Exploitation of Human RuvBL1 by HAdV E1A to inhibit interferon response for efficient viral growth

by

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A Thesis submitted to the Faculty of Graduate Studies
of The University of Manitoba in partial fulfilment of
the requirements of the degree of

MASTER OF SCIENCE

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Abstract

E1A of human adenovirus is the first gene product expressed during viral infection and serves as a preliminary step for efficient viral replication. Other early gene products include early proteins designated as E2, E3, and E4 are also made, which together with E1A prepare the infected cell for replication of the viral genome. Various studies have elucidated that E1A functions largely as a transcriptional regulator and can interact with a host of cellular modulators to enhance replication of the virus. RuvBL1, a chromatin remodeling protein involved in a host of cellular functions such as transcriptional regulation and host cell immune response has been shown to bind to E1A. My results identify RuvBL1 as an E1A binding protein and show that E1A is a direct binding partner of RuvBL1. I demonstrate that RuvBL1 plays a role in the growth of adenovirus, as knockdown of RuvBL1 negatively affects the growth of the virus and reveals that RuvBL1 functions as a viral growth enhancer. Finally, I identify a possible role of E1A's inhibition of interferon response in a RuvBL1 dependent manner.

Acknowledgements

I would like to thank Dr. Peter Pelka for the opportunity to work in his Lab for the past two years. I appreciate his assistance and the support during my time in the lab. I have gained a lot of insight about the research field that I would have not had otherwise.

I would like to thank my committee members, Dr Georg Hausner and Dr. Steve Whyard for their input and time during my research project.

I would like to thank my fellow colleagues in the Pelka lab, for their assistance and support during my time in the lab. They have also been instrumental to the completion of my research project.

I would like to thank the Department of Microbiology for their assistance during my time at the school and for enabling me to access several instruments for my experiments.

I would also like to extend my appreciation to my funding sources, National sciences and Engineering Research Council of Canada (NSERC) and Manitoba Health Research Council.

To my parents Olagbaye and Foluwake Olanubi and my

sister Timilehin Olanubi for their love and never ending support.

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1. Introduction

1.1 Adenovirus

Adenovirus (AdV) was first isolated in 1953 by Wallace Rowe in adenoids (tonsils) of sick army recruits (Berk, 2007). In humans, adenoviruses cause about 5-10% of upper respiratory illness in children including bronchitis and the common cold (Rowe et al., 1953). They have also been shown to cause cystitis, conjunctivitis, gastroenteritis, and pharyngitis (Lynch et al., 2011; Rocholl et al., 2004). Although, adenovirus is primarily asymptomatic, they can cause serious illness in immunocompromised individuals.

Adenovirus belongs to the *Adenoviridae* family and the *Mastadenovirus* genus (Davidson et al., 2003). There are over 57 serotypes of human adenovirus currently identified, which belong to the seven distinct species ranging from A to G (Bailey, and Mautner, 1994). There are many other serotypes that have also been identified in animals as well. Classification is based on hemagglutination properties, DNA homology, and oncogenic properties (Table 1.1). Adenovirus has been shown to induce oncogenic transformation in rodents such as hamsters and rats; however, the ability of AdV to cause malignant transformation in humans has not been recognized (Gallimore, 1972). AdV has therefore proven to be a useful model to study cancerous pathways because of its ability to induce cancer transformation.

Adenovirus also varies in its infective potential (Tollesfon et al., 2007). This variance is due to the type of host cell it is infecting, as well as the viral serotype. If the host cell is permissive, the virus will reproduce indefinitely until lysis occurs ultimately causing cell death (Eggerding & Pierce, 1986). Conversely, if the cell is non-permissive, the viral infection will not result in lysis or cell death because the virus will not reproduce. In humans, adenovirus produces a lytic infection because the cell is permissive and therefore the virus does not have enough time

to establish oncogenic transformation in cells (Doerfler, 1996). However, in animals, adenovirus infection does not result in a lytic infection because the host cell is non-permissive and therefore oncogenic transformation is possible.

Table 1.1: Classification of Human Adenovirus, Hemagglutination properties and oncogenic potential

		Hemagglutination		Oncogene		
Groups	Serotypes	Group	Results	Percentage of G + C[a] in DNA	Tumorigenecity in Vivo[b]	Transmation of cells
A	12,18,31	IV	None	48-49	High	+
B	3, 7, 11, 14, 16, 21, 34, 35, 50	I	Monkey(complete)	50-52	Moderate	+
C	1,2,5,6	III	Rat(Partial)	57-59	Low or none	+
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	II	Rat(Complete)	57-61	Low or none	+
E	4	III	Rat(Partial)	57	Low or none	+
F	40, 41	III	Rat(Partial)	57-59	Low or none	+

1.1.1 HAdV Structure

Adenovirus is a linear double stranded DNA virus capable of infecting a wide variety of host species (Rux and Burnette, 2004). The adenovirus particle has a molecular weight of 150 Mega-daltons and a genome of 36 kilobase pairs (Zubieta, 2005). Adenovirus contains an icosahedral and non-enveloped protein capsid (Fig 1.1). The capsid has three structural proteins protruding out of the edges of the virion (Smith et al., 2010). The proteins are fibers, hexons, and penton bases. The fibers help the virus to attach to cell surface receptors and enable entry of the virus into the cell. The capsid consists of 240 hexon capsomers, and each capsomer is comprised of three identical proteins that lies on the face of the virion particle (Rux and Burnette 2000). There are 12 vertices within the icosahedral structure of the virus, each of which contains units of pentons. Each penton has a fiber protruding out of its exterior. The adenoviral core particle consists of two major proteins polypeptide V and polypeptide VII (Harrison, 2010). The virion core consists of five other minor proteins termed VI, VIII, IX, IIIA, Iva (Merza and webber, 1982). The viral genome also contains a terminal protein (TP) that is attached to the 5' end of the genome. The virus is about 70-90nm in diameter and contains the DNA genome within the core of the virion.

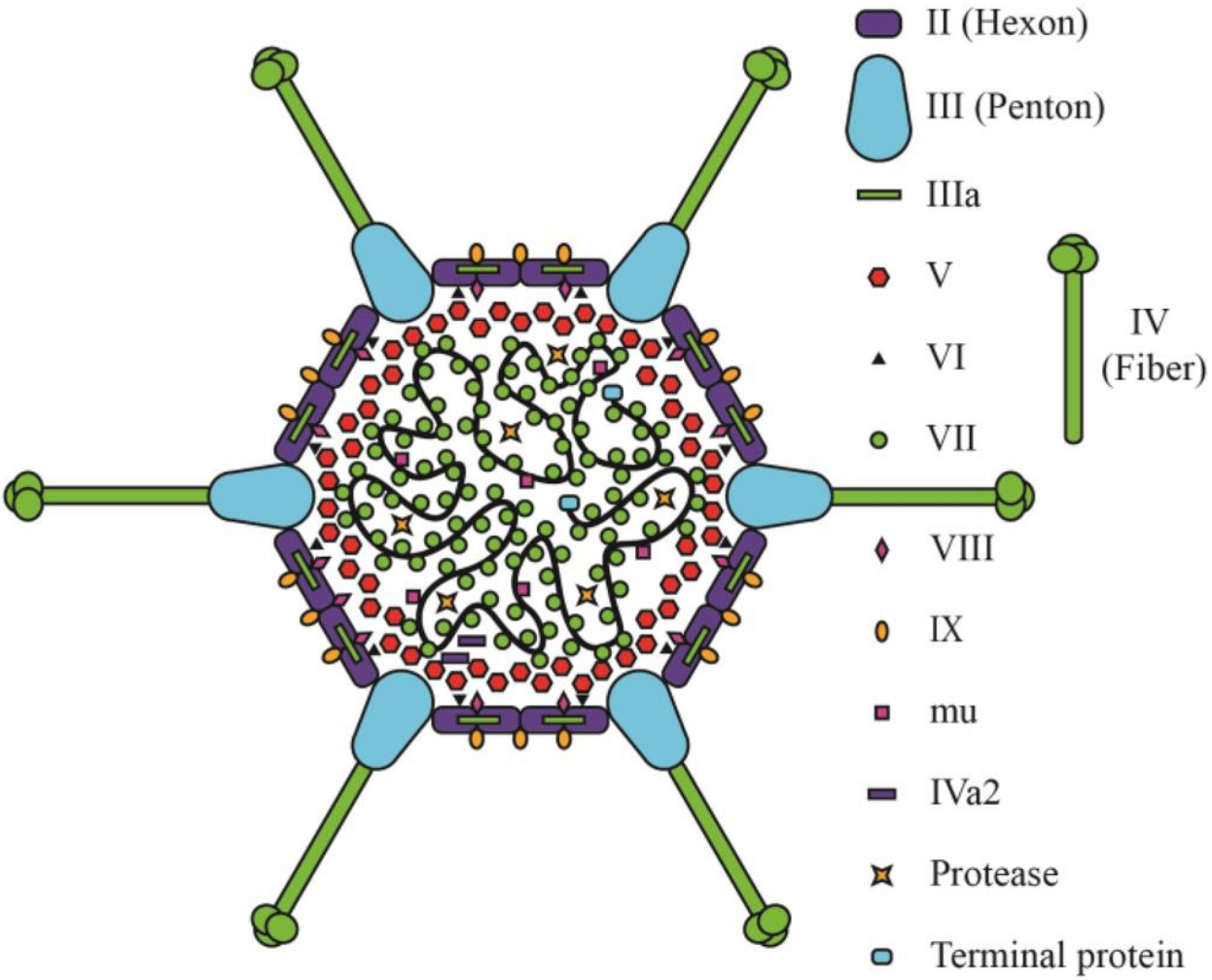


Figure 1.1 Structure of HAdV: Model of adenovirus virion and associated structural proteins (Saha et al., 2014).

1.1.2 HAdV Replication cycle

Adenovirus is able to infect host cells by interacting with a multitude of receptors that enable the internalization of the virus (Wu et al., 2003). The fibers on the surface of the viral particle are required for efficient attachment of the virus to the host cell receptors (Lonberg-holm and Lennart, 1969). The receptor responsible for binding to the fibers of the virus is the 40 kDa Coxsackie and Adenovirus receptor (CAR) type 1 membrane protein (Fig 1.2) (Hoeben and Uil, 2013). The CAR receptor contains a transmembrane protein with two immunoglobulin domains and a cytoplasmic domain (Walters et al., 2002). The CAR receptor is expressed primarily in epithelial and endothelial cells, and are also expressed in lesser magnitude in the heart, liver, and lungs.

However, not all HAdV depends on the CAR receptor for entry. For example, HAdV 11 has been shown to recognize and bind to a CD46 receptor (complement regulatory) protein and membrane cofactor protein, which is a protein expressed on many cell types such as hematopoietic cells (Segerman et al., 2003). Other HAdV serotypes like 8, 19, and 37 utilize sialic acid as a receptor for their entry into host cell.

Binding of AdV is not sufficient for the entry of the virus into the host cell. The penton base of the virus also needs to interact with type αvβ3 and αvβ5 integrins for efficient internalization (Wickham et al., 1993). Integrins are heterodimeric transmembrane proteins consisting of alpha and beta subunits that recognize tripeptide Arg-Gly-Asp (**RGD**) motifs that are found on AdV penton bases (Li et al., 1998). Binding of the penton base to integrins activates two signaling pathways, specifically the P13K and the GTPases family (Li et al., 1998). The

signaling pathways induce polymerization of actin filaments, a process required for the internalization of the virus into clathrin-coated vesicles (Varga et al., 1991). The vesicles mature into endosomes where acidification occurs. Once acidification commences, it results in the removal of the capsid vertex, hexon, protein VII and other structural proteins in a process known as uncoating (Mellman, 1992). These processes leads to the release of the virion into the cytoplasm. The virus is then transported to the nuclear pore complex via host cell microtubules (Greber et al., 1997). The viral DNA is then released into the nucleus through the nuclear pore, where viral DNA replication can begin.

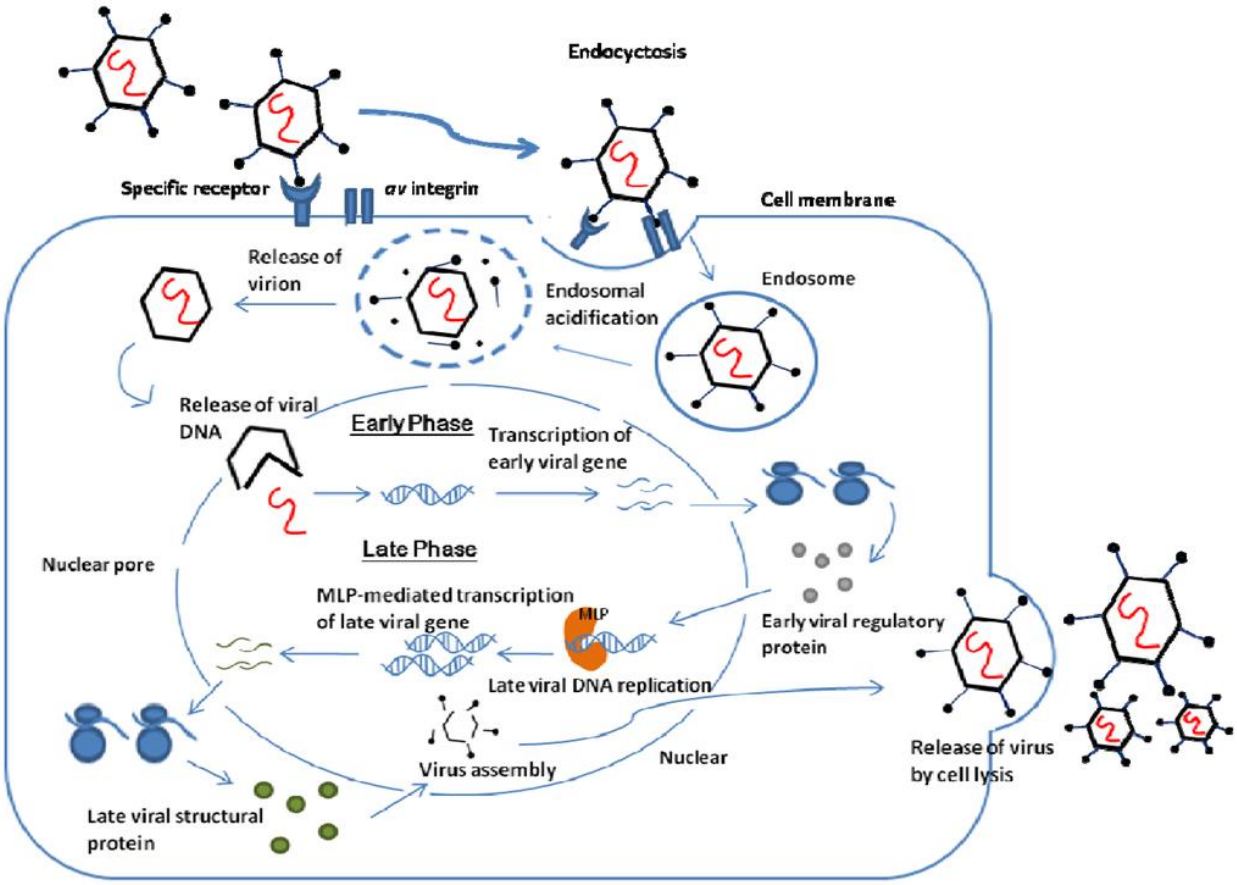


Figure 1.2 HAdV 5 Replication cycle: Schematic representation of HAdV infection and replicative life cycle (Waye and Sing, 2010)

1.1.3 HAdV 5 genome

The adenovirus genome consists of five early genes called E1A, E1B, E2, E3, E4 that are transcribed early on in infection (Fig 1.3) (Stephens and Harlow, 1987). The primary function of the early genes is to activate the transcription of other viral genes necessary for the efficient replication of the virus. Once the early genes have been expressed to a sufficient level, the replication of the viral genome will be initiated leading to the late phase. During the late phase, the early gene products are expressed at a lower level and results in the production of late genes encoded by L1, L2, L3, L4, L5 (Morris et al., 2010). There are also other viral products later in infection and they include structural protein IX and IVa2, which help package the viral genome into viral particles (Davison et al., 2003). The virus also encodes for VA RNA I and II, which act to inhibit activation of interferon response via inhibition of the RNA-dependent Protein Kinase R (O'Malley et al., 1986). Lastly, there are inverted terminal repeats of about 100 base pairs at both ends of the genome, which serve as the origin of replication for the virus.

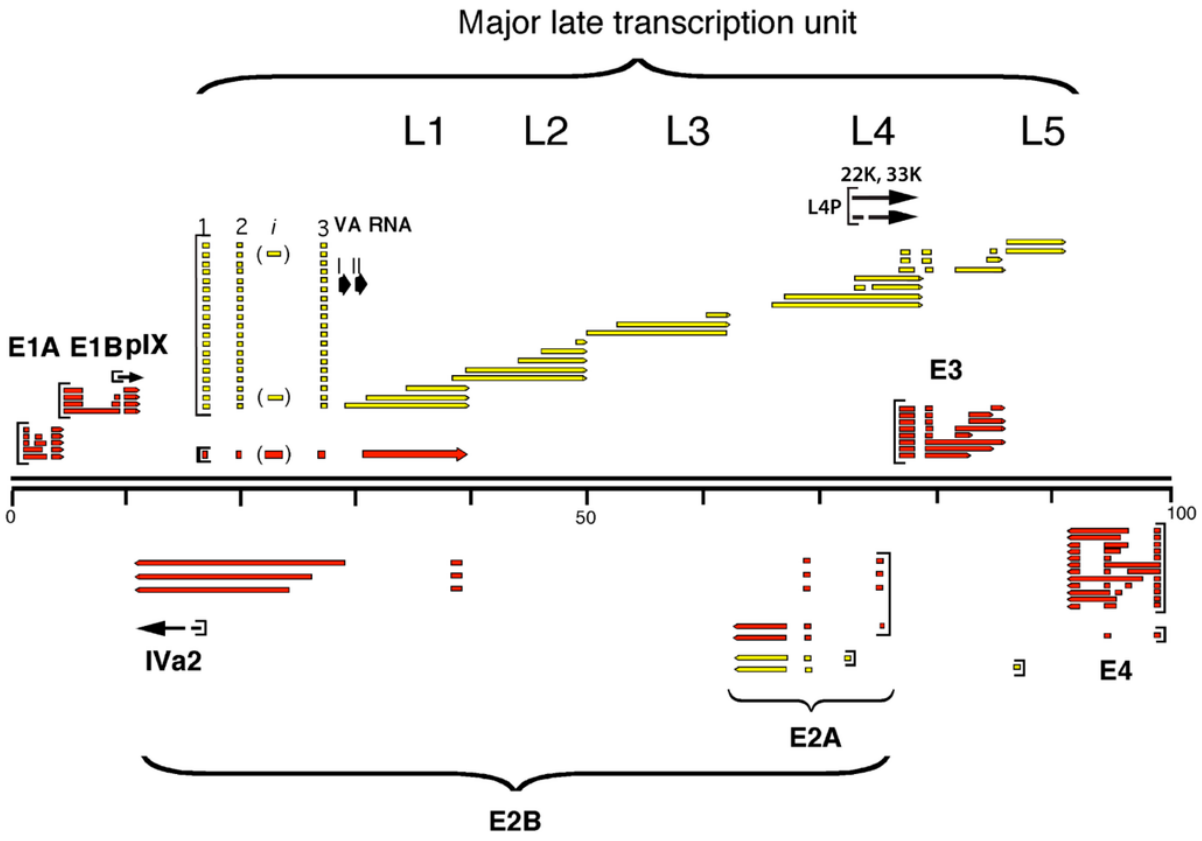


Figure 1.3 HAdV 5 genome: Representation of the HAdV type 5 genome and encoded viral genes. The early viral genes are shown in red and late genes are shown in yellow. Intermediate genes are shown as black arrows (Biasiotto and Akusjarvi, 2015).

1.1.4 HAdV E1A

There are other viruses with similar ability to cause cellular transformation such as polyomavirus and papillomavirus. These viruses are able to carry out their function with the help of associated proteins such as the large T antigen of polyomavirus and E7 of papillomavirus. Both viruses have been used to study interactions within the cellular protein network (Liao, 2006). Likewise, adenovirus E1A has been used to discover novel cellular proteins and their regulatory functions within the cellular environment (Pelka et al., 2008). E1A functions as an oncogene because together with the E1B gene, they are both sufficient enough to drive the cell to a transformed state. There are several binding partners of E1A that have been identified, which qualifies E1A as a hub detector protein. Hub detector proteins are generally known to bind to multiple proteins and complexes within the cell. E1A has highly disordered regions that exists within the E1A sequence that can allow E1A to adopt multiple conformations and therefore has the ability to bind to several DNA binding domains. Within the structure of E1A there are segments called molecular recognition features (MorFs) that allow for diverse binding partners because of the different and unique sequences within these MorFs (Fig 1.4). Individual E1A MorFs do not display any structural properties until their interaction with target proteins. These individual MorFs also have independent functions as well as coordinated activities within the cellular network. E1A MorFs have been analysed and several interacting protein partners have been discovered which includes pRb, CBP, Cyclin A, CDK3, and many others.

After infection, the adenovirus E1A gene is transcribed and five differing transcripts are produced by differential splicing which includes transcripts 13S, 12S, 11S, 10S and 9s (Fig. 1.5) (Subramanian, 2006). The 10S mRNA is the most abundant during infection, followed by the

13S and 12S transcripts (Radko et al., 2015). The 11S and 9S mRNA are expressed at lower levels throughout the course of infection. The 13S and 12S transcript are transcribed into 289 and 243 amino acid residues respectfully. The only difference between the 289 and 243 residue is a 46 amino acid segment also termed the "unique region", because it is found only in the 289 protein but missing in the 243 aa fragment. The other transcripts 11S, 10S, 9S are transcribed into 217, 171, 55 amino acid residues respectively.

There are four conserved regions within the E1A protein termed CR1, CR2, CR3 and CR4 (Berk, 2005). The CR1 Region has been shown to bind to the Retinoblastoma protein via a LXE sequence (Barbeau et al., 1992). This region along with the N-terminus both retain strong transcriptional activity when interacting with a promoter. The CR1 region also binds other proteins that are involved in transcriptional regulation such as pRb, E2F and p300/CBP.

The CR2 region is required for several functions including induction of cell cycle, expression of viral genes, apoptosis and regulation of tumor suppressor protein p53. CR2 has a strong affinity for the pRb protein and is responsible for disrupting the retinoblastoma protein from binding to the E2F factor by binding strongly to the pRb protein via a LXCXE sequence together with the N-Terminus and the CR1 region of E1A (Pelka et al., 2008). Therefore, it is thought that this region including CR1 is responsible for driving quiescent cells into S phase. E1A CR2 also interacts with other cellular proteins including p107, p130 and UBC9.

The CR3 region of E1A functions as a transcriptional activator of the viral genome by binding to DNA binding domains of transcription factors found at early promoters of the virus (Berk, 2005). Adenovirus E1A is not a DNA binding protein, therefore it carries out its cellular regulatory activities such as initiating transcription by interacting with transcriptional factors through their DNA-binding domain. The CR3 region interacts with Med23, a component of the mediator

complex. The interaction of CR3 with Med23 is partly responsible for the assembly of the preinitiation complex at the start of DNA replication, which includes RNA polymerase II. CR3 also binds to p300/CREB binding protein (CBP) acetyltransferase as well as the p300/CBP Associated Factor (pCAF), which are both crucial for the transactivation of CR3 (Pelka et al., 2009a and Pelka et al., 2009b). C-terminal binding protein (CtBP) also binds to CR3 and causes the repression of CR3 transactivation (Bruton et al., 2008).

The function of the CR4 region is not clearly understood but it is known to bind to the c-terminal binding protein (CtBP) through a conserved PXDLS motif. CtBP is a 48 kDa protein that functions as a corepressor (Berk, 2005). It has also been observed that CR4 also contains a nuclear localization signal for E1A and may also have the ability to regulate transcription (Gallimore, 2001). CR4 is able to inhibit transcription through CtBP and its ability to bind to histone deacetylases. CR4 has also been shown to bind to DNA replication-related element binding factor (DREF) and Ku70 (XRCC6), both this interaction contributes to activation of viral gene expression (Radko et al., 2014, and Frost et al., 2017).

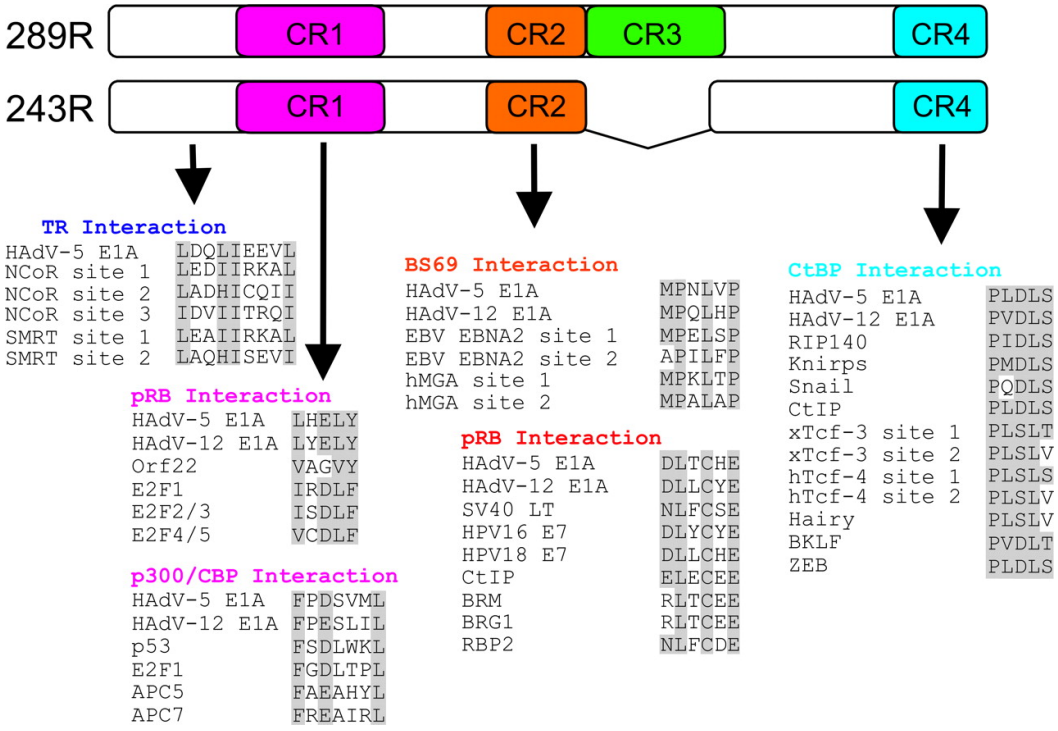


Figure 1.4 Map of E1A conserved regions and location of MorFs: Comparative analysis of the sequences of the E1A proteins from different HAdV serotypes are shown. Both sequences of viral and cellular proteins with similar MoRF analogous to E1A's are also specified. Residues that are shaded represent consensus within the MoRF (Pelka, et al, 2008).

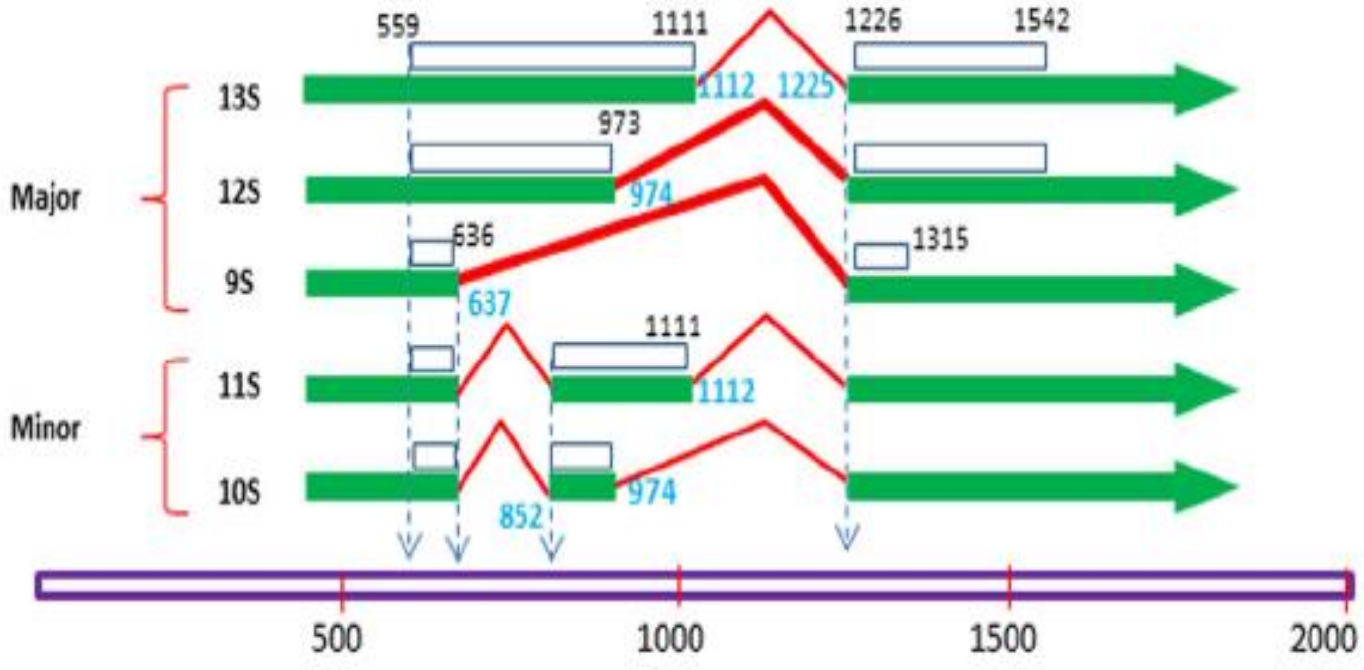


Figure 1.5 E1A Isoforms: Green arrow represents all the isoforms. Red lines indicate the splice site and blue number specifies position of splice sites within the E1A transcripts. The blue boxes represent open reading frames and black numbers represent positions of open reading frames (Zhao, 2014)

1.1.5 HAdV Early genes

There are other early viral genes that HAdV expresses to carry out efficient replication such as E1B, E2, E3, and E4 (Berk, 2007).

E1B encodes for two unrelated proteins of different function; a 55kDa protein which corresponds to a 496 aa residue and a 19kDa protein (176 aa) (Sarnow et al., 1984). E1B proteins are required for full cellular transformation of rodent cells with E1A. E1B functions by inhibiting apoptosis and cell cycle arrest, which results in uncontrolled growth. It is able to inhibit apoptosis by initiating the ubiquitination of p53, the tumor suppressor responsible for cell cycle arrest.

E2 encodes for three protein products, a DNA dependent polymerase (140kDa), a terminal protein and a single stranded DNA binding protein (Stillman et al., 1981). The DNA dependent polymerase functions by attaching the post-translationally modified terminal protein to the 5' end of the viral genome. AdV DNA polymerase also contains proofreading abilities because it carries an exonuclease domain. The terminal protein functions as a primer for the DNA dependent polymerase by allowing the DNA strands to separate and anchoring the template strand to the DNA dependent polymerase. The single stranded DNA binding protein promotes separation of the DNA strands and chain elongation to allow complete synthesis of new DNA strands.

E3 encodes multiple proteins that are involved in inhibiting the innate immune response (Wold, et al., 1995). For example, one of the E3 protein products, which is a 19 kDa protein localizes to the endoplasmic reticulum where it binds the major histocompatibility complex class

1 (MHC 1) preventing viral antigen presentation on the cell surface and blocking cytotoxic T-cell mediated cell killing of the infected cell.

E4 encodes for proteins with multiple functions, which are all named after E4's open reading frame. E4ORF1 and ORF4 activates the mTOR (mammalian target of rapamycin protein) signalling pathway, which is needed for the start of cell cycle (Leppard, 1997). ORF3 functions to inhibit the cell DNA damage response from interfering with viral genome replication. ORF6, in combination with E1B 55kDa protein, functions to inhibit p53 induced apoptosis and also promotes the transport of viral mRNA to the cytoplasm while blocking the translocation of cellular mRNAs to the cytoplasm (Dobner et al., 1996). In doing this, viral proteins can be synthesized in abundance compared to cellular proteins. ORF6/7 binds to E2F transcription factor and recruits it to the E2 promoter and leads to transactivation of E2 genes. Therefore, orf6/7 is able to initiate cell cycle independent of E1A (Marton et al.,1990).

1.1.6 HAdV E1A Functions and Interactions with Cellular Factors

One of the first protein binding partners to interact with E1A that was identified, is the retinoblastoma protein (pRb) (Egan et al., 1989). pRb is a tumor suppressor protein and one of its most important functions is to prevent the cell from entering the cell cycle (Whyte et al., 1988). pRb prevents the cell cycle by binding to and sequestering the E2f transcription factor. E2f are transcription factors, first identified by the virtue of them activating the viral E2 promoter, that are responsible for the regulation of expression of S phase proteins such cyclin dependent kinase 2 and cyclin E (Berk, 2005). Usually, the function of the pRb protein is controlled by multiple signals such as cell to cell signaling, mitogens and inherent cellular signals. These various signals modulate the expression of G1 CDK cyclins, which are responsible for the phosphorylation of E2F. Therefore, the phosphorylation of pRb relieves the suppression of E2F, which enables the initiation of cell cycle (Fig. 1.6) (Ikeda et al., 1993). In infected cells, E1A is responsible for disrupting the interaction of pRb to E2F by binding strongly to the pRb via a LXCXE motif found in CR2 (Dahiya et al., 2000). The free E2F factor can then resume transcription, and thereby drive the cell from G0 to S phase. The disruption of pRb and E2F binding by E1A is one of the preliminary steps of cellular transformation but not the sole step.

It has also been shown that E1A can bypass the step of binding to pRb, and instead bind directly to the E2F/DP1 complex (Pelka, 2011). E1A 13S bur not 12S can activate cell cycle by binding to the N-terminus DP1 and therefore can recruit itself to E2F regulated promoters to regulate E2F dependent genes.

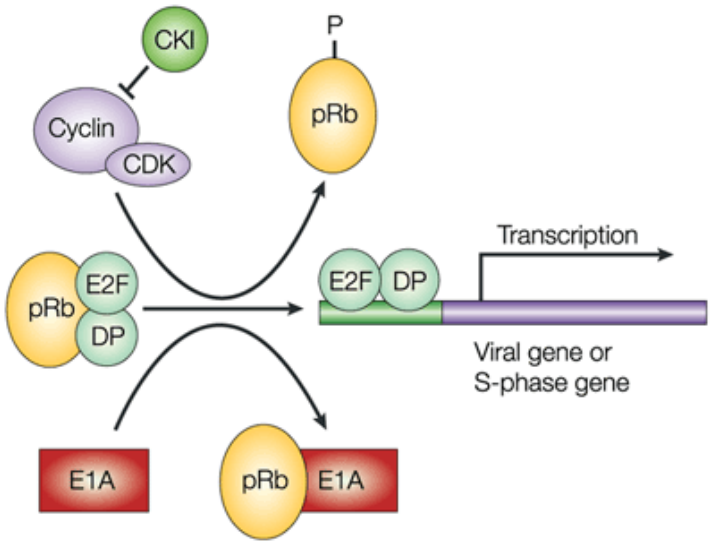


Figure 1.6 E1A inhibits the interaction between retinoblastoma protein and E2F transcription factor. In normal cells pRb is bound to E2F until the cell is ready to divide. The phosphorylation of pRb cause the disassociation of pRb from E2F and E2F can be recruited to the promoter to commence cell cycle. However, in infected cells, E1A sequesters pRb from associating with E2F, which leaves E2F free to initiate cells cycle progression and S phase (Steven et al., 2002).

1.2 Human RuvBL1

RuvBL1 is a 50 kDa eukaryotic protein belonging to a family of ATPases (AAA +) and are found ubiquitously throughout the cell (Cvackova et al., 2008). RuvBL1 is highly related to the bacteria RuvB, a protein involved in genetic recombination. However, RuvBL1 contains an additional domain II, in comparison to bacteria RuvB.

RuvBL1 has previously been characterized as Tip 49, TAP54 alpha, and more commonly as Pontin. RuvBL1 was first identified through co-immunoprecipitation, as it was discovered as being part of a RNA polymerase II complex. RuvBL1 is commonly found in a complex with a similar protein called RuvBL2 also known as Reptin (Matias, 2006). RuvBL1 and RuvBL2 are either found in hexameric or dodecameric arrangements. The dodecameric structure consists of two hetero-hexamers with interchanging RuvBL1 and RuvBL2 monomers that are bound to ATP/ADP molecules. Both RuvBL1 and RuvBL2 contain walker A and B domains that function to hydrolyse ATP.

RuvBL1 has been shown to be part of several chromatin-remodelling complexes such as Ino80, SRCAP and TIP60/NuA4 (Wood et al., 2000). Its functions include chromatin remodelling, mitosis, telomerase assembly, DNA damage repair, and transcriptional regulation. RuvBL1 is also able to regulate metastasis by modulating the expression of KAI1, a metastasis suppressor gene. RuvBL1 is known as a hub protein because it has the ability to interact with multiple cellular proteins through its domain to carry out a wide array of functions (Qiu, 1998). The role of RuvBL1 in oncogenic transformation has also been identified. Through affinity purification, RuvBL1 was discovered as one of the primary cofactors that binds to the transactivation domain of c-myc, which is an oncoprotein (Wood et al., 2000). A mutation in the

RuvBL1 gene was shown to inhibit the oncogenic effect of c-myc and therefore reveals that RuvBL1 is an important facilitator of oncogenic transformation. RuvBL1 and RuvBL2 have been shown to play a role in the regulation of β-catenin (Bauer et al., 2000). β-catenin is primarily known to function as a regulator of the wg/wnt signaling pathway, which is responsible for transducing signal to a cell via cell surface receptors. RuvBL1 and RuvBL2 however has an antagonistic effect on the transactivation of β-catenin.

RuvBL1 has also been implicated in the regulation of interferon stimulated genes (ISGs) which is affected by interferon alpha (Gnatovskiy, 2013). Interferon genes encodes cytokines that activate the antiviral response via the Janus kinase and signal transducer and activator of transcription pathway (JAK-STAT) (Matias, 2015). Upon stimulation by type 1 interferon, ISG proteins such as ISGF3 (composed of STAT1, STAT2 and DNA binding protein IRF9) are phosphorylated and move to the nucleus where they bind the interferon stimulated elements located on ISG promoters (Gnatovskiy, 2013). RuvBL1 was observed to bind to the transcriptional activation domain of STAT2 and was required for a high level of ISG induction. Furthermore, it is hypothesised that RuvBL1 along with RuvBL2 are required for the recruitment of RNA polymerase II to regulate ISG transcription.

1.3 Interferon stimulated genes and Interaction with E1A

During viral infection, the host cell switches on antiviral responses to inhibit the production of viral product and spread. Adenovirus has evolved mechanisms to evade antiviral responses by interferons, specifically interferon alpha (Leonard, 1996). Adenoviruses possesses a virus associated RNA I, which is double stranded and is able to inactivate the interferon induced double stranded RNA dependent protein kinase (Anderson, 1987). Adenovirus E1A functions by inhibiting the phosphorylation of STAT1 and prevents its translocation to the nucleus. However, a study has also showed that E1A is able to cause the inactivation of the STAT1 protein by causing phosphorylated STAT1 protein to be accumulated at viral centers and prevents its binding to interferon stimulated elements (Sohn, S.-Y., & Hearing, P. 2011). E1A has also been shown to inhibit ubiquitination of the lysine residue on the histone H2B (Fonesca, 2012). Ubiquitination of this lysine residue is required for activation of interferon stimulated genes, therefore E1A is able to inhibit expression of ISGs in this way.

1.4 Hypothesis and Objective

Through mass spectrometry and co-immunoprecipitation RuvBL1 has been identified as a binding partner of E1A. Therefore, there might be an importance in the interaction of RuvBL1 and E1A, considering RuvBL1 is a hub protein and adenoviral E1A protein is known to target hub proteins.

The working hypothesis is that E1A alters the activity of RuvBL1 in order to drive viral growth.

In order to study this hypothesis, there are different objectives that will be investigated.

Objectives

1. Characterize the interaction between E1A and RuvBL1
    -Indirect or direct binding
    -Identify regions required for binding
2. Investigate the effect of RuvBL1 on viral replication and growth
3. Explore the effect of E1A on RuvBL1-regulated interferon stimulated genes

2. Material and Methods

2.1 Cell cultures

Ht1080 human fibosarcoma cells, IMR-90 primary human lung fibroblast cells, U20S human osteosarcoma epithelial cells, and 293 Human Embryonic Kidney cells were grown in Dulbecco's modified Eagle's medium (Hyclone) supplemented with either 10% or 5% fetal bovine serum (Hyclone), 100 units/ml streptomycin and penicillin(Hyclone).

2.2 Antibodies

Table 2.1 contains all antibodies used. Primary and secondary antibodies are either diluted in Tris-Buffered Saline-Tween20 (TBS-T) containing 5% milk or Tris-Buffered Saline-Tween20 containing 3% Bovine serum albumin.

2.3 Plasmids

The expression plasmid for pcDNA3-HA-RuvBL1 was created by Dr. Peter Pelka. Plasmids for expression of E1A243R and E1A289R were previously described (Pelka, 2007). pCAN-myc-RuvBL1 was made by subcloning RuvBL1 in frame with the N-terminal myc tag. pGEX-6-P1-RuvBL1 was created by cutting the vector pGEX-6-P1 and inserting pCAN-myc-RuvBL1 with BamH1 and Xho1.

Table 2.1 Table of Antibodies

Antibodies	Description	Use	Dilution Factor	Source	Catalogue #
E1A (M73)	Mouse Monoclonal	Primary	WB: 1:400	In house	N/A
E1A (M58)	Mouse Monoclonal	Primary	WB: 1:400	In house	N/A
RuvBL1	Rabbit Polyclonal	Primary	WB: 1:100 CHIP: 1:20	Invitrogen	PA5-24579
HA	Rat Monoclonal	Primary	WB: 1:5000	Roche	11867423001
Actin	Mouse Monoclonal	Primary	WB: 1:1000	Abcam	ab3280
Adenovirus Type 5	Mouse Monoclonal	Primary	WB:1:10,000	Abcam	ab6982
9E10 (c-myc)	Mouse Monoclonal	Primary	WB: 1:300	In house	N/A
Mouse IgG	Goat	Secondary	WB: 1:200,000	Jackson ImmunoResearch	115-035-003
Rabbit IgG	Goat	Secondary	WB: 1:200,000	Jackson ImmunoResearch	111-035-003
Rat IgG	Goat	Secondary	WB: 1:200,000	Jackson ImmunoResearch	112-035-003

Notes: WB-Western Blot.

2.4 Transfections

$10cm^2$ plates (Sarstedt) were plated with $2x10^6$ HT1080 cells for Immunoprecipitation experiments. 6-well plates (Sarstedt) were plated with U20S for Luciferase assay at a density of 300,000 cells/per well. Cells were plated 24 hours prior to transfection. Transfections were carried out using Polyethylenimine (**PEI**) (Sigma) according manufacturer's guidelines.

2.5 Viruses and Viral Infections

All viruses were grown in house. All viral infections were carried out for 1h with serum free media and incubated at 37^o C with 5% CO_2 , after which complete media was added to the infection. Viruses used include wild type virus (dl309), which has a mutation within the E3 region. dl312 virus was used as a mock virus for infection, which has the E1A region mutated.

2.6 siRNA Knockdown

Small interfering RNA (SiRNA) knockdown was carried out as previously described (Pelka, 2009). IMR-90 cells were transfected with RuvBL1-specific Silencer Select siRNA (Life Technologies no s16369) using silentFect reagent (Catalogue # 170-3361, Bio-Rad) according to the manufacturer's protocol using a 5nM final siRNA concentration. Silencer Select negative-control siRNA no. 1 (Life Technologies, catalogue # 4390843) was used as the negative siRNA control. Fresh complete media was added to plated cells 15 minutes prior to addition of siRNA and silentFect. Transfection of siRNA was carried out in serum free media and incubated for 20 minutes. After which, transfection media was added to the cells in a drop wise fashion.

2.7 Viral Plaque Assay

RuvBL1 was knocked down in IMR90 cells using siRuvBL1. After which, cells were infected with HAdV-5 dl309 wildtype virus at a multiplicity of infection of 10. The viruses were then harvested at 48, 72 and 96 hours post infection. In order to lyse and release the virus from the cells, freezing/ thawing cycles were carried out using both a dry ice bath and room temperature water bath. To quantify the virus, plated 6 well plates of Human Embryonic Kidney (293) cells were infected with the harvested virus. Serial dilutions from 10^{-3} to 10^{-8} of the harvested viruses were used for the infections. The infection was carried out for 1h and an overlay of 2X DMEM (Gibco) and 1% Agarose was added to the cells. After 3-5 days the visible plaques were then counted and quantified to determine viral titres.

2.8 Luciferase Assays

Approximately 300,000 U20S cells were plated on to 6-well plates twenty-four hours prior to transfection. U20S cells were co-transfected with 1ug of the reporter plasmid pG-E4-Luc together with both pCAN-myc-RuvBL1 and pcDNA3-E1A 13S or individually. Total DNA concentration was equivalent to 4ug per well using an empty vector plasmid. 36 hours after transfection, cells were harvested and lysed with 300ul of 1X lysis buffer (Promega, E397A). The cells were harvested and transferred into Eppendorf tubes. The tubes were centrifuged at 13,000rpm for 10 minutes. 50ul of the lysate and 50ul of the luciferase substrate (Promega E151A) was transferred into 96 well white opaque plates (ThermoScientific). Luciferase activity was measured using FlexStation3 (Molecular Devices).

2.9 Protein Purification and GST-Pulldown

Glutathione *S*-transferase (GST) fusion of RuvBL1 was made by subcloning the cDNA into pGEX-6P1 (GE Healthcare Life Sciences) in frame with the N-terminal GST tag. The fused GST-RuvBL1 plasmid was then transformed into BL21 Rosetta cells to express the protein. Liquid culture of the bacteria was grown to an OD_{600} = 0.7 at 37^{o} C. The culture was then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4h at 37^{o} C. The culture was then centrifuged at 4000 rpm at 4^{o}C for 10 minutes to obtain a pellet. The pellet was then suspended and lysed in 20ml of NTEN buffer (100mM NaCl, 20mM Tris-Cl pH 8, 1mM EDTA, 1% Triton) supplemented with protease inhibitor and lysozyme. Samples were then sonicated three times for 10 seconds with 30 seconds on ice in between each sonication. The samples were centrifuged at 4000 rpm at 4^{o}C for 10 minutes. 2ml of glutathione s-transferase beads were prewashed with 4ml of PBS (phosphate buffer solution) three times was added to the clarified lysate and incubated together at 4^{o}C for 1h. After which, the sample was centrifuged at 4000 rpm for 10 minutes. The supernatant was removed and the pelleted beads were washed three times with PBS by centrifuging at 4000 rpm for 5 minutes. The beads were then resuspended with 5ml of PBS and packed into a gravity column. 500ul of fractions were collected through gravity filtration using 50mM Tris-Cl pH 8 and 20mM of reduced glutathione. To verify which fractions contained proteins, 25 ul of the samples from each fraction was boiled together with 25ul of 2X sample buffer and DTT 4:1 ratio) at 100 ^{o}C. The samples were resolved on SDS gels and stained with Coomasie blue. To visualize the presence of the protein, destain solution (50 volumes of water, 40 volumes of methanol and 10 volumes of acetic acid) was used. Fractions with proteins present were then pooled together and dialysed over night with 1X PBS buffer. Samples were

collected and Bradford assay was carried out to determine protein concentration. His-tagged E1A289R was created by sub-cloning the cDNA into a pET42 vector (Novagen) in frame with a C-terminal 6X His tag. GST pull downs were carried out as previously described (Pelka, 2009), using 3ug of protein for immunoprecipitation and GST pull down Buffer (50mM HEPES/KOH pH 7.5, 150mM KCl, 1mM EDTA, 10% glycerol, 0.1% NP-40 and 2ug/ul BSA). Western blots were carried out to visualize interactions.

2.10 Co-immunoprecipitation and Western Blot

Cells are scraped and harvested after transfection/infection and lysed in cold NP-40 lysis buffer (0.5% NP40, 100mM NaCl, 50mM TRIS pH 7.8) supplemented with protease inhibitor. 1ml of lysis buffer is used for cells plated on $10cm^2$ plates and 300 ul of lysis buffer is used for cells plated on 6-well plates. Samples are then lysed for 10 minutes on ice and centrifuged at 13,000 rpm for 10 minutes at 4^oC. For western blot, 25ul of lysate is boiled together in a 1:1 ratio of 2X Sample Buffer to DTT (4:1 ratio) at 100^oC for 10 minutes.

For Immunoprecipitation, 950ul of lysate is incubated with antibodies and 125 ul of protein A sepharose B or 150ul of M73-Protein A sepharose crosslinked beads. Samples are incubated for 1h at 4^oC. After which, samples are centrifuged at 13,000 rpm to pellet beads. Bead are washed three times for 30 seconds with 1 ml of NP-40 lysis buffer. Beads are the boiled with 25ul of 2X Sample Buffer to DTT (4:1 ratio) at 100^oC for 10 minutes. 25ul of lysate is also collected and boiled with Sample Buffer to DTT (4:1 ratio) at 100^oC for 10 minutes as input for the western blot.

Samples are loaded and run on a 10% SDS-PAGE self-casts gels. Gels were run in a BIORAD mini-PROTEAN Tetra System cell with SDS-PAGE running buffer (25mM Tris, 250mM

glycine pH 8.3, 0.1% SDS) at 200V for 60 minutes. Proteins were then transferred onto a PVDV membrane (BioRad) in a BIORAD mini-PROTEAN Tetra system cell using transfer buffer (25mM Tris, 250mM glycine pH 8.3, 0.1% SDS and 5% methanol). Transfer was carried out at 100v for 1h and 10 minutes at 4^{o}C. The membrane was blocked with blocking buffer (5% skim milk, 10% Tween 20 in TRIS-buffered saline solution pH 7.6 (TBS-T)) shaking for 1h at room temperature. 20ml of primary antibody is added to the membrane overnight at 4^{o}C. After which, the membrane is washed three times with TBST for 10 minutes with shaking. 20ml of diluted secondary antibody in blocking buffer is added and incubated with the membrane for 30 minutes. Additional three washes with TBST are carried out for 10 minutes. Luminata Forte Western HRP substrate (Millipore) was added to the membrane for 5 minutes. Amersham Hyperfilm was exposed to the membrane in the dark at varying exposure times as needed. The film was developed using the automated film developer (Konica Minolta Medical Imaging Model SRX-101A).

2.11 Chromatin Immunoprecipitation

HT1080 cells were plated on 15cm^2 plates. HT1080 were then infected with HAdV-5 dl309 at a MOI of 10. Twenty-four hours post infection; cells were either treated with 1000units/ml of interferon α for 8 hours. Cells were cross linked directly on the plate in the original growth media by adding formaldehyde to a final concentration of 1%. The cells were crosslinked for 10 minutes at room temperature with agitation. Cross-linking reaction was stopped by adding glycine to a final concentration of 125mM and allowed to continue to rock at RT for 5 minutes. Cells were washed with PBS three times and scraped off the dishes in PBS and transfer to 50 ml falcon tubes. The cells were centrifuged at 1500 rpm for 5 minutes. Cell pellet was resuspended in 400ul of ChIP cell lysis buffer (5mM PIPES, pH 8 (pH with KoH), 85mM KCl, 0.5% Np-40) containing protease inhibitors and incubated on ice for 10 minutes. The nuclei were pelleted by centrifuging at 5000 rpm for 5 minutes. The pelleted nuclei were resuspended in 400 ul of ChIP nuclear lysis buffer (50mM TRIS, pH 8.1, 10mM EDTA, 1% SDS) supplemented with protease inhibitors and incubated on ice for 10 minutes. The lysed nuclei were sonicated on ice using the Covaris Focused- UltraSonicator M220. The samples were then centrifuged after sonication at 5000 rpm for 10 minutes at 4^{o}C. 150ul of the supernatant were transferred into 2 eppendorf tubes (for the IP and the IgG negative control) and diluted 10-fold (up to 1.5mL) with ChIP dilution buffer (0.01% SDS, 1.1% Triton x-100, 1.2mM EDTA, 16.7 mM TRIS pH 8.1, 167mM NaCl AND protease inhibitors). The sample was pre-cleared with 60ul of salmon sperm DNA/protein A sepharose slurry for 30 minutes at 4^{o}C with agitation. The supernatant was collected and transferred into a new tube. 50ul from each sample was saved for IgG control as input. The IP tube was incubated with 10ul of RuvBL1 antibody was used as well as 25ul of M73 and M58 E1A antibody overnight at 4^{o}C with agitation. The same was done for the IgG negative control. 125ul of Protein A sepharose beads were added to the tubes and incubated for 2 hours with

shaking at 4ºC. Beads were washed for 5 minutes with agitation with the following buffers and in order of:

a. Low salt wash buffer (o.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM TRIS pH 8.1, 150mM NaCl).

b. High salt wash buffer (0.1% SDS, 1% Triton X-100, 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 8.0)

d. 2X in 1X TE buffer (TRIS-EDTA buffer)

The complexes were eluted with 300ul of elution buffer (1% SDS, 0.1M $NaHCO_3$). The samples were vortexed and agitated for 15 minutes. The beads were then pelleted and the supernatant was transferred to new eppendorf tubes. The crosslinking was reversed by adding 5M NaCl to a final concentration of 0.3M to the eluted complexes and incubating at 65ºC overnight. The DNA was pelleted and resuspended in 100ul of water. 2ul of 0.5M EDTA, 4ul of 1M TRIS pH 6.5 and 1ul of 20mg/ml Proteinase K was added and incubated for 2 hours at 45ºC. The DNA was purified using the PCR Purification Kit (ThermoScientific) according to manufacturer's guidelines. PCRs were carried out for HAdV5 early promoters, IFIT1 (Interferon Induced Protein with Tetratricopeptide Repeats 1) promoter, and IFI6 (Interferon Alpha Inducible Protein 6) promoter using SYBR select master mix for CFX (Applied Biosystems) according to the manufacturer's directions, with 3% of total ChIP DNA as the template and a CFX96 real-time PCR instrument (Bio-Rad). The annealing temperature used was 60°C, and 40 cycles were run.

2.12 PCR & PCR primers

IMR-90 cells were infected with dl309, dl312 (mock), dl1132, dl1133 (MOI of 50) for 24 hours and treated with interferon for 8 hours (Interferon assays only). Total cellular RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNA was used in reverse transcriptase reactions using SuperScript VILO reverse transcriptase (Invitrogen) according to the manufacturer's guidelines and random hexanucleotides for priming. The cDNA was used for real-time expression analysis using the Bio-Rad CFX96 real-time thermocycler. Fold changes in expression were determined by comparing expression levels with levels for control knockdown cells or mock infection and analyzing expression data using the Pfaffl method.

Table 2.2 PCR Primers

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
E1A	GCTCAGGTTCAGACACAGGACTGTA	CTTACCCCCAACGAGTTTGA
E1B	TCAAACGAGTTGGTGCTCATG	GCCGCCACAAGTGCTTTG
E2A	GCGGATGAGGCGGCGTATCGAG	TCGGCCTCCGAACGGTAAGA
E3A	CTCGGAGAGGTTCTCTCGTAGACT	CTGCTGCCCGAATGTAACACT
E4orf6/7	TCCACCTTGCGGTTGCTTAA	GGGGGTGGTTTCGCGCTGCTCC
Hexon	GGAGTACATGCGGTCCTTGT	CGCGCTGAGTTTGGCTCTAG
E2p	AGAATTCGGTTTCGGTGGGC	CGCGGGACCCCACATGATAT
E3p	CGCCCTCTGATTTTCAGGTG	TAAACACCTGAAAAACCCTCCTGCC
E4p	GGCTTTCGTTTCTGGGCGTA	AGCAAATACTGCGCGCTGAC
hGAPDH	TTGATTTTGGAGGGATCTCG	GAGTCAACGGATTTGGTCGT
ISG56	AAAAGCCCACATTTGAGGTG	GAAATTCCTGAAACCGACCA
IFI6	CTCGCTGATGAGCTGGTCT	TGCTGGCTACTCCTCATCCT
ISG56p	TTTCACTTTCCCCTTTCGGTTTCC	GGCTCCTCTGAGATCTGGCTATTC
IFI6p	CTGGGCGGAGCTGGGAGAG	TGGGCACAGCAGCGAGTAAAC
ISG563'p	TCTGAACATTGAAAGGAACAAACTC	ACTCACTGCTTGGCGATAGG

3. Results

3.1 Investigating the interactions of RuvBL1 and E1A

3.1.1 Interaction of E1A with RuvBL1

To identify cellular proteins that bound to E1A within the C-terminal region, affinity purification and mass spectrometry was performed. RuvBL1 was one of the binding partners of E1A that was identified. To verify the interaction between RuvBL1 and E1A, co-immunoprecipitation experiment was carried out to see if endogenous RuvBL1 interacted with E1A. Endogenous RuvBL1 was found to interact with wild type E1A (Fig 3.1).

It was also important to map the regions within E1A that are required for its interaction with RuvBL1. To do this, various deletion mutants of E1A were used. The mutant viruses used are dl116, dl1132, dl1133, dl1134, dl1135 and dl1136 (Mymryk and Bayley, 1993; Boyd et al., 1993 ;), which are all mutations within the C-terminus of E1A. A pcDNA-HA-RuvBL1 plasmid was transfected, along with infection of mutant viruses expressing mutated E1A within the C-terminus. The two mutant viruses that showed the least affinity for RuvBL1 is dl1132 and dl1133 (Fig. 3.2).

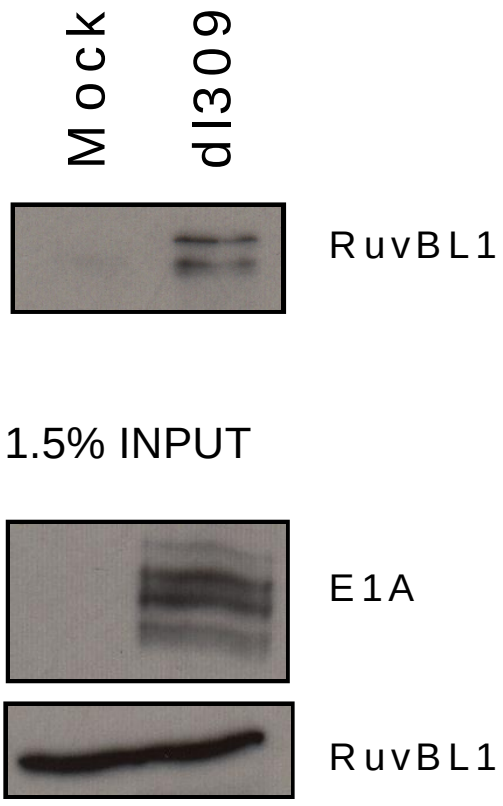


Figure 3.1 E1A and RuvBL1 interact: HT1080 human fibrosarcoma cells were infected with dl309 (wild type virus) at an MOI of 10. Twenty-four hours after transfections cells were harvested and immunoprecipitated with cross linked beads protein A sepharose beads to M73 E1A antibody. Samples were subjected to western blot to blot for endogenous RuvBL1. 1.5% of total sample lysate were used for input.

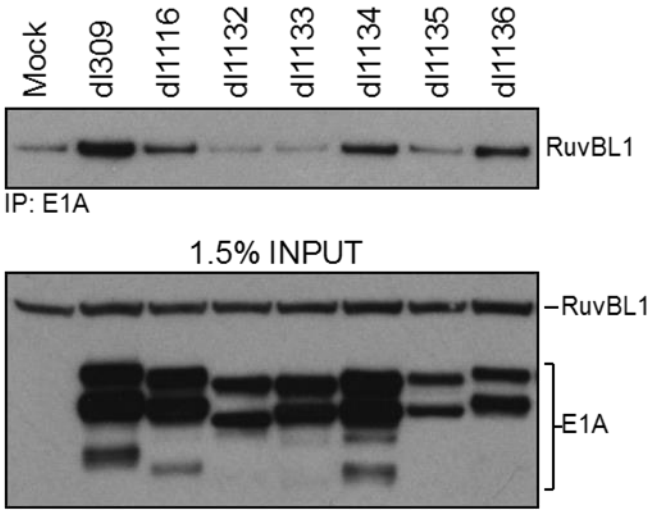


Figure 3.2 RuvBL1 interacts with E1A. HT1080 cells were infected with HAdV-5 deletion mutants or wild type virus (dl309). Twenty-four hours after infections cells were lysed and immunoprecipitated for E1A using either M73 or M58 antibodies, M58 was used for mutants dl1135 and dl1136 as these do not have the M73 epitope. Associated protein complexes were eluted from the beads using sample buffer, resolved by SDS-PAGE and detected by western blot using anti-RuvBL1 antibody. 1.5% of total sample lysate was used for input.

3.1.2 E1A interacts directly with RuvBL1

The above result verified the interaction of endogenous RuvBL1 and E1A, however we need to determine whether the interaction is through direct or indirect means. The mode of interaction between E1A and RuvBL1 will provide insight into the function of these proteins. In order to determine this, GST pull down assays were performed. This assay was carried out by creating a construct with the RuvBL1 gene sub-cloned into a GST-fusion vector and the E1A sub-cloned into a 6xHis-tagged vector. The proteins were expressed and purified, after which pull down assays were carried out to observe if a direct interaction occurs between the two proteins. E1A and RuvBL1 were found to interact *in vitro*, verifying that this is a direct association (Fig. 3.3). I decided to do both a pull down of GST-RuvBL1 with a glutathione resin and blot for E1A and also did the converse by pulling down 6xHis-E1A with a nickel resin and blotting for RuvBL1. Both experiments showed the same conclusion that E1A does directly bind to RuvBL1.

3.2 RuvBL1 enhances E1A-mediated activation of viral E4 promoter

One of RuvBL1's functions is to regulate gene expression as it is part of several complexes involved in transcriptional regulation such as Ino80 and NuA3 histone acetyltransferase (HAT) (Cvăková, 2008). Marcelo A. Wood et al had previously shown that RuvBL1 functions as a transcriptional activator for the regulation of the c-Myc gene. Therefore, I wanted to investigate if E1A is able to recruit RuvBL1 to act as a trans-activator of a promoter such as the E4 promoter. Analysis of luciferase activity showed that when RuvBL1 and E1A are present together there is about a two-fold increase of transcriptional activation compared to when RuvBL1 and E1A are present individually (Fig. 3.4). Both E1A and RuvBL1 showed

approximately the same level of transcriptional activity when alone with E1A showing a slightly higher level of transcription. I also performed a western blot analysis to detect protein expression of both RuvBL1 and E1A (Fig. 3.4**)**

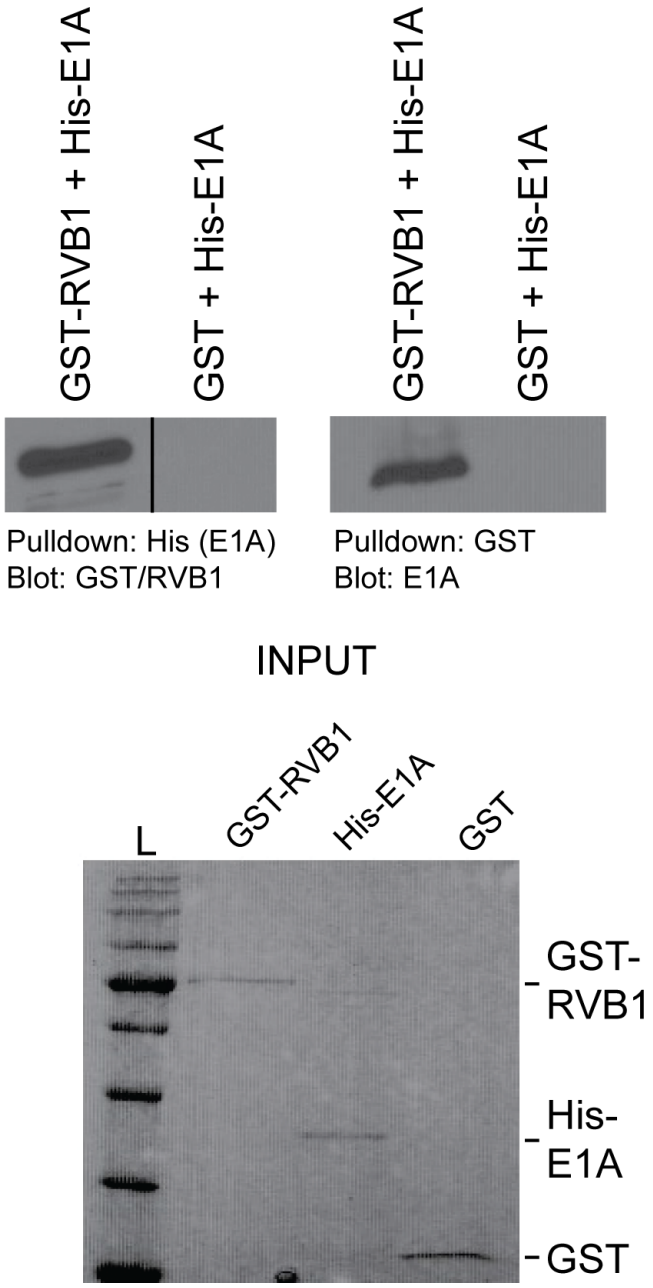


Figure 3.3 RuvBL1 and E1A interact directly: GST pulldown assays were carried out with purified RuvBL1 and purified E1A289R or and E1A243R alone. GST was used as a negative control. E1A pull down was performed on nickel bead, while RuvBL1 pull down was performed with GST beads. E1A was detected using M73, while RuvBL1 was detected using a polyclonal anti-RuvBL1 antibody.

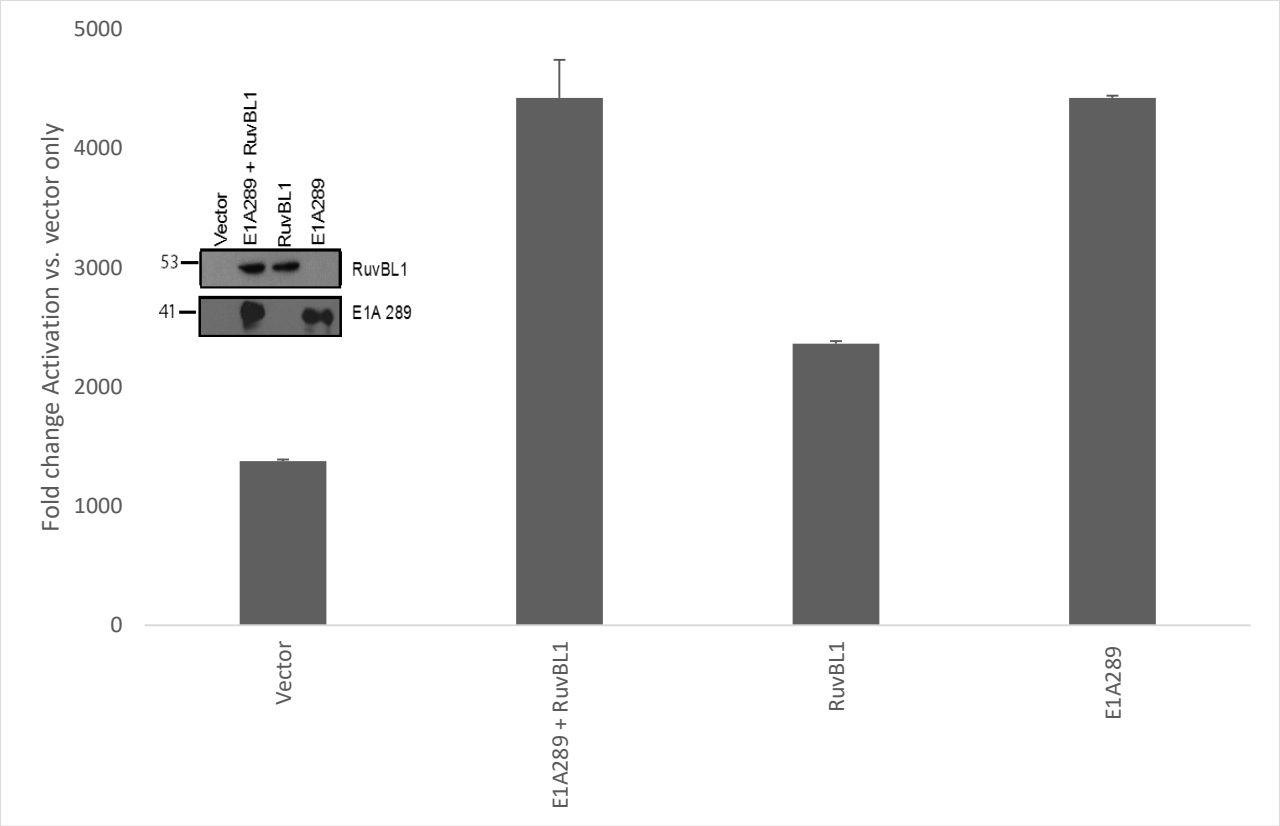


Figure 3.4 RuvBL1 enhances E1A-mediated activation of viral E4 promoter: RuvBL1 and E1A289 plasmid were transfected with a E4 luciferase reporter vector to measure level of activation. U2OS cells were co-transfected with the reporter plasmid pGL3-E4 together with RuvBL1 and E1A289R. Luciferase assays were performed 48 h after transfection, and results were plotted as fold change vs vector activation.

3.3 RuvBL1 is required for Efficient Adenovirus Growth

3.3.1 RuvBL1 is a positive regulator of viral growth

To observe the effect of viral growth when RuvBL1 is depleted via siRNA, RuvBL1 was knocked down in IMR-90 cells and was infected with wild type (dl309) virus at an MOI of 10. At 48h, 72h, 96h the viruses were harvested. There are no 24-hour time points because it would be too early to see any significant changes in viral growth. According to the results, the dl309 virus was not able to grow efficiently when RuvBL1 has been depleted. There was about a two-fold decrease at 96 hours post infection in dl309 growth after RuvBL1 was knocked down by siRNA (Fig. 3.5).

I also wanted to look at the phenotypic effect of the viral infections on IMR-90 by observing the cytopathic effects (CPE) which are structural changes to the cell due to infection taking place (Fig 3.6). At 48 hours post infection, there are no differences in the cells of both control knock down and RuvBL1 knockdown. However, at 72 hours, there are observable differences in the morphology of the cells. The control cells start to shown signs of CPE at 72 hours compared to RuvBL1 depleted cells. By 96 hours, CPE is readily visible in control knock down cells compared to RuvBL1 depleted cells.

3.3.2 RuvBL1's effect on viral gene expression

Next, I wanted to investigate if the reduced growth of the virus in RuvBL1-depleted cells was due to reduced viral gene expression. Since expression of most viral early genes are regulated by cellular transcription factors and RuvBL1 has been shown to function as a transcriptional regulator, then perhaps RuvBL1 plays a role in early viral gene expression. To also observe the effect on the expression of viral early genes when RuvBL1 has been knocked down, real time PCR was performed. RuvBL1 was first knocked down in IMR-90 cells and infected with the wildtype dl309 virus. The virus was then harvested at 24h, 48h, and 72h time points. RNA was then isolated using TRIzol by phase separation. Viral genes were quantified using real time PCR. The results show that at 72hr, most of the viral genes are not substantially affected when RuvBL1 has been knocked down (Fig. 3.7). However, a significant down regulation of the hexon gene at 24hrs is not observed.

Likewise, I wanted to investigate if knockdown of RuvBL1 effected viral proteins such as E1A, DNA binding protein (DBP) and viral structural proteins. I knocked down RuvBL1 in IMR90 and infected the cells with dl309 and harvested the samples. There is some reduction in protein levels of DBP and E1A (Fig. 3.7B). However, for viral structural proteins such as hexon, penton, protein v, and protein VII, were reduced in expression in RuvBL1 depleted cells (Fig 3.7B).

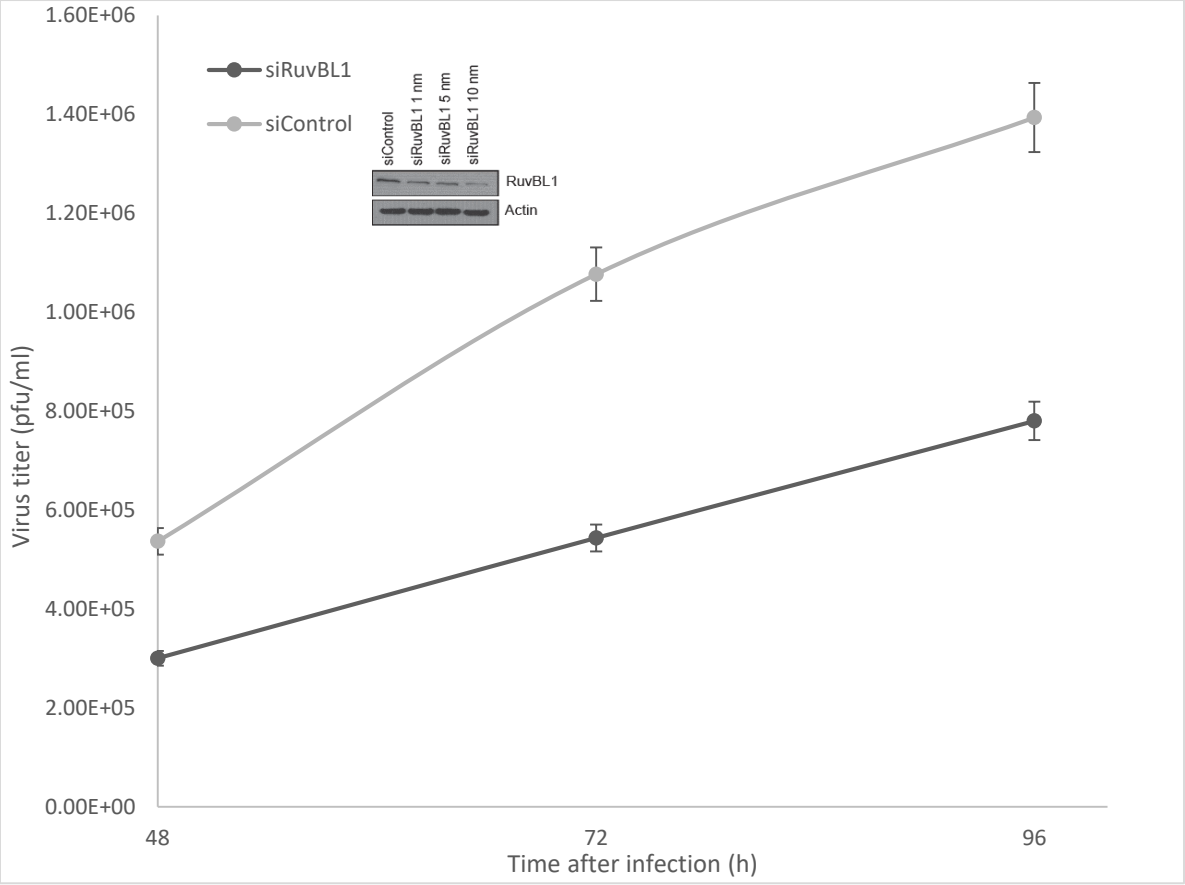


Figure 3.5 RuvBL1 knockdown decreases viral growth: RuvBL1 was knocked down in IMR-90 cells using RuvBL1-specific siRNA (siRuvBL1); nonspecific siRNA was used as a negative control (siControl). Twenty-four hours after siRNA transfection, cells were infected with HAdV5 (*dl*309) at an MOI of 10, and viral titres were determined on 293 cells at the indicated time points.

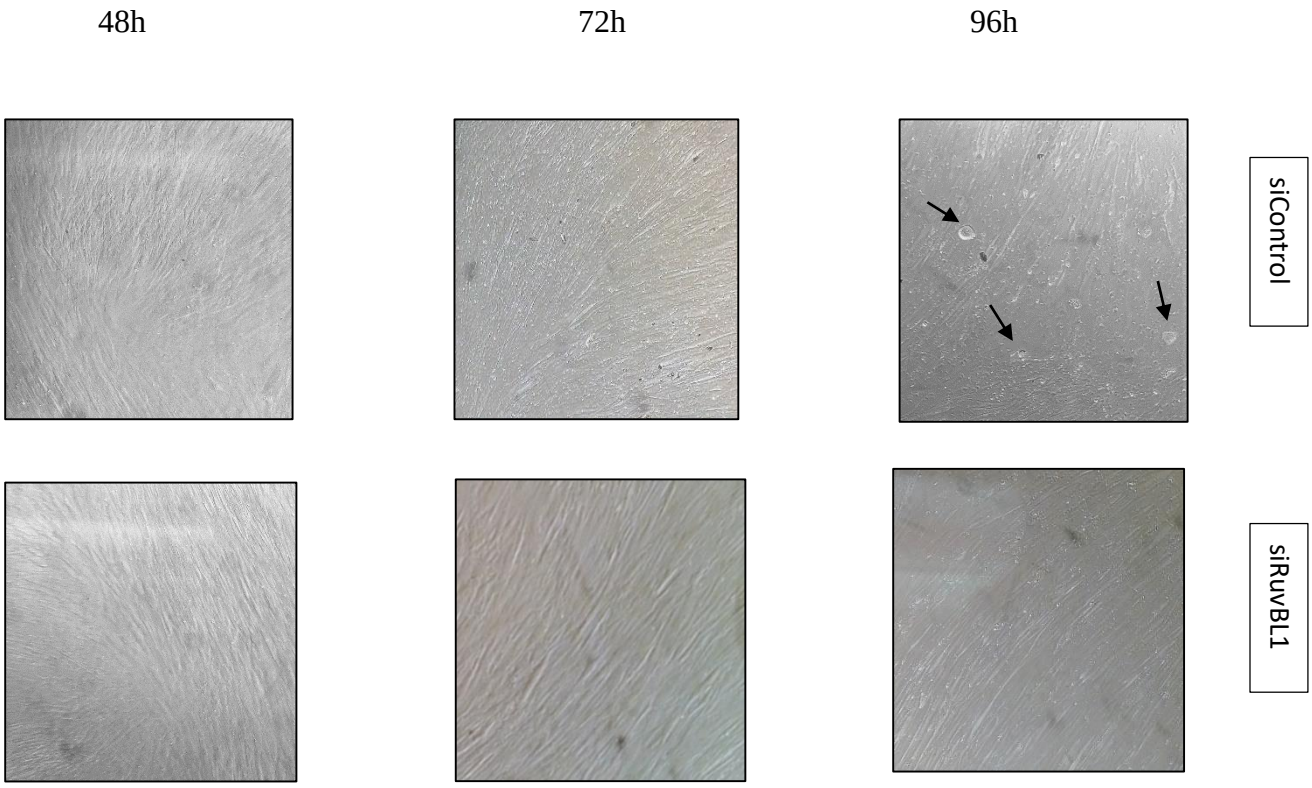


Figure 3.6 RuvBL1 knockdown delays cytopathic effect during viral growth: Representative images of infected cells in both control and RuvBL1 depleted cells Images were taken prior to harvest of cells for titre determination and were taken at 100X magnification. *Arrows indicate CPE (cytopathic effect)

A.

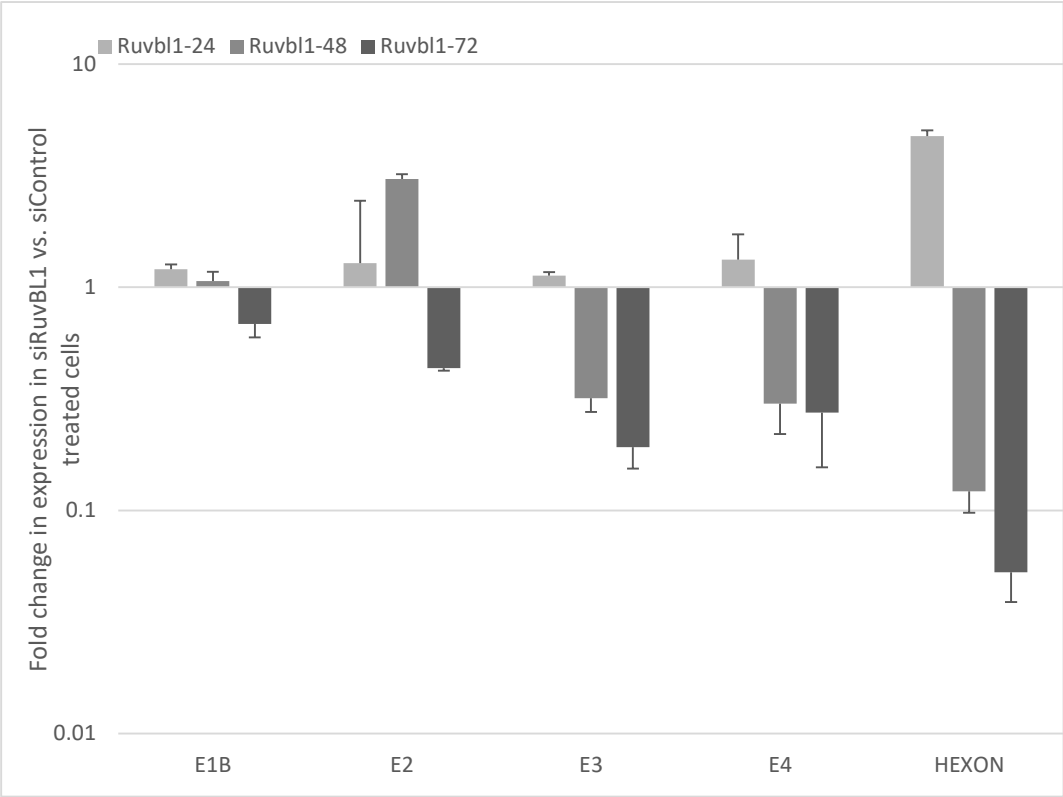


Figure 3.7 RuvBL1 knockdown and effects on viral gene expression: IMR90 cells were treated with siRNA for RuvBL1 or a negative-control siRNA. Forty-eight hours after siRNA transfection cells were infected with HAdV5 (*dl*309) at an MOI of 10, and total RNA was extracted 24h, 48h and 72 h after infection using TRIzol reagent. mRNA levels were quantified using real-time quantitative PCR and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The levels were plotted as fold changes versus the level for control siRNA-treated cells

B.

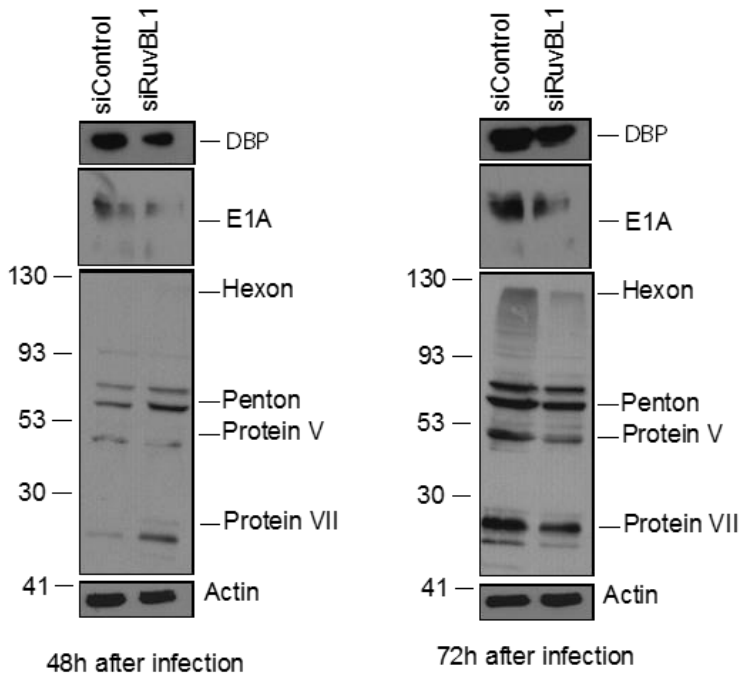


Figure 3.7B RuvBL1 knockdown decreases viral protein expression: RuvBL1 was knocked down in IMR-90 cells using RuvBL1-specific siRNA (siRuvBL1); nonspecific siRNA was used as a negative control (siControl). Twenty-four hours after siRNA transfection, cells were infected with HAdV5 (*dl*309) at an MOI of 10. Viral expression of protein was determined by western blot and anti-HAdV5 structural antibody.

3.4 Investigating Interferon Effect of E1A on RuvBL1-regulated interferon stimulated genes

3.4.1 Effect of dl309 on interferon genes in RuvBL1 depleted cells

So far we have seen that knock down of RuvBL1 reduces viral growth, however the reduction is not entirely due to RuvBL1's ability to regulate early viral gene expression due to the minor differences in viral gene and protein expression. Therefore, I hypothesised that perhaps the effect observed could be due to RuvBL1's association with interferon response. RuvBL1 plays a role in the regulation of interferon stimulated genes (Gnatovskiy et al. 2013). They showed that when RuvBL1 was knocked down, there was a significant reduction in the expression of interferon stimulated genes, specifically ISG56 and IFI6. It is also well known that HAdV5 is able to escape interferon responses in multiple ways. Therefore, I wanted to determine whether RuvBL1 plays a role in interferon evasion mediated by E1A. To test this, RuvBL1 was knocked down in IMR-90 cells and infected with dl309 for 24hrs and treated with interferon for 8 hrs to stimulate the interferon response. RNA was then extracted from these cells and real time PCR was carried out to observe the degree at which E1A is able to still inhibit interferon when RuvBL1 has been knocked down. I plotted the data as expression of interferon genes in control cells and RuvBL1 depleted cells versus mock infection (Fig. 3.8). Mock infection was done with dl312, which is a mutant virus with an E1A deletion. According to the data, HAdV dl309 wt virus can no longer inhibit interferon genes, specifically for ISG56 when RuvBL1 is absent from cells.

A. B.

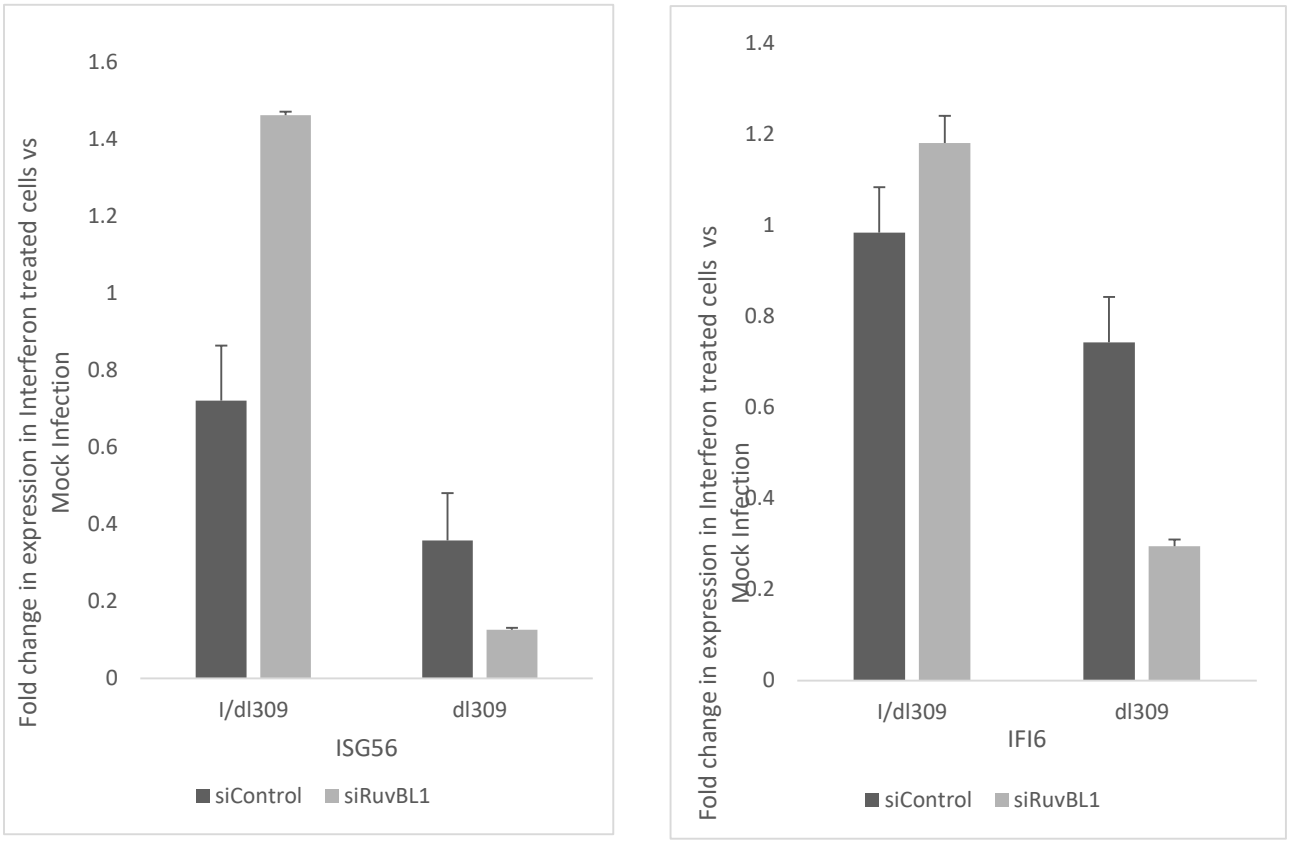


Figure 3.8 Effect of dl309 on interferon genes in RuvBL1 depleted cells: RuvBL1 was knocked down in IMR90 cells and infected with dl309 for 24h (MOI of 50) and treated with interferon for 8h. RNA was then harvested from these cells using TRIzol reagent. mRNA levels of ISG56 (A) and of IFI6 (B) were quantified using real-time quantitative PCR and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Note * I = interferon treatment.

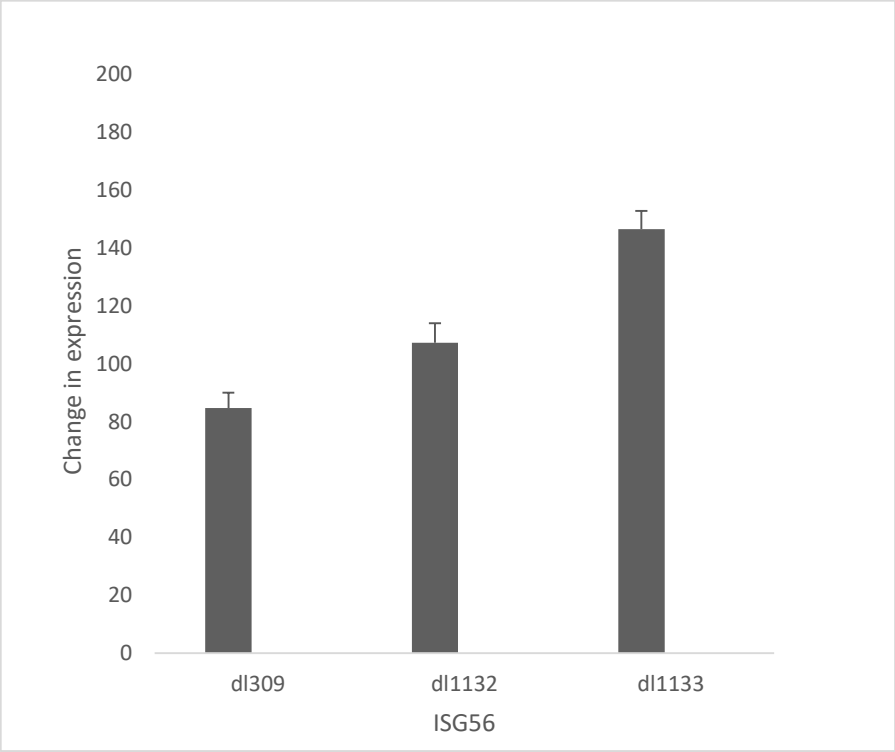


Figure 3.9 Effect of RuvBL1-binding mutant viruses on ISG56 expression: IMR90 cells were infected with wild type virus dl309 and mutant virus dl1132 and dl1133 for 24h (MOI of 50) and treated with interferon for 8h. RNA was then harvested from these cells using TRIzol reagent. mRNA levels of ISG56 were quantified using real-time quantitative PCR and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

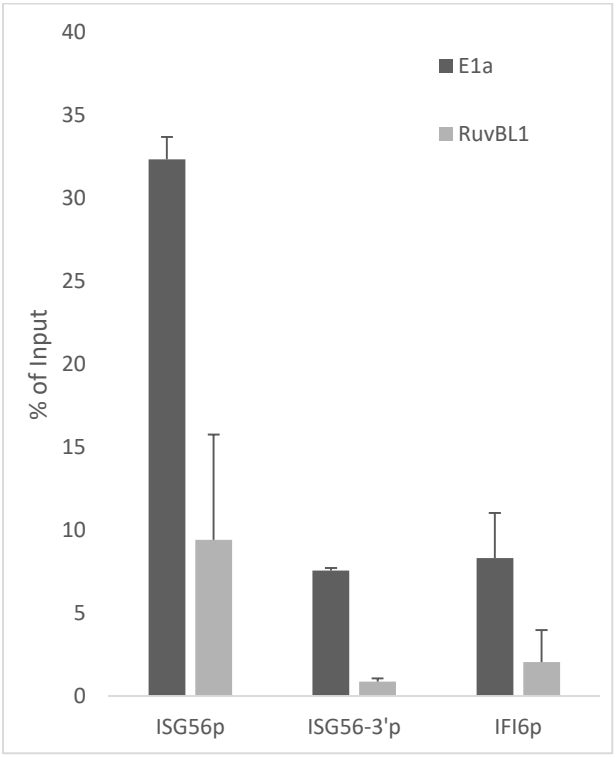
3.4.2 Effect of RuvBL1-binding mutant viruses on ISG56

I wanted to determine if viruses expressing E1A unable to bind to RuvBL1 (dl1132, dl1133) are deficient for interferon suppression. I infected IMR-90 cells with dl1132, dl1133 and dl309 and performed real time PCR to measure the expression of ISG56 gene only since I did not observe significant differences with the IFI6 gene. The two mutant viruses that express E1A that is no longer able to bind RuvBL1 were unable to inhibit expression of the ISG56 gene (Fig. 3.9)

3.5 RuvBL1 is recruited to ISG56 promoter upon adenovirus infection

I also wanted to see if the ability of HAdV to reduce interferon response was due to E1A being recruited by RuvBL1 to the ISG56 promoter. Chromatin immunoprecipitation for E1A and RuvBL1 was carried out at the ISG56 and IFI6 promoters. E1A and RuvBL1 were found on the ISG56 promoter only after interferon stimulation, but not on the IFI6 promoter (Fig. 3.10). I observed a substantial enrichment over an IgG negative-control immunoprecipitation that was performed. It is also important to point out that neither E1A nor RuvBL1 was found on ISG56 promoter in the absence of interferon treatment (Fig. 3.9B).

A.



B.

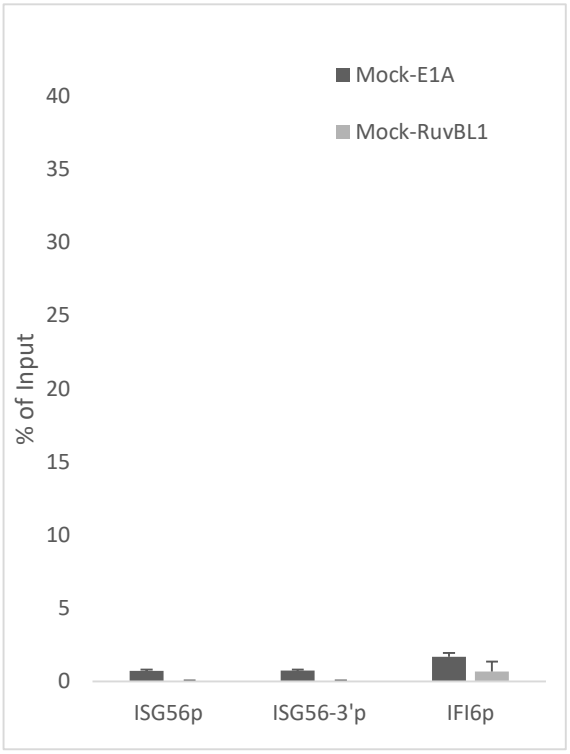


Figure 3.10 RuvBL1 is recruited to ISG56 promoter upon adenovirus infection: HT1080 cells were infected with HAdV5 (*dl*309) at an MOI of 10, and ChIPs were carried out 24 h after viral infection (A). E1A was immunoprecipitated using a cocktail of anti-E1A antibodies (M73 and M58), and RuvBL1 was immunoprecipitated using anti- polyclonal antibody. 12CA5 anti-HA monoclonal antibody was used as a negative IgG control. B. Mock infection with dl312 (E1A deleted).

4. Discussion

E1A's effect on the cell involves its interactions with a multitude of host regulators that are critical controllers of gene expression and cellular growth. E1A has proven to be valuable in identifying numerous regulatory proteins that are involved in crucial cellular processes. More importantly, other proteins have been shown to interact to the least characterized region of E1A, the C-terminus. By identifying essential regulators that bind to E1A within the C-terminus and discover how E1A is able to alter their function, it will lead us to a better understanding of the function of the C-terminus region of E1A.

4.1 Interaction of RuvBL1 and E1A

It was important to establish that E1A binds to endogenous RuvBL1 in order to establish that the interaction between the two proteins is genuine. Through co-immunoprecipitation I was able to decipher that RuvBL1 did in fact bind to E1A (Fig. 3.1 Mapping the interaction site that is crucial for binding identified two viruses (dl1132 and dl1133) with deletions in amino acids 224-238 and 241-254 of E1A. RuvBL1 showed reduced binding to both mutant E1As compared to wildtype E1A and the other mutant viruses with deletions of other regions within the E1A C-terminus (Fig. 3.2.). The dl1133 mutant has been shown to have important functions in immortalization and tumorigenesis (Boyd et al., 1993). Therefore, RuvBL1 may play a role in functions carried out by the regions required for binding to E1A such as immortalization). RuvBL1 seems to also interact with E1A directly as well, as we observed an interaction using the GST pulldown assay with purified His-E1A being able to pull down purified GST-RuvBL1 (Fig. 3.3).

4.2 RuvBL1 activation of AdV E4 promoter

E1A is primarily known to utilize cellular transcriptional regulators as activators for viral early gene expression (Mymryk, 1993). By studying the E4 promoter, many factors that interact with E1A have been identified (Jones and Lee., 1991). Consequently, it was important to establish if E1A is able to utilize RuvBL1 as a trans-activator of viral promoters, such as the HAdV E4 promoter. My results show that RuvBL1 affected E1A-mediated transactivation of the E4 reporter, validating RuvBL1as trans-activator (Fig 3.4). When RuvBL1 and E1A are both present we see an increase in the activation of the promoter of approximately two folds. This leads to the conclusion that perhaps E1A is able to either recruit RuvBL1 to the E4 promoter and act as a transcriptional activator or possibly E1A uses RuvBL1 by another means to enhance transcription. We can also deduce from the western blot that increase in transcriptional activity of E1A and RuvBL1 was not due to an increase in the expression of RuvBL1. We also can observe that from the assay, E1A is also able to induce transcription, however not to the same level as when present together with RuvBL1. This also solidifies that E1A utilizes RuvBL1 in some way to activate transcription.

4.3 Analyzing Effects of RuvBL1 on viral growth and expression

Considering the fact that we know E1A utilizes RuvBL1 to enhance transcription, it was only logical to assess how HAdV replicates in cells when RuvBL1 has been depleted using small interfering RNA targeted to RuvBL1. This will enable us to better understand the connection between RuvBL1 and E1A in regards to the growth of HAdV.

Knockdown of RuvBL1 acts as a restrictor of viral growth as seen with a decrease in viral titres of approximately 2 fold (Fig. 3.5). This observation corroborates with the phenotypic effects of the viral infection with regards to CPE. By seventy-two hours post infection, we start to observe a change in the morphology of the cells and signs of CPE are evident in control cells but absent in RuvBL1 depleted cells (Fig. 3.6). This observation was made more apparent at ninety-six hours post infection, where we see more roundness of cells, a clear indication of CPE, which is more apparent in control cells than RuvBL1 depleted cells.

RuvBL1 knockdown has some effect on viral gene expression. Observations show that knockdown of RuvBL1 did show reduction of viral mRNAs or protein levels **(**Fig. 3.7). I observed some reduction of the viral genes that were examined of about 10 folds (E1B, E2, E3, and E4orf6/7). More significantly, I did observe a reduction in viral mRNAs of the hexon gene at seventy-two hours, which is a component of the structural make up of HAdV.

This result was also verified with levels of viral structural proteins, where we see reduced expression of hexon as well as the other structural protein in RuvBL1 depleted cells (Fig. 3.7B). It was expected that there will be reduction of viral mRNAs when RuvBL1 was knocked down

since RuvBL1 is required for HAdV growth as observed from the growth assay. RuvBL1 does play a role in regulating activation and transcription of HAdV early genes.

4.4 Analyzing Effect of dl309 on interferon genes in RuvBL1 depleted cells

RuvBL1 plays a role in the regulation of interferon stimulated genes, specifically ISG56 and IFI6 (Gnatovskiy, 2013). Gnatovskiy et al showed that knockdown of RuvBL1 was related to a decrease in interferon response because RNA polymerase II could no longer be recruited to interferon stimulated genes. They also established that recruitment of RuvBL1 to ISG promoters was dependant on its interaction with STAT2. It is also well known that HAdV5 is able to escape interferon responses in multiple ways (Reich et al., 1988). Real time results show that when RuvBL1 is depleted, HAdV dl309 infection could no longer inhibit expression of ISG56 compared to control cells (Fig. 3.8). We see the same observations for IFI6 but to a lesser degree than that of ISG56. Clearly, RuvBL1 is required for E1A to suppress interferon stimulated genes upon HAdV infection in cells.

In addition, when I used the two mutant virus defective in binding to RuvBL1 we see a similar result. The virus dl1132 and dl1133 are both unable to bind to RuvBL1 compared to wildtype dl309. Therefore, when we infected the cells with these mutant viruses and looked at expression of ISG56, we see an increase in expression of interferon stimulated genes since these mutant viruses do not interact with RuvBL1 (Fig. 3.9). This observation also indicates that the ability of E1A to decrease interferon stimulated genes depend on E1A's interaction with RuvbL1. This results elucidates the reason as to why RuvBL1 increases viral growth. It appears that E1A is able to evade interferon response in some parts by its interaction with RuvBL1 and therefore is able to replicate and grow more efficiently in infected cells.

4.5 Investigating the effect of E1A on RuvBL1 recruitment to ISG56 Promoter

To solidify the above hypothesis that E1A perhaps modifies the function of RuvBL1 to evade interferon response, I decided to look at both E1A and RuvBL1 occupancy at the ISG56 promoter. According to the data, I observed that when cells are infected with HAdV dl309 virus, both E1A and RuvBL1 are found on the ISG56 promoter (Fig. 10). However, in the absence of dl309 infection, RuvBL1 is not recruited to ISG56 promoter (Fig. 10B). This suggests that E1A is somehow altering the function of RuvBL1 by using RuvBL1 to recruit itself to the ISG56 promoter and somehow inhibiting expression of interferon stimulated genes. As I mentioned earlier RuvbL1 was observed to bind to the transcriptional activation domain of STAT2 and is required for a high level of ISG induction (Gnatovskiy, 2013). Perhaps, E1A functions by interfering with RuvBL1's interaction with STAT2 and thereby represses STAT2 activity and subsequent activation of ISG56 transcription.

A similar observation was seen in a report where they showed that E1A interfered with the association of STAT2 and p300/CBP (CREB binding protein), a target of E1A (Bhattacharya, 1996). They identified that p300/CBP interacted with the transactivation domain of STAT2 and was able to induce expression of interferon stimulated genes. They also showed that E1A was able to inhibit the binding of p300/CBP to STAT2 and thereby inhibit interferon response. Clearly, E1A has evolved multiple ways to evade host immune interferon response to further replicate efficiently and its interaction with RuvBL1 could be a novel mechanism of doing so.

5. Summary

E1A has proven to be an ideal model to identify major cellular regulators involved in cellular growth as well as elucidating several key players and processes involved in oncogenic transformation. Likewise, E1A has shown to be an important modulator of RuvBL1's function. Through the series of experiment, I have performed, we have identified multiple roles of RuvBL1 in HAdV growth and replication. I have shown that E1A and RuvBL1 both interact with one another in a direct manner. We also have established that RuvBL1 is important for the growth of the virus, as the depletion of RuvBL1 resulted in a two-fold decrease of viral titres. The knockdown of RuvBL1 affected the phenotype of the infected cells because the wild type virus produced reduced cytopathic effect compared to control cells. However, the effect of RuvBL1 on viral gene expression was minimal as we saw that knockdown of RuvBL1 only generated a slight down regulation of the viral early genes, which might have been a side-effect of reduced viral growth. To establish the mechanistic reason of RuvBL1's interaction with E1A, the interaction of E1A and interferon stimulated genes were examined. Through real time analysis, I was able to establish that RuvBL1 is used by E1A to effectively evade interferon response. Knockdown of RuvBL1 diminished E1A's ability to properly inhibit interferon stimulated genes. Likewise, mutant HAdV viruses with an inability to bind to RuvBL1 could also not inhibit interferon stimulated genes in comparison to the wild type virus. Interestingly, when the promoter occupancy of ISG56 was observed, we see that both RuvBL1 and E1A are both recruited to the promoter. However, in the absence of infection or interferon treatment, RuvBL1 does not appear to be found at the ISG56 promoter. Therefore, we can infer from the results that E1A employs RuvBL1 to evade interferon response to enable the virus to efficiently replicate in the host cell. Future direction of this work would be to look at how E1A is able to

recruit RuvBL1 to inhibit interferon genes, since RuvBL1 has generally been shown to bind to STAT2 and initiate the activation of interferon genes. It would seem that E1A is altering the function of RuvBL1 to act as an inhibitor of interferon rather than an activator. Nevertheless, we have been able to deduce that RuvBL1 is an important player in the growth and replication of human adenovirus.

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