
DBP

3.7 Ku70 is important for the efficient induction of S-phase by HAdV

It has been shown that Ku70 is involved in signalling in the DNA damage response pathway and blocking of cell cycle entry (Fell & Schild-Poulter, 2015). Therefore, I wanted to determine if Ku70 played a role in the ability of HAdV to induce S-phase in previously arrested cells. IMR-90 cells were arrested via contact inhibition for 72h after depletion of Ku70 via siRNA or treatment with control siRNA. Cells were then infected with wild type *dl*309 for 24h and analyzed for cell cycle entry via EdU incorporation assay (Figure 3.7.1). Depletion of Ku70 in arrested primary cells led to a deficiency in the virus's ability to drive S-phase, with only 20% of infected cells entering S-phase. In contrast, 45% of arrested cells treated with control siRNA entered the S-phase after HAdV infection. (Figure 3.7.1) These results demonstrate that Ku70 is required for the efficient induction of S-phase by HAdV.

Next, I wanted to explore the mechanism by which Ku70 is able to influence S-phase induction by HAdV. To do this, the difference in activation in cell cycle genes known to regulate S-phase induction was examined in cells depleted of Ku70 vs. control cells. Cells depleted for Ku70 showed a significant reduction in cell cycle genes, specifically *BLM, CCNB1, CDC25C, MCM3,* and *MCM4*. (Figure 3.7.2). Interestingly, certain genes including *CDK6, CDKN1A,* and *PCNA* genes were found to be up-regulated as compared to cells with normal levels of Ku70. *CDKN1A* encodes for the cyclin dependent kinase inhibitor p21. This protein is responsible for negatively regulating cell cycle entry at G1 by binding to and inhibiting the activity of cyclin-dependent kinase 2 and cyclin-dependent kinase 1 complexes. It has been shown that p21 plays a role in S-phase DNA replication and DNA damage repair.(Abbas & Dutta, 2009) Together these

results suggest that Ku70 may be responsible for inhibiting the activation of the DNA damage response pathway during viral infection. In the absence of Ku70, the activation of the DNA damage pathway may lead to cell cycle arrest, subsequently resulting in a reduction in viral growth.

3.8 Ku70 is recruited to cellular promoters during HAdV infection

Ku70 depletion leads to a reduction of most cell cycle genes, with the exceptions of *PCNA*, *CDK6* and *CDKN1A* which were up-regulated. Based on this data, as well as earlier observations that Ku70 is recruited to the viral genome, I wanted to see if Ku70 is also recruited to cell cycle promoters during infection. To determine this, IMR-90 cells were infected with *dl*309 for 24h, and then ChIP was performed for Ku70, E1A or with rabbit anti-rat negative control antibody. As seen in Figure 3.8, prior to infection no Ku70 is observed at the cellular promoters *PCNA, CDK6, MCM4* and *CDKN1A*. After infection with HAdV, Ku70 enrichment at these promoters was observed. In particular, Ku70 was most significantly enriched at the *CDK6* and *PCNA* promoters. This suggests that Ku70 may play a role in transcriptional regulation during infection, this would be a novel role for the Ku70 protein, among its many other functions.

Figure 3.7.1: Ku70 affects HAdV's ability to drive S-phase induction. Arrested IMR-90 cells that were transfected with siRNA targeting Ku70 or control siRNA were infected with *dl*309 at an m.o.i. of 20 for 23h, pulsed with EdU for 1h, and then stained with Click-It EdU labeling kit. Cells were subsequently stained for E1A using the M73 monoclonal antibody and quantified using a fluorescent microscope. Data is represented as percentage of infected cells that were also positive for EdU. Error bars represent standard deviation of 5 random fields of view. (Frost *et al.*, 2017)

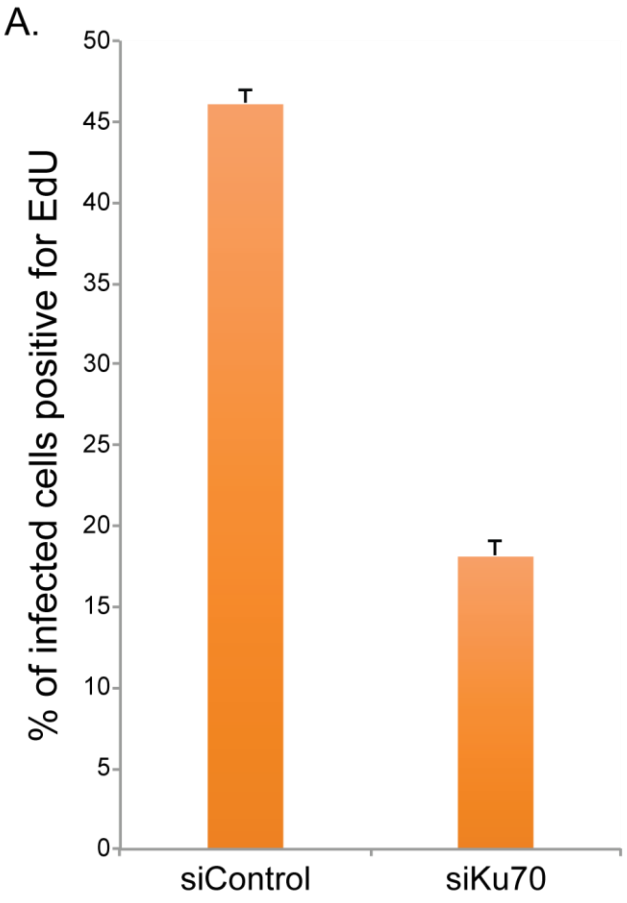

A.
% of infected cells positive for EdU
50
45
40
35
30
25
20
15
10
5
0
siControl
siKu70

Figure 3.7.2: Impact of Ku70 depletion on cell cycle genes. IMR-90 cells that were transfected with siRNA targeting Ku70 or control siRNA were infected with *dl*309 at an m.o.i. of 10 for 24h. Cells were harvested using the TRIzol method. Total RNA was extracted and quantified using reverse-transcriptase qPCR. GAPDH was used as a reference. The data is represented as fold change in expression versus cells transfected with control siRNA. Error bars represent standard deviation of 3 biological replicates.(Frost *et al.*, 2017)

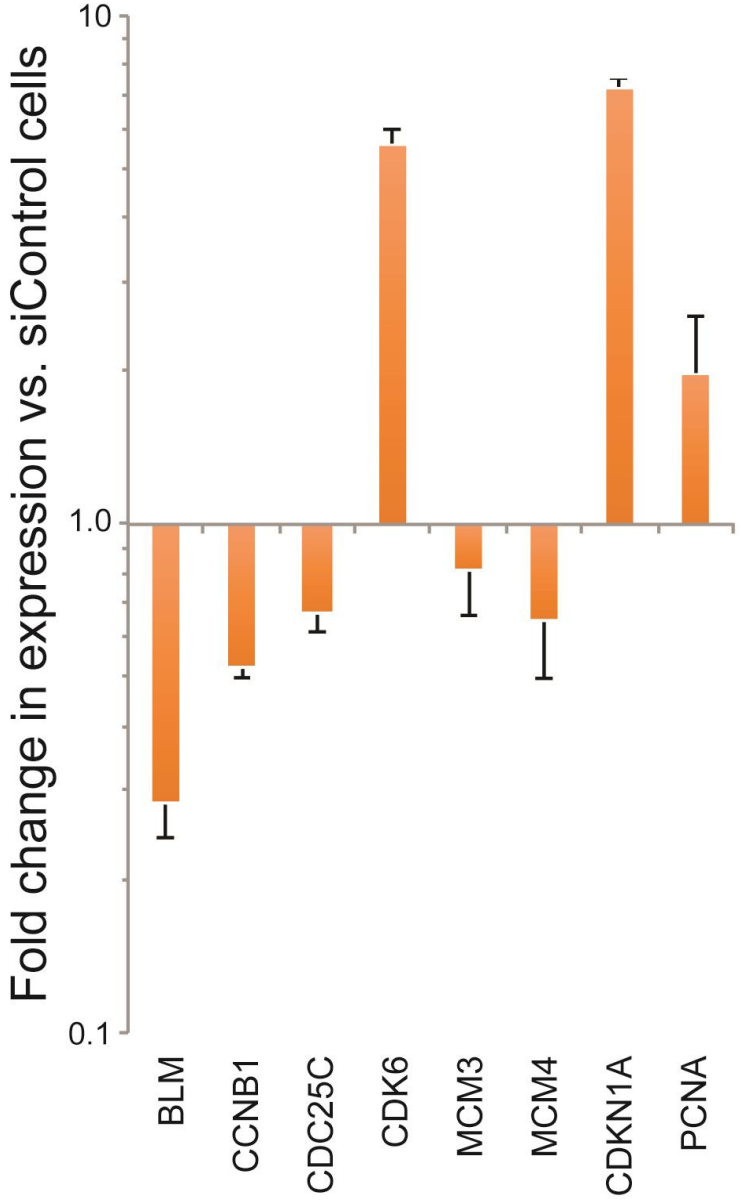
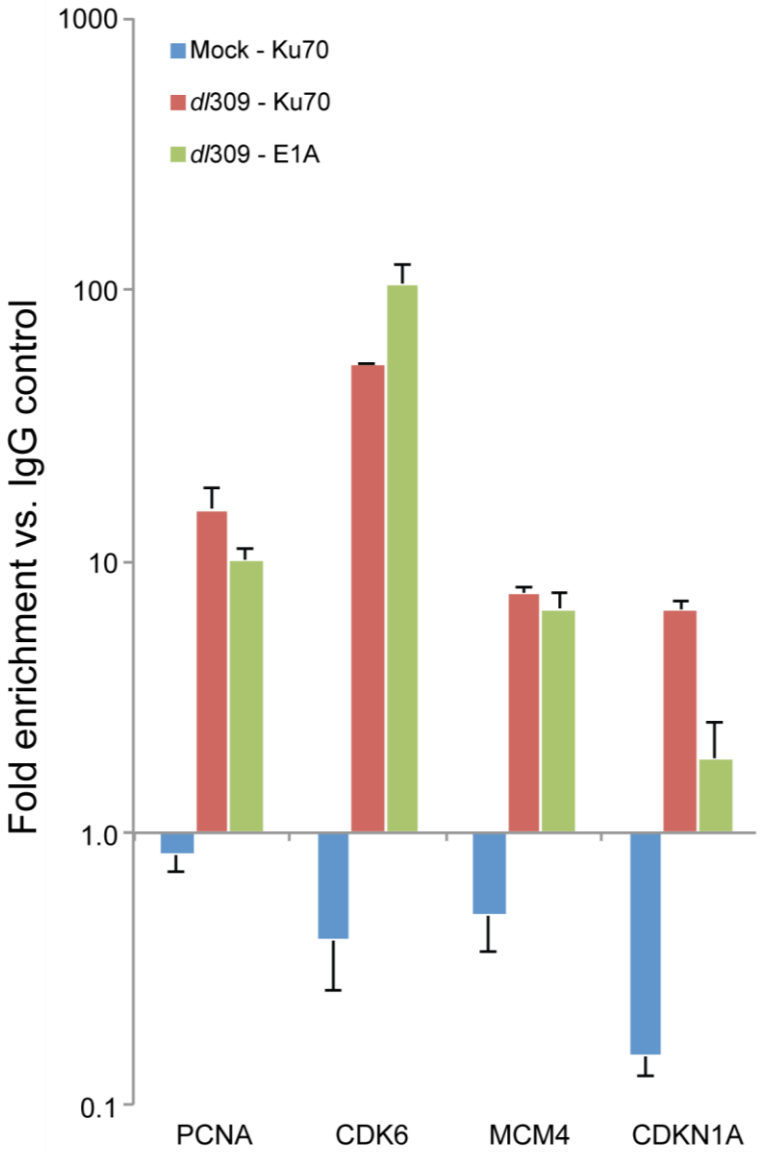

Fold change in expression vs. siControl cells
10
1.0
0.1
BLM
CCNB1
CDC25C
CDK6
MCM3
MCM4
CDKN1A
PCNA

Figure 3.8: Ku70 is recruited to cell cycle regulated promoters during infection. IMR-90 cells were mock infected or infected with *dl*309 at an m.o.i. of 10 for 24h and ChIPs were performed for Ku70, E1A, or IgG control antibody. Occupancy was quantified by qPCR and is represented as fold enrichment versus IgG negative control. Error bars represent standard deviation of 3 replicates. (Frost *et al.*, 2017)


1000
100
10
1.0
0.1
Fold enrichment vs. IgG control
Mock - Ku70
dl309 - Ku70
dl309 - E1A
PCNA
CDK6
MCM4
CDKN1A

FUBP1

3.9 FUBP1 binds directly to E1A

Through affinity purification and mass spectrometry, FUBP1 was one of the novel proteins found to bind to the C-terminus of the E1A protein. To determine whether this interaction was direct or indirect, GST-fused FUBP1 protein was used in a GST-pulldown assay. The GST-pull down assay was performed using bacterially expressed and purified GST-fused FUBP1 and His-tagged HAdV-5 E1A 289R (13S) protein (Figure 3.9.1). The results showed that E1A interacts directly with FUBP1.

To determine which region of E1A FUBP1 interacts with, HT1080 cells were co-transfected with plasmids expressing genomic HAdV-5 E1A isoforms or GFP-tagged HAdV-5 E1A regions and HA-tagged FUBP1. Twenty-four hours after transfection, cells were lysed and immunoprecipitated for E1A using the M73 monoclonal antibody or for GFP for those transfected with the E1A-GFP fusions. As seen in figure 3.9.2, FUBP1 is able to bind both the 289R isoform of E1A and the 243R isoform of E1A, although the affinity for the 289R appears to be stronger. The co-immunoprecipitation of the GFP-fused fragments of E1A with FUBP1 indicates that FUBP1 binds both the N-terminus and the CR3 region.

3.10 Effect of FUBP1 on viral growth and replication

To determine how FUBP1 affects viral growth, IMR-90 cells were treated with control siRNA that does not target any known human mRNA or a siRNA that targets FUBP1. After FUBP1 depletion, the cells were infected with HAdV *dl*309, and virus growth was assessed at 48h, 72h and 96h after infection. There was a reduction of *dl*309 growth in cells depleted of FUBP1 of

approximately 30%. (3.10 A) While this reduction in growth was seen consistently, the effect was not as dramatic as seen with other proteins such as Ku70. (Frost *et al.,* 2017).

Although the reduction of virus growth in cells depleted for FUBP1 was minimal, I wanted to determine whether viral gene expression was affected by depletion of FUBP1. IMR-90 cells depleted of FUBP1 via siRNA or control cells were infected with *dl*309 at a m.o.i. of 10. Figure 3.10 C shows that after FUBP1 depletion there is a minimal upregulation in viral genes.

Interestingly, when investigating if FUBP1 affects HAdV's ability to replicate its genome, a consistent decrease in the number of viral genomes per a cell was observed in IMR-90 cells depleted for FUBP1 as compared to control treated cells (Figure 3.10 B). While the effect of FUBP1 on viral growth and replication was minimal, the combined effects may have a significant impact on the virus's ability to proliferate efficiently.

3.11 FUBP1 is not critical for the induction of S-phase by HAdV

Since depletion of FUBP1 had minimal effect on HAdV growth and expression of viral genes, it was important to determine whether FUBP1 played a role in influencing E1A's ability to induce S-phase induction in arrested cells. Similarly to what was done for Ku70, IMR-90 cells arrested by contact inhibition were treated with siRNA targeting FUBP1 or control siRNA, and subsequently infected with *dl*309 at a m.o.i. of 20 for 24 hours. The cells were then analyzed for S-phase entry via an EdU incorporation assay (Figure 3.11). There were no significant differences observed between FUBP1 depleted cells and control cells, indicating FUBP1 does not play an active role in assisting E1A in the initiation of the cell cycle.

Figure 3.9.1: E1A binds directly to FUBP1. Bacterially purified GST-FUBP1 and His-E1A289R were incubated together for 1h at 4 °C, and pulled down using glutathione beads. Bound proteins were eluted from beads using sample buffer, and then resolved by SDS-PAGE. E1A was detected using M73 monoclonal antibody. Inputs represent a Coomassie-stained gel showing 1 μg of His-E1A and GST as used in the pull-down assay, while the GST-FUBP1 was detected via western blot due to low levels during purification.

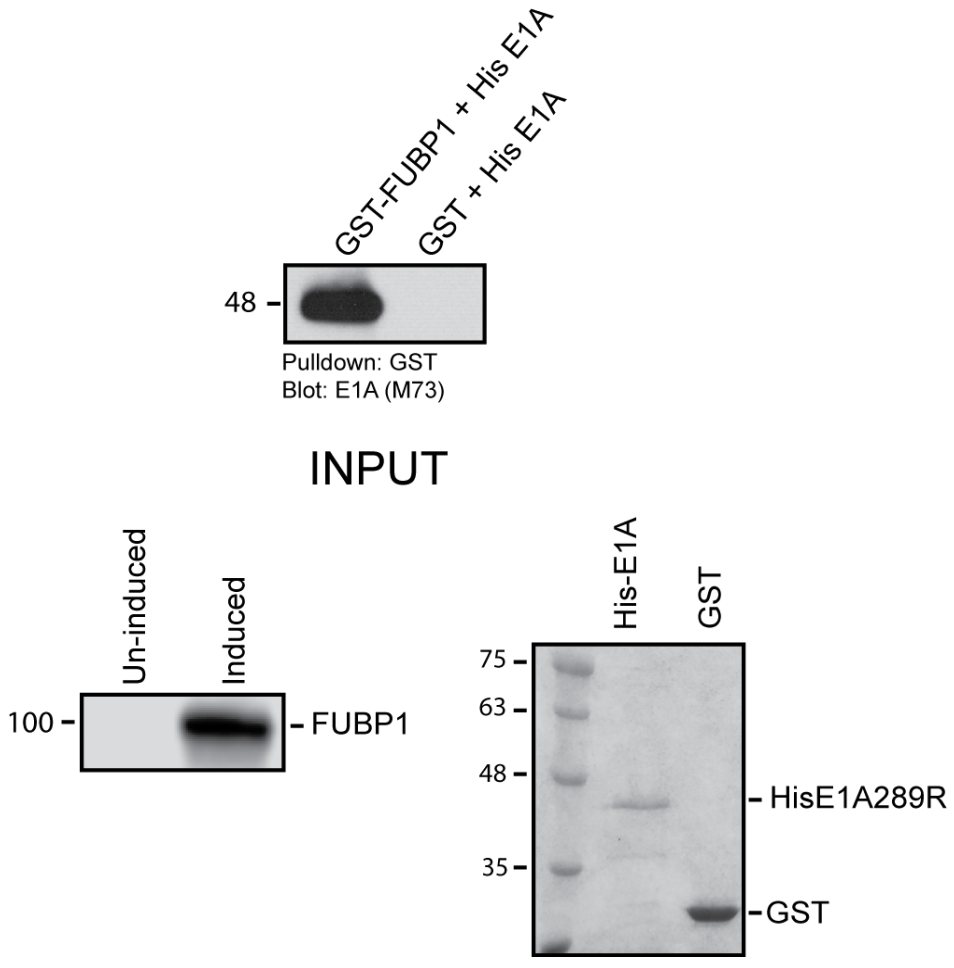

GST-FUBP1 + His E1A
GST + His E1A
48
Pulldown: GST
Blot: E1A (M73)
INPUT
Un-induced
Induced
100
FUBP1
His-E1A
GST
75
63
48
35
HisE1A289R
GST

Figure 3.9.2: FUBP1 interacts with the N-terminus and CR3 regions of E1A. HT1080 cells were co-transfected with plasmids expressing genomic HAdV-5 E1A isoforms or GFP-tagged HAdV-5 E1A regions and HA-tagged FUBP1. Twenty-four hours after transfection, cells were lysed and immunoprecipitated for E1A using the M73 monoclonal antibody or E1A-GFP fusions using anti-GFP antibody. Associated protein complexes were eluted from beads and resolved by SDS-PAGE and detected by western blot using anti-HA antibody. Inputs are shown.

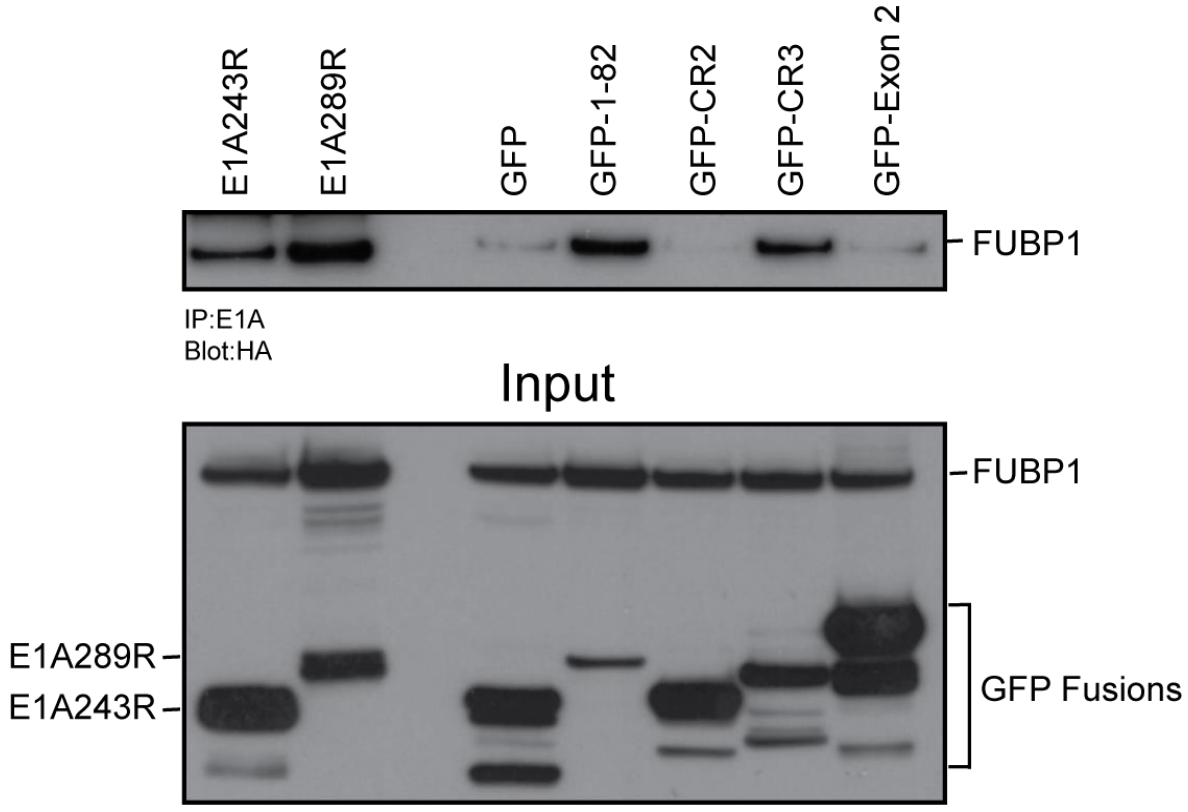

E1A243R
E1A289R
GFP
GFP-1-82
GFP-CR2
GFP-CR3
GFP-Exon 2
FUBP1
IP:E1A
Blot:HA
Input
FUBP1
E1A289R
E1A243R
GFP Fusions

Figure 3.10: Effect of FUBP1 on viral growth and replication. **A.** FUBP1-depleted IMR-90 cells were infected with wild-type HAdV-5 *dl*309 at an m.o.i. of 10 for the indicated times, after which cells were harvested and virus released by freeze-thaw. Virus titer was then quantified by plaque assay on 293 cells. Error bars represent standard deviation of 3 biological replicates. **B**. IMR-90 cells were treated the same way as in A and were harvested at the indicated times and assayed for viral genomes by qPCR. Error bars represent standard deviation of 4 biological replicates. **C.** IMR-90 cells treated the same way as in A. for 24h or 48h. Total RNA was extracted using the TRIzol method and viral gene expression was determined using reverse-transcriptase qPCR. GAPDH was used as a normalization control. Gene expression is represented as fold change in expression versus control siRNA transfected cells. Error bars represent standard deviation of 3 biological replicates.

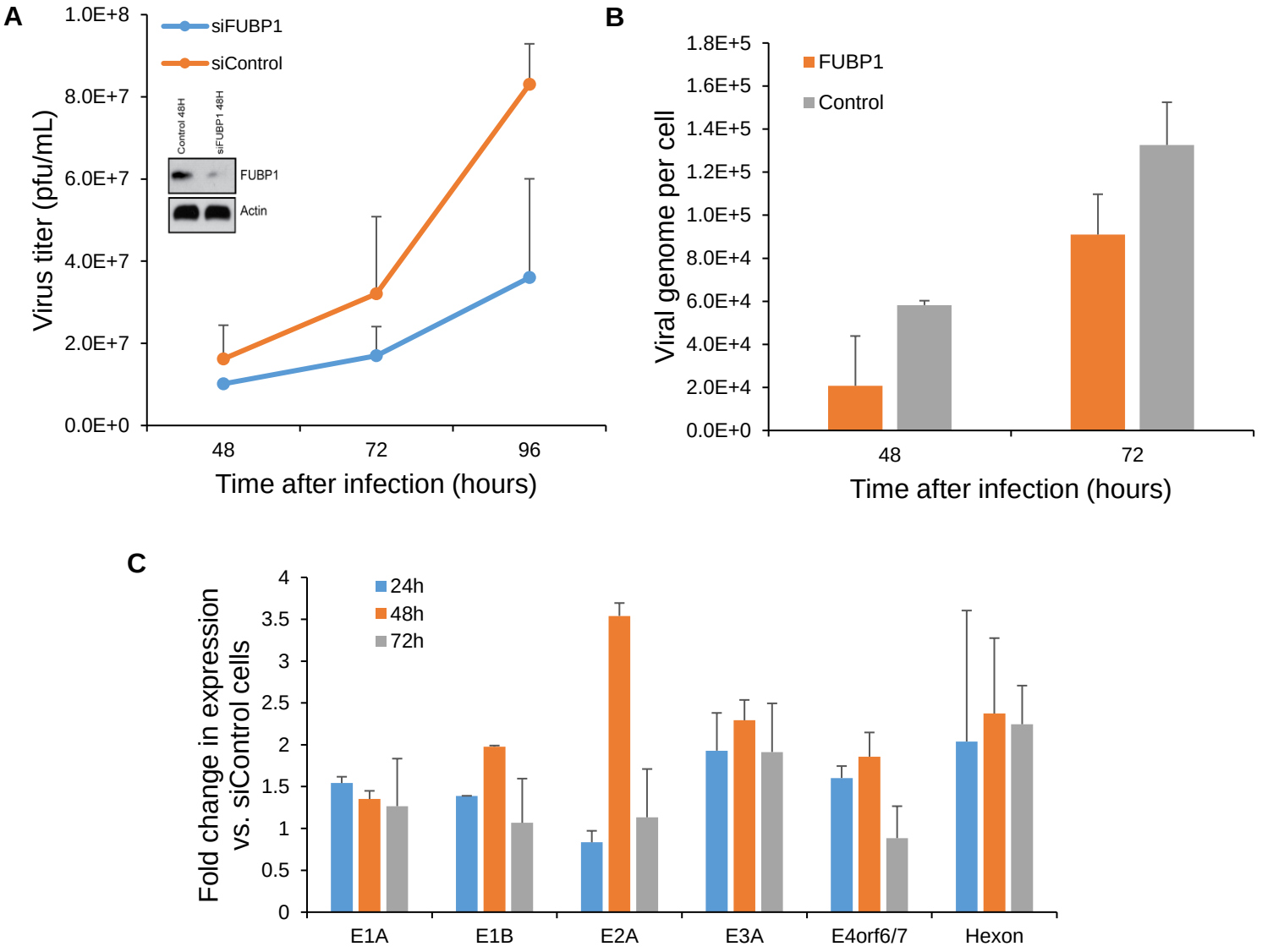
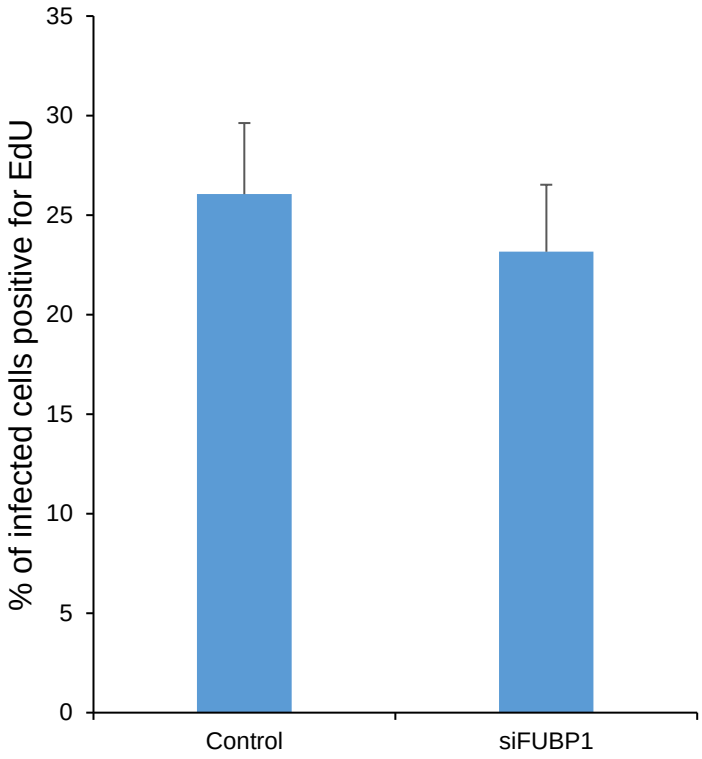

A
1.0E+8
8.0E+7
6.0E+7
4.0E+7
2.0E+7
0.0E+0
Virus titer (pfu/mL)
siFUBP1
siControl
Control 48H
siFUBP1 48H
FUBP1
Actin
48
72
96
Time after infection (hours)
B
1.8E+5
1.6E+5
1.4E+5
1.2E+5
1.0E+5
8.0E+4
6.0E+4
4.0E+4
2.0E+4
0.0E+0
Viral genome per cell
FUBP1
Control
48
72
Time after infection (hours)
C
Fold change in expression vs. siControl cells
4
3.5
3
2.5
2
1.5
1
0.5
0
24h
48h
72h
E1A
E1B
E2A
E3A
E4orf6/7
Hexon

Figure 3.11: FUBP1 does not affect S-phase induction. Arrested IMR-90 cells were transfected with siRNA targeting FUBP1 or control siRNA were then infected with *dl*309 at an m.o.i. of 20 for 23h, pulsed with EdU for 1h, and then stained with Click-It EdU labeling kit. Cells were subsequently stained for E1A using the M73 monoclonal antibody and quantified using a fluorescent microscope. Data is represented as percentage of infected cells that were also positive for EdU. Error bars represent standard deviation of 5 random fields of view.


% of infected cells positive for EdU
35
30
25
20
15
10
5
0
Control
siFUBP1

3.12 The role of FUBP1 in the regulation of cell cycle genes

Although it was observed that FUBP1 did not have an effect on E1A's ability to induce S-phase in arrested cells, I wanted to determine if there were any differences in the activation of cell cycle genes when FUBP1 was depleted. In particular, I was interested in the effect on p21 mRNA levels *(CDKN1A*), as it has been shown in the literature that the depletion of FUBP1 leads to an up-regulation of this protein. (Rabenhorst *et al.*, 2009) To determine this effect, the difference in activation in cell cycle genes including *CDKN1A* was examined using qPCR in IMR-90 cells depleted of FUBP1 vs. control cells. As seen in Figure 3.12, there is a consistent up-regulation of p21 (CDKN1A) in cells depleted of FUBP1 compared to control treated cells. This up-regulation increases with a longer duration of viral infection. Interestingly, there was also an upregulation of CDK6 at 72h, while other genes did not show a significant change in expression. These results suggest that FUBP1 may affect the translation of these cell cycle regulating genes, as the effect on p21 was similar to that shown in literature. (Abbas & Dutta, 2009) If FUBP1 is regulating these proteins by binding to their mRNAs, this could account for the small but consistent decrease seen in viral growth in cells depleted of FUBP1

3.13 FUBP1 sub-cellular localization is restructured during HAdV infection

FUBP1 has been shown in literature to play a role in binding to viral RNA as well as influencing viral replication. Therefore, I wanted to see if FUBP1 could be observed interacting with DNA replication centers (DBP) or if the cellular localization of FUBP1 was altered in the presence of E1A. To determine how the localization of FUBP1 in the nucleus becomes restructured during HAdV infection, IMR-90 cells were infected with *dl*309 for 24h, and then fixed and stained for E1A, DBP, FUBP1 and DAPI. As seen in Figure 3.13**,** during late infection, FUBP1 is re-

localized from being generally dispersed in uninfected cell nuclei, to being concentrated into punctate structures upon infection. While these structures do not seem to localize specifically to E1A or DNA binding protein (DBP), it is clear that FUBP1 is being reassigned from its normal localization after infection.

3.14 FUBP1 binds viral mRNAs

FUBP1 has previously been shown to bind to viral and cellular mRNAs in order to alter their regulation. (Zhang & Chen, 2012) Therefore, I wanted to see if FUBP1 was binding to HAdV viral mRNAs. To do this, HT1080 cells were infected at a m.o.i. of 20 for 48h and immunoprecipitation was carried out for FUBP1, followed by RNA extraction to isolated FUBP1-bound RNAs. Figure 3.14A shows that FUBP1 is able to bind certain viral mRNAs during infection. There is a significant fold enrichment of viral E1B, E3, and E4orf6/7 mRNAs. When this was repeated with E1A antibody (M73) to determine if E1A and FUBP1 were forming a complex to bind and recruit these mRNAs it was found that E1A did not form significant complexes with any of the viral mRNAs (Figure 3.14 B). As to what role FUBP1 is playing in binding to these viral mRNAs remains to be discovered.

Figure 3.12: FUBP1 in cell cycle gene regulation. IMR-90 cells that were transfected with siRNA targeting FUBP1 or control siRNA were infected with *dl*309 at an m.o.i. of 10 for 24h. Cells were harvested using the TRIzol method. Total RNA was extracted and quantified using reverse-transcriptase qPCR. GAPDH was used as a reference. The data is represented as fold change in expression versus cells transfected with control siRNA. Error bars represent standard deviation of 3 biological replicates.

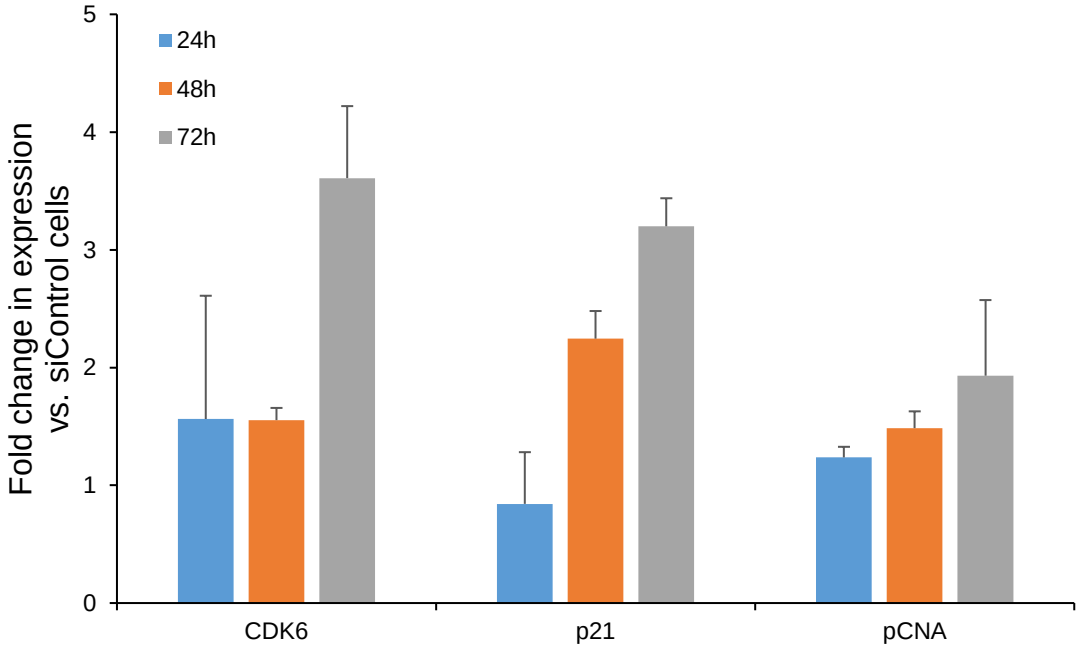

Fold change in expression vs. siControl cells
5
4
3
2
1
0
24h
48h
72h
CDK6
p21
pCNA

Figure 3.13: FUBP1 re-localization upon HAdV infection. IMR-90 cells grown in chamber slides were infected with *dl*309 24h, fixed and stained for E1A, DBP, FUBP1 and DAPI. Cells were visualized using Zeiss LSM700 laser confocal microscope and analyzed using Zeiss ZEN software and composed using Adobe Illustrator.

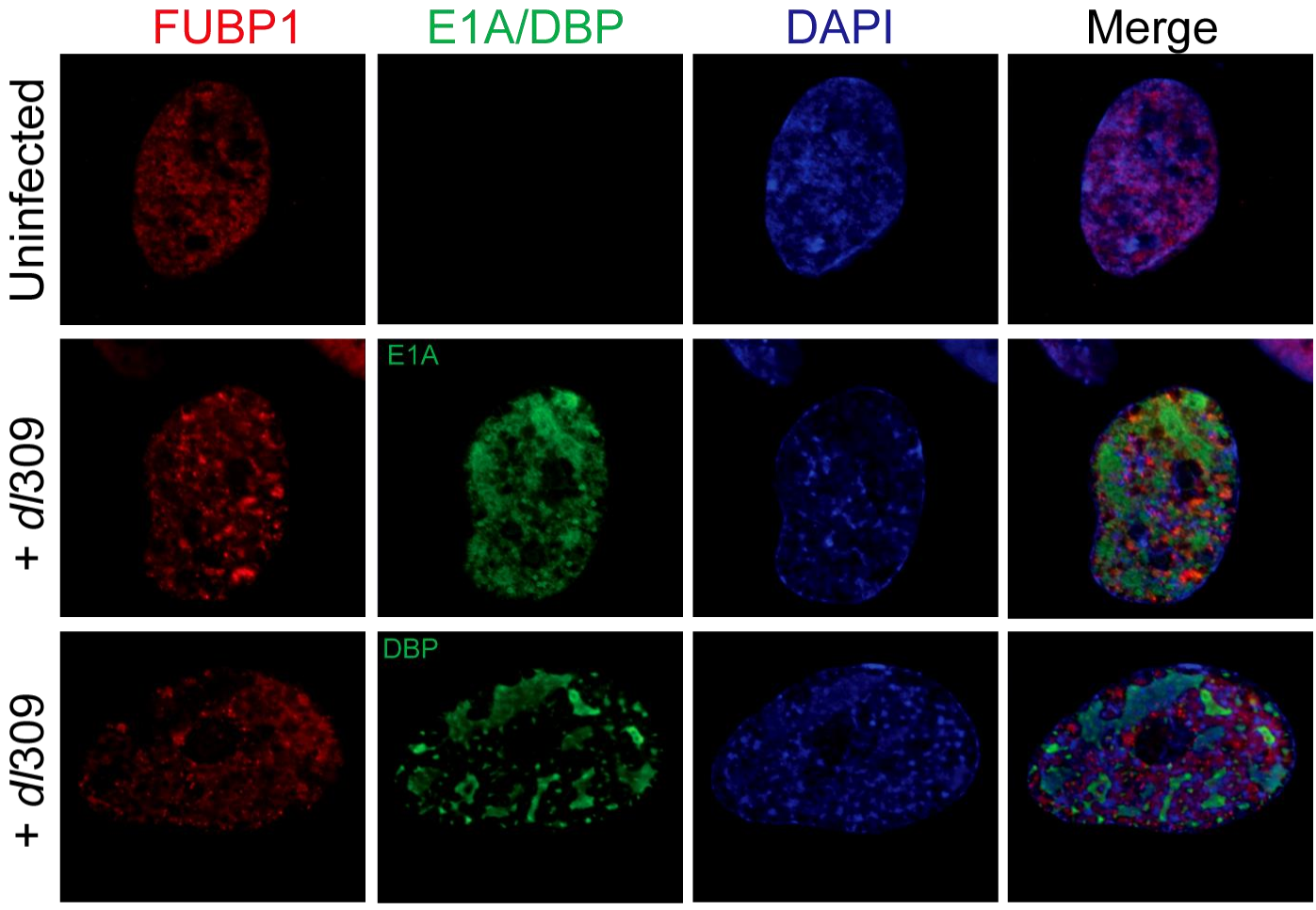
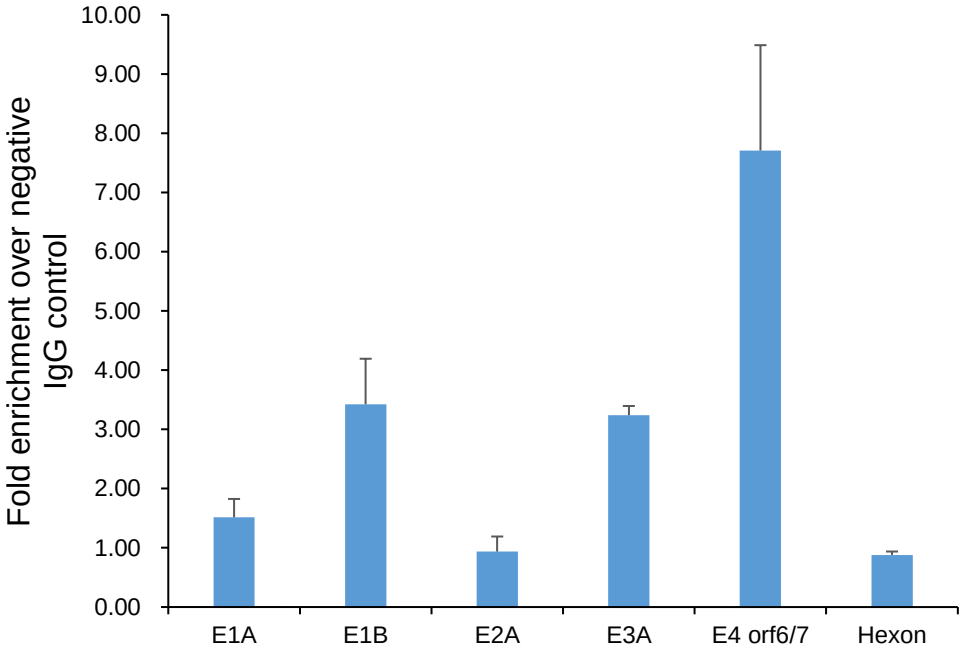
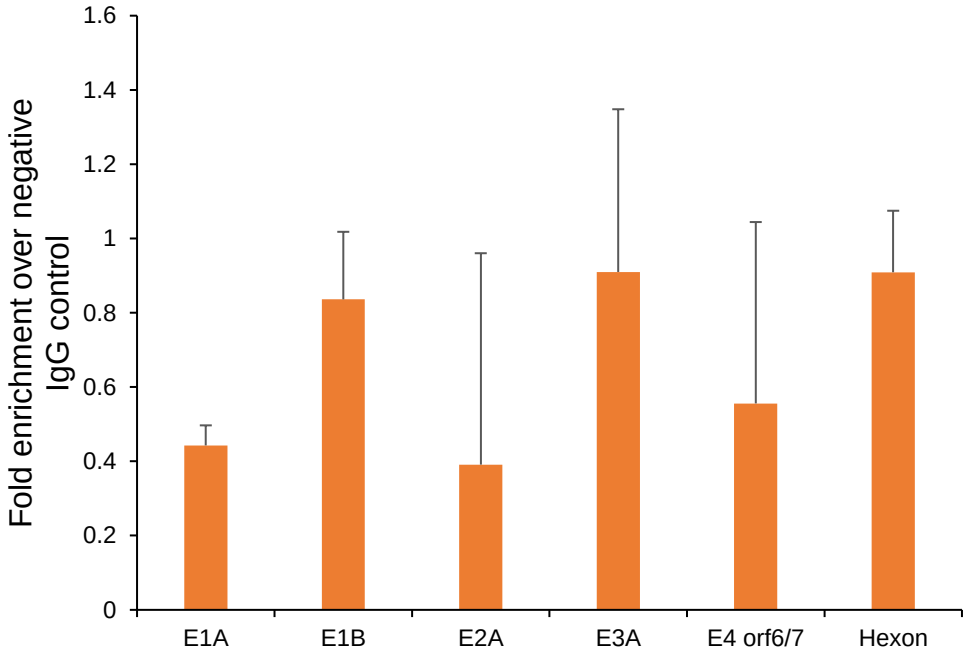

FUBP1
E1A/DBP
DAPI
Merge
Uninfected
+ dl309
+ dl309
E1A
DBP

Figure 3.14: FUBP1 binds viral mRNA's. A. HT1080 cells were infected with *dl*309 at a m.o.i. of 20 for 48h. Cells were then harvested and nuclei were collected and lysed using a homogenizer. Nuclear lysate was used for immunoprecipitation with FUBP1 antibody or control Rabbit anti-rat IgG antibody. Complexes were then harvested using the TRIzol reagent. Total RNA bound to beads was collected and quantified using reverse-transcriptase qPCR. IgG was used as a reference. The data is represented as fold change in expression versus immunoprecipitation with IgG antibody. Error bars represent standard deviation of 3 biological replicates. **B.** HT1080 cells were treated the same as in A, and were immunoprecipitated with E1A (M73) antibody or IgG control. Error bars represent standard deviation of 3 biological replicates.

A


Fold enrichment over negative IgG control
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
E1A
E1B
E2A
E3A
E4 orf6/7
Hexon

B


Fold enrichment over negative IgG control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E1A
E1B
E2A
E3A
E4 orf6/7
Hexon

4. Discussion:

4.1 Ku70

Ku70 is a multifunctional protein that plays a role in DNA repair, telomere maintenance and V(D)J recombination. (Fell & Schild-Poulter, 2015) Ku70's role in the DNA damage repair pathway and NHEJ has been well characterized. (Lees-Miller & Meek, 2003) Overall, Ku70 is a vital protein in human cells, and it participates in many roles. My studies have identified how human adenovirus uses Ku70 for its own benefit.

Ku70 interacts directly with the CR4 region of E1A

The novel interaction between Ku70 and E1A was first identified through affinity purification and mass spectrometry. This interaction was confirmed to be a direct protein-protein interaction. (Figure 3.1.1). This interaction was confirmed to occur during viral infection with the co-immunoprecipitation of Ku70 virus *pm*975 and *dl*520 which express the 289R isoform and 243R isoform of E1A respectively. It is worth noting that there seems to be a stronger interaction with *pm*975 that expresses the 289R isoform. (Figure 3.1.2)

Using a series of E1A mutants, the binding region of Ku70 to E1A was mapped to the CR4 region (Figure 3.2). Interestingly, any mutation in the CR4 region lead to a loss of binding (E1A mutant's *dl*1133-*dl*1136). This suggests that a defined structure may be required for the binding of Ku70 as most of the CR4 region has been predicted to be structured. The CR4 region contains a Pro-X-Asp-Leu-Ser (PXDLS) motif at residues 279-283, that has been shown to bind CtBP. (Pelka *et al.*, 2008) The residues N-terminal of this PXDLS motif have been shown to form an α-helix, which coincide with the mutated residues in the Ku70 binding deficient E1A mutants (residues 241-289), suggesting that structure is required for Ku70 binding. (Jelsma *et al.*, 1988; Molloy *et al.*, 2007) Alternatively, the structure of CR4 could be stabilized upon binding of Ku70

similarly to what has been observed with the CR1 region of E1A and binding of CBP (Ferreon *et al.*, 2009).

Ku70 was found to be recruited to most of the viral genome. This made mapping the binding location challenging, as distant Ku70 and E1A complexes bound to the viral chromosome were creating false positives in co-immunoprecipitation. To overcome this issue, cell lysates needed to be treated with DNase I prior to immunoprecipitation. This allowed for immunoprecipitation of complexes directly associated rather than those that were tethered by viral genomes.

CR4 has been shown to play a role in the immortalization and tumorigenicity functions of the E1A protein. Only a handful of binding partners have been identified for the CR4 region. For example, one of the well characterized interactions with the CR4 region is with CtBP. CtBP is a transcriptional corepressor that directs the formation of a silencing complex when recruited to a cellular promoter. HAdV E1A inhibits CtBP by competing with repressors for the repressor-binding site on CtBP, inhibiting the repression of transcription. (Frisch & Mymryk, 2002; Schaeper *et al.*, 1995) Another important CR4 binding protein is the dual-specificity Yak1-related kinases (Dyrks). Dyrk1A/B have been associated with the regulation of cell survival, proliferation and differentiation. When E1A interacts with Dyrk1A/B, it increases their kinase activity in vitro. Interestingly, an increase in Dyrk1A has been shown to be present in malignant HPV-positive cervical cancers, where it has been suggested to function as an anti-apoptotic factor. It is possible that E1A may also use Dyrk1A in a similar manner. (Zhang *et al.*, 2001) Overall, the few binding partners that have been identified for the CR4 region have been shown to play roles in the tumorigenicity and immortalization functions of HAdV E1A. Ku70 binding in the CR4 region

could indicate that it also contributes to E1A's ability to immortalize/transform cells. (Pelka *et al.*, 2008)

Ku70 affects viral growth and genome copy numbers

As seen in Figure 3.3 A, when Ku70 is depleted, there is an approximate 3-fold reduction in viral growth at the later time points compared to wild type cells. This indicates that Ku70 has a positive effect on viral proliferation, and is needed for efficient propagation of the virus. To confirm that the decrease in viral titre is due to the loss of Ku70 and the ability of E1A to modulate Ku70-regulated activities, the viral growth assay was repeated with viral E1A mutant *dl*1134, as seen in Figure 3.2, is deficient for binding to Ku70. Figure 3.3 B shows that there is a similar reduction in virus growth for *dl*1134 as is observed in cells depleted of Ku70 (72h). Ku70 depleted cells also showed a reduction in the number of viral genomes per a cell at 72h, again suggesting that Ku70 contributes to viral growth. (Figure 3.4.1) Taken together, the results on viral growth and genome copy numbers indicate it is likely that Ku70 is required for maximal viral growth.

Ku70 and viral genes

Interestingly, although there is a modest but consistent decrease in viral growth and viral genomes upon depletion of Ku70 as virus infection progresses, this is not correlated with the expression of viral genes. It was found that upon Ku70 depletion, most viral genes were actually upregulated to some extent. This was particularly true for the *E4orf6/7* gene which was upregulated approximately 3-fold at 48h. (Figure 3.4.2) *E4orf6/7* is able to bind to the cellular transcription factor E2F, resulting in the transactivation of the HAdV E2 promoter. The activation of the E2 promoter is favorable to viral DNA replication as the targets of the E2 promoter promote cell cycle S-phase. (Huang & Hearing, 1989) It is possible that the induction of the *E4orf6/7* gene is an

attempt by HAdV to compensate for the loss of S-phase induction seen when Ku70 is depleted. Overall, the effects seen in viral growth cannot be explained by Ku70s effect on the expression of viral genes, but it is interesting that the *E4orf6/7* gene is upregulated in the absence of normal Ku70 levels.

Ku70 associates with the viral genomes but not viral promoters

Regardless of the minimal effect seen on viral growth and the lack of effect on viral gene expression, I wanted to observe if Ku70 was recruited to viral promoters, as the Ku70 protein is known for its DNA binding abilities. (Fell & Schild-Poulter, 2015) ChIP assays showed that, recruitment of Ku70 to viral promoters was low. (Figure 3.5) This correlates with the lack of effect of the depletion of Ku70 on viral gene expression. However, Ku70 is recruited to viral open reading frames, such as *hexon*. This association with the viral genome may be a defense mechanism to prevent the detection of viral genomes within an infected cell. It is possible that Ku70 is replacing the viral protein VII after the initiation of viral genome replication. Protein VII is an important protein responsible for the establishment of viral chromatin structure. Protein VII is present in the nucleus with the viral DNA until the beginning of DNA replication, at which point the levels of protein VII decrease. (Chatterjee *et al.*, 1986; Chen *et al.*, 2007) E1A may assist in the exchange of protein VII to Ku70 as, it has been shown that E1A also interacts with protein VII. (Johnson *et al.*, 2004).

Ku70 re-localization upon HAdV infection

ChIP results suggested that Ku70 was associating with the viral genome, therefore I wanted to see if the normal localization of Ku70 in the nucleus was visually re-structured upon infection. Results shown in Figure 3.6 show that Ku70 is localized to viral replication centers upon late infection of HAdV. While Ku70 does not co-localize perfectly with DBP, there is a significantly amount of overlapping observed. This localization is similar to previous studies showing localization of viral RNAs to areas around replication centers. (Aspegren & Bridge, 2002; Berscheminski *et al.*, 2014; Puvion-Dutilleul *et al.*, 1998) This may suggest that Ku70 plays a role in viral RNA metabolism which may explain the up regulation seen in the *E4orf6/7* gene after Ku70 depletion. (Figure 3.4.2)

Ku70 S-phase induction

S-phase induction assay results indicate that Ku70 is needed for the efficient induction of S-phase by HAdV E1A. (Figure 3.7.1) One of the many roles Ku70 contributes to is the signalling of the DNA damage pathway which can block cell cycle entry. (Fell & Schild-Poulter, 2015) Therefore, the absence of Ku70 may induce a block to cellular DNA replication, inhibiting the induction of S-phase.

The expression of cell cycle genes showed a reduction in most genes upon Ku70 depletion, interestingly the genes *CDK6, CDKN1A,* and *PCNA* were shown to be upregulated. (Figure 3.7.2) *CDKN1A* which encodes for the cyclin dependent kinase p21 gene, was found to be significantly upregulated, over seven-fold in Ku70 depleted cells. p21 has been shown to negatively regulate cell cycle progression and has been shown to often be deregulated in cancers, which makes its role with Ku70 a point of interest. *CDKN1A* induction is due to genotoxic stress,

often by the p53 tumor suppressor protein. (Abbas & Dutta, 2009) Interestingly, p21 also has a significant role in controlling the DNA repair processes. It is able to positively regulate replication by slowing down the cell cycle or negatively affect replication by competing for PCNA binding with PCNA-reliant proteins that are involved in the DNA repair process, thus inhibiting the DNA repair process. (Abbas & Dutta, 2009) As noted earlier, Ku70 is recruited to the viral genome, potentially to prevent the detection of the viral genomes. (Figure 3.5A) Therefore, it is possible that the depletion of Ku70 stimulates a DNA damage response pathway due to the detection of these viral genomes, thus increasing the expression of *CDKN1A*.

Ku70 is recruited to cellular promoters during HAdV infection

As seen in Figure 3.8, Ku70 is recruited to cellular promoters, after adenovirus infection. This recruitment was most significant at the *CDK6* and *PCNA* promoters. This, together with the data representing the change in expression of certain cell cycle genes hints that Ku70 may have a novel role in the expression of cell cycle genes. It is possible that Ku70 may function as a transcriptional regulator for these genes upon genotoxic stress such as infection. The possibility of this novel role requires further studies.

4.2 FUBP1:

FUBP1 was first identified as a regulator of c-Myc, since its discovery, FUBP1 has been found to be upregulated in a variety of cancers. (Duncan *et al.*, 1994) While the role of FUBP1 in cellular networks remains unclear, it has been shown to play roles in mRNA regulation, translational regulation and even the regulation of viral replication. (Zhang & Chen, 2012) My studies begin outlining the possible roles FUBP1 may have in human adenovirus growth and proliferation. Accumulating evidence suggests that FUBP1 may be a proto-oncogene, as it has

been shown that there is a positive correlation between FUBP1 expression and tumor development. (Weber *et al.*, 2008) This upregulation of FUBP1 can be seen even in cancers where the level of c-Myc is not also upregulated, indicating FUBP1 has a role in carcinogenesis independent of c-Myc. FUBP1 has also been identified as a potential biomarker for cancers, as the difference observed in tumor tissues compared to background expression is significant. (Matsushita *et al.*, 2009; Rabenhorst *et al.*, 2009) Therefore, FUBP1 is exceedingly important to study; as it has ties with cancer, and has been suggested that FUBP1 itself may be a proto-oncogene which makes discovering what cellular pathways FUBP1 is involved in critical.

FUBP1 binds directly to E1A

FUBP1 was identified as a novel binding protein to the C-terminus of the E1A protein through affinity purification and mass spectrometry. This was confirmed to be a direct protein-protein interaction. (Figure 3.9.1) Through co-immunoprecipitation, it was found that FUBP1 interacts with the N-terminus and the CR3 region of E1A. Both of these regions have been shown to have transcriptional activation domains, or be involved in the regulation of gene expression. (Pelka *et al.*, 2008) The N-terminus of E1A is involved in transformation, the suppression of differentiation, the induction of cell cycle progression as well as the regulation of gene expression as a transactivator or a repressor. There have been multiple binding partners identified for the N-terminus, including p300/CBP, p400, and transformation/transcription domain-associated protein (TRRAP). (Bayley & Mymryk, 1994; Berk, 2005; Pelka *et al.*, 2008) The CR3 region contains a C4 zinc finger that is an influential transcriptional activation domain. (Webster & Ricciardi, 1991) The presence of this zinc finger suggests that the CR3 region is a structured domain. This is similar to the N-terminus, which is predicted to contain an α-helix. (Avvakumov *et al.*, 2004) This suggests that perhaps, FUBP1 binding is dependent on a structure. Regardless, as FUBP1 is a regulator of

transcription and translation, it seems logical that it binds in regions that are transcriptional activation domains, as these domains often contain binding sites for transcriptional co-regulators like FUBP1. (Zhang & Chen, 2012) It remains to be seen exactly where in the N-terminus and CR3 region FUBP1 binds, this will have to be determined through the use of double E1A mutants to eliminate both E1A binding sites of FUBP1.

Effect of FUBP1 on HAdV growth and replication

As the role of FUBP1 in cellular networks is still unknown, I wanted to begin my investigation into the association of FUBP1 to E1A by seeing if FUBP1 affected the virus's ability to grow efficiently. It was determined that FUBP1 had a small effect on viral growth and the number of genome copies per a cell. (Figure 3.10 A & B) While the effects seen when FUBP1 is reduced are marginal and combined effects may have a significant impact on HAdV's proliferation, these results suggest FUBP1 is not directly involved in pathways related to viral genome replication.

Interestingly, even though a reduction in viral growth and viral genome copy number was observed upon reduction of FUBP1, this did not correlate with the expression of viral genes. It was found that samples treated with siRNA targeting FUBP1 showed a minor upregulation of viral genes at 48h. (Figure 3.10 C) This indicates that the reduction seen in the viral growth and viral genomes cannot be explained by FUBP1 affecting the expression of viral genes, suggestion FUBP1 is involved in another pathway.

FUBP1 is not critical for S-phase induction

One of the critical roles E1A plays in the viral life cycle, is to initiate cell cycle in previously arrested cells so the virus will have ample materials to replicates its genome with, and

so I wanted to see if FUBP1 potentially involved in pathways related to this process. (Pelka *et al.*, 2008) The EdU incorporation assay showed that the depletion of FUBP1 had no effect on HAdV E1As ability to induce S-phase. (Figure 3.11)

FUBP1's role in the regulation of cell cycle genes

FUBP1 has been shown to regulate cyclin dependent kinase p21 (*CDKN1A*), which has been shown to repress cell cycle progression. (Abbas & Dutta, 2009) Therefore, I wanted to determine if HAdV interfered with this regulation of p21, as well as determining the expression of other cell cycle genes. HAdV does not interfere with FUBP1s regulation of p21 mRNA, as upon depletion of FUBP1 the expected increase in p21 is observed. (Figure 3.12). Interestingly, an upregulation of *CDK6* was observed. *CDK6* (Cell division protein kinase 6) is important for the G1/S-phase transition during the cell cycle. CDK6 also plays a role in phosphorylating Rb, resulting in E2F being released and the activation of the viral early genes to occur. (Lim & Kaldis, 2013) FUBP1 may be responsible for influencing the translation of these cell cycle genes, assisting in viral replication.

FUBP1 sub-cellular localization is restructured during HAdV infection

Since FUBP1's role within cellular pathways has yet to be determined, I wanted to observe if the localization of FUBP1 in the nucleus was altered upon HAdV infection. FUBP1 is re-localized into concentrated punctate structures upon HAdV infection, indicating it is being sequestered by the virus. The purpose of this sequestering has yet to be determined, however, literature has shown that FUBP1 regulates viruses such as JEV and HCV via mRNA binding. (Zhang & Chen, 2012) It is possible that FUBP1 is also carrying out a regulatory role by binding HAdV mRNA.

FUBP1 binds viral mRNAs

After observing the re-localization of FUBP1 upon adenovirus infection, I wanted to see if FUBP1 was binding to viral mRNA in order to carry out regulatory roles, similar to what has been noted in the literature. Through RNA immunoprecipitation, it was observed that FUBP1 was able to form complexes with the E1B, E3A and E4orf6/7 mRNA. (Figure 3.14). While, E1A was not found to be involved in these complexes, it raises the question of what role FUBP1 is participating in by binding to these viral mRNAs. This may help explain the upregulation of certain viral genes when FUBP1 is depleted; if FUBP1 is regulating the expression of these genes, reducing its presence would subsequently lead to a dis-regulation of said genes. This avenue requires further study.

5. Summary of findings:

5.1: Ku70

My studies have found Ku70 to be a novel E1A binding protein that interacts with the CR4 region during HAdV infection. Ku70 was found to influence viral replication, as the depletion of Ku70 resulted in lower viral titres, as well as a reduction in the number of viral genomes per cell at later infection time points. This was further confirmed by data representing Ku70 localization to viral replication centers later in infection. In addition to this, the depletion of Ku70 was found to inhibit efficient S-phase induction by E1A. This lead to the discovery that Ku70 had an unexpected role in the expression of cell cycle genes such as *CDKN1A* and *CDK6.* Furthermore, it was found that Ku70 was recruited to the cell cycle promoters only after infection by HAdV, suggesting a novel role in the regulation of transcription of these genes. Finally, it was found that Ku70 associates with the viral genome, potentially as a defense mechanism by the virus to prevent the detection of viral genomes within the host cell. Overall, my studies have shown that Ku70 is a direct binding partner of E1A, and contributes to multiple roles to assist in efficient viral proliferation.

5.2: FUBP1

The role of FUBP1 within the cell is still largely unknown. My studies have shown that FUBP1, a novel E1A C-terminus binding protein is involved in the adenovirus life cycle. FUBP1 appears to have multiple binding locations within E1A, as it was shown to immunoprecipitate with both the N-terminus and the CR3 region. This seems to correlate with previous data in the literature showing FUBP1 is a transcriptional and translational regulator, and both of these regions of E1A

have been shown to play a role in gene regulation. FUBP1 is not involved in S-phase induction by E1A, and its effect on viral growth as well as genome copy number per a cell is minimal. My studies have also shown that FUBP1 potentially has a role in the translational control of cell cycle genes. It is possible that these small effects are contributing to a larger overall outcome on the viral life cycle. Interestingly, I have also shown that FUBP1 is able to bind viral mRNAs, suggesting that its role in the viral life cycle may be regulating the expression of these viral genes. Further study needs to be conducted to determine what role FUBP1 has in binding the mRNAs, and how this affects the virus. Overall, my studies have identified FUBP1, a poorly understood protein that is often up-regulated in cancers, to be a novel E1A binding protein that may play a role in the regulation of viral mRNAs.

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