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Human Adenovirus E1A binds and retasks cellular hBre1, blocking interferon signalling and activating virus early gene transcription

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Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Human Adenovirus E1A binds and retasks cellular hBre1, blocking interferon signalling and activating virus early gene transcription

(Thesis format: Integrated Article)

by

Gregory Joseph Fonseca

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Upon infection, human adenovirus (HAdV) must block interferon signaling and activate the expression of its early genes to reprogram the cellular environment to support virus replication. During the initial phase of infection, these processes are orchestrated by the first HAdV gene expressed during infection, early region 1A (E1A). E1A binds and appropriates components of the cellular transcriptional machinery to modulate cellular gene transcription and activate viral early genes transcription. We have identified hBre1/RNF20 as a novel target of E1A. hBre1 is an E3 ubiquitin ligase which acts with the Ube2b E2 conjugase and accessory factors RNF40 and WAC1 to monoubiquitinate H2B at K120 (H2B-ub), a mark of chromatin which is highly transcriptionally active. hBre1 and the activity of the hBre1 complex to monoubiquitinate H2B, was found to be critical for interferon mediated induction of interferon stimulated genes (ISGs) and the establishment of an anti-viral state. During infection, E1A targets hBre1 at ISG gene bodies and blocks the catalytic component of the hBre1 complex, Ube2b from being recruited to ISGs. As a result, E1A can antagonize the innate antiviral response by blocking H2B monoubiquitination and, as such, ISG transcription. In contrast to blocking hBre1 activity at ISGs, E1A is able to recruit hBre1 to viral chromatin where hBre1 participates in the transactivation of HAdV early genes. As E1A blocks the catalytic activity of the hBre1 complex, E1A retasks hBre1, altering hBre1 function from an E3 conjugase to a scaffold which recruits the cellular transactivator, hPaf1. hPaf1 is recruited by hBre1 and E1A to HAdV early genes to induce activating histone post-translational modifications, H3K4 trimethylation and H3K79 trimethylation. The ability of E1A to target hBre1 to simultaneously repress cellular IFN dependent transcription while activating viral transcription represents an elegant example of the incredible economy of action accomplished by a viral regulatory protein through a single protein interaction.
Keywords

Human adenovirus, Early region 1A, E1A, interferon, adenovirus early gene, hBre1, RNF40, Ube2b, ISG, H2B-ub, H2K4me3, H3K79me3, hPaf1, early region 3, early region 4, early region 2e, transcription, hPTM

Co-Authorship Statement

Chapter 2 of this thesis was published in Cell Host & Microbe 11(6):597-606, June, 2012. I was involved in performing all of the experiments.

Chapter 3 of this thesis was published in . I was involved in performing all of the experiments with help from Michael Cohen in Figure 2B.
Acknowledgements

Firstly, I would like to thank my boss, Dr. J. Mymryk, for taking a chance on me. The time in your lab has been incredible as a learning experience. You have imparted on me so much of your knowledge and time and I strive to emulate the scientist that you are. I would also like to thank all of the lab members who have fought the vast array of metaphorical monsters in the many literal realms of science that we have had the good fortune to defeat. We have seen each other through to the other end. Specifically, I would like to thank Dr. Peter Pelka for his fault in training me into what I am now. As well, I would like to thank Dr. Ahmed Yousef for being a mentor beyond what anyone could expect. You are a true friend. Dr. Hon Leong, you have delayed my inevitable visit to the mental ward by 4 years. Dr. Matt Miller, though we were of the same vintage, your example changed my path. Dr. Biljana Todorovic, we served many years together, grew together and will now depart together. We have grown together so much and spent so much time together and for that I am sorry for you. Dr. Jai Ablack, your help was instrumental and your hair always immaculate. Dr. Gobi Thillainadesan, no matter where we go from now, we will always have the shared pain of the great incubator collapse of ’11. One day Dr. Michael Cohen, it has been a delight being your friend and watching science grow in you as I am sure all the previously mentioned saw it in me. Gloria Thomson, thanks for your endless patience, militant efficiency and song-birdyness. Newbs…do well.

Thank you caffeine. We have known each other only a short time, but it’s been great.

I would also like to thank my friends for all of the times they listened to me rant, watched
me burn myself at both ends and eventually collapse in a seemingly neverending and never learning cycle. Thank you Sarah, for being the one who dealt with me working on weekends and taking care of me when I was too exhausted to do anything buy lie on the couch. Thank you for encouraging my video game addiction, being a nerd yourself and spending time doing nerd things with me. I’m happier when you are around and have access to far more appa. Unfortunately, I don’t have the space to list you all, but we have shared life, happiness and sushi. So long, and thanks for all the fish.

Lastly, I would like to thank my family. You mean a lot to me. Your support and all around being-thereiness coupled with my drive to make you all proud has pushed me further then I could possibly go. I have succeeded beyond expectations and have covered up some of the most amazingly bone headed mistakes. Mom and Dad, you have always been there for me with your love, kindness, and support. Big brother Mikey, you’ve been a great big brother and I have always looked up to you. Having a niece for me has been a pleasant bonus. Jessica. You married Jay and to date, have had an amazing nephew and niece for me to dote over. You’ve been a big part of bringing the family together for events, you have been a wonderful host and I am so proud of you. You are a model mother, a caring wife and a loving sister. And I really like Jay. Brad…you are the youngest. Thank you all for the support.

“I was a victim of a series of accidents, as are we all.” Kurt Vonnegut
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## List of Abbreviations

### General Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>COMPASS</td>
<td>Complex of proteins associated with Set1</td>
</tr>
<tr>
<td>CR</td>
<td>Conserved region</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOT1 complex</td>
<td>Disruptor of telomeric silencing complex</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>FACT</td>
<td>Facilitates chromatin transcription complex</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>HAdV</td>
<td>Human adenovirus</td>
</tr>
<tr>
<td>hPIV2</td>
<td>Human parainfluenza viruses</td>
</tr>
<tr>
<td>hPTM</td>
<td>Histone post-translational modification</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HSRV</td>
<td>Human respiratory syncytial virus</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon response element</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>LIM</td>
<td>Linear interaction motif</td>
</tr>
<tr>
<td>me3</td>
<td>Trimethylation</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen-associated molecular profiles</td>
</tr>
<tr>
<td>PONDR</td>
<td>Predictor of Naturally Disordered Regions</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real time quantitative polymerase chain reaction</td>
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<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5 acetyltransferase</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SET1 complex</td>
<td>SET domain-containing complex</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SV5</td>
<td>Simian virus 5</td>
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<tr>
<td>UVK</td>
<td>Ultra-violet killed</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<tr>
<td>WT</td>
<td>Wildtype human adenovirus</td>
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<td>α-body</td>
<td>Antibody</td>
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<tr>
<td>ΔE1A</td>
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### Cell lines

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<td>HT 1080</td>
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<td>IMR-90</td>
<td>Non-transformed human Fetal Lung Fibroblast cell</td>
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<tr>
<td>MEF</td>
<td>Non-transformed mouse embryonic fibroblast cell</td>
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<td>U-2 OS</td>
<td>Human osteosarcoma cell</td>
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### Viral Protein Names

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<td>ADP</td>
<td>Adenovirus death protein</td>
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<td>E1A</td>
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</tr>
<tr>
<td>E1B</td>
<td>Early region 1B</td>
</tr>
<tr>
<td>E2e</td>
<td>Early region 2 early</td>
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<tr>
<td>E2l</td>
<td>Early region 2 late</td>
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<td>Early region 3</td>
</tr>
<tr>
<td>E4</td>
<td>Early region 4</td>
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<tr>
<td>RIDαβ</td>
<td>Receptor internalization and degradation αβ</td>
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<tr>
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<td>Terminal protein</td>
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<tr>
<td>UXP</td>
<td>U-exon protein</td>
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<td>VARNA</td>
<td>Viral associated RNA</td>
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### Cellular Protein Names

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<td>B-cell CLL/lymphoma 2</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>Blm</td>
<td>Bloom syndrome, RecQ helicase-like</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLL</td>
<td>Myeloid/lymphoid or mixed-lineage leukemia</td>
</tr>
<tr>
<td>MUPP1</td>
<td>Multi-PDZ domain protein 1</td>
</tr>
<tr>
<td>Mx1</td>
<td>Myxovirus (influenza virus) resistance 1</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells</td>
</tr>
<tr>
<td>Nup107</td>
<td>Nucleoporin 107kDa</td>
</tr>
<tr>
<td>OAS</td>
<td>2′-5′-oligoadenylate synthetase</td>
</tr>
<tr>
<td>p300</td>
<td>Protein 300</td>
</tr>
<tr>
<td>p400</td>
<td>Protein 400</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>p57kip2</td>
<td>Protein 57 kip2</td>
</tr>
<tr>
<td>pCAF</td>
<td>P300/CBP-associated factor</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma-associated protein</td>
</tr>
<tr>
<td>RNA pol ii</td>
<td>RNA polymerase ii</td>
</tr>
<tr>
<td>RNF40</td>
<td>Ring finger domain protein 40</td>
</tr>
<tr>
<td>SetD1B</td>
<td>SET domain containing 1B</td>
</tr>
<tr>
<td>Slc22a18</td>
<td>Solute carrier family 22, member 18</td>
</tr>
<tr>
<td>SMARCE1</td>
<td>SWI/SNF related, matrix associated, actin dep. regulator of chromatin, subfamily e, member 1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Sug1</td>
<td>26S protease regulatory subunit 1</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAAP</td>
<td>Transformation/transcription domain-associated protein</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRIM24</td>
<td>Tripartite motif containing 24</td>
</tr>
<tr>
<td>TYK1</td>
<td>Tyrosine kinase 2</td>
</tr>
<tr>
<td>Ube2b</td>
<td>Ubiquitin conjugating enzyme E2b</td>
</tr>
<tr>
<td>Ubp8</td>
<td>Ubiquitin specific protease 8</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>USP22</td>
<td>Ubiquitin specific peptidase 22</td>
</tr>
<tr>
<td>WAC1</td>
<td>WW domain-containing adapter protein with coiled-coil</td>
</tr>
<tr>
<td>ZO-2</td>
<td>Zonula occludens protein 2</td>
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</tbody>
</table>
Prelude

“In the beginning there was nothing, which exploded.” Terry Pratchet.

This thesis is built on independent experiments that produced negative or confusing results which, for a long time, amounted to nothing understandable. After a time (three years), all of these confusing results came together to form a coherent hypothesis and this lead to an explosive expansion of positive results and information. All of the experiments that didn’t work or didn’t provide publishable information and are not displayed anywhere in this thesis were likely just as important as the data contained within. In the end, displayed herein are a compendium of positive results and an accurate depiction of only what was discovered and not necessarily everything that was learned. What I have learned is largely represented by all of the mistakes I have made. These have been ignored and are now only hearsay tales of the past on which my future is built.
Chapter 1: Introduction

1.1 General Introduction

Viruses, being obligate intracellular pathogens, are incapable of independent replication. Instead, they must parasitize a host and subvert the host's cellular machinery to propagate. As a result, viruses have coevolved with higher organisms and typically make a complex network of interactions within the host cell to reorganize the cellular milieu into an environment favourable for the production of viral progeny. Historically, studies of virus-host cell interactions have proven invaluable in helping scientists elucidate the mechanisms regulating complex cellular processes such as replication, transcription, and immune responses.

With respect to using viruses to learn about the cell, the study of Adenovirus (AdV) is no exception. AdV has been used to study such processes as cell cycle control, DNA replication, transcription, mRNA processing, apoptosis and immunological responses (Shenk, 1996). Originally identified in 1953 (Rowe et al., 1953), AdV are small non-enveloped viruses with a linear double stranded DNA genome of approximately 35 kilobase pairs (Figure 1.1). The AdV family includes more than one hundred members infecting a range of species from mammalian to avian to reptilian. In 1954, human AdV (HAdV) was implicated as the causative agent for acute respiratory disorder (Hilleman and Werner, 1954). Currently, there are 52 members of HAdV which are separated into 6 species, and a 7th proposed species, based on various criteria (Figure 1.2) (Benkő et al., 2000; Jones et al., 2007). Members of the HAdV family have evolved specific niches with wide organ tropism (including the respiratory system, intestinal
Figure 1.1. A cartoon representation of the HAdV-5 double stranded DNA genome. The HAdV-5 genome has ten transcriptional units, five of which are expressed early during infection: E1A, E1B, E2e, E3, and E4, two are expressed delayed early: IX and VARNA, and three late during infection; Late genes, E2L, and UXP.
digestive system, and blood) causing an array of clinical diseases in humans, although many infections are asymptomatic. In 1962, HAdV type 12 was found to cause tumours in baby hamsters (Trentin et al., 1962). This was the first example of a human virus inducing cancers. Later, it was shown that HAdV 12 could induce tumours when injected into the retina of baboons (Mukai et al., 1980). Although not all subgroups of HAdV are capable of causing tumours in rodents, all have been shown to transform rodent cells in tissue culture (Shenk, 1996). Differences in tumorigenicity are attributed to differences in the ability of the various HAdV types to evade host immune responses, as types unable to induce tumours in immunocompetent rodents are capable of inducing tumours in immuno-compromised rodents (Gallimore, 1972).

1.2 Adenovirus

1.2.1 Physical properties of adenoviruses.

Structurally, adenoviruses are comprised of a non-enveloped icosahedral protein shell that ranges in size between 60-90 nm depending on the species. The icosohedral shell consists of 240 hexons and 12 pentons which make up the 252 subunits called capsomers (Ginsberg et al., 1966). As the names indicate, the pentons and hexons are surrounded by 5 or 6 other capsomers respectively. The pentons form a base for a protruding fibre of a length which is HAdV type dependent (Norrby, 1966; Norrby and Skaaret, 1967). The fibre knob in HAdV5 initially interacts with a host receptor, the coxsakie virus and adenovirus receptor (CAR) in HAdV5. A subsequent interaction between the arginine-glycine-aspartic acid (RGD) motif in the penton base and cellular integrins (namely αv-β3 and αv-β5), allows HAdV to be taken up via phagocytosis
Figure 1.2. Classification schemes for HAdV. HAdVs were originally separated into 6 species based on several criteria including hemagglutination of erythrocytes and oncogenic potential both in vivo and in vitro. Since then, genome sequencing of HAdVs has confirmed species classification and added a purported 7th species. Figure adapted from Fundamental Virology, 3rd edition.
<table>
<thead>
<tr>
<th>Group</th>
<th>Hemagglutination</th>
<th>Serotypes</th>
<th>Oncogenic Potential</th>
<th>% of G-C in DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(little or no agglutination)</td>
<td>12,18,31</td>
<td>High +</td>
<td>48-49</td>
</tr>
<tr>
<td>B</td>
<td>(complete agglutination of monkey erythrocytes)</td>
<td>3,7,11,14,16,21,34,35,50</td>
<td>Moderate +</td>
<td>50-52</td>
</tr>
<tr>
<td>C</td>
<td>(partial agglutination of rat erythrocytes)</td>
<td>1,2,5,6</td>
<td>Low or none +</td>
<td>57-59</td>
</tr>
<tr>
<td>D</td>
<td>(complete agglutination of rat erythrocytes)</td>
<td>8,9,10,13,15,17,19,20,22-30,32,33,36-39,42-49,51</td>
<td>Low or none +</td>
<td>57-61</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>4</td>
<td>Low or none +</td>
<td>57-59</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>40,41</td>
<td>Unknown +</td>
<td>50-51</td>
</tr>
<tr>
<td>G</td>
<td></td>
<td>52</td>
<td>Unknown N/A</td>
<td>55.1</td>
</tr>
</tbody>
</table>
(Meier and Greber, 2004). It should be noted, however, that not all adenoviruses contain the integrin targeting RGD motif and exchange of this region will change specificity to cellular receptors (Wickham et al., 1995; Cuzange et al., 1994; Albinsson and Kidd, 1999).

Held within the AdV capsid lies a linear double stranded DNA genome, ranging in size from 30-38 kbp, with a conserved organization throughout the HAdV family. Each end of the genome contains an inverted terminal repeat, which accounts for 103bp in HAdV5 (Steenbergh et al., 1977). The genome has arbitrarily been given directionality, with the left end of the genome containing a cis-acting packaging sequence located between 200-400bp that mediates interaction with the capsid proteins and encapsidation of the genome (Gräble and Hearing, 1992; Hearing and Shenk, 1983; Ostapchuk and Hearing, 2003). The 5’ end of each DNA strand of the genome is connected via a phosphodiester bond on the terminal viral nucleotide to a serine residue within the 55kDa adenovirus terminal protein (TP) (Rekosh et al., 1977). The TP functions as a primer during viral DNA replication, assists in viral packaging and has also been suggested to protect the virus from being recognized by DNA damage machinery as it covers the free ends of the linear viral genome (Weitzman and Ornelles, 2005; Stracker et al., 2002; Karen et al., 2009). Viral genes are encoded on both strands of the genome and can be separated into two classes: the early genes which are produced before the onset of viral genome replication and are involved in cellular restructuring for virus production, and the late genes which are produced after the onset of viral genome replication and include the structural proteins which make up the viral capsid (Figure 1.1) (Shenk, 1996).
1.2.2 Adenovirus transcripts.

As explained earlier, after viral attachment via the knob of the fibre protein, internalization occurs through binding of the RGD sequence found in the penton base to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins (Williams et al., 1975; Varga et al., 1991). This leads to receptor mediated endocytosis (Svensson, 1985). Acidification of the endosome causes release of pentons and the partially disrupted virus particle escapes to the cytosol through an, as yet, unknown mechanism (Mellman, 1992). Throughout the transportation process, viral uncoating is progressing, leaving, at this point, the genome associated with hexon (Greber et al., 1993; Meier and Greber, 2004). The viral genome is then transported to the nucleus by hexon using the cellular microtubule network. (Lonberg-Holm and Philipson, 1969; Philipson and Lonberg-Holm, 1969; Chardonnet and Dales, 1972; Dales and Chardonnet, 1973). Once the genome reaches the nucleus, the first gene transcribed is the early region 1A gene (E1A). Transcription of the other early genes follows closely after E1A and includes E1B, E2 early (E2e), E3, and E4. Though some of the virus structural proteins, including terminal protein, are encoded in the E2 late (E2L) gene and UXP, the majority of structural proteins are encoded by the Major Late gene. Many of the HAdV gene transcripts undergo extensive splicing, which substantially increases the number of possible protein products produced upon translation (Berget et al., 1977; Shenk, 1996). In fact, the process of splicing was initially described using the HAdV Major Late gene transcript and led to a Nobel prize for Drs. Richard J. Roberts and Phillip A. Sharp in 1993.

The E1 region, which includes E1A (discussed at length later) and E1B, is
necessary for oncogenic transformation. The E1B region encodes two proteins, both of which are anti-apoptotic, and are named for their observed molecular weights, 55kDa and 19kDa. E1B-55k acts early in infection to inhibit the guardian of the genome, the p53 tumour suppressor (Yew and Berk, 1992). Specifically, E1B-55k functions as a viral sumo ligase to modify p53, and force p53 nuclear export via an interaction with cellular PML (Muller and Dobner, 2008; Pennella et al., 2010). As well E1B-55k works in conjunction with the HAdV E4-ORF3 to sumoylate the Mre11-Rad50-Nbs1 complex involved in DNA damage response (Stracker et al., 2002; Sohn and Hearing, 2012). E1B-55k also co-operates with E4-ORF6 during infection to recruit the Nxf1/Tap export receptor for the transport of late viral RNAs from the nucleus to the cytoplasm, where the cellular translation machinery is hijacked into making late viral protein products (Gonzalez and Flint, 2002; Flint and Gonzalez, 2003; Yatherajam et al., 2011). Similarly to E1B-55k, E1B-19k works as an anti-apoptotic agent during infection. The E1B-19k protein is a viral mimic of Bcl2 which binds to BAX and blocks cytochrome c release from the mitochondria (Rao et al., 1992; Chiou et al., 1994; Farrow et al., 1995).

The E2 gene codes for three proteins that drive the replication of the viral genome. E2 encoded genes include the terminal protein (discussed earlier), the 140 kDa DNA-dependent DNA polymerase and the 59 kDa single stranded DNA binding protein. These three proteins work together to support the replication of the viral DNA. Replication occurs 5' to 3' with the terminal protein acting as a protein primer covalently attached to deoxycytosine monophosphate (Kelly and Lechner, 1979). The DNA binding protein binds single stranded viral DNA intermediates and then acts in conjunction with the viral DNA-dependent DNA polymerase to replicate the viral genome (Van Breukelen
et al., 2003; van der Vliet and Levine, 1973; Van Der Vliet et al., 1975; Levine et al., 1976; Challberg and Kelly, 1979a, 1979b; Challberg et al., 1980; Challberg and Kelly, 1981).

The E3 region contains one differentially spliced transcript which codes for 6 known protein products. These products are all seemingly involved in promoting cell survival, for the most part by antagonizing adaptive immune activity. These include Receptor internalization and degradation αβ (RIDαβ), gp19K, 14.7K, 12.5K, and 6.7K. While the adenovirus death protein (ADP) is involved in cell lysis. RIDαβ dimers are O-linked oligosaccharide modified intracellular plasma membrane linked proteins. From the plasma membrane and in conjunction with other E3 gene products, RIDαβ is involved in blocking extrinsic death ligand signals triggered by TNF (tumour necrosis factor) (Benedict and Ware, 2001; Friedman and Horwitz, 2002), Fas (TNF superfamily, member 6) ligand (Shisler et al., 1997; Elsing and Burgert, 1998; Tollefson et al., 1998), and TRAIL (TNF-related apoptosis-inducing ligand) (Tollefson et al., 2001). This is achieved through destruction of proapoptotic receptors, and blocking of TNF-mediated arachidonic acid release and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation (Friedman and Horwitz, 2002). gp19K is a transmembrane protein localized to the endoplasmic reticulum (Hermiston et al., 1993a, 1993b). It acts to obstruct CTL-mediated killing via blocking tapasin-mediated complex formation of MHC-class I molecules and this blocks the subsequent expression of MHC class I/antigen complexes on the cell surface (Bennett et al., 1999). 14.7K is the only known soluble E3 protein and is involved in binding and inhibiting the apoptotic activity of TNF, NF-κB and Caspase 8 (Klingseisen et al., 2012; Krajcsi et al., 1996). E3 6.7K is also a
transmembrane protein which acts in conjunction with RIDαβ in the downregulation of TRAIL and TNF signaling via blocking arachidonic acid release (Benedict and Ware, 2001; Moise et al., 2002). ADP is a transmembrane protein which, contrary to the rest of the E3 genes, is proapoptotic and also is predominantly expressed late during infection (Tollefson et al., 1992). ADP functions through an as yet unknown mechanism with MAD2B (Mitotic spindle assembly checkpoint protein) in viral lysis of infected cells (Ying and Wold, 2003). Despite much study of the E3 proteins, the 12.5K protein has no known function and is not required for virus replication. Interestingly, E3 12.5K is highly conserved throughout HAdV types suggesting that it serves and evolutionarily important function (Hawkins and Wold, 1992).

The E4 mRNA is differentially spliced to encode 7 potential protein products, 5 of which have known functions. Of the possible products, E4 open reading frame (orf) 2 has no known activity and E4orf3/4 has not been detected during infection. Mutant virus in which these orfs are deleted do not display even weak growth defects. E4orf1 is only detected in HAdV subgroup D viruses 9 and 26 (Javier, 1994; Weiss et al., 1997). Despite the low conservation of expression, E4orf1 shows a very pronounced phenotype. E4orf1 is the critical oncogenic determinant in the ability of subgroup D viruses to elicit mammary tumours in rodents (Thomas et al., 2001; Chung et al., 2007). This occurs via an interaction with cellular PDZ proteins MUPP1, MAGI-1, ZO-2, and Dlg1 through an unknown mechanism (Chung et al., 2007). E4orf3 is highly conserved both immunologically and by sequence comparison. The E4orf3 protein forms a unique nuclear polymer, which partitions off the nucleus into various compartments (Ou et al., 2012; Sarnow et al., 1982). This viral polymer is then able to inactivate multiple tumour
suppressors including p53, PML, TRIM24, interferon response and, in conjunction with E1B-55k as noted above, the MRN DNA damage complex (MRE11/RAD50/NBS1) (Ullman et al., 2007; Ou et al., 2012; Stracker et al., 2002; Sohn and Hearing, 2012). The E4orf4 protein binds and retargets the global cellular phosphatase PP2A. E4orf4 retargeting of PP2A induces p53 dependent cell death which may be important in virus release (Brestovitsky et al., 2012; Branton and Roopchand, 2001). As well, PP2A is redirected by E4orf4 to regulate alternative splicing of late genes through altering the phosphorylation of the cellular alternate splicing factor SR proteins (Kanopka et al., 1998; Estmer Nilsson et al., 2001). As previously mentioned, E4orf6 forms a multifunctional complex with E1B-55k. This drives viral DNA replication, RNA processing, and nucleo-cytoplasmic transport of late viral mRNAs. This complex also blocks host protein synthesis via prevention of cellular mRNA transport from the nucleus (Halbert et al., 1985; Huang and Hearing, 1989; Yatherajam et al., 2011; Bridge and Ketner, 1990). As well, both E4orf3 and orf6 are responsible for preventing concatamerization of the HAdV genome by DNA ligase IV (Weiden and Ginsberg, 1994; Baker et al., 2007). Lastly, the E4orf6/7 protein is involved in activation of cell cycle programming and HAdV E2 transcription via binding and redirection of dimers of the cellular E2F transcription factor, which is freed up by dissociation from pRb by E1A (Huang and Hearing, 1989; Cress and Nevins, 1994; Hardy et al., 1989; Reichel et al., 1989; Cress and Nevins, 1996; Helin and Harlow, 1994).

The Major Late transcript is differentially spliced and, along with U-exon protein (UXP) and the E2l genes, codes for the structural and non-structural proteins which are
Figure 1.3. Diagrammatic representation of E1A splice products. (A) The E1A transcript is differentially spliced into 5 products. Blocks represent the exons while lines represent introns. All splice events maintain the translational reading frame except in the 9S encoded product, which changes reading frame due to splicing and this is denoted by crosshatching. (B) Cartoon of the E1A protein products aligned to sequence expression as a result of the splicing events. Conserved regions (CR) 1-4 are labeled and denoted by orange, pink, blue, and yellow boxes, respectively. The novel sequence in the 55R protein produced by the 9S mRNA is denoted by crosshatching.
involved in virion assembly and packaging (Tollefson et al., 2007; Shenk, 1996). The virus is assembled in the nucleus and the cell is eventually lysed via several mechanisms including ADP production, releasing approximately 10,000 infectious virions per cell.

1.3 HAdV early region 1A (E1A)

1.2.3 Human adenovirus early region 1A (E1A).

The first gene expressed during infection is E1A. E1A is found at the extreme left end of the genome (Figure 1.1) (Nevins et al., 1979). The E1A genes are responsible for establishing a favourable environment for the replication of virus. During infection, E1A activities include modulating host cell transcription, such as in inducing quiescent epithelial cells to enter S-phase, and activating the transcription of the other viral early genes. The E1A transcript is differentially spliced into 5 variant products (Figure 1.3). The two largest mRNA products, 13S and 12S, are the major early splice products. They encode 289 and 243 residue E1A proteins in HAdV5, and these differ only by a 46 amino acid sequence that is unique to the larger protein (Figure 1.3). These early proteins display a predominantly nuclear or nucleocytoplasmic localization (Rowe et al., 1983; Turnell et al., 2000). Later during infection, a shift in splice site preference increases production of the 11S, 10S and 9S E1A mRNA species, which encode proteins of 217, 171, and 55 residues respectively in HAdV5 (Stephens and Harlow, 1987; Ulfendahl et al., 1987). All splicing events in E1A preserve the reading frame except in the 9S product, which contains a unique 29 amino acid sequence at its C-terminus (Figure 1.3). Comparisons of the sequences of the largest E1A proteins from different AdV types identified four regions of high conservation, termed Conserved Region (CR) 1 through 4.
Figure 1.4. Map of E1A conserved regions (CR) and the location of selected linear interaction motifs (LIM). Selected short linear protein interaction motifs that have been identified within E1A are shown. The E1A sequence and the sequences of other viral and cellular proteins that also use this LIM for interaction with the target protein are also indicated. Sequence shading indicates consensus with the indicated LIM. Figure adapted from Pelka et. al., 2008.
**pRB Interaction**

HAdV-5 E1A    LDIIEEVL
HAdV-12 E1A   LYELY
Orf22        VAVLY
E2F1         LADHICII
E2F2/3       LDLRITQ
E2F4/5       CVDLF

**BS69 Interaction**

HAdV-5 E1A    MPLNLVP
HAdV-12 E1A   MPOILHP
EBV EBNA2 site 1 MLPSELSP
EBV EBNA2 site 2 MPAFLBP
hMGA site 1    MPRKLTP
hMGA site 2    MPR4LAP

**p300/CBP Interaction**

HAdV-5 E1A    FPEGSLML
HAdV-12 E1A   FPEGSLIL
p53          FSLFLKDL
E2F1         LGDLTPL
APC5         EEEAEHYL
APC7         FRAAIRL

**TR Interaction**

HAdV-5 E1A    LDQLIEEVL
NCoR site 1   LEDIIRKAL
NCoR site 2   LADHICII
NCoR site 3   LDLRITQ
SMRT site 1   LCAHIRKAL
SMRT site 2   LAQHISEV

**BS69 Interaction**

HAdV-5 E1A    MPLNLVP
HAdV-12 E1A   MPOILHP
EBV EBNA2 site 1 MLPSELSP
EBV EBNA2 site 2 MPAFLBP
hMGA site 1    MPRKLTP
hMGA site 2    MPR4LAP

**p300/CBP Interaction**

HAdV-5 E1A    FPEGSLML
HAdV-12 E1A   FPEGSLIL
p53          FSLFLKDL
E2F1         LGDLTPL
APC5         EEEAEHYL
APC7         FRAAIRL

**CtBP Interaction**

HAdV-5 E1A    PLDLS
HAdV-12 E1A   PVDSL
RIP140        PIDLS
Knirps        PMDSL
Snail         PQDSL
CtIP          PLDSL
xTcf-3 site 1 PLSLT
xTcf-3 site 2 PLSVL
hTcf-4 site 1 PLSSL
hTcf-4 site 2 PLSLV
Hairy         PLSLV
BKLF          PVDSL
ZEB           PLDSL
The conservation of these sequences suggests that their functions are critical for the viral life cycle.

1.3.1 E1A is an unstructured protein which contains numerous discrete protein-protein interaction motifs.

E1A is able to act without any enzymatic or specific DNA binding capacities. Instead, E1A functions through the interaction and sequestration of key cellular factors and redirects the activity of these proteins to advance the infectious cycle. The promiscuous nature of the E1A protein interactions leads to a multitude of different effects, which virtually reprogram the entire regulatory circuitry of the host cell. To date, there has been neither a successful crystal nor solution structure for the full E1A protein. This is thought to be a result of the lack of intrinsic structure in the E1A protein. Indeed, programs such as PONDR (Garner et al., 1999; Romero et al., 2001), which determine the likely nature of tertiary protein structure, predict that E1A is almost entirely unstructured (Figure 1.5) (Pelka et al., 2008). This is not wholly unexpected, as many cellular proteins that interact with large numbers of target proteins are also largely unstructured (Dunker et al., 2005; Kim et al., 2008). This lack of intrinsic structure allows this class of proteins to bind many unrelated proteins through the use of short linear interaction motifs (LIMs) (Figure 1.4). LIMs can adopt multiple alternative conformations, increasing their ability to bind dissimilar targets (Dunker et al., 2005). The E1A proteins appears to utilize this strategy to excess; being effectively comprised of a string of LIMs that interact with many different types of proteins, including kinases, transcriptional activators and repressors,
Figure 1.5. Alignment of selected HAdV E1A proteins and prediction of intrinsic disorder. The amino acid sequences of the largest E1A proteins of HAdV-3, 4, 5, 9, 12 and 40, which represent members of each of the six HAdV subgroups, are shaded with respect to their predicted preference to form intrinsically unstructured regions (black) or structured domains (unshaded). The conserved regions (CR1-4) are indicated and sequences are aligned based on amino acid similarity. Figure adapted from Pelka et. al. 2008.
and cell cycle regulators (Pelka et al., 2008). It is believed that each separate binding motif within this viral protein may exhibit local and specific structure when interacting with a specific target protein as is seen with the interaction between E1A and the TAZ domain of p300 or in the case of a segment of the C-terminus of HAdV 12 E1A and CtBP (C-terminal binding protein) (Ferreon et al., 2009; Molloy et al., 2000). This, coupled with the high expression level of E1A protein during infection, aids this viral protein in interacting with a plethora of cellular proteins. This strategy leads to the greatest possible effect on the cellular protein network from the smallest allocation of viral coding capacity. Further, this characteristic of the E1A protein makes it ideal for mutational analysis, as insertions and deletions typically only affect processes dependent upon the specific LIMs that are perturbed in the targeted area, rather than having more global effects on E1A function via alterations in folding (Bayley and Mymryk, 1994). Furthermore, portions of the E1A protein still maintain their individual activities when they are subdivided into segments or modules (Bayley and Mymryk, 1994). This allows a complex protein, which normally disrupts a substantial number of cellular pathways through a plethora of different interactions, to be segmented into smaller portions that can be more easily studied in isolation.

1.3.2 E1A is a modulator of cellular hub proteins and is itself a viral hub.

E1A is indispensable for productive HAdV replication at a physiologically relevant multiplicity of infection (MOI) (Jones and Shenk, 1979) as it is required for the earliest stages of viral infection. Not surprisingly, the primary function of E1A is to initiate the viral replication cycle. This is achieved in two ways: by activating the transcription of
Figure 1.6. Model of IFN signalling in response to virus infection. Cellular receptors recognize virus upon entry into the cell and respond via downstream phosphorylation of IRF3. Upon phosphorylation IRF3 dimerizes and activates the transcription of genes required for anti-viral effects, including IFNβ1. IFNβ1 is then secreted into the extracellular matrix where it can act in an either autocrine or paracrine mode to activate the IFNβ receptor. The IFNβ receptor activates the TYK2 and JAK1 kinases which act to phosphorylate STAT1 and STAT2. Upon phosphorylation, STAT1 and STAT2 form a heterodimer and complex with IRF9 (called the ISGF3 complex). ISGF3, along with other factors, then activates transcription of >300 cellular genes.
IFNβ1 promoter

IFNβ

TYK1
JAK1

STAT1
STAT2

IRF9

IRF3

ISRE
other viral early genes and by altering the host cell environment via transcriptional modulation of host cell gene expression to create an environment favouring viral replication (Bayley and Mymryk, 1994; Gallimore and Turnell, 2001; Flint and Shenk, 1989). Of important note, E1A is recruited to more than 17,000 host cell promoters during infection and modulates the transcription of many of these (Horwitz et al., 2008b; Ferrari et al., 2008). To accomplish this, the relatively small 289 residue E1A protein must make the most of its interactions with the host cell protein interaction network. The most economical means by which the virus can achieve this is to target proteins which themselves have many cellular interactions, and therefore, modulate many cellular processes. Such proteins are known as cellular hub proteins, as they make multiple connections with the cell protein interaction network and function as critical regulators of cellular processes and activities (Batada et al., 2006; Haynes et al., 2006). These include proteins such as the G1 checkpoint protein pRb, the general cellular transcriptional activator p300/CBP, and the cellular repressor CtBP; all of which are targets of the E1A protein (Pelka et al., 2008). For example, pRb functions as a key regulator of the G1-S phase transition, as well as a modulator of cellular replication, angiogenesis, transcription and chromatin structure (Talluri and Dick, 2012). By targeting cellular hub proteins such as pRb, the relatively small E1A protein may affect a maximum number of pathways with a minimum number of interactions. As a result of this property, studies of E1A have been an invaluable tool to study these important cellular regulatory hub proteins and the pathways they control.

1.3.3 Depending on context, E1A can function as an oncogene or a tumour
The strategy of targeting cellular hub proteins is not exclusive to viral proteins such as E1A. Rather, this same result is seen in cancers, in which specific cellular hub proteins are repeatedly found to be affected. In fact, viral hub oncoproteins such as E1A target the same proteins found affected in cancers with striking conservation. These viral oncoproteins have been shown to predict cellular proteins involved in cancer with a reliability as great or greater than other approaches (Rozenblatt-Rosen et al., 2012).

With respect to cancer, the typical HAdV infection ends with lysis of the host cell and the release of progeny virus. Cell death precludes the possibility of malignancies resulting from human infection. As such, HAdVs are not generally thought to be a cause of human cancer. However, E1A is capable of immortalizing cells when introduced by stable transfection (Graham et al., 1977; Whittaker et al., 1984). This is indeed the case for the commonly used Human Embryonic Kidney (HEK) 293 cell line, which was transformed by the stable transfection of the left end of the HAdV genome expressing both the E1A and E1B oncogenes (Whittaker et al., 1984). Despite this, transformation of human cells with the HAdV genome is difficult and unreliable. Further, HAdV is not seen as a causative agent in cancers like other DNA tumour viruses, including human papillomavirus and polyomavirus.

In contrast, infection of immunocompromised rodents with HAdVs can cause aggressive and malignant tumors (Trentin et al., 1962). As well, rodent cells are readily immortalized by E1A and fully transformed in cooperation with a second oncogene, such as E1B or activated Ras (Bayley and Mymryk, 1994; Gallimore and Turnell, 2001; Flint and Shenk, 1989). In part, this difference is due to the fact that HAdV infections are non-
productive in rodents. Infected cells are not killed during the virus replication cycle due to a deficiency in viral DNA replication and late viral protein production (Eggerding and Pierce, 1986). However, this does not explain the difficulty in transforming human cells with pieces of the HAdV genome (mainly E1A and E1B) and would seem to suggest that E1A is unable to modulate a cellular process in human cells which it either can affect, or is already primed for transformation in rodents. This small evolutionary variation may be the key difference between HAdV, a very common infectious agent, causing or not causing cancer in humans. This question may be the next exciting avenue of research in the HAdV field. Nevertheless, in the context of rodents, the oncogenic potential of the E1A protein is readily apparent.

Paradoxically to the oncogenic effects of E1A on non-transformed cells, expression of E1A in previously transformed cell lines suppresses oncogenic phenotypes. This is seen with reductions in metastasis, angiogenesis and tumorigenicity \textit{in vivo}, a triggering of apoptosis, and induction of a phenotypic mesenchymal to epithelial transition; the opposite phenotypic cellular change from what is seen in cancers (Deng et al., 2002; Mymryk, 1996; Frisch and Mymryk, 2002). Thus, it is clear that E1A has multiple functions and that these influence many vital cellular processes. By using E1A as a tool to study these processes, we may better understand the mechanisms by which they are normally regulated in a cell, as well as understand how viruses and cancers subvert them.

1.3.4 \textit{E1A as a tool for identifying cellular regulatory proteins.}

E1A has an impressive track record as a tool for discovering and studying novel host
regulatory proteins. The proteins initially identified via their interaction with E1A have been shown to be important in critical cellular processes such as cell cycle control and transcriptional regulation, further confirming the hub targeting strategy of E1A (Pelka et al., 2008). As a viral hub protein, E1A will reorganize cellular protein interaction networks and divert their activity for the benefit of viral reproduction. Overall, the E1A proteins have been found to interact with over 50 different cellular proteins, all of which likely influence vital cellular processes (Pelka et al., 2008). As one example, the interaction between E1A and pRb was the first example of an oncoprotein interacting with a tumour-suppressor (Whyte et al., 1988). In this case, E1A uses two distinct binding sites to displace the E2F family of proteins from pRb and block further association (Whyte et al., 1988; Fattaey et al., 1993). The release of E2Fs from pRb has two distinct benefits for HAdV. Firstly, E2F proteins are freed to allow transcriptional activation of viral genes. Indeed, E2Fs derive their name from their initial identification as regulators of HAdV E2e transcription (Reichel et al., 1987). Secondly, the release of E2F proteins allows E2F family members to activate E2F responsive cellular genes, including cyclin A and cdc2 (Pagano et al., 1992), which control S-phase entry (Ghosh and Harter, 2003). However, the interaction of E1A with pRb is by no means the only manner in which E1A proteins affect cell cycle, as E1A mutants that are unable to bind pRb are still able to induce S-phase entry and activate viral early gene expression (Bayley and Mymryk, 1994; Gallimore and Turnell, 2001; Flint and Shenk, 1989; Frisch and Mymryk, 2002). This indicates that other cellular targets of E1A can also influence this key pathway.

E1A also has direct effects on transcription through the binding of cellular
transcription factors, such as p300/CBP. Rather than relieving the repressive effects on a transcription factor, such as the case of pRb, E1A directly binds the p300/CBP acetyltransferases to activate transcription (Eckner et al., 1994). This interaction was originally shown in the N-terminus/CR1 region where it is absolutely required for E1A dependent transcriptional activation, as well as E1A induced cellular transformation (Wang et al., 1993). Our lab has recently published a novel direct interaction between p300/CBP and the CR3 portion of E1A, which along with the N-terminus/CR1 region, make up the two independent transcriptional activation domains within E1A (Pelka et al., 2009b). Interestingly, depletion of p300 in serum starved breast cancer cells causes these cells to divide (Kolli et al., 2001), whereas the interactions with pRb and p300/CBP are required by E1A to force baby rat kidney cells through the mitotic phase of the cell cycle (Jelsma et al., 1989; Howe and Bayley, 1992). This difference may be explained by the discovery that E1A reorganizes p300/CBP on the cellular chromatin, removing it from promoters of genes involved in differentiation and targeting it to promoters involved in cell cycle (Horwitz et al., 2008b; Ferrari et al., 2008). Differentiation and cell cycle programming are opposing processes within the cell; as such, removing an activator of differentiation and transferring it to induce cell cycle promoters may have similar effects. Despite these advances, the role played by p300/CBP in E1A mediated transcriptional activation is still largely unknown.

E1A also interacts with a well characterized transcriptional repressor, CtBP, through CR4 in the C-terminus of E1A (Chinnadurai, 2002). In fact, CtBP was first discovered as an E1A interacting protein (Boyd et al., 1993). The interaction between E1A and CtBP was shown to sequester CtBP from sequence specific DNA binding
factors, relieving transcriptional repression at specific cellular genes (Chinnadurai, 2002). This interaction is absolutely necessary to initiate the mesenchymal to epithelial transition induced by E1A and contributes to the ability of E1A to block tumourogenesis and metastasis (Boyd et al., 1993). More recently, our lab in conjunction with the lab of Dr. R. Grand has shown a second independent interaction with CtBP which maps within the CR3 region (Bruton et al., 2008). This interaction can direct the transcriptionally active CR3 region of E1A to CtBP occupied promoters, relieving CtBP mediated repression and then further activating these promoters (Bruton et al., 2008). To the best of our knowledge, this may have been the first evidence of a viral protein exploiting a transcriptional repressor as a means of targeting specific promoters for activation.

The interactions of E1A with mammalian cellular proteins and the consequences of these interactions have been widely exploited and were critical in defining the role of many regulatory proteins within the cell. As well, the E1A protein has been heavily used to study the requirements of transcriptional activation and transformation of cells. Ergo, discovering novel interactions with E1A may identify proteins important in vital cellular processes and provide mechanistic insight into their activities.

1.3.5 E1A activates viral transcription.

The study of how E1A activates viral transcription has greatly increased our knowledge of cellular transcription activation complexes. In the late 1970’s, it was shown that the largest product of the E1A gene, 289R in HAdV5, was able to activate transcription. This was shown to require the region later deemed CR3 (Harrison et al., 1977; Berk et al., 1979). E1A CR3 is necessary to activate transcription from the viral genome, although it
is not alone sufficient as CR3 activates at a much reduced potency (Culp et al., 1988; Bayley and Mymryk, 1994; Berk, 2005; Flint and Shenk, 1997; Gallimore and Turnell, 2001; Ablack et al., 2010). Interestingly, unlike most of the E1A protein, CR3 does not appear to be intrinsically disordered (Garner et al., 1999; Romero et al., 2001). Considering this, it is not surprising that CR3 does not tolerate deletion mutants or contain short linear interaction motifs like the rest of the E1A protein (Egan et al., 1988; Fahnestock and Lewis, 1989; Pelka et al., 2008) (Figure 1.4). Indeed, CR3 is predicted to have defined tertiary structure coordinated by a novel zinc finger (Culp et al., 1988; Pelka et al., 2008). This may suggest that the purpose of this region is specifically defined for a single role; to be a stable scaffold for the recruitment of factors necessary for transcriptional activation. Consistent with this hypothesis, the zinc finger domain of CR3 was shown to interact with TBP and a component of the mediator adaptor complex, MED23 (Geisberg et al., 1994; Boyer et al., 1999; Stevens et al., 2002; Webster and Ricciardi, 1991). With these interactions, CR3 is able to recruit transcriptional activation complexes which are essential for E1A dependent virus early gene transcription. Further, the adjacent auxillary region 1 (AR1) region is necessary for activation of virus early genes through an as yet undefined means (Ström et al., 1998). CR3 recruits many additional factors for modulation of transcription. These include coactivators of CR3 dependent transcription, SUG1 and p300 (Rasti et al., 2006; Pelka et al., 2009b). As well, CR3 recruits repressors of CR3 mediated transcription, including GCN5 and BS69, presumably to allow for tight controls on transcriptional activation to maximize virus replication (Ablack et al., 2010; Hateboer et al., 1995; Masselink and Bernards, 2000). Overall, the structural components and mechanistic insight into CR3 function are fairly
well defined. However, as mentioned above, CR3 is necessary but not sufficient for transcriptional activation of early viral genes. Indeed, effective recruitment of p300/CBP to viral promoters requires the N-terminal region of E1A (Pelka et al., 2009b). In fact the N-terminal region of E1A, though unable to directly activate viral gene expression alone, is essential for optimal viral early gene transactivation in conjunction with CR3 (Duyndam et al., 1996; Wong and Ziff, 1994). Despite interacting with a plethora of known transactivators (AP2, TRAAP, TR, p300/CBP, p400, pCAF, and TBP, to name a few) (Pelka et al., 2008; Frisch and Mymryk, 2002), the mechanism by which the N-terminus of E1A participates in E1A dependent transcriptional activation of HAdV early genes is still not fully understood.

1.3.6 **E1A modulates cellular transcription.**

In 2008, two papers published in *Science* helped bring to light the global breadth with which E1A affects host cell transcription (Horwitz et al., 2008b; Ferrari et al., 2008). It was found that E1A is bound to the regulatory region of more than 17,000 host cell genes. Further, E1A modulates the expression of the host cell transcriptome by activating and repressing the expression of many of these genes. This is done by recruitment of either transcriptional activating complexes such as p300 (Horwitz et al., 2008b; Ferrari et al., 2008) or via recruitment of transcriptional repressor such as CtBP and pRb to promoter proximal regions (Sundqvist et al., 2001; Boyd et al., 1993; Ferrari et al., 2008; Whyte et al., 1989). However, E1A modulation of expression from the host cell genome is far more complex. In some circumstances, rather than recruiting these proteins to sites of active transcription to repress genes, E1A may use genes such as CtBP
and pRb to be recruit itself to sites of transcriptional repression. Upon recruitment, E1A may then direct transcriptional activation complexes to these previously repressed genes to initiate transcription (Bruton et al., 2008; Ferrari et al., 2008, 2009). Similarly, E1A uses transcriptional activators, such as p300, to recruit itself to the sites of transcriptionally active genes and repress transcription through the recruitment of repressor complexes (Pelka et al., 2009b; Ferrari et al., 2008, 2009). This adds many levels of complexity to the modulation of host cell gene expression by E1A, which allows this one viral protein to have a variety of effects on host cell transcription. Interestingly, 3 classes of genes may be found during adenovirus infection as seen in Ferrari et al., 2008: Class I genes which are shut off early in infection and include genes involved in pathogen response and inflammation, Class 2 genes which are activated early in infection and are involved in cell growth, division and DNA synthesis, and Class 3 genes which are repressed at later times during infection and are involved in development and differentiation (Ferrari et al., 2008). These results globally confirm the current working model of E1A in reprogramming the infected cell. Specifically, E1A blocks innate immunity, induces cell cycle and blocks/reverses differentiation. The net effect is that E1A optimizes the host cell environment for virus replication. However, despite a large compendium of published data, the means by which E1A distinguishes each class of targeted gene is not well understood.

1.3.7 E1A modulates the host immune system.

Class 1 genes, including genes involved in innate immunity, are of special interest during the early stages of infection. Innate immunity is the first line of defense against virus
infection. This is primarily mediated by TLRs (Toll-like receptors). TLRs recognize pathogen-associated molecular profiles (PAMPS), such as lipopolysaccharide from Gram-negative bacteria in the case of TLR4 or non-methylated CpGs in foreign DNA as in the case of TLR9. Recognition of viral infection by TLRs results in activation of type I (α and β) interferon (IFN) signaling (Hertzog et al., 2003; Stetson and Medzhitov, 2006b). Upon detection of foreign viral material, such as non-methylated DNA by TLR9 (Hemmi et al., 2000; Krug et al., 2001; Latz et al., 2004), cells activate the IRF3 and NF-κB transcription factors (Stetson and Medzhitov, 2006a). This, in turn, results in the transcriptional activation of several antiviral genes including IFNs α and β (Stetson and Medzhitov, 2006b; Perry et al., 2005). Upon secretion, IFNs may act as autocrine or paracrine ligands on the heterodimeric interferon αβ receptor (IFNαβR) (Raziuddin et al., 1984; Schwabe et al., 1988). Activation of the IFNαβ then leads to phosphorylation and activation of JAK1 and TYK2 which in turn leads to phosphorylation of the STAT1/STAT2 heterodimer (Kumar et al., 1994; Beadling et al., 1994). Upon phosphorylation and dimerization of active STAT1/2 and subsequent recruitment of the transcription factor IRF9 (interferon regulatory factor 9) (altogether denoted the interferon stimulated gene factor 3 [ISGF3]) (Levy et al., 1989; Fu et al., 1990). ISGF3 then binds at interferon stimulated response elements (ISRE) and activates transcription of hundreds of cellular interferon stimulated genes (ISGs) (Figure 1.6) (Levy et al., 1989). The combined expression of these ISGs results in the generation of an altered cell state that resists infection. As one example, PKR (protein kinase R) recognizes double stranded RNAs commonly present during viral infection and phosphorylates eIF-2alpha to inhibit new protein synthesis (Galabru et al., 1989). To successfully replicate, viruses
must evolve mechanisms to evade the innate immune system and the interferon response or face extinction by natural selection. Some viruses, such as VSV (vesicular stomatitis virus) and SARS (severe acute respiratory syndrome) replicate at a fast rate which overloads the endoplasmic reticulum and blocks IFN secretion, thereby evading this response by brute force. Most viruses have, however, required the evolution of specific mechanisms to block the IFN response. Some viruses have evolved receptor mimics to outcompete IFN ligand, such as vaccinia virus. Marburg virus, polyomavirus and human cytomegalovirus (HCMV) have evolved to block JAK1 activation, while EBV (Epstein-Barr virus), JEV (Japanese encephalitis virus), HPV (human papillomavirus), myxoma virus, hepatitis D and possibly dengue have evolved to block TYK2 activation. Further, a lengthy and growing list of viruses affecting STAT1/STAT2 activation and dimerization includes HRSV (human respiratory syncytial virus), dengue, SV5 (simian virus 5), measles, mumps, hPIV2 (human parainfluenza viruses), sendai, hepatitis C, nipah virus, west nile virus, vaccinia virus, rabies, HPV, reovirus and, importantly for this work, HAdV (Taylor and Mossman, 2012; Galligan et al., 2006; Perry et al., 2005; Sadler and Williams, 2008).

HAdV particles were initially discovered to induce IFN in chick cells and in vivo by two distinct groups in the late 1960’s (Ho and Köhler, 1967; Béládi and Pusztai, 1967; Pusztai et al., 1969). It was initially believed that differential induction of the IFN response by different HAdVs was a result of differences in the viral capsid (Tóth et al., 1983). Later, HAdV was found to specifically reduce MHC class-I antigens on the cell surface through HAdV E3-gp19K, resulting in a reduced sensitivity to IFN in vivo (Grand et al., 1987; Lichtenstein et al., 2004). Three years later, HAdV was shown to have
specific mechanisms for blocking the functions of specific ISGs. The viral VA RNA I blocks PKR activity through direct interference (Clarke et al., 1994; Ghadge et al., 1994; Katze et al., 1987; Kitajewski et al., 1986; O’Malley et al., 1989). In 1988, the lab of Dr. Darnell, through mutagenesis of the adenovirus genome, discovered that E1A specifically blocks IFN signaling in infected cells (Reich et al., 1988). This effect was later mapped to the N-terminus of E1A and shown to modulate transcriptional activation of IFN target genes (Kalvakolanu et al., 1991; Anderson and Fennie, 1987). The data was suggestive of changes in histone modifications being the means by which transcription of IFN target genes was affected. Interestingly, though most HAdVs modulate IFN sensitivity and transcriptional targets, some, such as HAdV 12, do not (Gallagher and Khoobyarian, 1972, 1969; Béládi et al., 1973; Tóth et al., 1983; Zhao et al., 2009). In the case of HAdV 12, the viral life cycle and replication progress much slower with lower viral titres at the end stage of infection (Zhao et al., 2009). This may result from antagonism by the increased IFN signaling due to the inability of this virus to block IFN target gene transcription.

1.4 Histone post-translational modifications (hPTMs) regulate gene expression.

Eukaryotic DNA is compacted into a highly order structure called chromatin. Chromatin is made up of structures known as nucleosomes. The nucleosome is the basic unit of chromatin, consisting of approximately 146 nucleotides of DNA wrapped around a positively charged protein core containing 2 copies each of histone 2A, 2B, 3 and 4 and separated by free linker DNA of up to 80 bp. Along with functioning in DNA compaction, the histones may be covalently modified with a set of moieties, called
histone post-translational modifications (hPTM), to signal the compaction of specific regions of DNA into euchromatin or heterochromatin. While heterochromatin is highly correlated with transcriptional silencing, euchromatin regions may be transcriptionally active in the presence of transcriptional initiation complexes (Chi et al., 2010). Further, condensation of DNA into chromatin is not static and the cell uses a large and complex array of machinery to modify and remodel chromatin (Chi et al., 2010; Jenuwein and Allis, 2001).

hPTMs are chromatin modifications in which moieties are covalently attached to the histones. These include phosphorylation, acetylation, citrullination, monoubiquitinylation, sumoylation, and mono, di, and tri-methylation. These moieties then either sterically alter the compaction of the chromatin, usually by modifications on the histone bodies, or signal the cell to alter chromatin compaction, usually through modifications on the histone tails (Chi et al., 2010). hPTMs, altogether called the histone code, are a key mechanism by which cells interpret and adjust the transcriptional state of the underlying chromatin (Jenuwein and Allis, 2001). The combinatorial makeup of the modifications, rather than any one particular modifications assists in the physical and transcriptional end state of the affected gene. As such, although changes to a single modification can have drastic effects on the transcriptional state of a gene, this effect may differ depending on the remaining chromatin modifications. For example, though H3K4 trimethylation and H3K9 and K14 acetylation are typically considered marks of transcriptional activation, in the case of IFN responsive genes (described in detail below), H3K4 trimethylation and H3K9 and K14 acetylation are present at non-transcriptionally active genes and are, in fact, marks of preactivation (Escoubet-Lozach et al., 2011).
Further, H3K79 methylation is very contextual, having strong activation and repression effects based on the surrounding hPTMs (Frederiks et al., 2008). As well, hPTMs are also involved in post-transcriptional effects including splicing (Enroth et al., 2012; Kim et al., 2011) and in DNA replication (Petruk et al., 2012). As such, hPTMs add a layer of complexity and control to DNA transcription, translation, and replication.

1.5 Innate immunity and histone post-translational modifications (hPTM).

As mentioned above, in recent years, the impact of chromatin state and the necessity of hPTMs in modulating transcription has begun to unfold. hPTMs act to modulate the activity of the underlying chromatin by providing complex information for the cells transcriptional machinery beyond the DNA sequence (Chi et al., 2010; Jenuwein and Allis, 2001). To further this complexity, it has been shown that hPTMs work in concert, rather than on their own (as described above). The overall composition of the hPTMs at a particular gene work together to specify the transcriptional state of the gene. With regards to innate immunity, IFN target genes have interesting and complex hPTM patterns. During non-stimulated conditions the promoters of many IFN target genes display the appearance of being fully operational based on hPTM status (Escoubet-Lozach et al., 2011). These genes are marked by hPTMs traditionally associated with active transcription in the form of H3K4me3. As well, RNA polymerase II is loaded on the transcriptional start site (Escoubet-Lozach et al., 2011). Interestingly, only barely detectable levels of transcription occur under these conditions despite the presence of chromatin marks typically indicative of active transcription. Furthermore, transcripts show defects in elongation, displaying premature termination and high levels of
transcriptional pausing (Escoubet-Lozach et al., 2011). Upon stimulation, hPTMs associated with elongation, such as histone 2B monoubiquitination (H2B-ub) or histone 4 acetylation, are introduced to the chromatin (Escoubet-Lozach et al., 2011; Fonseca et al., 2012). Thus, the primed hPTM status may allows for faster exchange to a fully active transcriptional state. As a result, expression of IFN target gene products and activation of innate immunity is able to occur in mere hours after stimulation by IFN through the ISGF3 complex (Waddell et al., 2010; Taylor and Mossman, 2012; Fonseca et al., 2012).

1.6 hBre1 and monoubiquitination of H2B.

hBre1 is an E3 ubiquitin ligase which acts in conjunction with the highly similar RNF40 protein, the Ube2b conjugase (E2), and an accessory factor of unknown function, WAC1, to monoubiquitinylate histone 2B (H2B) at lysine 120 in mammalian cells. This is done in the presence of the hPaf1c transcriptional regulatory complex (Hwang et al., 2003; Xiao et al., 2005; Wood et al., 2003; Zhang and Yu, 2011; Kim and Roeder, 2009; Krogan et al., 2002). hBre1 and the associated H2B-ub activity are not global hPTMs, but are only associated with small subsets of genes. H2B-ub has been found to be necessary for two important processes: transcriptional pre-initiation and efficient transcriptional elongation. Firstly, during formation of the transcriptional pre-initiation complex, H2B-ub, mediated by the hBre1 complex, acts as a signal for subsequent monoubiquitinylation and thereby activation of an integral structural component of the COMPASS complex, Cps35 (Lee et al., 2007). COMPASS, using the SET1 complex, then acts to trimethylate H3K4 (Wood et al., 2003; Lee et al., 2007). As well, H2B-ub acts as a signal to the DOT1 complex through as yet unknown means to trimethylate
Figure 1.7. hBre1 facilitates transcriptional initiation. PAF1 coordinates targeting of the Bre1 complex to target genes. The hBre1/RNF40/Ube2b complex subsequently monoubiquinates H2B at K120 (mammals). After this, the Cps37 component of the MLL complex, which is necessary for catalytic activity, is targeted by hBre1 for monoubiquitination. The MLL complex may then associate with the SET1 methyltransferase complex, which targets H3K4 for trimethylation. H2B monoubiquitylation also acts as a signal for the trimethylation of H3K79 by the DOT1 complex. These two methylation events are signals for transcriptional initiation by RNA Polymerase II.
H3K79 (Wood et al., 2003; Shahbazian et al., 2005; Mohan et al., 2010). Both of these chromatin modifications are markers for transcriptional activation. The combination of H2B-ub, and H3K4 and K79 trimethylation along with hPaf1c recruitment by hBre1 appear to cooperate to recruit the RNA polymerase II complex to initiate transcription (Figure 1.7) (Lee et al., 2007; Xiao et al., 2005). Secondly, hBre1 is necessary for efficient transcriptional elongation (Minsky et al., 2008; Xiao et al., 2005). In this process, the ubiquitin moiety of H2B-ub acts as a signal for the FACT complex (Pavri et al., 2006). Prior to transcriptional elongation by RNA polymerase II, FACT removes H2A/H2B-ub dimers from the nucleosome, effectively converting the histone octamer to a hexamer (Pavri et al., 2006). This effectively alters the compaction of DNA in the nucleosome, allowing RNA polymerase II to more efficiently transcribe the affected chromatin. FACT then acts with the Ubp8 component of the SAGA complex, to remove the ubiquitin moiety from H2B and replaces H2B in the nucleosome following the passage of the transcriptional elongation complex (Figure 1.8) (Henry et al., 2003; Wyce et al., 2007). The combination of these two activities has the effect of making the H2B-ub moiety an hPTM associated with not just actively transcribed chromatin, but with rapid increases in transcriptional activation (Henry et al., 2003; Pavri et al., 2006; Wyce et al., 2007). This is evidenced in the fact that hBre1 mediated H2B-ub is associated with the transcribed regions of highly expressed genes (Minsky et al., 2008). To date, mammalian H2B-ub has been implicated in the transcriptional activation of small subsets of genes including HOX, Notch, estrogen receptor, DNA damage response, MLL rearrangement events and p53 responses (Moyal et al., 2011; Bray et al., 2005; Mohan et al., 2010; Nakamura et al., 2011; Prenzel et al., 2011; Shema et al., 2008; Zhu et al.,
2005; Wang et al., 2013). Interestingly, recent reports have suggested that O-linked N-acetylglucosamine (GlcNAc) catalyzed by two enzymes, O-GlcNAc transferase and O-GlcNAcase, onto serine 112 of H2B may facilitate H2B-ub at K120 by functioning as a docking site for hBre1 (Fujiki et al., 2011).

1.7 Thesis Overview

The work within this thesis identifies and characterizes an interaction between hBre1 and E1A. Further, we show the mechanisms by which the viral E1A protein uses this cellular protein both for transcriptional repression of cellular transcription and activation of viral transcription.

1.7.1 Chapter 2: Adenovirus evasion of interferon mediated innate immunity by direct antagonism of a cellular histone post translational modification

In this study, we found an increase of H2B-ub upon infection with a virus lacking E1A as a result of an IFN regulated innate immune response. This increase correlated with an increase in ISG transcription. We then showed the ability of E1A to block these increases. This lead to the initial evidence for the interaction between E1A and hBre1, a member of the complex involved in H2B-ub, as well as defining the interaction to residues 4-25 on E1A. hBre1 was shown to be necessary for full ISG transcription through H2B-ub. The ability of E1A to block ISG transcription is a direct result of binding hBre1 and dissociating it from other members of the hBre1 complex, which are necessary for monoubiquitinating H2B. Lastly, growth of an HAdV lacking residues 4-25 of E1A, which was previously shown to be susceptible to IFN treatment, was partially rescued by knockdown of hBre1 and associated complex members.
Figure 1.8. hBre1 mediated transcript elongation. Upstream of the elongating RNA polymerase II transcription complex, monoubiquitination of H2B acts as a signal for the Facilitates chromatin transcription (FACT) complex to remove H2A/H2B-ub dimers from the nucleosome. This disturbs the tightly packed nature of the coiled DNA surrounding the nucleosome and facilitates transcriptional elongation by RNA polymerase II, along with human Polymerase Accessory Factor 1 (hPaf1). The ubiquitin moiety is then removed from H2B by the SAGA associated deubiquitinase Usp22. FACT then restores nucleosome integrity by reintroducing H2A/H2B dimers downstream of elongating transcript. This process is thought to be necessary for efficient transcription of highly transcribed genes.
1.7.2 Chapter 3: Viral retasking of hBre1/RNF20 to recruit hPaf1 for transcriptional activation

Following the previous chapter, we examined the effect of hBre1 on E1A mediated transcriptional activation of HAdV early genes. We found that hBre1 is necessary for E1A mediated transcription in an artificial Gal4 fusion system. We then found that hBre1 was localized to viral early genes in an E1A dependent manner and is necessary for full expression of several viral early genes. This activation did not occur through H2B-ub, which is disrupted by E1A during HAdV infection. Instead, hBre1 acted as a scaffold to recruit hPaf1 to HAdV early genes.

These results illustrate both the intricacy and economy with which the E1A protein works to modulate the cellular environment through specific cellular interactions. As well, these studies show the continuing value of studying viral proteins to identify critical regulators of cellular processes and understand their mechanism of action.

1.8 References


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Chapter 2:
Adenovirus evasion of interferon mediated innate immunity by direct antagonism of a cellular histone post translational modification

2.1 Introduction

Eukaryotic DNA is compacted via association with protein to form a highly organized polymer called chromatin (Campos & Reinberg, 2009). The fundamental subunit of chromatin is the nucleosome, which is formed by wrapping DNA around an octamer of histone proteins. hPTMs actively alter chromatin structure in a dynamic and often reversible process (Campos & Reinberg, 2009; Li et al., 2008; Rice et al., 2003). These enzyme mediated covalent histone modifications affect the compaction of cellular chromatin and are vital for any process requiring access to the DNA template (Braunstein, Rose, & Holmes, 1993; Rice et al., 2003; Sullivan & Karpen, 2004). In particular, many of these modifications directly or indirectly influence the recruitment of transcriptional regulatory factors to chromatin, providing an additional mechanism to control gene expression Garske et al., 2010; Li et al., 2008; Wysocka, 2006). Mounting evidence suggests that global changes in histone modifications lead to global changes in gene expression during disease processes including cancer and infection (Chi, Allis, & Wang, 2010; Ferrari, Berk, & Kurdistani, 2009; Lilley et al., 2010).

Viruses are obligate intracellular pathogens. As such, they have evolved simple, yet elegant mechanisms to reprogram cellular systems for the benefit of the virus. Not
surprisingly, many viruses target and utilize hPTMs to modify cellular and viral transcription and optimize the cellular environment for virus replication. Human adenovirus (HAdV) is a small DNA tumour virus that is able to transform cells and cause malignancies in rodents (Pelka, Ablack, Fonseca, Yousef, & Mymryk, 2008). As an infectious human pathogen, HAdV only has access to the terminally differentiated cells of the exposed epithelial surfaces in the respiratory and gastrointestinal tracts. These quiescent cells do not provide an ideal environment for virus replication. To overcome this difficulty, the E1A proteins of HAdV have evolved to reprogram this suboptimal cellular environment into one more conducive to virus replication. E1A is the first viral gene expressed during infection and it plays critical roles in modulating transcription, forcing infected quiescent cells back into the cell cycle and suppressing cellular innate anti-viral responses. The interaction between the viral E1A protein and several enzymes comprising the cellular hPTM apparatus, particularly the p300/CBP acetyltransferases, is well established and E1A exploits these interactions to alter gene expression and cell growth (Pelka et al., 2008). Indeed, recent work has established that E1A globally reprograms histone 3 lysine 18 (H3-K18) acetylation in order to coerce quiescent cells to replicate in a process akin to that observed in some cancers (Horwitz et al., 2008).

hBre1/RNF20 is a member of a complex involved in the monoubiquitination of H2B at lysine 120 (H2B-ub) and we detected it as an interacting partner of E1A in a yeast two-hybrid screen. In this study, we have focused on characterizing the effect of E1A on global H2B-ub. In eukaryotes, H2B-ub marks chromatin that is highly transcriptionally active and is involved in efficient transcript elongation (Lee et al., 2007; Xiao et al., 2005). H2B-ub is a precursor to several other epigenetic marks of transcriptional
Figure 2.1. Virus infection and IFN treatment induces a global increase in H2B-ub. (A) Western blot analysis of H2B and H2B-ub, E1A and actin proteins extracted from HAdV infected A549 cells over a 48 hour time course. (B) UV inactivated HAdV and myxoma viruses and IFN treatment stimulate a global increase in H2B-ub. A549 cells were infected with the indicated viruses or treated with IFNβ1. Cell extracts were analyzed as in (A) and also blotted for GFP to demonstrate successful Myxoma infection. (C) E1A expression is sufficient to block an IFN induced global increase in H2B-ub. Parental A549 and A549 cells stably expressing E1A were treated with IFNβ1 over 48 hours. Cells extracts were analyzed as in (A).
A

**Time (h)**

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**IB:E1A**

**IB:Actin**

Longer exposure

B

**UI**

**ΔE1A**

**WT**

**WT**

**WT**

**WT**

**WT**

**WT**

**WT**

**WT**

**WT**

**IFNβ1**

**IB:H2B-ub**

**IB:H2B**

**IB:E1A**

**IB:GFP**

**IB:Actin**

C

**IFNβ1**

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**IB:H2B-ub**

**IB:H2B**

**IB:E1A**

**E1A A549**

**wt A549**
activation including tri-methylation of histone 3 at lysines 4 (H3-K4) and 79 (H3-K79) by the COMPASS and DOT1 complexes (Lee et al., 2007). H2B-ub is also required for the displacement of H2B from the nucleosome by the FACT complex (Xiao et al., 2005). This is thought to loosen the surrounding DNA and allow for efficient passage of the RNA polymerase II complex during transcript elongation. These effects on transcription make regulation of the H2B-ub hPTM a candidate target for a strong viral transcriptional regulator such as E1A.

2.2 Results

2.2.1 Cellular levels of H2B-ub greatly increase in response to viral infection.

To assess the effects of HAdV infection on global H2B-ub levels, we infected human A549 lung adenocarcinoma cells with either wild type HAdV type 5 (WT) expressing the full length E1A protein or a virus lacking E1A (ΔE1A) and collected cells over the following 48 hours (Figure 2.1A). HAdV ΔE1A is severely impaired in the ability to reprogram the gene expression profile of the infected cell (Ferrari et al., 2008) and would not be expected to actively affect global histone modifications. Unexpectedly, infection with HAdV ΔE1A stimulated a strong global increase in H2B-ub by 8 hrs post infection that was sustained over the course of our analysis. This suggests that cells responded to the presence of ΔE1A virus by globally increasing H2B-ub levels. However, this increase was largely abrogated during infection with the wild type virus, which expresses E1A (Figure 2.1A). This was also observed during infection of murine embryonic fibroblasts and the human diploid lung fibroblast line WI-38 (Figures 2.2A, 2D). These results suggested that HAdV utilizes E1A to actively block the cellular upregulation of
Figure 2.2. Investigation of IFN signalling during HAdV infection. Data significantly different than control within groups is indicated (*P<0.05). (A) H2B-ub is not upregulated in response to infection in cells that are not IFN responsive. Western blot analysis of H2B and H2B-ub in WI-38 and Vero cells treated with IFN or infected with HAdV. (B) IRF3 is activated in response to WT HAdV infection. A549 cells were infected with WT HAdV and samples were collected over the course of 36 hours. Western blots were then performed on protein extracts with antibodies for IRF3, active IRF3 phosphorylated on S396 (IRF3(S396)) or Actin. (C) IRF3 associates with the IFNβ1 promoter during WT HAdV infection. A549 cells were infected with WT HAdV or HAdV ΔE1A or treated with IFNβ1 for 8 hours. Chromatin immunoprecipitation (ChIP) was then performed. Chromatin was analyzed for the presence of IRF3. DNA was quantitated via qReal Time PCR using primers specific to the IFNβ1 promoter. (D) Induction of a global increase in H2B-ub by viral infection requires IRF3 and the type I IFN receptors. WT mouse embryonic fibroblasts (MEFs) or MEFs lacking IRF3(IRF3−/-) or the IFNα and IFNβ receptors (INFRαβ−/-) were infected at an MOI of 20 plaque forming units/cell with either WT HAdV or HAdV E1A A4-25 or treated with IFNβ1 for 16 hours. Western blots were then performed on acid extracted chromatin to detect global levels of H2B-ub and total H2B. (E) Repression of NF-κβ activity requires a region of E1A not involved in blocking H2B-ub. U2OS cells were transfected with an NF-κβ responsive luciferase reporter and a constitutive β-galactosidase reporter. After 2 days, cells were infected with the indicated HAdV for 16 hours. Activity of the NF-κβ reporter was assayed via luciferase activity. Data was normalized to the constitutive β-galactosidase reporter activity determined via the ONPG colorimetric assay. A Western blot for the expression levels of the various E1A mutants is shown in the inset. (F) E1A dependent activation of HAdV E4 orf3 expression requires a region of E1A not involved in blocking H2B-ub. A549 cells were infected at an MOI of 5 plaque forming units/cell with virus expressing the indicated E1A mutants for 16 hours. Cell lysates were collected for protein analysis and RNA was extracted. E4 orf3 mRNA levels were determined by quantitative RT-PCR and normalized to E1A expression level. A Western blot for the expression levels of the various E1A mutants is shown in the inset. Error bars represent +/- SD.
A

WI-38  
Uninfected  
E1A  
ΔE1A  
IFN1  
ΔE1A  
WT

Vero  
Uninfected  
E1A  
ΔE1A  
IFN1  
ΔE1A  
WT

IB: H2B-ub  
IB: H2B

B

WT infection  
0  4  6  12  18  24  30  36

αIRF3(S396)  
αIRF3  
αActin

C

α-IRF3

D

WT MEF  
IFNRβ-/- MEF  
IRF3-/- MEF

Uninfected  
WT E1A  
ΔE1A  
IFN1  
ΔE1A  
WT

IB: αH2B-ub  
IB: αH2B

E

NfκB reporter activation

Fold Activation

F

E4 orf3

Fold Change
the H2B-ub modification that occurs in response to virus infection. Global H2B-ub was also stimulated upon infection by UV inactivated wild type HAdV (WT UVK) or the rabbit specific poxvirus, Myxoma virus (Myxoma UVK) (Figure 2.1B). H2B-ub was reduced or blocked completely when cells were treated with native viruses (Figure 2.1B). Interestingly, despite representing different virus families, both HAdV and Myxoma appear to have evolved strategies that antagonize this cellular anti-viral response to virus exposure. Thus, the observed global H2B-ub increase in response to virus infection likely results from a cellular innate immune response to virus particles, rather than from the activity of a viral protein.

2.2.2 Cellular levels of H2B-ub are greatly increased by type I IFN treatment.

Production of type I interferon (IFN) from virus-infected cells is the hallmark characteristic of innate antiviral immunity. Type I IFN exposure alters the expression of numerous cellular interferon-stimulated genes (ISGs) (Der, Zho, Williams, & R.H., 1998; Takaoka & Yanai, 2006), rendering the cell more resistant to viral infection (Sadler & Williams, 2008). We reasoned that the robust activation of over 300 cellular genes by type I IFN could be responsible for the observed global upregulation of H2B-ub levels in response to virus. When tested directly, treatment of A549 cells with the interferon β1 (IFNβ1) cytokine stimulated a strong global increase in H2B-ub levels similar to that induced by infection with defective viruses (Figure 2.1B). In addition, neither treatment with IFN nor infection with HAdV ΔE1A increased H2B-ub levels in Vero cells, which are unable to produce IFNβ1 in response to viral infection (Figure 2.2A). These data connect type I IFN induced antiviral immunity with a localized change in the H2B-ub
Figure 2.3. Infection upregulates global H2B-ub and ISG transcription in an hBre1 dependent manner and this is blocked by HAdV E1A via residues 4-25. (A) A549 cells were infected with HAdV expressing wild type E1A (WT) or the indicated E1A deletion mutants in the presence or absence of IFNβ1. Western blot analysis was performed as in Figure 2.1A. (B) A549 cells were infected with a panel of HAdV viruses alone or cotreated with IFNβ1 for 16 hrs and RNA was extracted. Data was normalized to GAPDH and set to fold increase over the uninfected control. A statistically significant decrease in the transcription of all tested ISGs was found in WT HAdV infected as well as HAdV E1A Δ26-35 and HAdV E1A Δ30-49 infected cells as compared to HAdV ΔE1A and HAdV Δ4-25 infected cells (P<0.001). No significant difference was detected virus alone or treated with IFNβ1. (C) A549 cells were treated with control siRNA or siRNA specific for hBre1 and infected with WT HAdV, HAdV ΔE1A, or IFNβ1 and RNA was extracted. RT-qPCR was performed with a panel of ISGs, normalized to GAPDH and fold change to uninfected ctrl siRNA treated cells was plotted. Control siRNA treated HAdV ΔE1A and IFNβ1 treated cells were found to be statistically significant from all other groups while all hBre1 siRNA treated groups were not found to be statistically upregulated as compared to uninfected control treated cells (P<0.001). Error bars represent +/- SD.
2.2.3 **E1A residues 4-25 are necessary to block H2B-ub and ISG upregulation by type I IFN.**

Transcription of ISGs has long been known to be induced by HAdV particles and E1A is known to block this type I IFN mediated response, although the exact mechanisms have not been elucidated (Anderson & Fennie, 1987; Kalvakolanu, Bandyopadhyay, Harter, & Sen, 1991; Reich, Pine, Levy, & Darnell, 1988). We confirmed that E1A alone is necessary and sufficient to block the IFNβ1 induced global increase in H2B-ub in A549 cells by producing an A549 line that stably expresses E1A (Figure 2.1C).

To map the region of E1A required for blocking the type I IFN induced global increase in H2B-ub levels, we infected cells with a panel of viruses containing deletions within the E1A protein in the presence or absence of IFNβ1 (Figures 2.3A, 4A). A virus lacking residues 4-25 of E1A behaved similarly to the ΔE1A virus and triggered a global increase in H2B-ub. Deletions in adjacent regions (Δ26-35 and Δ30-49) functioned in a manner similar to WT E1A expressing virus, as infection with these viruses did not induce H2B-ub. Thus, the N-terminal region of E1A is essential for inhibition of H2B-ub induction. Identical results were seen in the presence or absence of IFNβ1, demonstrating that E1A effectively abrogates the induction of H2B-ub whether it is indirectly induced by cellular recognition of the virus particle or directly by exposure to IFNβ1. We also compared the transcriptional effects of virus infection and type I IFN treatment on a representative subset of well established ISGs (Figure 2.3B) and a panel of control genes not known to be IFN responsive (Figures 2.5B-E). As expected, IFNβ1
Figure 2.4. Mutants of E1A and their properties. (A) Schematic depiction of HAdV5 E1A and the mutants used in this study. (B) Relevant protein interaction properties of the E1A mutants (p300/CBP, pRB (Mymryk et al., 1992), and pCAF (Pelka et al. 2009)) used in this study and their experimentally determined phenotypes.
**A**

![Figure A: Diagram of gene structure with exons and unique regions labeled.]

**B**

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<th>PCAF Binding</th>
<th>hBre1 or RNF40 Binding</th>
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treatment strongly induced expression of all six ISGs, but not the control genes. The effect of virus infection on ISG expression, but not the control genes, closely mirrored the effects seen on global H2B-ub levels during virus treatment. Specifically, infection with HAdV ΔE1A or the Δ4-25 virus caused a robust increase in ISG transcriptional activation similarly to IFNβ1 treatment. However, WT HAdV as well as viruses with deletions in E1A adjacent to 4-25 largely blocked activation of ISG expression. This data establishes a strong correlation between the ability of E1A to block IFN dependent transcription with the ability to block global increases in H2B-ub levels.

2.2.4 The early stages of the type I IFN response are not blocked by E1A.

During infection with WT HAdV, IRF3 became phosphorylated and was recruited to the IFNβ1 promoter (Figures 2.2B, C). Thus, the earliest stages of the type I IFN response induced by infection remain intact. However, the global increase in H2B-ub induced by E1A mutant HAdV were not detected in MEFs deficient for both the type I IFN receptors (IFNα/βR−/−) or MEFs lacking IRF3 (IRF3−/−) (Figure 2.2D). This confirmed that the increase in H2B-ub levels, observed upon infection with HAdV expressing mutant E1A was dependent on the IFN response. Given that NF-κβ is a key regulator of inflammation, we determined if the region of E1A necessary to repress NF-κβ activity is the same as that required to repress ISG transcription. We compared the ability of the panel of E1A mutant viruses to affect the NF-κβ response. While WT E1A could repress NF-κβ activation, all of the N-terminal E1A mutants have lost this ability (Figure 2.2E). As several E1A mutants are unable to repress NF-κβ dependent transcription but are still able to block type I IFN responses, this activity may be of secondary importance for viral
evasion of the innate immune response. As the viral E4 region encodes E4 orf3 which is known to antagonize the IFN-induced antiviral state (Ullman, Reich, & Hearing, 2007), we examined whether the effects of E1A could also be mediated via activation of viral E4 expression. As reported (Nevins, 1981), E1A does activate E4 expression. However, an E1A mutant (Δ30-49) unable to activate E4 orf3 expression was still able to block type I IFN responses (Figures 2.2F, 3B). This indicates that E4 mediated effects are not necessary for the inhibition of ISG expression, and this agrees with our observation that E1A expression alone was necessary and sufficient to block IFNβ1 effects (Figure 2.1C).

2.2.5 hBre1 is necessary for IFN induced gene transcription.

Human Bre1/RNF20 (hBre1) is the E3 ubiquitin ligase which acts in conjunction with the E2 ubiquitin conjugase Ube2b, and accessory factors RNF40 and WAC to monoubiquitinate H2B at lysine 120 (Hwang et al., 2003; Kim et al., 2009; F. Zhang & Yu, 2011). While H2B-ub is a hPTM that is generally associated with transcriptional activation, hBre1 depletion only affects the expression of a small subset of genes (Shema et al., 2008). However, the involvement of hBre1 in IFN-induced activation of ISG transcription has never been assessed. A549 cells were treated with either a nonspecific control siRNA or a siRNA specific for hBre1, and then exposed to WT HAdV, HAdV ΔE1A or IFNβ1. The expression level of six ISGs (Figures 2.3C) and a panel of control genes not responsive to IFNβ1 (Figures 2.5B-E) were determined. As expected, IFNβ1 and HAdV ΔE1A induced high levels of ISG transcriptional activation in cells treated with a nonspecific control siRNA, whereas wild type HAdV did not strongly stimulate ISGs (Figure 2.3C). In contrast, knockdown of hBre1 with specific siRNA blocked ISG
Figure 2.5. The hBre1 complex regulates expression of specific cellular genes. (A) RNF40 is required for ISG transcriptional activation. A549 cells were treated with siRNA specific for RNF40 or a non-specific control siRNA and infected with WT virus, ΔE1A virus, or treated with IFNβ1. cDNA was made from the extracted RNA and quantitative real-time PCR (RT-qPCR) was performed, normalized to GAPDH and fold change to uninfected cells was plotted. Control siRNA treated HAdVΔE1A and IFNβ1 treated cells were found to be statistically significant from all other groups, while all RNF40 siRNA treated groups were not found to be statistically upregulated as compared to uninfected control treated cells (P<0.001). (B) Knockdown of hBre1 or RNF40 does not reduce the expression of non-ISGs. RT-qPCR was similarly performed with a panel of non-ISGs which are not regulated by IFN or E1A. (C-D) Knockdown of hBre1 or RNF40 does not alter the ability of E1A to activate or repress the expression of non-ISGs. RT-qPCR was similarly performed with a panel of non-ISGs which are increased during HAdV infection (C) or decreased during HAdV infection (D). Interestingly, while none of these genes are reported ISGs, Mcm3 and fhl2 responded weakly to IFNβ1 and this was reduced by knockdown of hBre1 or RNF40. (E-F) E1A does not block the expression of hBre1 regulated genes that are not ISGs. RT-qPCR was similarly performed with a panel of non-ISGs which are regulated by hBre1. Results obtained from uninfected cells shown in panel (E) were normalized with respect to Ctrl siRNA values to visualize their dependence on hBre1 and RNF40 (F). Data significantly different than uninfected control within groups is indicated (*P<0.05). Error bars represent +/- SD.
transcriptional activation under any treatment condition (Figure 2.3C). Similar results were obtained with siRNA against RNF40 (Figure 2.5A). This data provides evidence that the hBre1 complex is an essential component of the IFN stimulated antiviral response and links transcriptional activation of ISGs and the ensuing innate immune response with hPTM changes in H2B-ub status. These effects appear specific, as expression of the non-IFN responsive genes were not decreased by knockdown of hBre1 or RNF40 (Figure 2.5B). Furthermore, knockdown of either hBre1 or RNF40 had no effect on a panel of non-ISGs known to be affected by E1A (Figure 2.5C, D) and little if any H2B-ub could be detected on these genes by Chromatin Immunoprecipitation (ChIP) assays (Figure 2.6). We also examined the effect of E1A on three genes reported to be regulated by hBre1 (HoxA10, fosL2 and p53). Although expression of each of these genes was reduced upon knockdown of hBre1 or RNF40 (Figure 2.5F) and had detectable levels of H2B-ub (Figure 2.6), their expression was not blocked by E1A (Figure 2.5E). Thus, the effects of E1A on hBre1 may not affect genes with H2B-ub that exists prior to infection or they may be restricted in some other fashion to ISGs. This may be related to the observation that recruitment of E1A to cellular promoters varies temporally, such that several distinct classes of promoters have been defined based on the kinetics of E1A occupancy (Ferrari et al., 2008).

2.2.6 The hBre1 complex is disrupted by E1A.

E1A is an intrinsically disordered protein that functions by binding to cellular proteins and affecting their normal functions (Ferreon, Martinez-Yamout, Dyson, & Wright, 2009; Pelka et al., 2008). We, therefore, tested the ability of E1A to physically interact with
Figure 2.6. E1A does not affect the H2B-ub status of non-ISGs. (A) The H2B-ub status of non-ISGs activated by E1A is not altered by infection. A549 cells were infected with HAdV ΔE1A or WT HAdV or treated with IFNβ1 for 16 hours. Chromatin immunoprecipitation (ChIP) was then performed using a nonspecific control antibody or antibodies specific for H2B, H2B-ub, hBre1, Ube2b or E1A. DNA was then quantitated via RT-qPCR using gene specific primers located within the transcribed region of each indicated gene. (B) The H2B-ub status of non-ISGs repressed by E1A is not altered by infection. Experiment was performed as in (A). (C) E1A does not affect H2B-ub levels at genes that are regulated by hBre1, but not IFN. Experiment was performed as in (A). Data significantly different than uninfected control within groups is indicated (*P<0.05). Error bars represent +/- SD.
members of the hBre1 complex, which includes hBre1, RNF40 and Ube2b. To do this, we infected A549 cells with HAdVs expressing either WT or deletion mutant E1A proteins. Lysates from infected cells were immunoprecipitated using an E1A specific antibody and the presence of hBRE1, RNF40 and Ube2b was assayed by immunoblotting using antibodies specific to these proteins (Figure 2.7A). Both hBre1 and RNF40, but not Ube2b co-precipitated with E1A. Furthermore, the association of E1A with hBre1 and RNF40 required E1A residues 4-25 (Figure 2.7A), the same residues which are required to block global increases in H2B-ub levels and IFN induced ISG transcription (Figures 2.3A, B). The interaction between E1A and hBre1 was further confirmed by co-immunoprecipitation of E1A with endogenous hBre1 (Figure 2.8A) or transfected epitope tagged hBre1 or RNF40 (Figure 2.8B). However, E1A did not co-immunoprecipitate with epitope tagged Ube2b (Figure 2.8B).

The inability of E1A to interact with Ube2b suggested that E1A interfered with the association between hBre1 and the Ube2b catalytic subunit. Perturbation of the interaction between the hBre1/RNF40 E3 ligase with the E2 conjugase Ube2b provides an attractive mechanism by which E1A could block type I IFN induced H2B-ub and antiviral gene expression. To test this directly, we transfected tagged hBre1 and Ube2b along with wild type E1A or E1A mutants into HT1080 cells. As expected, hBre1 was readily co-immunoprecipitated with Ube2b (Kim et al., 2009) in the absence of E1A (Figure 2.7B). However, this interaction was disrupted in the presence of E1A, and this, once again, required residues 4-25 of E1A (Figure 2.7B). Thus, the identical region of E1A (residues 4-25) that is required to abrogate type I IFN induction of H2B-ub levels and ISG expression (Figures 2.3A, B) was also required for the association with and
Figure 2.7. E1A binds hBre1 through residues 4-25 and this blocks hBre1 association with Ube2b. (A) A549 cells were infected with wild type HAdV or HAdV expressing the indicated E1A deletion mutants for 16 hours at an MOI of 5. E1A was immunoprecipitated and Western blots were performed using antibodies specific to the indicated hBre1 complex components. (B) HT1080 cells were transfected with E1A or an E1A deletion mutant as well as tagged hBre1 and tagged Ube2b. Co-immunoprecipitation was performed by IP with an antibody specific to tagged Ube2b. Western Blot analysis was performed with an antibody specific to the tagged hBre1.
A

IP:E1A

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2% Input

IB:hBre1
IB:RNF40
IB:Ube2b
IB:UbcH6
IB:E1A

B

IP:HA-Ube2b

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<tr>
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<tr>
<td>IB:myc-hBre1</td>
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disruption of the hBre1 complex (Figure 2.7B). Serial immunoprecipitation of endogenous E1A from HEK 293 cells showed that a substantial amount of hBre1 was not co-immunoprecipitated with E1A. This suggested that E1A interacted with only a small, and perhaps distinct, subset of the total cellular hBre1 pool (Figure 2.8C). This data supports a model whereby the interaction of E1A with the hBre1 complex specifically blocks H2B-ub, which is required for gene expression, by excluding the catalytic Ube2b ubiquitin conjugase subunit from the hBre1 complex.

2.2.7 hBre1 and Ube2b are recruited to ISGs during innate immune stimulation.

H2B-ub is considered to be a hPTM that is preferentially associated with chromatin that is transcribed at high levels (Batta, Zhang, Yen, Goffman, & Pugh, 2011; Shema et al., 2008). Given the requirement of hBre1 for type I IFN induced gene expression, we predicted that H2B-ub would be present in the transcribed chromatin of actively expressed ISGs. Indeed, chromatin immunoprecipitation assays (ChIPs) determined that H2B-ub was present within the transcribed regions, as well as the promoter regions, of a panel of ISGs upon infection with HAdV ΔE1A or treatment with IFNβ1 (Figures 2.9, 10). Furthermore, this chromatin modification was not present during wild type HAdV infection (Figures 2.9, 2.10) suggesting that expression of E1A was locally inhibiting this histone modification as a mechanism to block type I IFN induced antiviral gene expression. Unlike wild type virus, infection with HAdV E1A Δ4-25 induced H2B-ub in the transcribed regions of ISGs (Figure 2.9) further indicating that this region was essential for antagonizing IFN induced H2B-ub. ChIP analysis also demonstrated strongly increased Bre1 occupancy of the ISGs in response to infection with WT HAdV,
Figure 2.8. Interaction of E1A with the hBre1 complex. (A) E1A is co-immunoprecipitated with endogenous hBre1. A549 cells were infected with a panel of HAdV at an MOI of 5 plaque forming units/cell. Cells were lysed and 5mg of protein was used to perform co-immunoprecipitation. Lysate was immunoprecipitated with 3µg of α-hBre1 specific antibody. Membranes were then blotted with E1A specific antibodies (M73). (B) hBre1 and RNF40, but not Ube2b, interact with E1A and this requires residues 4-25 of E1A. HT1080 cells were transfected with wild type E1A or the indicated E1A N-terminal deletion mutants and epitope tagged hBre1(left), RNF40 (middle), or Ube2b (right). After 24 hours, E1A was immunoprecipitated and Western blots were performed using antibodies specific to the indicated epitope tagged hBre1 complex components. (C) E1A targets a fraction of the cellular pool of endogenous hBre1. HEK 293 cells, which endogenously express E1A, were lysed with NP40 lysis buffer and 5 mg of lysate was serially immunoprecipitated 3 consecutive times with an α-E1A specific antibody. Input corresponds to 2% of the total lysate at the start of each round. Membranes were then blotted with antibodies specific for hBre1 and E1A.
HAdV ΔE1A or treatment with IFNβ1. This occurred regardless of the presence or absence of E1A (Figure 2.11A). This suggested that E1A does not interfere with recruitment of hBre1 to the target chromatin. Indeed, hBre1 and E1A were found to co-localize to ISGs during innate immune stimulation by ChiP reChIP experiments (Figure 2.11B). Furthermore, E1A was localized to the transcribed regions of ISGs during WT HAdV infection and could potentially be recruited via its interaction with hBre1. Ube2b was found to localize with hBre1 within the transcribed region of ISGs after IFN treatment or exposure to HAdV ΔE1A (Figures 2.11A, 11C). In contrast to hBre1, the Ube2b ubiquitin conjugase was absent in the presence of wild type E1A (Figure 2.11A). H2B-ub or hBre1 were not found within the transcribed regions of genes regulated by E1A, but not IFN (Figure 2.6). Taken together, these data support a model whereby IFN treatment stimulates the localized formation of an enzymatically competent hBre1 complex and subsequent H2B-ub modification of the chromatin of IFN regulated genes. E1A antagonized this process by displacing Ube2b from the hBre1 complex. Furthermore, H3-K4 and H3-K79 trimethylation in the promoter regions of ISGs was blocked under conditions in which H2B-ub was antagonized by E1A (Figure 2.10). This was expected as these hPTMs are marks of transcriptional activation that require H2B-ub (Kim et al., 2009; Mohan et al., 2010). This was not the case with H3 acetylation (H3ac) status, which has not been shown to depend on H2B-ub (Figure 2.10). Specifically, changes in H3K18 acetylation of ISG promoters, which is another mark altered by E1A during infection, did not consistently correlate with transcriptional activation of ISGs or H2B-ub status (Figure 2.11D).
Figure 2.9. The H2B-ub hPTM is present at ISGs upon IFN induction and this is blocked by E1A. The identity of each gene tested is indicated at the top of each figure. A549 cells were infected with WT HAdV, ΔE1A HAdV, E1A Δ4-25 HAdV or treated with IFNβ1 for 16 hours. Chromatin immunoprecipitation (ChIP) was then performed. Chromatin was analyzed for the presence of the following proteins with specific antibodies: nonspecific control, H2B, and H2B-ub. DNA was then quantitated via qReal Time PCR using the indicated gene specific primers. Data significantly different than uninfected control within groups is indicated (*P<0.001). Error bars represent +/- SD.
Mx1

Percent Input

mouse  H2B  H2B-ub

H2B  H2B-ub

OAS2

Percent Input

mouse  H2B  H2B-ub

IFNβ1

Percent Input

mouse  H2B  H2B-ub

IRF9

Percent Input

mouse  H2B  H2B-ub

OAS1

Percent Input

mouse  H2B  H2B-ub

GAPDH

Percent Input

mouse  H2B  H2B-ub
2.2.8 *hBre1 is required for activation of the type I IFN mediated innate immune response against virus infection.*

To establish the biological significance of the interaction of E1A with the hBre1 complex, we assessed the effect of type I IFN on virus replication. A549 cells were treated with siRNA specific to hBre1, RNF40, or both hBre1 and RNF40 prior to infection in the presence or absence of IFNβ1. Production of infectious viral progeny was assayed at various time points over 48 hours (Figures 2.12, 13). WT virus was unaffected by IFNβ1 treatment or knockdown of hBre1 or RNF40 alone or in combination. In stark contrast, growth of virus expressing E1A Δ4-25 was completely abrogated by IFNβ1 treatment (Figures 2.12, 13). Importantly, knockdown of hBre1 or RNF40 alone or in combination by siRNA relieved the hypersensitivity of the E1A Δ4-25 virus to IFN, allowing substantial virus replication in the presence of IFNβ1 (Figures 2.12, 13). Thus, the inability of this mutant to interact with the hBre1 complex greatly sensitized this virus to the IFNβ mediated innate immune response. These data confirm the importance of the hBre1 complex in establishing the IFN mediated anti-viral state.

2.3 Discussion

This study has identified a previously unsuspected requirement for the hBre1 complex and its ability to enzymatically modify chromatin in the type I IFN response (Figure 2.14). hBre1 functions as a ubiquitin ligase in conjunction with ubiquitin conjugase Ube2b, and accessory factors RNF40 and WAC to monoubiquitinate H2B at lysine 120 (Hwang et al., 2003; Kim et al., 2009; F. Zhang & Yu, 2011). H2B-ub marks chromatin that is highly transcriptionally active, is involved in efficient transcript elongation
Figure 2.10. hPTMs related to H2B-ub are similarly induced by IFN and reduced by E1A. Cells treated as above were ChIP’ed with a nonspecific control antibody or antibodies recognizing H2B, or the indicated hPTMs. RT-qPCR was performed using primers specific to the promoter region. Data significantly different than uninfected control within groups is indicated (*P<0.001). Error bars represent +/- SD.
(Lee et al., 2007; Xiao et al., 2005) and is a precursor to several other epigenetic marks of transcriptional activation including tri-methylation of histone 3 at lysines 4 (H3-K4) and 79 (H3-K79) (Lee et al., 2007).

hBre1 depletion only affects the expression of a small subset of genes (Shema et al., 2008), suggesting that its serves a regulatory role that may be restricted for specific purposes. Studies in yeast similarly support the concept that the H2B-ub hPTM is not necessary for the general control of transcription, as yeast unable to monoubiquinate H2B do not have obvious global defects (Hwang et al., 2003). We directly confirmed that the hBre1 complex was required for the cellular IFN response by knocking down the hBre1 ubiquitin ligase or the accessory factor RNF40 (Figures 2.3C, 5A). Specific knockdown of either factor using siRNA greatly reduced ISG expression that is directly induced in response to treatment with IFNβ1 or indirectly induced by infection with attenuated virus. In contrast, targeted knockdown did not alter the expression of a panel of genes not known to be regulated by IFN (Figures 2.5B, C, D). Furthermore, we detected each of the hBre1, RNF40 and Ube2b components of the hBre1 complex co-occupying the coding regions of ISGs in response to activation of IFN signalling (Figures 2.11A, C). These results directly demonstrate that the hBre1 complex is necessary for the IFN induced program of gene expression and further confirm that the activity of hBre1 is confined to specific transcriptional programs. Our discovery that hBre1 is required for the transcriptional program induced by type I IFN adds substantially to the short list of pathways for which H2B-ub is an epigenetic modification required for expression. To date, this has included HOX, Notch, estrogen
Figure 2.11. Status of various hPTMs at ISG loci. (A) The hBre1 complex is recruited to IFNβ1 activated genes and E1A blocks the recruitment of Ube2b to chromatin. A549 cells were infected with WT HAdV, HAdV ΔE1A, or HAdV E1A Δ4-25 or treated with IFNβ1 for 16 hours. Chromatin immunoprecipitation (ChIP) was then performed for the indicated genes. Chromatin was analyzed for the presence of the following proteins with specific antibodies: nonspecific control, hBre1, Ube2b or E1A. DNA was then quantitated via RT-qPCR using the indicated gene specific primer. Data significantly different from uninfected control within groups is indicated (*P<0.01). (B) ChIP reChIP was performed on WT HAdV infected cells. Chromatin was initially precipitated with an anti-E1A antibody and then precipitated with either an hBre1 or mouse IgG control antibody. Interaction was then determined on ISGs with a panel of primers specific to ISG transcribed regions. Data is presented as an increase in enrichment of ChIP E1A, reChIP hBre1 over ChIP E1A reChIP mouse specific IgG control. (*P<0.001). (C) ChIP was performed on IFNβ1 treated cells using an antibody recognizing Ube2b. Samples were reChIP’ed with antibodies specific for hBre1 or RNF40 to determine co-occupancy within the transcribed regions of a panel of ISGs during IFN stimulation. Data is presented as an enrichment of ChIP Ube2b, reChIP hBre1 or RNF40 over ChIP Ube2b reChIP mouse specific IgG control. (*P<0.01). (D) H3K18 acetylation at ISGs does not correlate with gene activation or H2B-ub. H3K18 acetylation status of ISG was determined via ChIP and RT-qPCR. Data significantly different than uninfected control within groups is indicated (*P<0.05). Error bars represent +/- SD.
receptor and p53 responses (Bray et al., 2005; Mohan et al., 2010; Moyal et al., 2011; Nakamura et al., 2011; Prenzel et al., 2011; Shema et al., 2008; Zhu et al., 2005). Like the type I IFN response, in each of these cases, rapid and substantial increases in the transcription of target genes occur.

Interestingly, a global increase in H2B-ub was not observed during infection with live HAdV expressing E1A or when cells stably expressing E1A were exposed to IFNβ1. Thus, the viral E1A protein is necessary and sufficient to overcome this IFNβ1 dependent global upregulation of the H2B-ub hPTM. Inhibition of the global increase in H2B-ub stimulated either by adenovirus infection or IFNβ1 treatment specifically required HAdV E1A residues 4-25 (Figure 2.3A). E1A residues 4-25 were also required to block transcriptional activation of ISGs (Figure 2.3B). However, adjacent regions of E1A necessary for binding other chromatin modifiers were not required to inhibit H2B-ub upregulation and ISG transcription (summarized in Figure 2.4B). This excludes indirect effects of E1A mediated by its interaction with general and ubiquitous co-regulators of transcription. We reasoned that E1A may instead function via direct interference with the cellular apparatus responsible for creating the H2B-ub hPTM.

We identified a physical interaction between E1A and the hBre1 complex, which required the exact same region of E1A necessary for blocking H2B-ub upregulation and ISG transcription (Figure 2.7A). In addition, the interaction of E1A with hBre1 blocked its association with Ube2b, the catalytic component of the complex involved in monoubiquitination of H2B (Figure 2.7B) and prevented the association of Ube2b with ISG coding regions in response to IFN signalling (Figure 2.11A). This provides an elegant mechanism by which E1A inhibits H2B-ub and subsequent epigenetic marks
Figure 2.12. Growth of a mutant adenovirus unable to bind the hBre1 complex is abrogated by type I IFN treatment and hBre1 or RNF40 knockdown complement this replication defect. A549 cells were treated with control siRNA or siRNA specific to hBre1, RNF40 or both in combination prior to infection with WT virus (MOI of 10) or HAdV E1A Δ4-25 (MOI of 20). Infected cells were treated with or without IFNβ1. Cells were collected at various time points up to 48 hrs post infection to prepare virus lysates. Production of infectious progeny virus was quantitatively assayed by plaque formation on HEK 293 cells. Data is shown over 24-48 hrs. Growth of WT HAdV is unaffected by IFNβ1 treatment, whereas growth of HAdV E1A Δ4-25, which is unable to target the hBre1 complex, is abrogated by IFNβ1 treatment. Knockdown of hBre1 or RNF40 partially restores growth of HAdV E1A Δ4-25 in the presence of IFNβ1.
dependent on this modification at ISGs (Figure 2.14), including H3 K4 and K79 trimethylation (Figure 2.10). Similarly to what we have observed with H2B-ub, E1A globally alters H3-K18 acetylation by targeting several cellular histone acetyltransferases to alter cell growth (Ferrari et al., 2008). Clearly the versatile E1A protein has evolved to exploit interactions with cellular chromatin modifying enzymes as a mechanism to efficiently reprogram transcription in the infected cell.

Wild-type HAdV is impervious to type I IFN in ex vivo experiments and it is known that this virus encodes multiple proteins that counteract the activities of specific IFN induced antiviral pathways (Thimmappaya, Weinberger, Schneider, & Shenk, 1982; Weitzman & Ornelles, 2005). Each of these known viral countermeasures is produced later in infection than E1A. As E1A is the first protein expressed during an HAdV infection, it likely represents the first line of defence against the IFN response. In order to establish if the interaction of E1A with the hBre1 contributes to evading the type I IFN response, we tested the effects of IFN on growth of WT HAdV and a mutant lacking residues 4-25 of E1A. As expected, IFN treatment had no effect on growth of WT HAdV. Although the mutant virus lacking residues 4-25 of E1A grew more poorly than WT virus, its growth was completely abrogated by IFN treatment (Figures 2.12, 14). These growth characteristics confirm that a virus lacking the ability to bind hBre1 and interfere with H2B-ub is hypersensitive to IFN. Importantly, knockdown of hBre1, RNF40 or both hBre1 and RNF40 simultaneously prior to HAdV infection partially restored growth of the Δ4-25 virus in the presence of IFN (Figures 2.12, 14). Thus, knockdown of the hBre1 complex genetically complements the inability of this mutant virus to target hBre1 and abrogate the IFN response. Furthermore, these experiments
Figure 2.13. Growth of a mutant adenovirus unable to bind the hBre1 at 48 hours only. A549 cells were treated with control siRNA or siRNA specific to hBre1, RNF40 or both in combination prior to infection with WT virus (MOI of 5) or HAdV E1A Δ4-25 (MOI of 20). Infected cells were treated with or without IFNβ1. Cells were collected at various time points up to 48 hrs post infection to prepare virus lysates. Production of infectious progeny virus was quantitatively assayed by plaque formation of on HEK 293 cells. Here, data from Figure 2.6 is shown as a bar graph at 48 hrs only. Growth of WT HAdV is unaffected by IFNβ1 treatment, whereas growth of HAdV E1A Δ4-25, which is unable to target the hBre1 complex, is abrogated by IFNβ1 treatment. Knockdown of hBre1 or RNF40 partially restores growth of HAdV E1A Δ4-25 in the presence of IFNβ1. Data significantly different WT virus untreated with IFN within siRNA treatment groups is indicated (*P<0.01). Error bars represent +/- SD.
confirm the biological significance of the hBre1 complex in the IFN mediated anti-viral response.

It was recently reported that the N2 protein of influenza H3N2 contributes to suppression of the innate immune response by targeting the PAF1 transcriptional elongation complex (Marazzi et al., 2012). Although the PAF1 complex does not contain any enzymatic activities that directly modify chromatin, it plays a role in recruiting the hBre1 complex to enhance H2B-ub, transcriptional elongation and H3K4 methylation (Kim et al., 2009). Although the effect of influenza N2 on H2B-ub is not currently known, its interference with PAF1 function will likely reduce H2B-ub, similarly to what we observed with E1A. Thus, adenoviruses, influenza A and potentially other viruses have independently evolved mechanisms to interfere with the innate immune response by antagonizing hPTMs required for ISGs expression. This convergence of function highlights the importance of chromatin modification in the regulation of the type I IFN response.

hBre1 has also been suggested to function as a tumour suppressor gene (Blank et al., 2012; Kim et al., 2009; Moyal et al., 2011). This is intriguing, as the type I IFN response is important in resisting cancer, as well as in controlling infection. Indeed, many cancers lose the ability to respond to IFN (Domschke et al., 2009; Hirsch, Caux, Hasan, Bendarss-vermare, & Olive, 2010; Krishnamurthy, Takimoto, Scroggs, & Portner, 2006; Marozin et al., 2008) and the importance of immune evasion has been formally acknowledged by inclusion in the recently revised list of the hallmarks of cancer (Hanahan & Weinberg, 2011). Our data demonstrating that loss of hBre1 function confers a loss of IFN responsiveness may provide some mechanistic insight
Figure 2.14. The hBre1 complex is involved in the transcription of interferon stimulated genes (ISGs) and E1A blocks this process by disrupting the interaction between hBre1 and Ube2b. Exposure to type I IFN or the innate immune response to virus infection results in the recruitment of the hBre1 complex the transcribed regions of ISGs. Here, the catalytically active hBre1 complex monoubiquitinates H2B at K120 resulting in the efficient transcription of ISGs. To counteract this innate immune defence during virus infection, HAdV produces the E1A protein, which binds to hBre1, inhibiting the binding of Ube2b to the hBre1 complex. This results in a loss of Ube2b occupancy at the ISG, a loss of monoubiquitination of H2B within the chromatin of ISGs and a subsequent lack of ISG transcription. Blockade of this hPTM by the viral E1A protein abrogates the large scale changes in cellular gene expression induced by the IFN innate immune response.
IFNβ activates TYK2 and JAK1, leading to the expression of >300 ISGs (Interferon-Inducible Genes) through the ISRE (Interferon-Stimulated Response Element) in the cytoplasm. E1A inhibits Ube2b, preventing the expression of ISGs.
into its putative role as a tumour suppressor (Shema et al., 2008).

In summary, our studies of E1A have led to uncovering an essential role for the hBre1 complex in the type I IFN response, which is summarized in Figure 2.7. The type I IFN induced transcriptional program is comprised of >300 ISGs and the establishment of this antiviral state is accompanied by a local increase in H2B-ub at each ISG that collectively leads to a readily detectable global increase in H2B-ub. Mechanistically, we show that this hPTM requires the recruitment of the hBre1 complex to ISGs, resulting in additional hPTM modifications and culminating in efficient IFN induced gene expression. We also identify a unique and elegant mechanism by which a viral oncoprotein subverts the type I IFN mediated cellular antiviral response by specifically antagonizing this hPTM. This work also exemplifies how studying interactions between viruses and their hosts can provide mechanistic insight into fundamental biological processes.

2.4 Experimental Procedures

2.4.1 Cell lines and plasmids

Human adenocarcinoma A549 and human fibrosarcoma HT1080 cells were grown at 37°C, 5% CO₂ in DMEM (Multicell) and supplemented with 10% fetal bovine serum (Gibco). Plasmids were transfected into HT1080 cells using Superfect reagent (Qiagen) following the manufacturer's recommendations. Transfection efficiency was typically, 60-70%. After 24 hours in culture, transfected cells were used for experimentation. hBre1 and RNF40 were cloned as a myc tag fusion into pCAN-myc. Ube2b was cloned as an HA tag fusion into pcDNA4.
2.4.2 Quantitative RT-PCR

Total RNA was prepared with Trizol extraction (Invitrogen). A total of 1μg of RNA was reverse transcribed into cDNA by random priming using the One step RT-PCR kit (Qiagen) following the manufacturers' instructions. Quantification of cDNA was done using SYBR-Green supermix for real-time qPCR (MyIQ, BioRAD) with oligonucleotide sequences that specifically recognize GAPDH, IFITM1, IFNβ1, IRF9, Mx1, OA2, and OA4. GAPDH was used as a control for total cDNA. Controls without reverse transcriptase were done for each RNA sample alongside the cDNA control. Results were normalized to the GAPDH and uninfected sample. The oligonucleotide sequences are listed in Table 2.1.

2.4.3 RNAi knockdown

Downregulation of hBre1 and RNF40 was performed using Silencer Select siRNA (Ambion). siRNA was delivered to cells via transfection with silentfect (BioRad) following the manufacturer’s instructions, 3 hours after seeding cells, for a period of 48 hours. A scrambled siRNA was used as a control.

2.4.4 Virus and type I interferon treatment of cells

Cells were infected with human adenovirus (HAdV5) wild type (dl309), or a panel of HAdV containing the indicated E1A deletion mutations: ΔE1A (dl312), E1A Δ4-25 (dl1101), E1A Δ26-35 (dl1102), E1A Δ30-49 (dl1103) (1). HAdV was used at a multiplicity of infection (MOI) of 5 pfu/cell. Myxoma was used at an MOI of 20. Cell cultures were infected at 50% confluence and left for 16 hours. Virus infection was
Table 2.1: A List of Oligonucleotide Primers used in this chapter.

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found to be near 100% by fluorescence microscopy under these conditions using virus expressing GFP. Interferon β1 (IFNβ1) (Cedarlane 11420-1) was added at 1 unit/mL to the cell media for 16 hours. In co-treatment experiments, cells were first infected with HAdV at an MOI of 5 for 4 hours before the addition of 1 unit/mL of IFNβ1 for an additional 14 hours. Subconfluent cells were collected for further experimentation.

2.4.5 Acid extraction of histones

4x10^5 cells were collected after treatment and lysed with NP40 lysis buffer (0.5%NP-40, 150mM NaCl, 50mM Tris pH 7.8). Cells were pelleted and pellets were then treated overnight with 0.1 M HCl. Protein concentrations were then determined using the BioRad protein assay reagent using BSA as a control after neutralizing the HCl.

2.4.6 Western blotting and co-immunoprecipitation

Cells were lysed with NP40 lysis buffer and protein concentrations were determined with BioRad protein assay reagent using BSA as a standard. 0.5mg of protein lysate was immunoprecipitated with myc or HA hybridoma supernatant or 6µg of anti-mouse IgG (Sigma M-7023) at 4ºC for 4 hours. 25 µg of protein was kept as 5% input, except as noted in the Figure 2.legend of individual blots. After 3 washes in NP40 lysis buffer, complexes were boiled in 25 µL of sample buffer for 5 min. Proteins were separated on NuPage 4-12% Bis-Tris gradient gels (Invitrogen) and transferred onto a nitrocellulose membrane (Amersham). Membranes were blocked in TBS with 0.1% Tween-20 and 5% skim milk or BSA and blotted with the indicated primary overnight at 4ºC. Details for
Table 2.2: A List of Antibodies used in this chapter.

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<th>Description</th>
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<td>Millipore</td>
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<td>Mouse monoclonal</td>
<td>Millipore</td>
<td>05-1312</td>
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<tr>
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<td>Mouse monoclonal</td>
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<td>M73 (E1A)</td>
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<td>In house</td>
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the primary antibodies may be found in Table 2.2. Horseradish peroxidase conjugated secondary antibodies were detected using ECL plus western blotting detection system (Amersham).

2.4.7 Chromatin immunoprecipitation (ChIP) /ChIP-reChIP

Approximately $10^7$ cells per sample were cross-linked with 1% formaldehyde at room temperature for 10 min. Cells were washed twice with ice cold PBS and harvested. Cell pellets were lysed in 1mL of cell lysis buffer (50 mM Tris-HCl [pH8.1], 10 mM EDTA, 1% SDS, and a protease inhibitor cocktail (Sigma) on ice for 10 min. Lysates were sonicated in an ultrasonic biorupter bath (Diogenode XL-2006 TO) to yield DNA fragment sizes of 200-500 base pairs. Samples were then centrifuged at 10,000xg for 10 min. 1 mg of protein was used for ChIP, 100 μg of this was kept as 5% input. Supernatants were then diluted 10-fold in dilution buffer (20 mM Tris-HCl [pH8.1], 1% Triton X-100, 2 mM EDTA, 150mM NaCl and protease inhibitors) and precleared with 50 μL of ChIP protein-A Sepharose (50% slurry of protein A-Sepharose containing 2.5 μg of salmon sperm DNA and BSA /mL) for 50 min at 4°C. Immunoprecipitations were performed overnight at 4°C using 5 μg of the indicated antibody found in Table 2.2. The next morning, 50 μL of ChIP protein-A Sepharose was incubated with each sample for 2 hrs. Beads were then washed once each with 500 μL of wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 150 mM NaCl), wash buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 500 mM NaCl), and wash buffer III (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH8.0) respectively and then washed twice with Tris-EDTA buffer.
Immunocomplexes were extracted twice with 150 μL of elution buffer (1% SDS, 0.1 M NaHCO₃). For ChIP-reChIP, samples were then rediluted 10x with dilution buffer and immunoprecipitation was repeated with a second antibody as indicated (2). After final elution, 12 μL of 5M NaCl was added to the 300 μL pooled elution and incubated at 65°C overnight to decrosslink the complexes. DNA was then purified using Quigen PCR purification spin columns. Conditions for qRT PCR using SYBR Green were as per the manufacturers' directions. Briefly, each 15 μL reaction contained 80 nM oligos and 0.5 uL of ChIP DNA.

### 2.4.8 Statistical analysis

All numerical values represent means ± S.E.M. Each experiment was done in three replicates and a representative replicate is shown for each blot. Statistical significance of the differences was calculated using one way Anova and Holm-Sidak to all other treatments in the experiment.

### 2.5 References


Horwitz, G. a, Zhang, K., McBryan, M. a, Grunstein, M., Kurdistani, S. K., & Berk, A. J.


Chapter 3:
Viral retasking of hBre1/RNF20 to recruit hPaf1 for transcriptional activation

3.1 Introduction

Viruses are obligate intracellular pathogens as they require cellular machinery to replicate. Indeed, viruses often subvert the functions of cellular machinery to support their life cycle. Human adenovirus (HAdV) is no exception, and during infection must appropriate the host cellular transcriptional apparatus to begin transcription of the viral genes necessary to reprogram the cellular environment (Frisch and Mymryk, 2002; Ferrari et al., 2009). This is done in large part by the viral products of Early Region 1A (E1A), the first gene transcribed after infection. The E1A proteins bind and redirect the activity of transcriptional regulators to initiate transcription of the HAdV early genes (Hearing and Shenk, 1986; Ferrari et al., 2009). The HAdV 5 E1A mRNA has five splice variants. The two largest isoforms, 13S and 12S, encode 289 and 243 residue (R) proteins, respectively. These proteins predominate at the early stages of virus infection. Sequence alignment of E1A from a variety of HAdVs shows four regions of conservation, and have been designated CR1-4 (Avvakumov et al., 2002). The 289R and 243R E1A proteins of HAdV 5 are identical except for the presence of an additional 46 amino acid sequence within the 289R (Pelka et al., 2008). This unique 46 amino acid region encompasses CR3 (Geisberg et al., 1994). Both the CR3 region and N-terminal 82 residues of E1A are sufficient to activate transcription when fused to a heterologous DNA binding domain (Yousef et al., 2009; Bondesson et al., 1994). Although each region
can separately recruit a plethora of transcriptional activators (Stevens et al., 2002; Geisberg et al., 1994; Bondesson et al., 1994; Mazzarelli et al., 1997; Grand et al., 1999; Duyndam et al., 1996), they function together to recruit cellular transcriptional complexes for the activation of viral transcription (Geisberg et al., 1994; Wong and Ziff, 1994; Pelka et al., 2008; Ablack et al., 2012). CR3, specifically, activates transcription through interactions with the mediator complex component Med23 (mediator complex subunit 23) (Stevens et al., 2002; Ablack et al., 2010; Berk, 2005). CR3 activity is further modulated by pCaf (CREBBP-associated factor), Gcn5 (general control of amino-acid synthesis, yeast, homolog), p300 (E1A binding protein p300), BS69 (bone morphogenetic protein receptor-associated molecule 1) and Sug1 (26S proteasome AAA-ATPase subunit RPT6) (Pelka et al., 2009a; Ablack et al., 2010; Hateboer et al., 1995; Grand et al., 1999; Pelka et al., 2009b). Likewise, the N-terminus of E1A interacts with transcriptional activators, such as p300, CBP (CREB-binding protein), p400 (E1A binding protein p400), pCaf, TBP (TATA binding protein), and TRAAP (transformation/transcription domain-associated protein) (Frisch and Mymryk, 2002). Although there exists a large body of research focusing on the role that CR3 plays in virus transcription, the requirement for the N-terminus, which is conserved in both the 289R and 243R E1A proteins, and the mechanisms through which it cooperates with CR3 to activate viral transcription, are poorly understood.

Previously, we identified a novel interaction between the N-terminus of HAdV 5 E1A and hBre1(human BREfeldin A sensitivity)/RNF20 (Ring finger protein 20) (Fonseca et al., 2012). hBre1 is a cellular ubiquitin ligase, which functions in concert with the accessory factor RNF40 (Ring finger protein 40) and the Ube2b (Ubiquitin-
conjugating enzyme E2B) ubiquitin conjugase to monoubiquitinate histone 2B (H2B) (Kim et al., 2009; Osley, 2004). The monoubiquitination of H2B (H2B-ub) is an epigenetic mark of genes that are highly transcriptionally active (Shema et al., 2008). H2B-ub is a precursor to other activation marks, such as the trimethylation of histone 3 lysines 4 and 79, which are promoted by the interaction of the hBre1 complex with the hPaf1 (human RNA polymerase II associated factor 1) complex (Kim and Roeder, 2009; Wood et al., 2003). During infection, E1A binds to hBre1 and blocks the interaction between hBre1 and the catalytic subunit Ube2b. This specifically antagonizes the ability of the hBre1 complex to monoubiquitinate H2B at interferon (IFN) responsive genes (Fonseca et al., 2012). In this way, the interaction of E1A with hBre1 blocks transcriptional activation of IFN responsive genes and this contributes to inhibition of the cellular innate immune response to HAdV infection via an epigenetic mechanism (Fonseca et al., 2012).

Here, we investigate whether the interaction of HAdV 5 E1A with hBre1 influences transcription from the HAdV genome. We have found that the interaction of E1A with hBre1 contributes to the activation of early viral gene expression. Specifically, E1A utilizes hBre1 as a scaffolding protein to recruit the hPaf1 complex to HAdV early genes. This retasking of the hPaf1 complex, which is known to promote RNA polymerase II transcription elongation and transcription-coupled histone modifications, contributes to E1A mediated activation of HAdV early gene expression. Thus, in addition to antagonizing the ability of hBre1 to function as an E3 ubiquitin ligase involved in transcriptional activation of the innate immune response, the interaction of E1A with hBre1 serves a second distinct purpose as a novel means of enhancing viral
gene transcription by recruiting the hPaf1 complex.

3.2 Results

3.2.1 hBre1 contributes to Gal4 mediated transcriptional activation by the N-terminus.

We previously showed that the N-terminus of E1A interacts with the cellular ubiquitin ligase hBre1 (Fonseca et al., 2012). To determine if hBre1 influences transcriptional activation by the N-terminus of E1A, human U-2 OS osteosarcoma cells were treated with either a control (Ctrl) siRNA or one of 4 hBre1 specific siRNAs. Cells were then transfected with a constitutive β-galactosidase reporter, a Gal4 responsive luciferase reporter and a vector expressing the Gal4 DNA binding domain (DBD) alone or Gal4 DBD fused to the N-terminus of E1A. As an additional control, cells were similarly transfected with a vector expressing the Gal4 DBD fused to E1A CR3. E1A CR3 also functions as a strong transcriptional activation domain, but does not interact with hBre1. Luciferase activities were measured and results were normalized to β-galactosidase activity. The effects of siRNA treatment were calculated as a relative fold change with respect to cells transfected with a vector expressing the Gal4 DBD alone. Treatment with each of the 4 hBre1 specific siRNAs resulted in decreased hBre1 expression, which was accompanied by a decrease in E1A N-terminal dependent transcriptional activation (Figure 3.1). Based on this result, we used hBre1 siRNA 3 for further experiments. In contrast to its effects on transactivation by the N-terminus of E1A, knockdown of hBre1 had only modest effects on transcriptional activation by E1A CR3. These data suggest that hBre1 is involved transcriptional activation mediated by the N-terminus of E1A, but not by CR3.
Figure 3.1. The N-terminus of E1A specifically requires hBre1 for full activation of transcription. U-2 OS cells were transfected with a negative control siRNA or 1 of 4 siRNAs specific for hBre1. Cells were then transfected with a constitutive β-galactosidase reporter, a Gal4 responsive luciferase reporter and a vector expressing the Gal4 DNA binding domain (DBD) alone, the Gal4 DBD fused to the N-terminus of E1A, or the Gal4 DBD fused to E1A CR3. Luciferase activity was measured. Results were normalized to β-galactosidase activity and siRNA treated groups were set as a fold change to the Gal4 only transfected counterpart. A statistically significant decrease from control siRNA treatment is indicated (* P<0.01). n=3
Figure 3.2. Kinetics of virus infection under the conditions used for all experiments. (A) Viral genome replication is detected at 36 h.p.i. RT-qPCR was performed on DNA collected from the time course of infected cells using primers for the E1A promoter to detect copies of the viral genome and GAPDH to detect copies of the cellular genome. Results of WT infected cells were normalized to ΔE1A HAdV and set as fold to the cellular genome via the GAPDH promoter. (B) HAdV late gene expression is not detected until 36 h.p.i. A549 cells were infected at an MOI of 5 with WT or ΔE1A HAdV. Cells were collected at the indicated time points and RNA and DNA were collected. RT-qPCR was performed for the HAdV early transcripts E1A and the late genes E2l and L5, and normalized to GAPDH. Fold change to GAPDH was plotted. (C) HAdV late gene protein expression is detected at 48 h.p.i. A549 cells infected with WT virus were collected after 20 h.p.i. and protein was detected via western blotting with the indicated antibodies (E1A represents early gene expression and hexon represents late gene expression). n=2
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**E1A promoter**

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3.2.2 *E1A requires hBre1 and likely p300/CBP to fully activate transcription of viral early genes.*

To determine if the hBre1 complex is required for E1A dependent activation of viral early gene expression, human A549 lung epithelial cells were treated with Ctrl siRNA or siRNA specific to either hBre1 or the hBre1 complex member RNF40 and were then infected with either wildtype (WT; dl309) HAdV or a series of HAdV E1A deletion mutants at a multiplicity of infection (MOI) of 5. These viruses express both the 289R and 243R E1A proteins. cDNA was prepared from cells collected 20 hours post infection (h.p.i). Under these conditions of infection, this time point precedes the onset of viral late gene transcription and expression, as well as amplification of the viral genome (Figure 3.2). The expression of a panel of HAdV early genes known to be activated by E1A was determined by quantitative real time PCR. Knockdown of hBre1 did not consistently reduce expression from the viral E1A or E1B transcription units over the panel of viruses (Figure 3.3A,B). However, mRNA levels were significantly decreased from both the E3 and E4 transcription units in cells treated with hBre1 specific siRNA (Figure 3.3C and D). In contrast, treatment with siRNA specific for the RNF40 component of the hBre1 complex did not significantly decrease mRNA levels from any of the HAdV early genes (Figure 3.3, all panels). This result was unexpected, as RNF40 is essential for monoubiquitination of H2B by the hBre1 complex (Kim et al., 2009). Infection with a virus containing a deletion of E1A that is unable to bind hBre1 (E1A Δ4-25), also showed a reduction in transcription of E3 and E4, but not E1A and E1B (Figure 3.3). Interestingly, the decreased early gene expression observed with this mutant was not further exacerbated by knockdown of hBre1. This suggested the inability of the E1A
Figure 3.3. hBre1 contributes to E1A mediated activation of E3 and E4 expression during infection with HAdV. A549 cells were treated with Ctrl siRNA, siRNA specific to RNF40 or siRNA specific for hBre1 (Shown in the inset panel) and infected at an MOI of 5 with WT HAdV, or a series of HAdV containing the indicated deletions within the N-terminus of E1A (Shown in the inset panel). RT-qPCR was performed with a panel of HAdV early genes, normalized to GAPDH and fold change to uninfected Ctrl siRNA treated cells was plotted. Results for the HAdV E1A (A), E1B (B), E3 (C), and E4 (D) transcription units are shown. A statistically significant decrease from siCtrl is indicated (* P<0.01). n=3
Δ4-25 mutant to bind hBre1 and the knockdown of hBre1 might have redundant effects on E1A dependent transcription of E3 and E4 (Figure 3.3C and D).

A reduction in the transcriptional activation of E3 and E4 was also observed during infection with virus containing E1A deletions in adjacent regions not required for interaction with hBre1 (E1A Δ26-35, E1A Δ30-49; Figure 3.3C and D). In contrast to E1A Δ4-25, transcription of E3 and E4 during infection with these mutants was further decreased by knockdown of hBre1 (Figure 3.3C and D). Taken together, these results suggest that, while hBre1 is specifically targeted by E1A to enhance transcriptional activation of E3 and E4, other factors bound at adjacent regions of E1A also contribute to full transcriptional activation.

It should be noted that residues 4-25 of E1A are also required for interaction with p300 and CBP as well as hBre1 (Egan et al., 1988; Mymryk et al., 1992). p300/CBP may also play a role in E1A-dependent transactivation as the mutant lacking residues 30-49, which are also required for p300/CBP interaction (Egan et al., 1988; Mymryk et al., 1992) but not hBre1 binding (Fonseca et al., 2012), also shows a reduction of E3 and E4 expression (Figure 3.3C, 2D).

3.2.3 hBre1 is recruited to HAdV early genes by E1A, but this does not lead to H2B monoubiquitination.

As hBre1, but not RNF40 is required for maximal E1A dependent activation of E3 and E4 expression (Figure 3.3), we next determined whether hBre1 complex members or H2B-ub could be detected on the E3 and E4 promoters. A549 cells were infected with WT HAdV or a HAdV containing a deletion of E1A (ΔE1A) at an MOI of 5. Chromatin
Figure 3.4. E1A recruits hBre1 to HAdV early gene promoters, but HAdV chromatin is not monoubiquitinated by hBre1. A549 cells were infected with WT HAdV or ΔE1A HAdV for 20 hours. Chromatin immunoprecipitation (ChIP) was then performed with antibodies specific for the indicated proteins. DNA was probed via qRT-PCR for the presence of HAdV early gene promoters. Data was normalized to ΔE1A HAdV and a non-specific antibody control. Occupancy by RNA pol II and E1A (A), hBre1 complex members (B), and the indicated histone post-translational modifications (C) at HAdV early gene promoters are shown. In B, a statistically significant increase from mouse antibody ChIP is indicated (* P<0.01). n=3
immunoprecipitation (ChIP) was then performed using an antibody control, E1A specific antibody, RNA polymerase II (RNA pol II) specific antibody, and antibodies specific to hBre1 complex members hBre1, RNF40, and Ube2b. As expected (Rasti et al., 2006), E1A was present at all HAdV early gene promoters and this correlated with the transcriptional status as determined by occupancy of RNA pol II (Figure 3.4A). E1A was not detected on the cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter, as has been previously published (Fonseca et al., 2012; Ferrari et al., 2008). Similarly, hBre1 and RNF40 were specifically associated with the promoters of the HAdV early genes E2e, E3, and E4, but not with E1A or E1B (Figure 3.4B and 3.5). In contrast, Ube2b, the catalytic member of the hBre1 complex involved in H2B monoubiquitination, was not recruited to the HAdV genome. Given that RNF40 is not required for transcriptional activation of HAdV early genes, and Ube2b is not present on their promoters, it seems unlikely that the hBre1 complex could perform its best characterized function, the monoubiquitination of H2B. This was confirmed by ChIP analysis, which demonstrated that H2B-ub was not present on viral chromatin at the E3 and E4 promoters (Figure 3.4C). Despite an absence of H2B-ub, marks of transcriptional activation such as H3K18 acetylation, and H3K4 and K79 trimethylation were detected by ChIP on the E3 and E4 promoters (Figure 3.4C), which is consistent with the presence of RNA pol II (Figure 3.4A).

3.2.4 hPaf1 contributes to transcriptional activation by the N-terminus of E1A, but not by CR3.

In addition to its function in H2B monoubiquitination, hBre1 interacts with several other
Figure 3.5. HAdV early genes E1A, E1B, and E2e display varied hPTM states which do not include H2B-ub association. A549 cells were infected with WT HAdV or ΔE1A HAdV for 20 hours. Chromatin immunoprecipitation (ChIP) was then performed with antibodies specific for the indicated histone or hPTMs. DNA was probed via qRT-PCR for the presence of HAdV early gene promoters E1A, E1B or E2e. Data was normalized to ΔE1A HAdV and a non-specific antibody control. n=3
complexes involved in transcriptional regulation (Lee et al., 2007; Wood et al., 2003; Kim and Roeder, 2009; Hahn et al., 2012). To determine if any of these hBre1 interacting complexes were similarly required for E1A dependent transcriptional activation, U-2 OS cells were treated with siRNA specific to hPaf1, SetD (SET domain containing) 1A, SetD1B, MLL (myeloid/lymphoid or mixed-lineage leukemia) 2, or MLL3 & 4. Cells were then transfected with a constitutive β-galactosidase reporter, a Gal4 responsive luciferase reporter and a vector expressing Gal4 or Gal4 fused to the N-terminus of E1A. Gal4 fused to E1A CR3 was again used as a control, as it functions as a strong transactivator, but does not bind to hBre1. Knockdown of hPaf1 did not affect hBre1 expression (Figure 3.6, inset), but nevertheless resulted in significant decreases in transcriptional activation by the N-terminus of E1A, but not CR3 (Figure 3.6). This result is similar to what was observed for knockdown of hBre1 (Figure 3.1) and suggests that the hPaf1 complex is specifically recruited by the N-terminus of E1A to enhance transcriptional activation. None of the other knockdowns specifically reduced activation by the N-terminus of E1A as they also reduced activation by CR3, which does not bind to hBre1 (Figure 3.6).

3.2.5 hPaf1 is recruited to the viral E3 and E4 gene promoters in an E1A and hBre1 dependent manner.

To determine whether hPaf1 is recruited to the HAdV genome, we performed ChIP analysis. A549 cells were infected with WT HAdV and a series of HAdV containing deletions in E1A at an MOI of 5. ChIP was then performed using an antibody control and a hPaf1 specific antibody. hPaf1 was found to specifically localize to the E2e, E3
Figure 3.6. The N-terminus of E1A, but not CR3 requires hPaf1 for efficient transcriptional activation. U-2 OS cells were transfected with a negative Ctrl siRNA or siRNAs specific for known hBre1 interacting proteins. Cells were then transfected with a constitutive β-galactosidase reporter, a Gal4 responsive luciferase reporter and a vector expressing the Gal4 DBD alone, Gal4 DBD fused to the N-terminus of E1A, or Gal4 DBD fused to E1A CR3. Luciferase activity was measured. Results were normalized to β-galactosidase activity and siRNA treated groups were set as a fold to the Gal4 only transfected counterpart. A statistically significant decrease from Ctrl siRNA treatment is indicated (* P<0.01). n=3
and E4 viral early gene promoters in an E1A dependent manner, and this required the same region of E1A (residues 4-25) necessary for interaction with hBre1 (Figure 3.7A and B). Reduced hPaf1 occupancy of the E3 and E4 promoters was also observed during infection with virus expressing E1A lacking residues 30-49 of E1A. Although this reduction was not as substantial as with the Δ4-25 mutant, the Δ30-49 mutant retains binding to hBre1, suggesting that other factors such as p300/CBP may influence hPaf1 recruitment. ChIP analysis did not detect hPaf1 within the E3 and E4 gene transcribed regions (Figure 3.7A), the E1A and E1B promoters (Figure 3.7B), or IFN responsive genes (Figure 3.8) during infection suggesting a specific recruitment and localization to the E2e, E3 and E4 promoters. Furthermore, ChIP reChIP experiments demonstrated that hPaf1 co-occupied the E2e, E3 and E4 promoter regions with both hBre1 and E1A (Figure 3.7C) but did not show any co-localization with either hBre1 or E1A at E1A or E1B promoters (Figure 3.9A). This colocalization on HAdV early genes suggests that E1A, by binding hBre1, is recruiting hPaf1 to participate in the transcriptional activation of the viral E3 and E4 early genes.

Next, we tested whether hPaf1 recruitment to HAdV early genes required hBre1. A549 cells were treated with either a non-specific control siRNA or an siRNA specific to hBre1 and infected with WT HAdV at an MOI of 5. ChIP was then performed using an antibody control or an antibody specific to hPaf1. Knockdown of hBre1 substantially affected hPaf1 recruitment to the E2e, E3 and E4 promoters during HAdV infection, but not E1A, E1B or GAPDH (Figure 3.9B). In contrast, siRNA knockdown of hPaf1 did not reduce hBre1 recruitment to the HAdV genome during HAdV infection (Figure 3.9B). These results indicate that E1A is utilizing hBre1 as a scaffold to recruit hPaf1 to
Figure 3.7. E1A recruits hPaf1 to HAdV early gene promoters via hBre1. (A, B) hPaf1 is localized at the E2e, E3 and E4 promoters. A549 cells were infected with WT HAdV, ΔE1A HAdV or HAdV containing deletions within E1A for 20 hours. Chromatin immunoprecipitation (ChIP) was performed with the indicated antibodies and DNA was probed via qRT-PCR for the presence of HAdV early gene promoters or transcribed regions. hPaf1 localization requires residues 4-25 of E1A and is found on the E2e, E3 and E4 but not the E1A or E1B promoter regions. A statistically significant decrease from WT in A is indicated (* P<0.01). (C) hPaf1 colocalizes with E1A and hBre1 on the E3 and E4 promoters. hPaf1 ChIP was followed by re-ChIP with either hBre1 or E1A specific antibodies to determine co-occupancy. Data was normalized to ΔE1A HAdV and a non-specific antibody control. (D and E) hPaf1 recruitment to the E3 and E4 promoter requires hBre1. A549 cells were treated with a non-specific siRNA, or siRNA specific for hBre1 (D), or hPaf1 (E) prior to virus infection. ChIP assays were then performed using hBre1 or hPaf1 specific antibodies. hPaf1 and hBre1 occupancy was then determined at the HAdV E3 and E4 promoters as described above. n=3
the E3 and E4 promoters. Further, hPaf1 was shown to interact with E1A by co-immunoprecipitation. This interaction was dependent on hBre1 expression, as knockdown of hBre1 reduced hPaf1 co-association with E1A (Figure 3.9C).

3.2.6 **hPaf1 is required for expression of the viral E3 and E4 transcription units.**

To determine if hPaf1 is involved in the transcriptional activation of HAdV early genes, A549 cells were treated with control or hPaf1 specific siRNAs and infected with WT or ΔE1A virus. Knockdown of hPaf1 compared to control siRNA treatment caused a reduction in the ability of WT virus to activate expression of the viral E2e, E3 and E4 (Figure 3.10A), but not the E1A and E1B early genes during infection (Figure 3.10A). Thus, hPaf1 knockdown affected the expression of same subset of early genes affected by knockdown of hBre1 (Figure 3.3). These data confirm that hPaf1 is involved in E1A dependent activation of viral E2e, E3 and E4 gene expression.

3.2.7 **Recruitment of hPaf1 is required for H3K4 and H3K79 tri-methylation of the HAdV E3 and E4 promoters.**

Recruitment of the hPaf1 complex to a transcriptional template has been reported to be necessary for several histone post-translational modifications associated with active transcription, including trimethylation of H3K4 and H3K79 (Kim et al., 2009; Mohan et al., 2010). To determine if hPaf1 is required for the observed E1A dependent increase in H3K4 and H3K79 tri-methylation at the viral E3 and E4 promoters, A549 cells were infected as before with WT or ΔE1A virus and ChIP was performed using antibodies
Figure 3.8. hPaf1 is excluded from IFN regulated genes during WT HAdV infection. ChIP assays were performed on A549 cells 20 hours after infection with the indicated viruses at a MOI of 5. ChIP was then performed using a control anti-mouse antibody or antibodies specific for hBre1, Ube2b, E1A, or hPaf1. hBre1, Ube2b and hPaf1 associate with IFN regulated gene bodies during IFN stimulating conditions (ΔE1A virus infection). In contrast, in the presence of E1A, Ube2b and hPaf1 are excluded from IFN stimulated gene bodies (WT virus infection). A statistically significant decrease from ΔE1A infected is indicated (* P<0.01). n=3
specific for H3K4 or H3K79 trimethylation. Knockdown of hPaf1 significantly reduced H3K4 and H3K79 trimethylation at the viral E2e, E3 and E4 promoters, but not the E1A, E1B or GAPDH promoters (Figure 3.10B). This suggests that hPaf1 plays an essential role in generating these two histone modifications at the E3 and E4 promoters, but not the E1A promoter.

3.2.8 The E1A proteins from multiple HAdV types target hBre1.

Although it is known that the HAdV 5 E1A protein binds hBre1, it is not known whether this interaction is a feature of all E1A proteins. To determine if the interaction with hBre1 is evolutionarily conserved among the different HAdV types, we co-transfected human HT1080 fibrosarcoma cells with hBre1 and a representative E1A from six different HAdV groups with a C-terminal GFP. Co-immunoprecipitation was performed on lysates by immunoprecipitating the representative E1As with GFP antibody and any co-precipitated hBre1 was detected with hBre1 specific antibody. hBre1 interacted strongly with all of the E1A proteins tested, with the exception of HAdV 12 (Figure 3.11A). The conservation of the E1A-hBre1 interaction across multiple HAdV species suggests that targeting of hBre1 is an important aspect of E1A function.

3.2.9 hBre1 and hPaf1 are required for transcriptional activation by the E1A proteins of multiple HAdV types.

The evolutionary conservation of the E1A-hBre1 interaction (Figure 3.11A) suggested that there could also be an evolutionary conservation of E1A-hBre1 function as well. To test this, U-2 OS cells were treated with non-specific siRNA, hBre1 specific siRNA or
Figure 3.9. hPaf1 recruitment to the E2e, E3 and E4 promoters requires E1A and hBre1 and the interaction of E1A with hPaf1 requires hBre1. (A) hPaf1 ChIP was followed by re-ChIP with either hBre1 or E1A specific antibodies to determine co-occupancy at the E1A and E1B promoters. Data was normalized to ΔE1A HAdV and a non-specific antibody control. hPaf1 is not co-associated with E1A and hBre1 at these promoters. (B) hPaf1 recruitment to the E2e, E3 and E4 promoters requires hBre1. A549 cells were treated with a non-specific siRNA, or siRNA specific for hBre1, or hPaf1 prior to virus infection. ChIP assays were then performed using hBre1 or hPaf1 specific antibodies. hPaf1 and hBre1 occupancy was then determined at the HAdV E1A, E1B, E2e, E3 and E4 promoters as described above. A statistically significant decrease from Ctrl siRNA treatment is indicated (* P<0.01) for B. (C) hPaf1 interacts with E1A and this is hBre1 dependent. A549 cells were treated with Ctrl or hBre1 specific siRNA and infected with WT or ΔE1A virus. Cells were collected after 20 hours. Lysates were immunoprecipitated with anti-E1A antibody and probed for hPaf1. n=2
A

ChIP: hPaf1
reChIP: E1A
hBre1

Fold Increase

E1A E1B

B

Percent Input

α-hBre1 α-hPaf1 α-hBre1 α-hPaf1
siCtrl sihPaf1 sihBre1

C

IP-αE1A sICtrl sihBre1

E1A - + - +

IB-αhPaf1 2% Input

IB-αhBre1

IB-αhPaf1

IB-αE1A
hPaf1 specific siRNA. Cells were then transfected with a constitutive β-galactosidase reporter, a Gal4 responsive luciferase reporter and a vector expressing the Gal4 DBD or the Gal4 DBD fused to the N-terminus of E1A. The results from the luciferase assays indicate that activation by the N-terminus of the HAdV 3, 4, 5 and 9 E1A proteins types was reduced when either hPaf1 or hBre1 are knocked down (Figure 3.11B). Thus, the E1A proteins from multiple HAdV types appear to utilize both hBre1 and hPaf1 for transcriptional activation.

3.3 Discussion

HAdV E1A is an unusually strong and multifarious regulator of gene expression. E1A is the first protein expressed during viral infection and acts to reprogram cellular gene expression, as well as activate viral early gene expression. As such, E1A serves as a paradigm of eukaryotic transcriptional control. As one particular example, the ability of E1A to bind and sequester the p300/CBP acetyltransferases is a well established mechanism by which E1A can repress cellular transcription (Jelsma et al., 1989; Horwitz et al., 2008a; Ferrari et al., 2009). We previously demonstrated that HAdV 5 E1A targets the cellular hBre1 complex. E1A disrupts the interaction between the hBre1 ligase and the Ube2b conjugase, leading to a global reduction in H2B-ub, decreased occupancy by hPaf1 and a consequent abrogation of type I IFN dependent gene expression (Fonseca et al., 2012) (Figure 3.8). The interaction of E1A with hBre1 provides a mechanism by which E1A antagonizes expression of cellular genes required for the innate immune response to viral infection in addition to the sequestration of p300/CBP. In the present work, we have determined that the interaction of E1A with hBre1 is further exploited by
Figure 3.10. hPaf1 is required for expression of the HAdV E2e, E3 and E4 genes during infection and chromatin marks associated with active gene expression. (A) Knockdown of hPaf1 does not affect E1A and E1B expression, but greatly reduces E3 and E4 expression during infection. A549 cells were treated with Ctrl siRNA or hPaf1 specific siRNA and infected with wildtype (WT) or ΔE1A HAdV. qRT-PCR was then performed for E3 and E4 expression. Data was normalized to GAPDH and fold was set to the WT CTRL siRNA of the same gene. (B) hPaf1 is required for H3K4 and H3K79 trimethylation at the E3 and E4 promoters. ChIP assays were performed on A549 cells treated with siRNA as indicated and infected for 20 hours at a MOI of 5. ChIP was then performed using antibodies specific for trimethylated H3K4 or H3K79 and qRT-PCR was performed for the indicated HAdV early gene promoter region. A statistically significant decrease from Ctrl siRNA treatment is indicated (* P<0.01). n=3
the virus to activate expression of the viral E2e, E3 and E4 transcription units. Thus, subversion of the hBre1 complex by E1A results in two distinct and opposite effects: inhibition of transcription from cellular IFN responsive genes and activation of viral gene expression (Figure 3.12).

Our findings here show a novel means by which hBre1 facilitates transcriptional activation, specifically via recruitment and modulation by the N-terminus of E1A. More importantly, the contribution made by this interaction is completely independent of the known ability of the hBre1 complex to monoubiquitinate H2B. Indeed, neither the Ube2b nor RNF40 components of the hBre1 complex are required for E1A dependent activation of viral early gene expression. This reveals an alternative and unexpected method by which hBre1 can activate transcription. We hypothesized that during infection, E1A functionally retasks the catalytically inactive hBre1 complex, converting it into a scaffold to recruit additional factors that enhance expression of the viral E2e, E3 and E4 early gene promoters. Our results indicate that the outcome of viral retasking of the hBre1 complex is the recruitment of the cellular hPaf1 complex. The hPaf1 complex functions as an important regulator of RNA pol II transcriptional regulation, primarily by promoting transcription elongation and transcription-coupled histone modifications (Tomson and Arndt, 2012). We demonstrate that hPaf1 was specifically required for transcriptional activation by the N-terminus of HAdV 5 E1A (Figure 3.6), was recruited to HAdV early genes in an identical pattern to hBre1 and required hBre1 and E1A for this recruitment (Figure 3.4 and 3.7). In addition, hPaf1 shared co-occupancy on the viral E2e, E3 and E4 promoters with both E1A and hBre1 and was required for efficient expression of these genes during infection (Figure 3.7 and 3.10). In contrast, hPaf1 was
Figure 3.11. hBre1 is a conserved target of the E1A proteins of multiple types of HAdV. (A) Evolutionary conservation of hBre1 binding with the E1A proteins of multiple HAdV types. HT1080 cells were transfected with hBre1 and either empty control vector, or the E1A genes from representative members of the 6 types of HAdV. E1A was immunoprecipitated and Western blots were performed using hBre1 specific antibody or HA to detect E1A proteins. (B) The E1A proteins from several types utilize hBre1 and hPaf1 for transcriptional activation. U-2 OS cells were treated with Ctrl non-specific siRNA, or an hBre1 or hPaf1 specific siRNA (shown in the inset panel). Cells were then transfected with a constitutive β-galactosidase reporter, a Gal4 responsive luciferase reporter and a vector expressing the Gal4 DBD alone, or Gal4 DBD fused to the N-terminus of the indicated HAdV E1A. Luciferase activity was measured. Results were normalized to β-galactosidase activity and siRNA treated groups were set as a fold to the Gal4 only transfected counterpart. A statistically significant decrease from Ctrl siRNA treatment within the Gal4 fusion group is indicated (* P<0.01). n=3
A

IP-αGFP

IB-αBre1

IB-αGFP

2% Input

IB-αBre1

IB-αGFP

IB-αBre1

IB-αGFP

E1A-GFP

GFP

B

Fold increase

Gal4 fusion

CTRL HAdV 3 HAdV 4 HAdV 5 HAdV 9 HAdV 12 HAdV 40

IB:hPaf1

IB:hBre1

IB:Actin

siCtrl sihBre1 sihPaf1

CTRL HAdV 3 HAdV 4 HAdV 5 HAdV 9 HAdV 12 HAdV 40

* * * * * * *
not recruited to the viral E1A and E1B promoters and was also not required for their expression (Figure 3.9B, 3.7B and 3.10A), suggesting that additional factors are involved in activating expression of these genes.

CR3 is known to be the primary activation domain of viral transcription in HAdV 5 E1A. This is evidenced in that the 289R E1A product activates viral transcription far more strongly than the 243R E1A product, which lacks CR3 (Ablack et al., 2010; Berk, 2005). Also, CR3 alone is able to activate viral transcription (Ablack et al., 2010; Mazzarelli et al., 1997). However, activation of early gene transcription is greatly enhanced by the presence of the N-terminus of E1A (Ablack et al., 2010; Wong and Ziff, 1994). This functional cooperation may be mediated in part by the co-recruitment of the p300/CBP histone acetyltransferases by the N-terminus and CR3 regions of E1A HAdV 5 (Pelka et al., 2009b; Wong and Ziff, 1994). The data presented here suggests that, in addition to p300/CBP, the N-terminus also contributes to viral early gene activation by recruiting hBre1. E1A dependent promoter occupancy by hBre1 in turns recruits the hPaf1 complex and in turn recruits additional factors that further modify viral chromatin, including the appearance of transcriptional activation marks such as H3K4 and H3K79 trimethylation (Figure 3.10B).

The N-terminus of the E1A proteins from multiple HAdV species interact with hBre1 (Figure 3.11A). This preservation of E1A interaction suggests a strong evolutionary conservation of functional utility. Knockdown of hBre1 or hPaf1 reduced transactivation by HAdV3, 4 and 9 (Figure 3.11B), corroborating that their conserved ability to bind hBre1 contributes to transcriptional activation, as we observed with HAdV 5 E1A. Although HAdV 40 E1A bound hBre1, knockdown of hBre1 or hPaf1 did not
Figure 3.12. The interaction of E1A with hBre1 serves two completely different purposes during HAdV infection. By disassociating the catalytically active Ube2b component from the hBre1 complex, E1A inhibits H2B monoubiquitination and suppresses transcription of IFN responsive genes (left panel). However, E1A then retasks the catalytically inactive hBre1 complex by using it as a scaffold to recruit hPaf1, leading to localized H3K4 and H3K79 trimethylation and stimulation of viral gene expression.
Cellular Chromatin (IFN gene bodies)

Viral Chromatin (promoters)
affect its transactivation ability. Interestingly, HAdV 40 E1A exhibits a deficiency in activating transcription of other early viral genes during infection (Van Loon et al., 1987), which could arise from its inability to productively utilize hBre1 to activate transcription. The interaction of HAdV 40 E1A with hBre1 may instead be primarily involved in perturbing the composition of the hBre1 complex to antagonize cellular IFN dependent gene expression, rather than enhancing E1A dependent transcriptional activation. Interestingly, HAdV 12 E1A differs from all the other E1A proteins tested in that it did not bind to hBre1 and similarly showed no requirement for hBre1 or hPaf1 in transcriptional activation (Figure 3.11B). The inability HAdV 12 E1A to bind hBre1 would be predicted to lead to weaker transactivation, and this may explain why HAdV 12 infection progresses more slowly and yields less virus compared to species C viruses, such as HAdV2/5 (Zhao et al., 2009). In addition, HAdV 12 infection also differs from species C infection in that it is also less able to evade the type I IFN response (Zhao et al., 2009), which would also be expected based on HAdV 12 E1A’s inability to bind hBre1.

The region spanning residues 4-25 on E1A, which is required for interaction with hBre1 (Fonseca et al., 2012) and to recruit Paf1 to early gene promoters (Figure 3.7A), is also required for binding to the p300 and CBP acetyltransferases (Egan et al., 1988; Mymryk et al., 1992). Both p300 and CBP are global cellular transcriptional regulators that function through their ability to acetylate proteins including histones and to recruit additional transcriptional regulators (Eckner et al., 1994; Ferrari et al., 2009, 2008; Horwitz et al., 2008b; Chen and Li, 2011; Bedford et al., 2010). As mentioned above, p300 and CBP have documented roles in transcriptional regulation of viral early genes (Pelka et al., 2009b; Wong and Ziff, 1994). It remains possible that the interaction of
E1A with p300/CBP may also assist hBre1 with the recruitment of hPaf1 or function independently to recruit hPaf1. This is supported by a number of our observations. Firstly, knockdown of hBre1 does not completely abrogate hPaf1 recruitment to viral early genes (Figure 3.9B). Secondly, an E1A mutant lacking residues 30-49, which binds to hBre1 but not p300/CBP, also exhibited reduced hPaf1 recruitment to HAdV early genes (Figure 3.7A). This reduction was not as pronounced as for the Δ4-25 mutant, which binds neither hBre1 nor p300/CBP, supporting roles for both hBre1 and p300/CBP in hPaf1 recruitment. Finally, knockdown of hPaf1 reduced viral early gene transcription to a greater extent than did knockdown of hBre1 (Figure 3.3 and 3.10A). Taken together, these data suggest that one function of p300/CBP in early viral gene expression may be to assist hBre1 with the recruitment of hPaf1. This could occur via direct interaction, alterations in chromatin acetylation that influence hPaf1 binding, or acetylation of other non-chromatin factors involved in hPaf1 recruitment. Although both hBre1 and p300/CBP require residues 4-25 for interaction with E1A, p300/CBP also requires residues 36-69 for binding (Egan et al., 1988; Mymryk et al., 1992). It remains to be determined if E1A can simultaneously interact with both hBre1 and p300/CBP, as has been shown for p300/CBP and pRb (Ferreon et al., 2009), or whether E1A interacts sequentially with these factors to prepare the local chromatin environment for efficient early viral gene expression.

Although no other viral proteins besides E1A have been shown to bind hBre1, several others target hPaf1. Recently, hPaf1 was shown to bind the Influenza A H3N2 NS1 protein (Marazzi et al., 2012). This interaction provides a mechanism by which Influenza may block the IFN response (Marazzi et al., 2012), similarly to what we have
reported to result from the interaction of E1A with hBre1 (Fonseca et al., 2012). It is interesting that these very different viruses have independently evolved to target the IFN response via these two interlocking epigenetic mechanisms. In addition to Influenza NS1, the HIV gene product Tat also interacts with hPaf1 (Sobhian et al., 2010; Marazzi et al., 2012). Tat recruits the hPaf1 complex along with numerous interacting partners to trimethylate H3K4 and activate viral gene expression from the HIV LTR (Sobhian et al., 2010). This is a very similar function to what we have observed with hPaf1 recruitment by E1A. It remains possible that NS1 may also utilize its interaction with hPaf1 to regulate viral transcription in a manner similar to HAdV E1A and HIV Tat, but this has yet to be confirmed. Correspondingly, the interaction of Tat with hPaf1 may also affect the type I IFN response. Nevertheless, HAdV, Influenza A and HIV have convergently evolved to target the cellular hBre1/hPaf1 complexes as a strategy to regulate transcription during infection. The roles of these two complexes in the transcription process are clearly separable, as removal of hPaf1 dependent methylation patterns has similar effects on both cellular and viral genes, whereas hBre1 dependent monoubiquitination of H2B is required for IFN dependent transcription, but is unnecessary for E1A mediated activation of E2e, E3 and E4 gene expression. How E1A compensates for the lack of active H2B monoubiquitination at the E2e, E3 and E4 transcription units remains unknown, but may be related to differences between HAdV chromatin and normal cellular chromatin.

In summary, we have determined that the interaction of E1A with hBre1 serves two completely different purposes during a HAdV infection (Figure 3.12). E1A inhibits H2B monoubiquitination and suppresses the type I IFN response by disassociation of
hBre1 from Ube2b. However, E1A then retasks the catalytically inactive hBre1 complex by using it as a scaffold to recruit hPaf1 and stimulate viral early gene expression. Achieving an inhibition of innate immunity, while simultaneously repurposing a target to enhance viral transcription, represents an elegant example of the incredible economy of action accomplished by a viral regulatory protein through a single protein interaction.

3.4 Materials and Methods

3.4.1 Cell lines and plasmids

Human adenocarcinoma A549, human fibrosarcoma HT-1080, and human osteosarcoma U-2 OS cells were grown at 37°C with 5% CO₂ in DMEM (Multicell) supplemented with 10% fetal bovine serum (Gibco). Plasmids were transfected into HT1080 and U-2 OS cells using Superfect reagent (Qiagen) following the manufacturer's recommendations. Transfection efficiency was typically, 70-80% for HT1080 and 40-50% for U-2 OS cell lines. After 48 hours in culture, transfected cells were used for experimentation. E1A’s from the HAdV types were cloned as fusions with GFP located at the C-terminus. All E1A N-terminus and CR3 clones used in luciferase experiments were fused at their N-terminus with the Gal4 DNA binding domain.

3.4.2 Quantitative RT-PCR

Total RNA was prepared with Trizol extraction (Invitrogen). A total of 1μg of RNA was reverse transcribed into cDNA by random priming using the One step RT-PCR kit (Qiagen) following the manufacturers' instructions. Quantification of cDNA was done...
Table 3.1. A List of Oligonucleotide Primers used in this chapter.

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<th>Region</th>
<th>Location</th>
<th>Forward</th>
<th>Reverse</th>
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using SYBR-Green Supermix for real-time qPCR (MyIQ, BioRAD) with oligonucleotide sequences that specifically recognize the indicated target. GAPDH was used as a control for total cDNA. Controls without reverse transcriptase were done for each RNA sample alongside the cDNA control. Results were normalized to the GAPDH and uninfected sample. The oligonucleotide sequences are listed in Supplementary Table S1.

3.4.3 RNAi knockdown

Downregulation of hBre1, RNF40, and hBre1 interacting proteins, including hPaf1 was performed using Silencer Select siRNA (Ambion). siRNA was delivered to cells via transfection with Silentfect (BioRad) following the manufacturer’s instructions: 3 hours after seeding cells, for a period of 48 hours. A scrambled siRNA was used as a control.

3.4.4 Virus infection of cells

All viruses are derived from the HAdV 5 dl309 background (Jones and Shenk, 1979) and express both the larger 289Rand smaller 243R E1A proteins (Egan et al., 1988). Cells were infected with WT (dl309), or a panel of HAdV containing the indicated E1A deletion mutations: ΔE1A (dl312), E1A Δ4-25 (dl1101), E1A Δ26-35 (dl1102), E1A Δ30-49 (dl1103). HAdV was used at a multiplicity of infection (MOI) of 5 pfu/cell. Cell cultures were infected at 50% confluence and left for 20 hours. Virus infection was found to be near 100% by fluorescence microscopy under these conditions using virus expressing GFP. Subconfluent cells were collected for further experimentation.
Table 3.2. A List of Antibodies used in this chapter.

<table>
<thead>
<tr>
<th>Reactivity</th>
<th>Description</th>
<th>Company</th>
<th>Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG</td>
<td>Rabbit polyclonal</td>
<td>Sigma</td>
<td>M-7023</td>
</tr>
<tr>
<td>Actin</td>
<td>Rabbit polyclonal</td>
<td>Sigma</td>
<td>A 2066</td>
</tr>
<tr>
<td>GFP</td>
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<td>Clonetech</td>
<td>632592</td>
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<tr>
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<td>Rabbit polyclonal</td>
<td>Bethyl Labs</td>
<td>A300-173A</td>
</tr>
<tr>
<td>H2B</td>
<td>Rabbit polyclonal</td>
<td>Millipore</td>
<td>07-371</td>
</tr>
<tr>
<td>H2BK120-monoubiquitination</td>
<td>Mouse monoclonal</td>
<td>Millipore</td>
<td>05-1312</td>
</tr>
<tr>
<td>H3K4 tri methylation</td>
<td>Mouse monoclonal</td>
<td>Abcam</td>
<td>Ab1012</td>
</tr>
<tr>
<td>H3K18 acetylation</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>Ab1191</td>
</tr>
<tr>
<td>H3K79 tri methylation</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>Ab2621</td>
</tr>
<tr>
<td>hBre1/RNF20</td>
<td>Mouse monoclonal</td>
<td>Sigma</td>
<td>R8904</td>
</tr>
<tr>
<td>M73 (E1A)</td>
<td>Mouse monoclonal</td>
<td>In house</td>
<td></td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>Ab26721</td>
</tr>
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<td>DSHB</td>
<td>TC31-9C12.C9</td>
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<tr>
<td>27F11 (hexon)</td>
<td>Mouse monoclonal</td>
<td>DSHB</td>
<td>TC31-27F11.C2</td>
</tr>
</tbody>
</table>
3.4.5 *Gal4 Luciferase assay*

Cells were transfected with a β-galactosidase reporter plasmid for normalization, a plasmid containing the luciferase gene driven by a 6xGal4 binding sequence, and the indicated Gal4 DNA binding domain fusion plasmids. After 48 hours, cells were lysed in 200µL using the supplied lysis buffer (Promega E397A). For detection of luciferase production, 50µL of lysate was mixed with 50uL of Luciferase Substrate (Promega E151A) immediately before detection of light as measured using a Berthold Lumat LB 9507. Results were then set to an empty plasmid control and further normalized via β-galactosidase activity as detected by ONPG (Bioshop) assays.

3.4.6 *Western blotting and co-immunoprecipitation*

Cells were lysed with NP40 lysis buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 0.1% NP-40) and protein concentrations were determined with BioRad protein assay reagent using BSA as a standard. 0.5mg of protein lysate was immunoprecipitated with 1 µg of GFP rabbit polyclonal (Supp. Table 2) at 4°C for 4 hours. 25 µg of protein was kept as 5% input, except as noted in the Figure legend of individual blots. After 3 washes in NP40 lysis buffer, complexes were boiled in 25 µL of sample buffer for 5 min. Proteins were separated on NuPage 4-12% Bis-Tris gradient gels (Invitrogen) and transferred onto a nitrocellulose membrane (Amersham). Membranes were blocked in TBS with 0.1% Tween-20 and 5% skim milk or BSA and blotted with the indicated primary overnight at 4°C. Details for the primary antibodies may be found in Table S2. Horseradish peroxidase conjugated secondary antibodies were detected using ECL Plus western
3.4.7 Chromatin immunoprecipitation (ChIP) and ChIP-reChIP assays

Approximately $10^7$ cells per sample were cross-linked with 1% formaldehyde at room temperature for 10 min. Cells were washed twice with ice cold PBS and harvested. Cell pellets were lysed in 1mL of cell lysis buffer (50 mM Tris-HCl [pH8.1], 10 mM EDTA, 1% SDS, and a protease inhibitor cocktail (Sigma) on ice for 10 min. Lysates were sonicated in an ultrasonic biorupter bath (Diogenode XL-2006 TO) to yield DNA fragment sizes of 200-500 base pairs. Samples were then centrifuged at 10,000xg for 10 min. 1 mg of protein was used for ChIP, 100 μg of this was kept as 5% input. Supernatants were then diluted 10-fold in dilution buffer (20 mM Tris-HCl [pH8.1], 1% Triton X-100, 2 mM EDTA, 150mM NaCl and protease inhibitors) and precleared with 50 μL of ChIP protein-A Sepharose (50% slurry of protein A-Sepharose containing 2.5 μg of salmon sperm DNA and BSA /mL) for 50 min at 4°C. Immunoprecipitations were performed overnight at 4°C using 5 μg of the indicated antibody found in Supp. Table 2. The next morning, 50 μL of ChIP protein-A Sepharose was incubated with each sample for 2 hrs. Beads were then washed once each with 500 μL of wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 150 mM NaCl), wash buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 500 mM NaCl), and wash buffer III (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH8.0) respectively and then washed twice with Tris-EDTA buffer. Immunocomplexes were extracted twice with 150 μL of elution buffer (1% SDS, 0.1 M NaHCO₃). For ChIP-reChIP, samples were then rediluted 10x with dilution buffer and
immunoprecipitation was repeated with a second antibody as indicated. After final elution, 12 μL of 5M NaCl was added to the 300 μL pooled elution and incubated at 65°C overnight to decrosslink the complexes. DNA was then purified using Quigen PCR Purification Spin Columns. Conditions for qRT PCR using SYBR Green were as per the manufacturers' directions. Briefly, each 15 μL reaction contained 80 nM oligos and 0.5 μL of ChIP DNA.

3.4.8 Statistical analysis

All experiments were carried out in duplicate with three replicates, with the exception of those shown in Figure 3.2 and Figure 3.9. Figure 3.2 and Figure 3.9 were carried out in duplicate with two replicates. Graphs represent the mean and standard error of the mean (S.E.M) of all experiments, while western blots are representative experiments. All numerical values represent means ± S.E.M. For blots, a representative example of the replicates is shown. Statistical significance of the differences was calculated using one way ANOVA and a Holm-Sidak post-hoc comparison to all other treatments in the experiment.

3.5 References


Hearing, P., and Shenk, T. (1986). The adenovirus type 5 E1A enhancer contains two functionally distinct domains: one is specific for E1A and the other modulates all early units in cis. Cell 45, 229–236.


Chapter 4:

Discussion

4.1 Thesis Summary

This thesis describes my studies of the HAdV E1A protein. Specifically, I describe the interaction of the N-terminus of E1A with the cellular E3 ubiquitin ligase hBre1 and the consequences thereof. In chapter 2, hBre1 was shown to monoubiquitinate H2B throughout the transcribed region of ISGs after stimulation of the innate immune response by IFN treatment or infection with defective virus (Figure 2.1). The monoubiquination of H2B was so highly upregulated by these stimuli, that large changes in the global levels of H2B-ub were easily detectable by western blot analysis (Figure 2.1). I showed that H2B-ub was required for transcriptional activation of a panel of ISGs (Figure 2.3). Further, introduction of E1A into the system resulted in global reductions in H2B-ub (Figure 2.4). I showed that this was a result of E1A binding hBre1 and blocking the recruitment of the E2 conjugase of the hBre1 complex, Ube2b, to ISG gene bodies (Figures 2.7, 2.9). This inability to properly recruit Ube2b resulted in a parallel reduction of H2B-ub at ISGs and corresponding decreases in ISG transcription, dampening the IFN response and enhancing virus growth (Figure 2.12). In chapter 3, I observed that hBre1 was also recruited to the viral genome by E1A and plays a role in early viral transcription. My initial experiments using a Gal4-E1A fusion with a luciferase reporter system showed a requirement for hBre1 in E1A dependent transcriptional activation (Figure 3.1). From here, I observed both a localization of hBre1 on the promoters of several HAdV early genes as well as a requirement for hBre1 in the transcriptional
activation of these early genes during infection (Figures 3.3, 3.4). As E1A disrupts the catalytic activity of the hBre1 complex in the context of cellular chromatin (Figure 2.7, 2.9) and recruitment of hBre1 to viral chromatin is not accompanied by H2B-ub (Figure 3.5), it appeared that the Bre1 complex was not functioning directly to modify viral chromatin. Instead, I hypothesized that hBre1, in the absence of its well characterized activity, may function in this context as a scaffold to make interactions with other cellular transcriptional activators. I tested the requirement of known hBre1 interacting proteins for their effects on E1A dependent transactivation in the Gal4-E1A fusion luciferase system. I discovered that E1A uses hBre1 to recruit the hPaf1 complex to viral genes for transactivation (Figure 3.6). A reduction in transcription of viral early genes by hPaf1 knockdown was observed with concomitant reduction in several hPaf1 linked hPTMs, including H3K4me3 and H3K79me3 (Figure 3.10). Overall, my results have uncovered a novel subset of genes affected by H2B-ub and hBre1, as well as a novel mechanism by which E1A rewires the cellular network by retasking cellular transcriptional regulatory proteins (Figures 2.14, 3.12).

4.2 The interaction between E1A and hBre1

Yeast Bre1 (yBre1) was originally identified through a susceptibility screen for Brefeldin A, a drug that inhibits transport of proteins from ER to Golgi (Murén et al., 2001). Later, in 2003, yBre1 was identified as an E3 ubiquitin RING ligase that recruits an E2 conjugase, Rad6 (yeast Ube2b homologue), to cellular chromatin. Along with the accessory factor Lge1, yBre1 and Rad6 are involved in the monoubiquitination of H2B at K123 in yeast (K120 in mammals) (Wood et al., 2003; Hwang et al., 2003). Building on
the work published in these seminal yeast papers, this complex was further characterized and the homologous mammalian complex was identified. The mammalian complex, is comprised of hBre1/RNF20, RNF40/RBP95, Rad6A&B/Ube2A&B and the understudied WAC1 subunit. The hBre1 complex does not appear to function at all transcribed genes, but instead has been reported to catalyze the formation of H2B-ub on several distinct gene sets (Kim et al., 2005, 2009; Zhang and Yu, 2011). These include genes regulated by p53, MLL, HOX, Notch and estrogen receptor transcription factors, as well as directly influencing important cellular processes such as the DNA damage response and stem cell differentiation (Moyal et al., 2011; Bray et al., 2005; Mohan et al., 2010; Nakamura et al., 2011; Prenzel et al., 2011; Shema et al., 2008; Zhu et al., 2005; Chen et al., 2012; Karpiuk et al., 2012; Wang et al., 2013). In essence, hBre1 is required for the efficient transcription of a very specific subset of highly important cellular genes, many of which are highly relevant to cancer. As such, the targeting of hBre1 by a viral oncoprotein like E1A is perhaps not unexpected.

The N-terminal region of E1A was found to interact with yBre1 in a yeast 2-hybrid library screen (Appendix Figure 4.11). This interaction was further confirmed with hBre1 in mammalian co-immunoprecipitation experiments (Figures 2.7, 2.8). Using a panel of well characterized E1A deletion mutants, hBre1 was shown to interact with E1A through residues 4-25. Binding did not require the adjacent region, as seen by its ability to interact with E1A containing deletions of residues 26-35 and 30-49 (Figure 2.7, 2.8). Importantly, this pattern of interaction differentiates the hBre1 interaction from other well defined E1A interactors, such as p300/CBP (Figure 2.4). The same interaction profile was seen for the RNF40 component of the hBre1 complex. RNF40 is an
orthologue of hBre1, and the two proteins share 57% identity and 72% similarity (Figure 4.1). Considering this, the interaction surface on E1A could potentially directly bind one or both of these proteins. However, studies of co-precipitation using purified protein will have to be performed to conclusively determine which is directly targeted by E1A.

Of particular interest, the mechanism by which E1A blocks the interaction between the hBre1/RNF40 and Ube2b subunits of the hBre1 complex remains to be determined. The most probable explanation is direct steric hindrance via a competition between E1A and Ube2b for interaction with hBre1. The interaction between hBre1/RNF40 and Ube2b has been mapped to between residues 230 and 381 of hBre1 (Kim et al., 2009). Going forward, it will be important to determine the interaction site on hBre1 utilized by E1A and further map the Ube2b binding sites on hBre1. Other possible explanations for the ability of E1A to block Ube2b association with hBre1 include a change in conformation as a result of E1A binding to hBre1 or indirect steric hindrance by the large multi-protein complexes with which E1A may be associated with (Figure 4.2).

4.3 The modulation of IFN signaling by E1A

E1A has previously been shown to block the IFN response (Anderson and Fennie, 1987; Kalvakolanu et al., 1991; Reich et al., 1988). In my studies, I have uncovered a novel hPTM based mechanism which serves to block ISG transcription. The interaction of E1A with the hBre1 complex inhibits H2B-ub by disrupting the catalytic activity of the hBre1 complex (Figure 2.14). However, other papers have implicated effects of E1A on
Figure 4.1. Alignment of hBre1/RNF20 and RNF40. Sequence alignment shows 57% identity and 72% similarity using Blosum 35 score tables. Sequence identity is indicated by black shading, while sequence similarity in the absence of identity is indicated by grey shading.
p300/CBP, Stat1 and reductions in expression/activity of trans-acting factors necessary for the phosphorylation events preceding the formation of ISGF3 (Leonard and Sen, 1996; Look et al., 1998; Zhang et al., 1996; Bhattacharya et al., 1996). It should be noted that these papers all attribute the ability to downregulate IFN signaling to the E1A Δ4-25 deletion mutant. In addition, this work is generally correlative, without showing direct evidence of p300/CBP or Stat1 involvement. Further, they analyze signaling by type II IFN (gamma or gamma associated site genes) rather than type I IFN, which are mostly non-overlapping gene sets with different transcriptional regulation (Samarajiwa et al., 2009; Rusinova et al., 2013). Lastly, another publication has shown that p300/CBP, as well as the H3K18ac hPTM which these proteins catalyze, is found at type I IFN genes before stimulation (Escoubet-Lozach et al., 2011). This is thought to be a preactivation mechanism that allows for faster initiation and, ultimately, faster production of an antiviral state. However, p300 was not found to have been displaced from type I IFN genes until after the repression of these genes by virus infection, noted as "Class I" in these studies (Horwitz et al., 2008b; Ferrari et al., 2009). These data suggest that, although blocking p300/CBP and Stat1 may play a role in the repression of ISGs during HAdV infection, interference with their function by HAdV may not necessarily be sufficient to block IFN response. As such, our observation that the interaction of E1A with hBre1 blocks ISG transcription is not contradicted by previously published data. Indeed, antagonism of H2B-ub by E1A may cooperate with the mechanisms proposed in those studies to provide a more complete understanding of what is actually occurring during infection. In fact, the possibility remains that these are interconnected modalities of ISG suppression by HAdV. As the mechanism by which the hBre1 complex and Stat1
Figure 4.2. Possible mechanisms by which E1A blocks the hBre1 interaction with Ube2b. (A) Under normal conditions, Ube2b is able to bind hBre1 between residues 230 and 381 (white on hBre1). However, this is blocked in the presence of E1A. The possible mechanisms which may attribute this result could include (B) direct steric hindrance, whereby E1A competes for the same binding surface as Ube2b, (C) the binding of E1A causes a conformational change, altering the binding site for Ube2b on hBre1 in such a way that Ube2b can no longer interact, or (D) indirect steric hindrance where E1A binds to a different site on hBre1 from Ube2b, but masks the Ube2b binding site due to the recruitment of large E1A interacting complexes.
A

Cellular Chromatin (IFN gene bodies)

Normal conditions

B

Cellular Chromatin (IFN gene bodies)

Direct steric hinderance

C

Cellular Chromatin (IFN gene bodies)

Indirect steric hinderance

D

Cellular Chromatin (IFN gene bodies)

Conformational change
are recruited to ISGs remains unclear, it is possible that these complexes interact and are recruited together to ISGs. What is clear in these separate studies is that Stat1, p300, and the hBre1 complex are all required for efficient ISG transcription. Going forward, even more detailed studies of transcriptional control of ISGs will remain an important avenue of research. These may allow a reactivation of a suppressed IFN response that will help clear a serious infection or help limit an overactive life threatening IFN response.

4.4 hPaf1 interaction with hBre1

Previous work has established an interaction between hBre1 and the hPaf1 complex (Kim and Roeder, 2009). This interaction is necessary for H2B-ub by the hBre1 complex as well as H3K4me3 and K79me3, which are induced by hPaf1 through the MLL/SET1 and DOT1 complexes (Figure 1.7) (Kim and Roeder, 2009; Marazzi et al., 2012; Wood et al., 2003). It has been found that the hBre1 complex co-precipitates with the full hPaf1 complex in in vitro and ex vivo cell culture models through an interaction with hPaf1 (Kim et al., 2009; Kim and Roeder, 2009; Wood et al., 2003; Mulder et al., 2007). In yeast, only the histone modification domain of the Rtf1 component of the hPaf1 complex has been shown necessary for all of these hPTM effects (Tomson and Arndt, 2012). Interestingly, this effect is independent of the Rtf1 DNA binding domain. In fact, removal of the DNA binding domain causes non-specific hPTM induced activation of genes (Piro et al., 2012). This may suggest that the hPaf1 complex is a controller of H2B-ub, H3K4me3 and K79me3 through regulating the hBre1 complex or via scaffolding of these genetically interacting complexes. We have found that hBre1 is able to, in the absence of Ube2b, recruit the hPaf1 complex to virus early genes (Figures 3.7,
Given these previously stated findings, the hBre1 interaction with Rtf1, through hPaf1, may operate independently of H2B-ub to recruit the MLL/SET1 and DOT1 complexes. Recruitment could, in turn, lead to the local generation of the respective hPTMs created by these complexes. To confirm this model, future work demonstrating that recruitment of the hPaf1 complex member, Rtf1, by E1A is essential for the observed hPTMs induced on the viral genome would be necessary.

These results do not, however, explain the lack of hPaf1 recruitment to cellular ISGs in the presence of E1A. Under inducing conditions, hBre1 was found to be associated with ISGs and this resulted in H2B-ub throughout the gene body and H3K4me3 and H3K79me3 on the gene promoter (Figure 2.3). In contrast, during viral infection, E1A was able to block these hPTMs by disrupting the hBre1 complex. However, it should be noted that hBre1 is still present at ISGs, co-localizing with E1A (Figure 2.9). Considering the interaction of the hBre1 complex with hPaf1 and the localization to ISGs, I expected that hPaf1 would be recruited to ISGs and be able to perform its activating functions with hBre1. This is, however, clearly not seen (Figure 3.8). In the context of cellular ISGs, hPaf1 is, along with Ube2b, specifically blocked from association with ISGs in the presence of E1A. No mechanistic evidence exists to explain this finding. It is possible that there may be a difference in the 3-dimensional loading of the hBre1 and E1A proteins on the ISG and viral chromatin that accounts for this difference. As hBre1 is found on ISGs in the absence of E1A, it would seem prudent to assume that hBre1 loads onto the chromatin first in this context and that E1A targets hBre1 bound at ISGs. In contrast, on the viral chromatin, hBre1 is not present in the absence of E1A. Indeed, hBre1 appears to be recruited by E1A to viral chromatin. The
altered order of recruitment between these two sets of hBre1 regulated genes suggests that in the context of cellular chromatin, hBre1 may not be completely exposed to the cellular environment due to being coated by E1A and its associated complexes. However, in the context of viral genes, E1A may be proximal to the viral chromatin, with hBre1 placed in a more exposed location that allows for interaction with the hPaf1 complex.

Future functional studies of the roles of the hPaf1 complex in viral transcription will need to also focus on the means of regulation by the hPaf1 complex. My data shows a positive effect on general transcription by hPaf1 recruitment. However, the hPaf1 complex is involved in several stages of transcriptional control, including transcriptional initiation and elongation (Jaehning, 2010). This leaves four distinct possibilities for the mechanism behind the positive influence of the hPaf1 complex in viral early gene transcription: increased transcriptional initiation, more efficient elongation, a combination of both initiation and elongation or through transcriptionally independent processes such as altered mRNA processing.

### 4.5 E1A control of viral early gene transcription

HAdV E1A mediated control of transcription has been historically used as a paradigm of transcriptional activation of cellular genes. E1A contains two regions that independently function as transcriptional activation domains when fused to a heterologous DNA binding domain: the N-terminus and CR3. Although the N-terminus of E1A contains a transactivation domain, CR3 is considered to be the primary transactivation domain. Formation of the transcriptional activation complex by E1A CR3 includes binding of
activation factors including mediator and TBP and modulators of transcription including the acetyltransferases Gcn5 and pCaf, the proteasome and other transcription factors (Geisberg et al., 1994; Boyer et al., 1999; Stevens et al., 2002; Webster and Ricciardi, 1991; Ablack et al., 2010, 2012; Rasti et al., 2006). These allow for transcriptional activation and modulation for optimal levels of target gene transcription. As well, the N-terminus assists CR3 in recruiting some of these factors, as in the case of p300/CBP, pCaf, and TBP (Pelka et al., 2009b, 2008; Frisch and Mymryk, 2002). E1A also associates with many other transcriptional regulators. Indeed, targets of several regions of E1A may be used for localizing the E1A associated transcriptional machinery to cellular chromatin for modulating expression of specific groups of genes. These include p300 and pRb which are targeted by the N-terminus and CR2 respectively (Horwitz et al., 2008b; Ferrari et al., 2009). It has been hypothesized that E1A uses these cellular proteins as landmarks to target regions where activation states need to be changed. In the case of pRb, E1A targets repressed cell cycle regulatory genes for activation. In the case of p300/CBP, E1A targets activated differentiation specific genes for repression (Horwitz et al., 2008b; Ferrari et al., 2009). A similar situation has been reported for the E1A target CtBP. E1A targets the CtBP co-repressor through CR4 and blocks the repressive effects of CtBP at these genes (Johansson et al., 2005; Zhao et al., 2006). Then, in association with a second CtBP interaction found within CR3, E1A can induce CtBP target genes by recruiting cellular activation machinery for further activation of these initially repressed genes (Bruton et al., 2008). The mechanisms by which E1A modulates cellular gene transcription are still not fully developed, but are being actively pursued by multiple groups of investigators.
Regulation of transcription from the E1A gene itself is well characterized. As E1A is produced immediately after viral DNA is transported into the nucleus, the initial stage of E1A transcription occurs in the absence of virally encoded transactivators and relies on cellular cis acting factors on the viral DNA (Hearing and Shenk, 1983, 1986). This initial level of transcription from the E1A gene is augmented by E1A itself, in a positive feedback loop. E1A then similarly activates the other viral early genes.

Despite the plethora of research on E1A mediated transcription, little is known as to how E1A specifically activates viral transcription. Multiple studies have shown that this is a dynamic and complex process, which appears to be carefully orchestrated and involves many factors. Early results showed that regulation of HAdV early genes may not be through a single mechanism. As one specific example, the HAdV5 host range 440 mutant (lacking E1A Δ141-289) is able to activate the E1B and E4 transcripts to wildtype levels, however, the E2 and E3 transcripts were not detectable (Solnick, 1981). Despite previous findings showing that E1A is readily detectable by ChIP analysis at all viral early genes (Rasti et al., 2006), each is differentially affected by E1A mediated transcriptional activation (Figures 3.3, 3.4, 3.7, 3.10). As such, viral early genes are individually regulated in a very context dependent manner. This is similar to the observation by the labs of Berk and Kurdistani, which found that E1A modulates transcription of cellular genes in many ways, and demonstrated that these can be broadly grouped into three categories based on temporal changes in expression and occupancy by E1A (Horwitz et al., 2008b; Ferrari et al., 2009). It seems that the E1A complexes at each viral early gene are not equivalent and this may be a result from different combinations of cis-acting elements at HAdV early genes. These may affect
transcription by either assisting in the recruitment of factors directly or in co-operation with E1A. Previous data has shown that the E3 and E4 genes are differentially affected in the absence of p300/CBP recruitment (Pelka et al., 2009b). Indeed, my studies of the role of hBre1 in regulating expression from viral early genes shows similar results. Specifically, hBre1 knockdown drastically reduces E4 expression (15% of wt), only moderately reduces E3 (57% of wt) and has no statistical effect on E1A (95% of wt) (Figure 3.1). This provides further evidence that E1A differentially regulates early gene expression via a variety of distinct mechanisms. Similar results can be seen for endogenous cellular transcriptional regulators. As one example, ZNF217 can activate or repress gene expression in a context dependent manner. In most cases, ZNF217 represses transcription by recruiting repressive complexes such as CoREST to actively methylate DNA CpG islands (Thillainadesan et al., 2012) and LSD1 to demethylate H3K4me3, which is an activating hPMTs (Murray-Stewart et al., 2013; Musri et al., 2010). However, ZNF217 may also participate in transcriptional activation by recruiting CoREST and LSD1 to demethylate H3K9me3 and H3K56me3, which are repressive hPTMs (Musri et al., 2010; Sakane et al., 2011). Indeed, HIV Tat recruits ZNF217 and LSD1 for the purpose of transcriptional activation by demethylating H3K51 monomethylation at viral LTRs (Sakane et al., 2011). Similarly to E1A modulation of hBre1 and hPaf1, the context dependent activation or repression functions of ZNF217 are not well understood. Overall, transcriptional activation of viral genes by E1A may provide a useful model of non-static, context dependent transcriptional activation in which hPTMs and cis acting factors regulate transcriptional complexes.
4.6 E1A control of cellular transcription

As described above, a significant amount of research has been applied to study the function of E1A on cellular transcription. A seminal set of papers published by the laboratories of Drs. Berk and Kurdistani have placed the mechanistic implications of p300 and pRb binding by E1A into a global context using ChIP and cDNA microarray experiments (Horwitz et al., 2008b; Ferrari et al., 2009). Despite the intensive characterization of these interactions in these and other publications, including structural interaction data (Pelka et al., 2009b; Whyte et al., 1988; Loewenstein et al., 2012; Kadeppagari et al., 2009; Ferreon et al., 2009), and their importance in E1A mediated transcriptional control, no perfectly generalizable global effect was observed. Instead, genes were categorized into classes dependent on their temporal patterns of activation or repression of transcription. However, the initial activation state of a given gene did not necessarily correlate with transcriptional outcomes, as some inactive genes were left inactive and some active genes were left active. Further, many cellular genes repressed by pRb prior to infection remained repressed despite E1A recruitment. Similarly, many genes which were transcriptionally activated by p300/CBP before infection remained active post infection despite E1A localization. These results clearly indicate that E1A does not simply function to alter the state of transcription on the cellular chromatin by overriding all aspects of regulation. Similarly to viral chromatin, the effects of E1A appear context dependent, likely being differentially regulated by cellular cis elements and transacting factors. The most probable situation is that E1A is able to globally modify the transcriptional state of cellular genes, while being further regulated, biochemically and genetically, by context dependent interactions with cellular and viral
proteins. This would put specific subsets of genes into a more controlled context, which would better benefit viral infection. As such, although p300/CBP and pRb may be general factors with which E1A alters global cellular transcriptional profiles, other interacting proteins may help to modulate these activities on specifically targeted gene sets. This would lead to the exquisite and reproducible control of cellular transcription despite infection of a wide range of cell types with different cellular milieus and growth conditions. Further, this suggests that additional study of the more transient or less globally affected targets of E1A may provide insight into the complex control of cellular transcription in which these proteins participate.

4.7 Significance of this Research

An important driver in research is often the satisfaction of the innate human need for discovery, knowledge and answers to new, previously unanswered questions. Importantly, in a societal context, these discoveries should, and often will, lead to improvements in the human condition and positive life outcomes. My data has provided insight into the cellular activities of both the hBre1 complex as well as the hPaf1 complex. Further, they have provided valuable understanding of the requirement for hBre1 in E1A mediated evasion of the IFN response and transcriptional activation of viral early gene transcription. More abstractly, this study underlines the vast wealth of undiscovered knowledge with which HAdV may be a useful tool in probing the complexities of transcriptional control. As well, my work exposes new aspects of cellular transcriptional control and reveals how immature our grasp is of the intricacies of some important aspects of cellular gene expression. Moving forward, a deeper
understanding of cellular processes including cellular transcription will allow for more
discrete and directed manipulation of physiological and cellular activity in the treatment
of disease. Furthermore, it would seem that HAdV and E1A still have much more
information to share with us in our goal to further our knowledge of biological science.

4.8 References

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Appendix 1. BLAST results for yeast 2-hybrid sequencing data for extracted clones S1 and S4. Positive results from yeast 2-hybrid library screen of the yeast genome were picked and plasmids were purified and sequenced. BLAST results showed that clones S1 and S4 were identical copies of yBre1.

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Appendix 2. Permission to publish Fonseca et. al., 2012, Cell Host & Microbe.

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Curriculum Vitae

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Education

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* Award-winning publication (see ‘Research Awards and Accomplishments’ section for details)
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**Conference Poster Presentations:**


**Conference Oral Presentations:**


3. The Adenovirus E1A Oncogene Modulates hBre1 Chromatin Modification Activity and Blocks Interferon Signaling. *Fonseca GJ,* Thillainadesan G, Yousef AF, Torchia J, and Mymryk JS. International DNA Tumor Virus Conference. ICGEB.
Trieste, Italy. July 2011.


*Award-winning presentation (see ‘Research Awards and Accomplishments’ section for details)

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Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, Canada, Effective: 09/2010, Ending: 08/2011, $7,204

Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, Canada, Effective: 09/2009, Ending: 08/2010, $6,672

Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, Canada, Effective: 09/2008, Ending: 08/2009, $6,672
Schulich Graduate Enhancement Scholarship, Schulich School of Medicine and Dentistry, Canada, Effective: 09/2007, Ending: 08/2009, $10,000
Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, Canada, Effective: 09/2007, Ending: 08/2008, $6,672

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September 2009-December 2009
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