

**Identification and validation of small molecules inhibiting
human adenovirus replication**

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Abstract

Human adenovirus (HAdV) mainly causes minor illnesses, but can lead to severe disease and death in both immunocompromised and immunocompetent patients. In such cases, the current standards of treatment often do not improve disease outcome and no approved antiviral therapy against HAdV exists. Since HAdV relies on cellular machinery to assist in the progression of the virus lifecycle, we hypothesized that small molecules targeting certain cellular proteins/pathways, without severely affecting cell health, may serve as effective anti-HAdV compounds. Thus, we aimed to identify novel inhibitors of HAdV, and investigate the molecular mechanism to determine new therapeutic targets for intervention in HAdV infection. We first examined the antiviral properties of pan-histone deacetylase (HDAC) inhibitor SAHA and found that the drug affects multiple stages of the HAdV lifecycle, resulting in significant reductions in virus yield. SAHA was effective in decreasing gene expression from clinically relevant HAdV serotypes. Subsequent investigations on the role of HDACs in HAdV infection led us to determine that class I HDAC activity, mainly HDAC2, is necessary for optimal viral gene expression. Using a wildtype-like HAdV reporter construct that allows us to monitor virus replication by fluorescence microscopy, we then designed an efficient system for screening small molecules to identify novel HAdV inhibitors. We screened over 1300 small molecules, and the screen was sensitive enough to detect compounds with both robust and modest antiviral activity. Several positive hits were validated to reduce HAdV gene expression and yield from infected cells. Further investigation on the efficacy of these compounds and the mechanism behind their inhibition of HAdV can lead to the discovery of new pharmacological targets and the development of more effective antivirals.

Dedicated to the loving memory of my wonderful grandparents. You never stopped believing in me and encouraging me, and you were always proud of my smallest accomplishments. I know you would have been the most delighted at the successful completion of my Ph.D.

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List of Abbreviations

293: human embryonic kidney cells	MRC-5: human lung fibroblasts
A549: human lung adenocarcinoma-derived epithelial cells	mRNA: messenger RNA
ADP: adenovirus death protein	MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
bp: base pair	PBST: phosphate-buffered saline with Triton X-100
CAR: coxackie adenovirus receptor	PFU: plaque-forming units
CDK: cyclin-dependent kinase	pIX: adenovirus protein IX
cDNA: complementary DNA	Pol: adenovirus DNA polymerase
ChIP: chromatin immunoprecipitation	pTP: pre-terminal protein
CMV: cytomegalovirus	PVDF: polyvinylidene fluoride
CPE: cytopathic effects	pVII: adenovirus protein VII
DBP: DNA-binding protein	qPCR: quantitative polymerase chain reaction
DMEM: Dulbecco's modified eagle medium	RAR β : retinoic acid receptor β
DMSO: dimethyl sulfoxide	Rb: retinoblastoma protein
dsDNA: double-stranded DNA	RFP: red fluorescent protein
E1, E2, E3, E4: adenovirus early region 1, 2, 3 and 4	RIPA: radioimmunoprecipitation assay
FBS: fetal bovine serum	RNase: ribonuclease
FDA: Food and Drug Administration	RSV: respiratory syncytial virus
fgAd: first generation adenovirus vector	RT-PCR: reverse transcription polymerase chain reaction
GFP: green fluorescent protein	SAHA: suberanilohydroxamic acid
H3/4: histone 3/histone 4	SD: standard deviation
HAdV/Ad: human adenovirus	SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
HAT: histone acetyltransferase	siRNA: small interfering RNA
HCV: hepatitis C virus	siControl: control siRNA
HDAC: histone deacetylase	SIRT: sirtuins
hdAd: helper-dependent adenovirus vector	ssDNA: single-stranded DNA
HepG2: human liver epithelial cells	TAF: template-activating factor
hpi: hours post infection	TBST: tris-buffered saline with Tween 20
HSV: herpes simplex virus	tetrazolium
HRP: horseradish peroxidase	TP: adenovirus terminal protein
HTS: high throughput screen	TSA: trichostatin A
IgG: immunoglobulin G	TSS: transcription start site
IP: immunoprecipitation	UXP: adenovirus U exon protein
ITR: inverted terminal repeat	VA RNA: virus-associated RNA
kb: kilobase	VPA: valproic acid
kDa: kilodalton	wt: wildtype
MEF: mouse embryonic fibroblasts	
MEM: minimum essential media	
MLP: major late promoter	
MLV: murine leukemia virus	
MOI: multiplicity of infection	

Chapter 1:

General Introduction to Adenovirus Biology and Disease

Human adenovirus (HAdV) is a non-enveloped DNA virus that mainly causes self-limiting illnesses in most patients, but can lead to severe disease and death in immunocompromised individuals. HAdV-induced disease is prevalent in confined populations such as daycare centres, hospitals, retirement homes and military training venues (1).

HAdV was first isolated from the adenoid tissue and determined to be the causative agent in some respiratory infections in the 1950's (2, 3). Over 80 HAdV serotypes, which are grouped into seven species (A-G), have been identified since then (4). Of these, serotypes 2 and 5 (species C) have been extensively studied. Investigations on the association of viral proteins with cellular components have led to the elucidation of various host protein/pathway functions. For example, RNA splicing was first identified in HAdV, and the histone acetyltransferase (HAT) p300 and chaperone EP400 were discovered through their interaction with the HAdV E1A protein (5-8). Furthermore, examination of the HAdV infection process and lifecycle provided novel information on fundamental cellular processes, including DNA replication, cell cycle progression and RNA splicing (9). Thus, studies on HAdV have led to significant insights on virus biology, infection process, host-pathogen interactions and manipulation of cellular processes (10, 11).

1.1 HAdV biology: the viral genome

HAdV is a nuclear virus with replication and progeny virion formation occurring within the nucleus of the infected cell (10). The majority of our studies were conducted with HAdV-5, which has a ~36 kb double-stranded genome that encodes over 40 proteins within its “early” and “late” transcription units (figure 1.1), which are expressed before and after viral DNA replication, respectively (9, 12). Both ends of the linear viral genome contain inverted terminal repeats

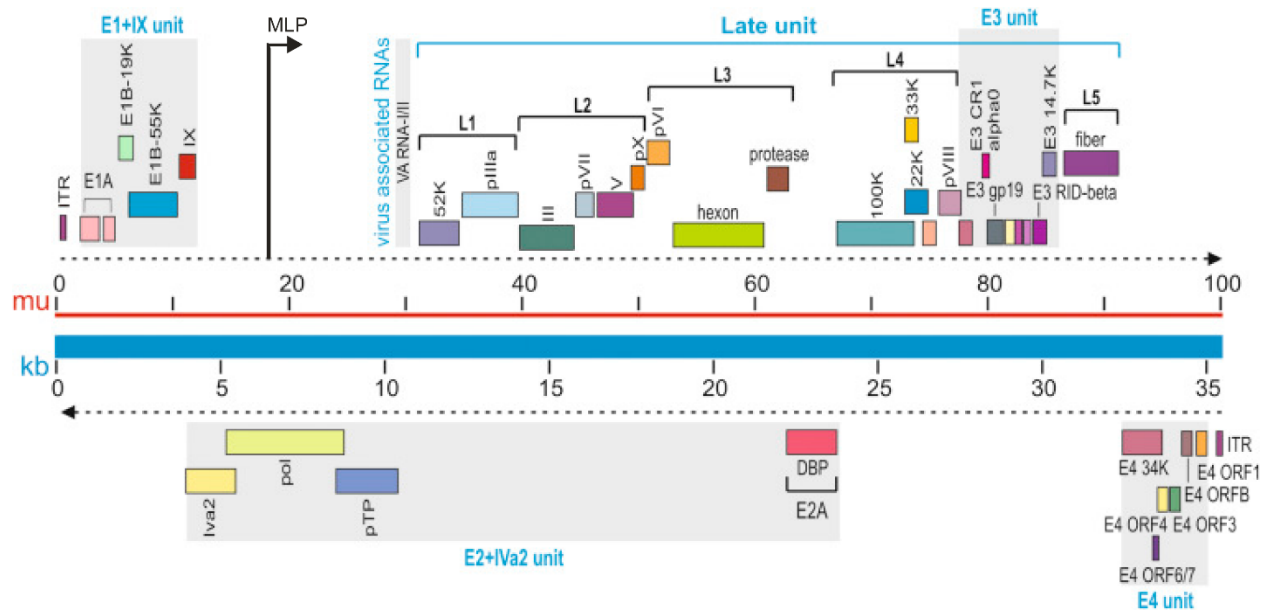


Figure 1.1 Schematic representation of the complex HAdV-5 genome (36 kb). Relative locations of the early (E1-E4) and late (L1-L5) coding regions are shown in terms of kb as well as mapping units (mu). The genome packaging sequence (not shown in this diagram) is located adjacent to the left inverted terminal repeat (ITR), before the E1 transcription unit. The major late promoter (MLP) is located at ~17 mu. This diagram has been adapted from Lee *et al.*, figure 1 (13).

(ITRs), which serve as the origins of replication (figure 1.1). A packaging sequence required for genome encapsidation is located beside the left ITR (14).

1.1.1 The early genes

The early regions (E1A, E1B, E2, E3 and E4) are the first to be transcribed during infection, and the proteins from these regions activate transcription of other viral regions and/or make the cellular environment more conducive to virus replication (15-17). The early region 1A (E1A) proteins are the first to be expressed from the infecting virus, and they are crucial for efficient viral gene expression and replication as they stimulate expression of all other viral genes (18). Differential splicing of the primary E1A transcript produces five different transcripts, of which, the 13S and 12S mRNAs are the most abundant in early infection and the 9S is the major species in late infection (19). However, all five forms of the E1A protein can be detected *in vitro* during late infection. The major forms, the 289 residue (R) and the 243R (resulting from the 13S and 12S mRNAs, respectively), interact with a variety of cellular proteins including cell cycle regulators, epigenetic regulators and transcription factors including p21, p300/CBP and E2F1 (5, 6, 20-24). These interactions, among other functions, serve to enhance viral gene expression, alter cellular gene expression profile, modulate host protein functions, and induce mitogenic activity within the host cell to promote a productive infection (9, 17, 25-27). The 55 kDa protein product from the E1B region has been implicated in ubiquitination of cellular proteins (e.g. p53), inactivation of cellular DNA damage response and regulation of viral mRNA export (28, 29).

The early region 2 (E2) consists of two transcriptional units, E2A and E2B, which have separate polyadenylation sites and encode proteins required for viral DNA replication (30). E2A

codes for the DNA-binding protein (DBP), while E2B codes for the precursor terminal protein (pTP) and the viral DNA polymerase (Pol). In the protein-primed HAdV DNA replication process, pTP-bound viral DNA serves as the template (figure 1.2). Formation of a pre-initiation complex consisting of the three E2 proteins and several cellular proteins precedes initiation of replication, and replication begins with Pol covalently attaching a dCMP molecule to the pTP in the complex (31, 32). The 3'-OH group of the dCMP then serves as a primer for elongation by Pol through a strand-displacement process, during which, DBP forms multimers on the displaced single-stranded DNA (ssDNA) (figure 1.2) (33, 34). Although the main function of DBP is to drive template unwinding, it may also protect the ssDNA by preventing immune and DNA damage responses that can be elicited in the cell against naked DNA from foreign species (31, 33, 35). During virion maturation in the late phases of infection, pTP is cleaved by an HAdV-encoded protease, resulting in the mature terminal protein (TP) that remains associated with each 5' end of the newly-replicated DNA within progeny virions (36, 37).

Proteins from the E3 region have immunomodulatory functions within the infected host cells (15, 38, 39). The E3 region also encodes the HAdV death protein (ADP), which increases the efficiency of cell lysis and virus progeny release (40). Since the E3 proteins are not essential for HAdV replication *in vitro*, this region is frequently deleted, and often replaced with foreign expression cassettes for generating viral vectors with therapeutic transgenes or fluorescent protein genes to monitor virus entry/localization. Lastly, the E4 proteins alter cellular protein stability/activity and signalling pathways to regulate viral RNA splicing, prevent DNA damage response, etc. (16).

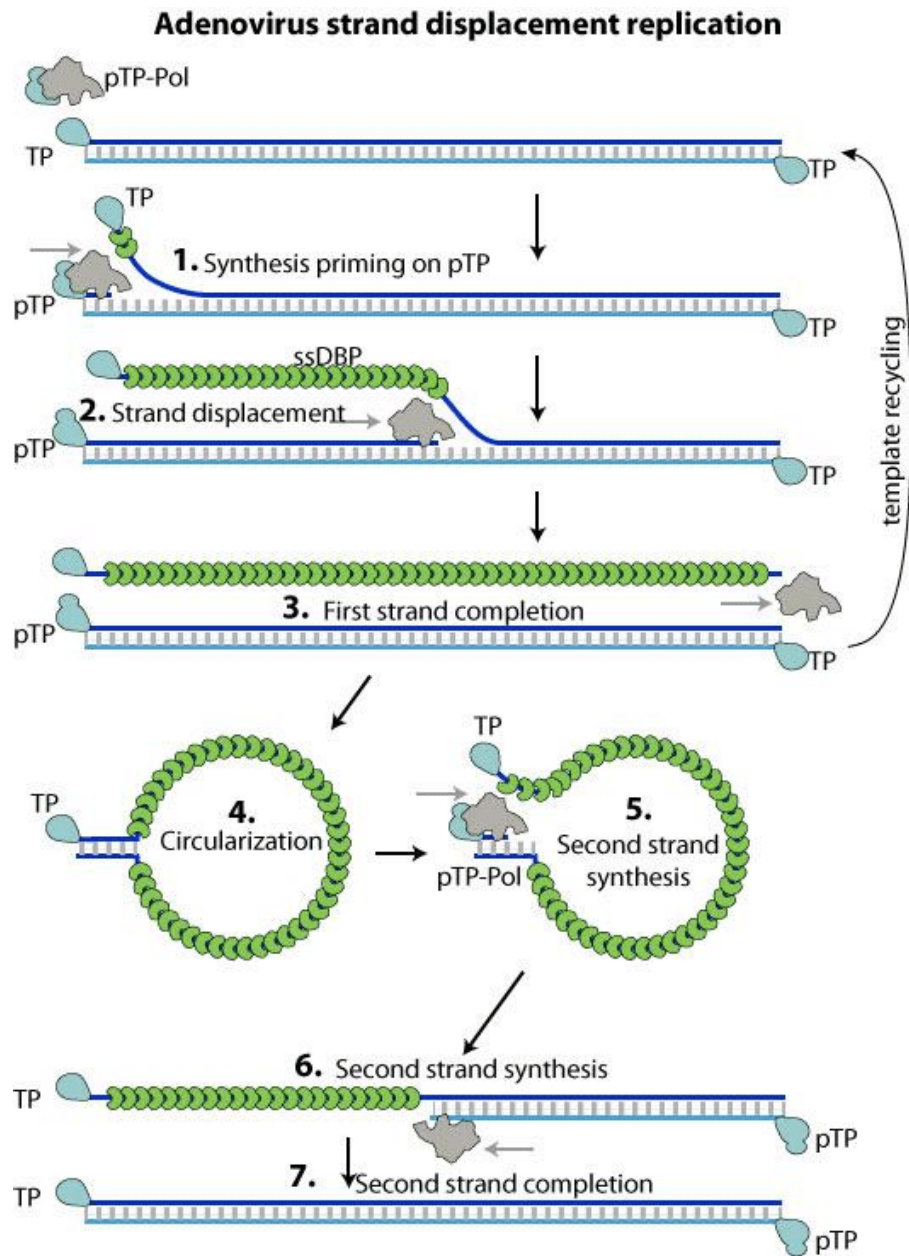


Figure 1.2 Illustration of HAdV DNA replication. The linear genome of HAdV is replicated through a protein-primed, strand-displacement method. Please see text in section 1.1.1 for details. This diagram has been obtained from the ExPASy Bioinformatics Resource Portal (41).

HAdV-based vectors are one of the most commonly used vehicles for delivery of transgenes in pre-clinical research and in gene therapy clinical trials (42, 43). Most of these vectors (known as first-generation or fgAd vectors) are deleted of both the E1 and E3 regions, making them replication-deficient in most cell types. The fgAd vectors are ideal for *in vitro* and short-term *in vivo* studies as low-level expression from the remaining coding regions of the viral genome eventually result in immune-mediated vector elimination (44). Thus, subsequent generations of HAdV-based vectors for gene therapy contain deletions in the E2/E4 regions, or are devoid of all viral protein coding sequencing (e.g. helper-dependent HAdV or hdAd vectors) (45, 46).

1.1.2 The late genes

The HAdV genome encodes five late transcription units, L1-L5 (figure 1.1), which code for virus structural/capsid (e.g. penton, hexon, fiber) and core (e.g. protein VII, protease) proteins (figure 1.3) (47). Regulation of late gene expression is under the control of a common major late promoter (MLP), which is active at a very low level during early infection (48, 49). The MLP is fully activated following the onset of viral DNA replication in late infection (50). A single transcript containing five different polyadenylation sites is first produced, and five separate transcripts are then generated by alternative splicing (51). Another late promoter within the L4 region was later identified, which initially allows low-level expression of the L4-22K and L4-33K proteins that are involved in the full activation of the MLP (52). The late structural proteins are further discussed in the following section (chapter 1.2).

In addition to the major early and late proteins discussed above, several other small products are produced from the HAdV genome during intermediate (protein IX or pIX, Iva2) and

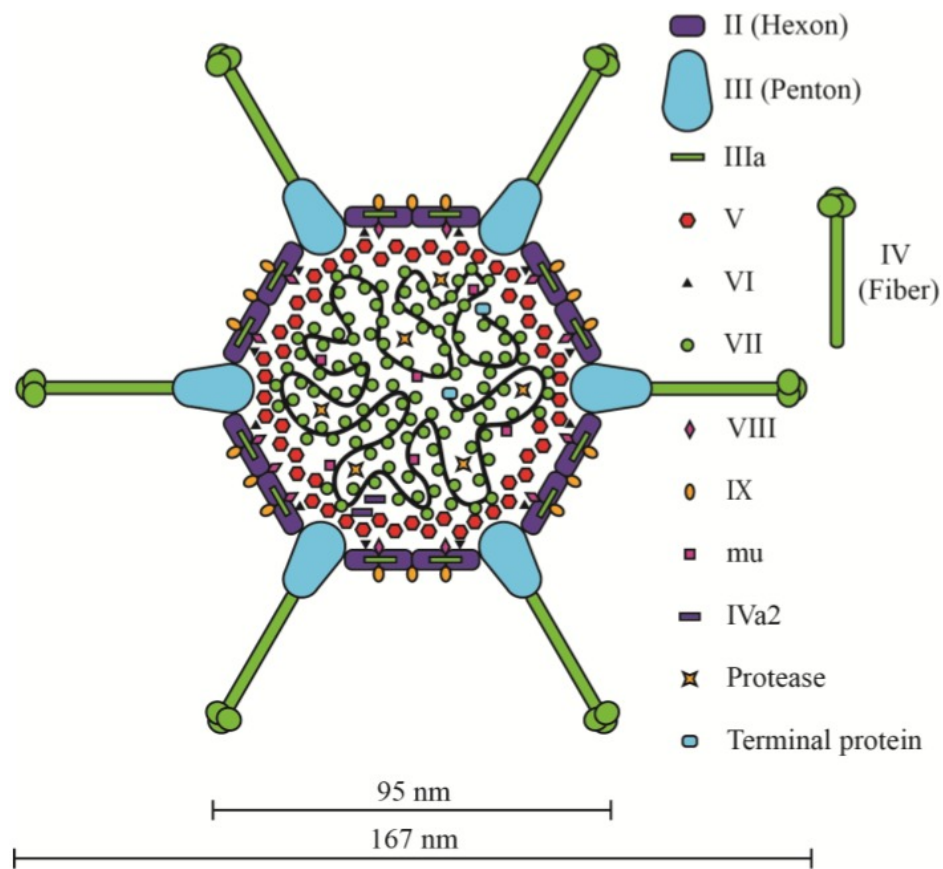


Figure 1.3 Structure of the HAdV virion. The majority of capsid proteins (e.g. penton, hexon, fiber) and core proteins (e.g. pVII, terminal protein, mu) associating with the viral DNA (black line) are shown. This illustration has been obtained from Saha *et al.*, figure 2 (53).

late (virus-associated RNA I, VA RNA II and the U exon protein or UXP) infection times (figure 1.1). pIX, a structural protein expressed after initiation of early gene expression and before late gene expression, plays a role in virion stability (54). VA RNA I and II are double-stranded RNA molecules that promote viral protein synthesis while impairing interferon-mediated antiviral responses and cellular micro-RNA processing (55-57). UXP has been suggested to play a role in virus DNA replication or RNA transcription (58, 59).

1.2 HAdV biology: the virion

The general structure of mature and infectious virions is relatively conserved among HAdV species. The non-enveloped, icosahedral capsid mainly consists of hexon, penton and fiber proteins (figure 1.3) (60-63). The latter is a long protein that emerges from each vertex of the icosahedron, increasing the overall capsid diameter (figure 1.3). Fiber is crucial for HAdV-5 attachment and entry into host cells (please see chapter 1.3).

Inside the virion, the HAdV DNA is bound to basic proteins VII, V and mu (figure 1.3) (64). Both protein VII (pVII) and mu condense and compact the DNA within the virion (65, 66). Interestingly, a recent study reported that viral DNA packaging and virion formation can occur normally in the absence of pVII, but efficient endosomal escape and virion maturation are impaired (67). Thus, it is possible that pVII is not absolutely essential for packaging the HAdV DNA within the capsid. Furthermore, pVII has been shown to interfere with DNA damage response and immune signalling through interaction with cellular proteins (68-70). Protein V is believed to surround the pVII-DNA complex and link the core to the capsid through interaction with capsid proteins (64, 71-73). Five other minor proteins (IIIa, IVa2, VI, VIII and IX; see figure 1.3) either

assist with stabilizing the capsid, connecting the capsid to the nucleoprotein core or packaging the viral genome (74, 75).

1.3 Infection and virus lifecycle

Cell surface receptors used by HAdV can vary based on the species, but the coxsackie and adenovirus receptors (CAR) are primarily used by species C serotypes including HAdV-2 and HAdV-5 (76). Binding of the fiber protein to the cellular CAR initiates virus entry into host cells (figure 1.4) (76, 77). Most cell types express CAR and HAdV-induced downregulation of CAR expression has been suggested as a mechanism to prevent super-infection of persistently infected cells (78). Subsequently, interaction of an RGD motif in the penton protein with cell membrane-associated $\alpha\beta3$ or $\alpha\beta5$ integrins induces virus internalization by receptor-mediated endocytosis into clathrin-coated vesicles (79-82). These vesicles eventually form endosomes, which the virus escapes from by lysing the early endosomal membrane (81). The virions then migrate to the cell nucleus using the cellular microtubule network (83, 84). The capsid proteins are removed during transport, such that only the pVII-wrapped viral DNA enters through the nuclear pore complex (85-87).

Although there is some debate regarding the fate of pVII after entry into the nucleus, data from our lab and others show that at least some of the pVII dissociate from the HAdV DNA, and cellular histones associate with the DNA as early as 1-hour post infection (88-91). Total pVII levels in infected cells appear to decrease significantly within the first few hours of infection, suggesting that the protein is likely targeted for degradation (90, 92). *In vitro* studies have shown that the highly basic pVII tightly condenses the viral DNA into a structure that is not permissive



Figure 1.4 A simplified representation of the HAdV lifecycle. Important stages of the lifecycle (e.g. attachment, internalization, nuclear entry, gene expression, DNA replication, virion formation and mature virus release) are illustrated in a step-wise manner. Following attachment of HAdV fiber proteins to CAR receptors on the cell surface, internalization occurs via clathrin-mediated endocytosis (steps 1, 2). The virus escapes endosomal degradation and uses the cellular microtubule network for transportation to nuclear pores (step 3). The capsid is disassembled en route and only the pVII-wrapped viral DNA enters the host cell nucleus (step 4), where early/intermediate/late gene transcription and DNA replication occur (steps 5, 9, 13-15). Although viral proteins must be translated in the cytoplasm, the newly synthesized proteins are then transported to the nucleus for regulatory functions (e.g. E1A) or formation of new capsids (steps 6-8, 10-12, 16-19). The final steps of the lifecycle consist of maturation and release of progeny virions (steps 20, 21). This diagram was obtained from Fenner's Veterinary Virology with permission (93).

of efficient transcription or replication (65). Consequently, “chromatinization” of the genome in the host cell nucleus may be necessary for transitioning the viral genome to a transcriptionally active state that allows for transcription of the early genes. On the contrary, Komatsu *et al.* found that exogenously supplied pVII is able to enhance transcription initiation, and further investigation is required to clarify the role of pVII during infection (88). The nucleosome density on the viral genome is significantly reduced during late infection (90, 94, 95). Due to the requirement for DBP during DNA replication and the high levels of DBP in late infection, DBP-bound DNA molecules may be more abundant at this time (96). In addition, the nascent viral DNA must associate with pre-pVII for packaging into newly formed capsids. Thus, viral/cellular protein association with the HAdV genome is dynamically regulated throughout infection, and it is possible that as infection proceeds, the infected cell nucleus contains an increasingly diverse pool of viral genomes associated with different proteins that facilitate gene expression, DNA replication or packaging.

In the cell nucleus, HAdV DNA localizes at transcription start sites (TSSs) and enhancers of highly expressed cellular genes, which likely promotes access to activating epigenetic regulators and transcription factors for viral gene expression (97). Viral DNA replication occurs after early gene expression, which allows full activation of the MLP and subsequently, the expression of late genes (50). Following their synthesis in the host cell cytoplasm, the structural proteins (e.g. fiber, penton, hexon) are transported into the nucleus for assembly of progeny virions (63, 98). HAdV capsids form first, followed by packaging of the DNA into the empty capsids which requires the ITR, IVa2 and other viral proteins (47, 99). Finally, the death of infected cells from virus-induced cytopathic effects (CPE) and release of progeny virions conclude the HAdV lifecycle.

1.4 Epigenetic regulation of HAdV gene expression

1.4.1 Overview of histone modifications and epigenetic regulators

The genomic DNA of eukaryotic cells associates with histones, forming a nucleosome, which is the basic unit of chromatin. Each nucleosome is composed of 147 bp of DNA wrapped around an octamer histone core containing two H2A/H2B dimers and a H3/H4 tetramer. Nucleosomes not only compact the DNA within the cell nuclei, they also carry epigenetic information in the form of covalent histone modifications (100). The N-terminal ends of histones undergo various post-translational modifications (e.g. acetylation, methylation, phosphorylation, ubiquitylation) that control access of transcription factors and other proteins to chromatin, thereby regulating gene expression (101, 102). HAT enzymes from the p300/CBP, MYST and GNAT families catalyze the acetylation of lysine residues, which neutralizes the positive charge on the lysines and prevents strong interaction with negatively-charged DNA (103). This allows chromatin unfolding and transcription factors increased access to specific genomic regions. As such, acetylation is enriched within promoters/TSSs of highly transcribed genes and is generally associated with transcriptional activation (103). Acetylation is reversible and the acetyl groups can be removed by histone deacetylases (HDACs), which are associated with transcriptional repression. There are four classes of HDACs: class I, II and IV (HDAC1-11) are zinc-dependent enzymes, while class III (SIRT1-7) are NAD⁺-dependent (104). Methylation of lysine residues are performed by methyltransferase enzymes, which are associated with both transcriptional activation and repression depending on the location and type of methylation (101).

1.4.2 Epigenetic regulation of HAdV genome

Like the herpes simplex virus (HSV), the HAdV genome also associates with histones

within the first few hours of infection, and adopts a repeating nucleosome-like structure similar to the host DNA (88-90). Preferential association with H3 variant H3.3 was observed in both early and late phases of infection, although nucleosome density on the viral genome was significantly reduced in late infection (89, 90, 94, 95). H3.3 is deposited onto various active regions of cellular DNA in a replication-independent manner by histone chaperones HIRA, DAXX/ATRX or EP400 (105-107). The DAXX/ATRX chromatin remodelling complex is known to repress HAdV gene expression and replication, and the virus targets DAXX for degradation (108). Although HIRA was implicated in H3.3 deposition on incoming HSV DNA and on hdAd vectors, the loss of HIRA did not affect H3.3 association with the wildtype HAdV genome (90, 92, 109). Thus, the chaperone(s) responsible for H3 deposition on wildtype HAdV DNA is yet to be determined. In addition, other cellular proteins involved in mediating the process of HAdV genome “chromatinization” and the role of chromatin modifying enzymes in the epigenetic regulation of HAdV gene expression remain unknown. We and others have recently shown that viral genome transcription is not required for pVII removal and nucleosome deposition (88, 90). Proteins from the template activating factor (TAF) family were found to play a role in pVII removal and pre-pVII deposition in cell-free systems, but the exact mechanism has not been elucidated in cells (88, 110). The viral E1A and E4orf3 proteins may also regulate the nucleosome density on the viral genome (111).

The presence of H3 acetylation at highly active HAdV-5 promoters was first observed almost a decade ago (88). Komatsu *et al.* found that the level of acetylation (H3K9 and/or K14) at the early promoters increased in a transcription-independent manner as the infection progressed. The overall trend was similar for the MLP and late regions, even though the extent of acetylation

was lower compared to the early regions and transcription was required. A second study provided evidence suggesting that the HAT Tip60 associates with E1A promoter and increases H4 acetylation, which suppresses E1A expression and HAdV replication (112). Furthermore, Tip60 was targeted for degradation by other viral proteins, likely to enhance virus replication. Several recent studies also reported that the nucleosomes associated with the HAdV-5 E2, E3 and E4 promoters were acetylated at K9, K18 and K27 of H3 (111, 113). Although the levels of these acetyl marks were found to be differentially regulated depending on region and infection time, acetylation generally served to recruit TBP/RNA polymerase and activate transcription from the early promoters (111, 113). Lastly, valproic acid (VPA)-mediated inhibition of HDAC activity was shown to inhibit HAdV-5 replication and spread in cell culture (114), which indicates the importance of HDAC activity for optimal virus replication and likely gene expression.

The literature on the epigenetic regulation of HAdV genome suggests that the virus may have a complex temporal/regional requirement for histone acetylation for efficient early promoter function. However, further investigation is necessary to fully understand these processes. In addition, the presence of other histone modifications (e.g. methylation, phosphorylation) and the role of epigenetic regulators remain to be uncovered.

1.5 Manipulation of cellular processes by HAdV

As with all other viruses, HAdV relies on cellular machinery for almost all steps of the virus lifecycle, and hijacks cellular pathways to promote viral gene expression and replication. Virus-encoded proteins interact with a multitude of cellular proteins to recruit transcriptional/epigenetic regulators to viral promoters; prevent access to cellular promoters;

impair cellular protein functions; and target them for degradation (5, 6, 11, 15-17, 20, 22-24, 28, 29, 38, 39, 68-70, 112, 115-119). This results in changes in host gene expression and protein activity, allowing optimal viral gene expression replication, establishment of a mitogenic state, prevention of immune/DNA damage response, etc. As infection progresses, the virus inhibits gene expression and protein synthesis in the cell, which serves as a virus production factory and eventually cell death occurs as a result of cytopathic effects (120).

Upon infection, HAdV early proteins drive the infected cells into S phase of the cell cycle (11). One of the main mechanisms by which this occurs (and of particular importance to this study) is antagonization of the cyclin-dependent kinase (CDK) inhibitor p21 by E1A. E1A has been shown to both reduce p21 expression and neutralize p21 activity through physical interactions (22, 121). This prevents p21 from inhibiting the kinase activity of CDKs (mainly CDK2), which phosphorylate pRB, allowing E2F to activate transcription of genes involved in cell proliferation and cell cycle progression (122, 123). E1A also directly interacts with pRB to release E2F1, and recruits E2F1 to its binding sites at the HAdV E2 promoter to allow maximum expression of the E2 proteins (21, 124, 125). Alongside the E2F/DP-1 complex, E1A interacts with other transcription factors (e.g. SP1, the ATF family) and chromatin modifiers (e.g. p300/CBP) to induce transcriptional activation of viral genes (6, 20, 24, 113, 116, 126).

Other HAdV-encoded early/late proteins also manipulate cellular processes and gene expression. For example, the E1B-55 kDa protein (together with E4orf6 and E4orf3) neutralizes tumour suppressor p53 activity, which promotes cell proliferation and prevents DNA damage responses (16, 28, 127). E4orf3 targets the MRN DNA repair complex for degradation (128). pVII

binding to SET and HMGB1 allows the virus to evade DNA damage and immune responses (68, 69). In summary, efficient HAdV replication and growth rely on the ability of the viral proteins to interfere with numerous cellular protein and pathway functions. Several excellent reviews on HAdV's control of cellular processes have been published (5, 11, 15-17, 29, 116, 118, 119, 129).

1.6 HAdV-induced disease and current treatment options

HAdV transmission mainly occurs via aerosol droplets and the fecal-oral route (130). The virus causes minor, self-limiting illnesses in most immunocompetent patients. Such mild infections are highly underreported, leading to an underestimation of HAdV infection and prevalence in the population (131). A voluntary reporting system, called the National Adenovirus Type Reporting System (NATRS), was created by the CDC in 2014 to collect information on HAdV serotypes circulating in the United States (132).

1.6.1 HAdV infections

HAdV has a remarkable capacity to spread – an infected patient can contract disease from only five virus particles (133). To place this in perspective, sputum/oral secretions from an infected adult and blood from patients with disseminated infection can contain between 10^6 and 10^7 virus particles per milliliter (134). It is not surprising that the virus can also cause severe disease, including hemorrhagic cystitis, hepatitis, myocarditis and neurologic complications, leading to respiratory/multiorgan failure and even death in pediatric, geriatric and immunocompromised patients (e.g. stem cell or organ transplant recipients) (130, 132, 135-141). In fact, HAdV-induced disease can be fatal in up to 80% of neonates with systemic infection and in ~40% of children with infection-related neurologic complications (139, 142). Recently, several cases of

immunocompetent patients succumbing to HAdV-induced respiratory failure was documented (143, 144).

The type, severity and tissue tropism of HAdV-induced diseases varies based on the virus serotype involved (132, 145, 146). Of the seven species of HAdV, species B, C and E are most frequently associated with upper respiratory tract infections: species C (serotypes 1, 2, 5) mainly causes mild infections in young children, while species B (serotypes 3, 7, 14, 21) and E (serotype 4) cause more severe infections in both children and adults (131, 132, 145). Although HAdV accounts for ~5% of all childhood respiratory tract infections, it can lead to bronchitis or pneumonia, requiring hospitalization in many cases (147, 148). Species D is associated with conjunctivitis, and species A and F are responsible for causing infections of the gastrointestinal tract (149). Overall, HAdV infection can be severe and fatal in some populations, and is a significant burden to society due to loss of work hours and associated medical expenses (estimated to be over 1,000 USD per patient) (148).

1.6.2 Current antiviral therapies

No approved antiviral therapy currently exists for the treatment of severe HAdV infections (138, 139, 146, 150). Cidofovir, a cytosine analogue that interferes with viral DNA synthesis and is an approved antiviral agent against cytomegalovirus (CMV), is considered to be the standard treatment in most cases (130, 137, 139, 141, 150, 151). Several other DNA/RNA synthesis inhibitors used as antivirals in other viral infections (such as ganciclovir and ribavirin) are also used to treat HAdV infections in the clinic (130, 137-139, 141). However, these drugs are often associated with serious adverse effects (130, 137, 150, 152) and poor disease prognosis in systemic

HAdV infections in immunocompromised patients (139, 141, 153). Brincidofovir, a phospholipid conjugate of cidofovir, is currently in clinical trials and has received FDA Fast Track status for treatment of HAdV-induced diseases (154, 155). This drug has several advantages over cidofovir, including oral delivery and increased cellular uptake. However, it provides only modest benefits and can cause gastro-intestinal toxicity in some patients (150, 155). Immunotherapies assessing virus-specific T-cells are also under clinical trials (150, 156). Thus, more effective anti-HAdV therapies are necessary to combat severe HAdV infections (151, 153).

1.6.3 Anti-HAdV vaccines

A live, oral vaccine against serotypes 4 and 7 was used by the U.S. military from 1971 to 1997 to prevent HAdV disease in new recruits (132). The vaccine (now mandatory) was later reintroduced in 2011 due to a substantial increase in HAdV infection within military personnel during the intervening period (1). This vaccine is very safe and effective, and it led to a 100-fold decrease in disease burden within the first two years of reintroduction (130). However, it is not available to the general public.

1.7 Discovery of new antiviral therapies

1.7.1 Existing drugs with anti-HAdV activity

The need for novel anti-HAdV therapies has been highlighted by many clinical and case studies, and several research groups have discovered novel compounds that exhibit considerable antiviral activity against HAdV in cell culture and in animal models. As previously discussed, the pan-HDAC inhibitor VPA was found to inhibit virus replication and spread in cell culture (114). The researchers speculated that this may be due to VPA-induced upregulation of CDK-inhibitor

p21 expression and subsequent cell-cycle arrest. This data suggests that pharmacological inhibition of chromatin-modifying enzymes may be useful in preventing/inhibiting HAdV infection. Indeed, this has been observed in HSV infections, where inhibition of histone demethylases prevented virus lytic replication and reactivation from latency (157-159). However, a better understanding of the role of HDACs in HAdV infection must be obtained first.

Anti-HAdV activity of steroid-based compounds has been reported by many independent research groups. Changes in the localization of SR proteins, which are involved in cellular RNA splicing, by cardiotonic steroids digoxin and digitoxin was shown to affect HAdV-5 RNA processing. Consequently, relative levels of the spliced E1A transcripts (and thus, E1A protein isoforms) were altered, leading to drastic reductions in virus yield (160). In another study by Marrugal-Lorenzo *et al.*, mifepristone, a commercially available synthetic steroid, reduced HAdV replication at micromolar concentrations in cells and in mice (161). The researchers provided evidence suggesting that the compound interfered with efficient nucleus entry and replication of the viral genome. On the other hand, natural steroid hormones epiandrosterone, dehydroepiandrosterone and their derivative compounds were reported to reduce HAdV replication by impacting upon protein production, while entry/adsorption was unaffected (162).

Since cidofovir is often an effective treatment for HAdV infections in the clinic, various analogues of the drug and other nucleoside/nucleotide inhibitors have been investigated. Many of these compounds were either comparable to or more effective in inhibiting HAdV than the parental drug *in vitro* and *in vivo* (163-166). These compounds may prove to be useful in cases where cidofovir fails to improve disease outcome, but thorough assessments of their efficacy and safety

in animal models are required prior to clinical testing. Piperazine-based compounds have shown promise in several investigations (167, 168). Tazarotene, a selective agonist of the retinoic acid receptor β (RAR β), was recently identified as an HAdV replication inhibitor after transcriptomic analysis of infected A549 cells revealed that the virus induced downregulation of RAR β mRNA (169). Other miscellaneous and broad-spectrum antiviral compounds with anti-HAdV activity (e.g. stavudine, cycloferon, lactoferrin) have been previously described (170, 171). Identification of such compounds and elucidation of their mechanism of inhibition may eventually lead to the discovery of more effective therapeutic agents to treat HAdV-induced disease.

1.7.2 Small molecule library screens

High-throughput screening of small molecules is a widely used, cost-effective drug-discovery approach that has led to the identification of novel therapeutics or the repurposing of existing therapeutic compounds against previously unexploited viral and host targets to combat a variety of viral infections (172-175). However, nearly all published studies on new HAdV inhibitors have been small-scale and only evaluated a single or a handful of select compounds (160, 161, 167, 169). Very few drug library screens have been performed to identify novel anti-HAdV compounds (168, 176-178). Many of these screens were conducted using non-replicating HAdV vectors that expressed the green fluorescent protein (GFP) from a heterologous promoter, which does not mimic wildtype virus gene expression and replication accurately (168, 178). A fluorescence-based high-throughput screen was designed by Duffy *et al.* to specifically identify small molecules that inhibited coagulation factor X (FX)-mediated uptake of HAdV-5 vectors by hepatocytes (177). 10,240 molecules were screened and three positive hits were found to be effective in blocking intracellular transport of virus at low micromolar concentrations. Since the objective of this

screening system was to discover HAdV transduction inhibitors, compounds impairing other stages of the virus lifecycle were not identified.

More recently, a screening platform using luminescence-based detection of cytotoxicity and CPE was developed by Hartline *et al.* This approach, where the antiviral activity of a given compound is quantified by analyzing the difference in drug-induced cytotoxicity and virus-induced CPE, allowed for broad-spectrum screening for antiviral compounds against various DNA viruses including HAdV (176). The investigators identified two compounds with previously unreported anti-HAdV activity: FCV and 4-thio-IDU. Although this assay offers the advantage of selecting antiviral compounds at a desirable concentration with low cytotoxicity, it relies on measuring effects induced by the virus on the host cell, instead of a more direct measure of virus growth/replication. This may limit the ability of the screen to detect compounds with modest effects on viral gene expression and replication.

1.7.3 Non-small molecule antivirals

Development of novel vaccines against HAdV is a relatively unexplored field. To date, a limited number of inactivated and recombinant multivalent vaccines against clinically relevant HAdV serotypes have been investigated. The majority of these vaccines contain hexon as the target antigen because host neutralizing antibodies are primarily directed against hexon (147). Fiber, penton and other capsid proteins are also immunogenic (179). Liu *et al.* showed that a trivalent vaccine against three serotypes (3, 7 and 55) responsible for acute respiratory disease, induced production of antibodies, reduced virus load and improved lung pathology in a mouse model of infection (180). Other types of non-small molecule antivirals against HAdV explored in non-

clinical research include RNA interference (e.g. siRNAs targeting viral mRNAs), intravenous immunoglobulin therapy and T-cell immunotherapy (170, 171, 181).

1.8 Rationale, hypothesis and objectives

As with all viruses, HAdV relies on the cellular machinery and pathways to assist with virus entry, viral gene expression, genome replication and production of progeny virions. We **hypothesize** that small molecules that modulate certain cellular pathways and protein activities, without severely affecting cell health, may serve as effective anti-HAdV compounds. Such small molecules can be efficiently discovered through screens of existing compound libraries, facilitating drug repurposing for HAdV-induced disease. Thus, our overall **objective** is to identify novel inhibitors of HAdV, and investigate the molecular mechanism to determine new therapeutic targets for disease intervention. The **specific aims** include:

- (a) Generate and characterize several HAdV constructs.
 - i. Ad-late/RFP for use in small-molecule screens.
 - ii. FLAG-tagged constructs to facilitate detection of E2 proteins.
- (b) Investigate HDACs as potential therapeutic targets in HAdV infection.
 - i. Determine efficacy of HDAC inhibitors against HAdV *in vitro*.
 - ii. Further elucidate the role of HDACs in HAdV infection.
- (c) Identify novel anti-HAdV compounds through small-molecule screens.
 - i. Develop an efficient screening method and screen small-molecule libraries.
 - ii. Validate positive hits by determining effects on virus lifecycle and cell health.

Chapter 2:

Human Adenovirus Type 5 Vectors Deleted of Early Region 1 (E1) Undergo Limited Expression of Early Replicative E2 Proteins and DNA Replication in Non-Permissive Cells

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2.1 Abstract

Adenovirus (Ad) vectors deleted of the early region 1 (E1) are widely used for transgene delivery in preclinical and clinical gene therapy studies. Although proteins encoded within the E1 region are required for efficient virus replication, previous studies have suggested that certain viral or cellular proteins can functionally compensate for E1, leading to expression of the early region 2 (E2)-encoded replicative proteins and subsequent virus replication. We have generated a series of E1-encoding and E1-deficient Ad vectors containing a FLAG-epitope tag on each of the E2-encoded proteins: DNA-binding protein (DBP), terminal protein (TP) and DNA polymerase (Pol). Using these constructs, we show that for the replication-competent virus, the expression level of each E2-encoded protein declines with increasing distance from the E2 promoter, with E2A-encoded DBP expression being ~800-fold higher than E2B-encoded TP. Pol was expressed at extremely low levels in infected cells, and immunoprecipitation from cell lysates was required prior to its detection by immunoblot. We further show that DBP was expressed 200- to 400-fold less efficiently from an E1-deficient virus compared to a replication-competent virus in A549 and HepG2 cells, which was accompanied by a very small increase in genome copy number. For the E1-deficient virus, late gene expression (a marker of virus replication) was only observed at very high multiplicities of infection. These data show that E1-deleted Ad gives rise to limited expression of the E2-encoded genes and replication in infected cells, but highlight the importance of considering viral dose-dependent effects in gene therapy studies.

2.2 Introduction

Human adenovirus (Ad) mainly causes self-limiting respiratory illnesses and can rapidly spread through confined populations such as day care centers, hospitals, retirement homes and military training venues (1). Since their identification in the 1950's, over 100 serotypes of Ad have been isolated from a variety of species. Of the human Ads, serotypes 2 and 5 (Ad5) have been extensively studied to gain a better understanding of virus biology, host cellular processes and virus-cell interactions during infection (2). The Ad5 genome (36 kb, double-stranded DNA) consists of “early” and “late” regions, which are expressed before and after viral DNA replication, respectively (Fig 2.1A). The early regions (E1A, E1B, E2, E3 and E4) encode proteins that are involved in activating transcription of other viral regions, altering the host cellular environment to enhance virus replication, or in replication of the viral DNA (3). The E1A gene products are the first proteins expressed from the infecting virus, and these proteins transactivate other viral coding regions, interact with a multitude of cellular proteins, and have a variety of other functions that ultimately promote infection (4). The major product from the E1B region, the 55-kDa protein, is required for selective export of viral late mRNAs from the host cell nucleus; it also acts (in cooperation with E1A and E4orf6) to induce degradation of certain cellular proteins and promotes transformation of the infected cells (5, 6). Together, the E1A and E1B coding regions are absolutely required for efficient viral gene expression and replication.

The E2 transcription unit consists of two regions, E2A and E2B, which have separate polyadenylation sites (7). The E2A region encodes the 72-kDa DNA-binding protein (DBP), which plays a crucial role during the elongation phase of Ad DNA replication: DBP multimerizes on the single-stranded DNA (ssDNA) template, and is mainly responsible for unwinding the template (8).

DBP association with ssDNA may also function to protect the viral DNA and prevent immune and DNA damage responses that can be induced in the cell against naked DNA from foreign species (9). The E2B region codes for the 80-kDa precursor terminal protein (pTP) and the 140-kDa Ad DNA polymerase (Pol), which, along with DBP and other cellular proteins, form a pre-initiation complex prior to Ad DNA replication (10). Initiation of DNA synthesis occurs via a protein-priming mechanism where Pol covalently attaches a dCMP residue to the pTP in the complex. The 3'-OH group of the dCMP then serves as a primer for elongation by Pol through a strand-displacement process (11). During virion maturation in the late phases of infection, pTP is cleaved by the Ad-encoded protease to produce the mature 55-kDa terminal protein (TP), which remains associated with each 5' end of the linear Ad DNA (12). The E3 and E4 proteins alter host immune responses and cell signaling, respectively (13, 14).

In general, the late transcription units, L1-L5, are produced from alternative splicing of a common major late transcript, and code for structural and capsid proteins (e.g. fiber, hexon) required for the production of progeny virions. In addition to these major early and late proteins, four other small products are produced by Ad: protein IX (pIX), virus-associated (VA) RNA I, VA RNA II and the U exon protein (UXP). pIX is a structural protein expressed at an intermediate time during infection (after initiation of early gene expression and before late gene expression), and it plays a role in virion stability (15, 16). VA RNA I and II are double-stranded RNA molecules produced during late infection, and they promote viral protein synthesis (17), inhibit activation of the interferon-mediated antiviral responses (18) and impede cellular micro-RNA processing (19). UXP, also expressed during late infection from a unique promoter, associates with viral replication

centers throughout the host nuclei and has been suggested to play a role in DNA replication or RNA transcription (20, 21).

Ad vectors are one of the most commonly used platforms for gene delivery in human gene therapy clinical studies, as well as in biochemical studies conducted *in vitro* (22). First generation Ad (fgAd) vectors are deleted of the entire E1 region, which is commonly believed to render the virus replication-deficient in most cell lines. These vectors are ideal for studies involving short-term gene expression either *in vitro* or *in vivo* (23). One major limitation of the fgAd vectors *in vivo* is the transient expression of the therapeutic transgenes due to rapid clearance of the transduced cells, caused in part by induction of immune responses to Ad proteins produced from the viral protein coding regions retained in the vector (24, 25). Furthermore, DNA replication and late gene expression of fgAd has been observed in many “non-permissive” cell lines (*i.e.* cells that do not complement the E1A/E1B deletion in these vectors) at multiplicities of infection (MOI) as low as 10 (26). In such cases, it has been speculated that activation of the E2 promoter by viral or cellular proteins and subsequent expression of the E2 replicative proteins likely led to the observed vector replication (27, 28). For example, the Ad E4-ORF6/7 fusion protein can transactivate the E2 promoter to a considerable degree, especially in the absence of E1A (29-31). Consequently, E4-ORF6/7-mediated activation of E2 expression has been implicated in partially circumventing the replication-defective nature of E1A-deleted vectors used in various gene therapy applications (31), allowing fgAd to give rise to virus replication at high levels in some cell lines (26, 27).

Although antibodies against the E2-encoded proteins have been generated by some research laboratories, they are not commercially available or readily accessible (32-34). Thus, to

facilitate studies of these proteins, we have inserted a FLAG-epitope tag individually into Ad5 DBP, TP and Pol. We show that the presence of the tag does not adversely affect virus replication, and that each protein could be easily detected by immunoblot of infected cell lysates or virions. We also examined the relative levels of DBP expression and DNA replication from an E1-deleted virus in cell lines that do not complement the E1-deletion. Although E2 proteins were expressed at low levels, removal of E1 severely compromised the ability of the virus to replicate.

2.3 Materials and Methods

Cloning FLAG-tags into the E2 region of Ad5. All Ad constructs were cloned using a combination of conventional and bacterial RecA-mediated cloning (35). Clones generated by polymerase chain reaction (PCR) were verified by DNA sequencing.

Generation of FLAG-tagged DBP

The coding sequence for the FLAG-tag was added to DBP using overlap PCR. pRP2014 (15) was subjected to PCR in two separate reactions using the following synthetic oligonucleotides: 3016F1-GCAACGCGTCGTCAGATAATGGCGAC and 3116R-CTTATCGTCGTCGTCCTTGTAATCGGGAGGCGGCGGCGACGGGGAC; 3116F-GATTACAAGGACGACGACGATAAGCGGGCGCCCCAAAAAGC and 3016R2-CCAACGCGTTTAGCAGGTCGGGC. The two resulting DNA products were combined into a single PCR reaction and re-amplified with 3016F1 and 3016R2. The DNA product from this second amplification was digested with MluI and cloned into MluI-digested pGEM7, generating pRP3116. A 5520 bp BamHI/SpeI fragment from pRP2014 was also cloned into BamHI/SpeI-digested pBluescript, generating pRP3037. An 1821 bp MluI fragment from pRP3116 was then used to replace the MluI fragment in pRP3037, generating pRP3118. A 5544 bp BamHI/SpeI fragment from pRP3118 was cloned into pRP2014, generating pRP3120 which is an Ad5 genomic plasmid lacking E1 and E3, but containing a FLAG-tag on the DBP. pRP3120 was recombined with pRP2483 (36) to produce pRP3121, an E1/E3-deleted Ad5 genomic plasmid containing a monomeric red fluorescent protein (RFP) reporter gene (37) under the regulation of the cytomegalovirus (CMV) immediate early enhancer/promoter and bovine growth hormone polyadenylation sequence (BpA) replacing the E1 region, and the FLAG-tagged DBP. pRP3120 was also recombined with pXC1 (38) to generate pRP3122, an E1⁺/E3⁻ infectious plasmid

containing the FLAG-tagged DBP. pRP3121 and pRP3122 were digested with PacI to liberate the inverted terminal repeats, transfected into 293 cells and recovered as viruses, which are designated AdE1⁻DBP-F and AdE1⁺DBP-F, respectively, in this study. In these viruses, the coding sequence for the FLAG-tag is located immediately before 23916 bp of the conventional human Ad5 genome (GenBank AC_000008). Please note that the E2 coding sequences runs right to left, so before 23916 bp actually places the FLAG-tag downstream of this position in the E2 open reading frame. The specific amino acid sequence of this region of DBP is 36-PPPP***DYKDDDD***KRAPP, where 36 refers to the position in the DBP of the first listed amino acid (in this case proline, P), the FLAG-tag is shown in italics and bold, and the native DBP sequence is in normal type.

Generation of FLAG-tagged TP

A 1615 bp MfeI fragment (8016 bp to 9631 bp of the Ad5 genome) containing a portion of the pTP coding sequence from pRP2014 was cloned into MfeI-digested pNEB193, generating pRP2982. The synthetic oligonucleotide CTTATCGTCGTCATCCTTGTA and its complement were ligated into EcoRV-digested pRP2982, generating pRP2988. A 1069 bp EagI/XbaI fragment from pRP2110a (39) was cloned into EagI/XbaI-digested pRP2982, which added an extra region of Ad genome homology and convenient restriction sites for the next step of cloning, and was designated pRP2994. A 2202 bp BsrGI/SnaBI fragment from pRP2982 was then cloned into BsrGI/SnaBI-digested pRP2110a, generating pRP2995. Finally, a 7124 bp XbaI fragment from pRP2995 was cloned into XbaI-digested pRP2014ΔXbaI, generating pRP2996, an Ad5 genomic plasmid lacking E1 and E3 but containing a FLAG-tag on the pTP. pRP2996 was recombined with pRP2483 to produce pRP2997, an E1/E3-deleted Ad5 genomic plasmid containing a CMV-RFP-BpA expression cassette replacing the E1 region and the FLAG-tagged pTP. pRP2996 was also recombined with pXC1 to obtain pRP3000, an E1⁺/E3⁻ infectious plasmid containing the FLAG-

tagged pTP. pRP2997 and pRP3000 were digested with PacI to liberate the inverted terminal repeats, transfected into 293 cells and recovered as viruses, which are designated AdE1⁻TP-F and AdE1⁺TP-F, respectively. In these viruses, the coding sequence for the FLAG-tag is located immediately before 9201 bp of the Ad genome. The specific amino acid sequence of this region of TP is 463-EALG**DYKDDDDK**INES. Of note, the 467th amino acid (D) is native to the TP protein and also constitutes the first residue of the FLAG-tag.

Generation of FLAG-tagged Pol

A 327 bp XhoI/NsiI fragment (XhoI at 8255 bp to NsiI at 8582 bp of the Ad5 genome) from pRP2014 was cloned into XhoI/NsiI-digested pGEM7, generating pRP2183. pRP2183 was amplified by PCR with synthetic oligonucleotides BRS100F-GCGAAGCTTCCGCCTGACCTGACGCCGC and BRS100R-GCGAAGCTTATCGTCGTCATCCTTGTAATCCTCCTGCAGGTTTACCTCGCATAGACG, which prime in opposite directions around the circular plasmid. The resulting DNA product was digested with HindIII and re-circularized, generating pBRS100. A 602 bp ApaI/XhoI fragment from pRP2110a was cloned into XhoI/ApaI digested pBRS100, generating pBRS101 and adding an extra region of Ad genome homology and convenient restriction sites for the next step of cloning. A 309 bp BsrGI/Sse8387I fragment from pBRS101 was cloned into pRP2110a, generating pBRS103. Lastly, a 7127 bp XbaI fragment from pBRS103 was cloned into XbaI digested pRP2014ΔXbaI, generating pBRS104, an Ad5 genomic plasmid lacking E1 and E3 but containing a FLAG-tag on the Pol. pBRS104 was recombined with pRP2483, generating pBRS105, an E1/E3-deleted Ad5 genomic plasmid containing a CMV-RFP-BpA expression cassette replacing the E1 region and the FLAG-tagged Pol. pBRS104 was also recombined with the pXC1, generating pBRS106, an E1⁺/E3⁻ infectious plasmid containing the FLAG-tagged Pol.

pBRS105 and pBRS106 were digested with PacI to liberate the inverted terminal repeats, transfected into 293 cells and recovered as viruses, which are designated AdE1⁻Pol-F and AdE1⁺Pol-F, respectively. In these viruses, the coding sequence for the FLAG-tag is located immediately before nucleotide 8404 bp of the Ad genome. The specific amino acid sequence of this region of Pol is 127-NLQEDYKDDDDKLPPD.

Cell lines and infections. 293 (a kind gift from Dr. Frank Graham, Professor Emeritus, McMaster University) (40) and A549 cells (CCL-185, ATCC) were grown in Minimum Essential Medium (MEM, Sigma Aldrich) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Sigma Aldrich), 2 mM GlutaMAX (Invitrogen) and 1x antibiotic-antimycotic (Invitrogen). HepG2 cells (HB-8065, ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented as above.

Viruses were propagated in 293 cells and purified by cesium chloride buoyant density centrifugation, using standard procedures (41). Viral titers were determined by plaque assay on 293 cell monolayers and scored as the number of plaque-forming units (PFU) per milliliter of purified virus (41). For all experiments, monolayers of A549 cells were infected at the indicated multiplicity of infection (MOI) with Ad5, AdE1⁺DBP-F, AdE1⁻DBP-F, AdE1⁺TP-F or AdE1⁺Pol-F diluted in phosphate-buffered saline (PBS, Sigma-Aldrich) for 1 hour at 37°C. Medium was then added to the cells and incubated in a humidified CO₂ incubator at 37°C until the indicated time point. For the quantitative PCR (qPCR) experiments, the viral inoculum was removed after the 1-hour incubation and the monolayers were washed once with PBS before adding medium.

Immunoblot analysis. To prepare protein samples, medium was removed from infected cells at the indicated time points, 2x protein sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β -mercaptoethanol) was added, and the resulting crude protein extract was collected. The protein samples were boiled prior to separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) and the membrane was probed with monoclonal antibodies to Ad5 fiber (1/10,000 dilution; MS-1027-P0, Neomarkers), FLAG (M2, 1/5,000; 200-301-383S, Rockland), Ad5 E1A (1/5,000; ab31686, Abcam) or a monoclonal antibody to human vinculin (1/10,000; ab73412, Abcam). The monoclonal antibody against Ad5 DBP (32) was a kind gift from Dr. Arnold J. Levine (antibody 72K-B6, 1/500 dilution).

Detection of 55-kDa TP within purified virions. For detection of the viral DNA-associated 55-kDa TP present within the virion, 3×10^{10} virus particles (VP) were diluted in 10 mM Tris pH 7.5 to a final volume of 25 μ l and denatured for 10 min at 60°C. The samples were cooled to room temperature, prior to adding reaction buffer (final concentrations: 50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM MgCl₂ 1 mM DTT) and 2 units of DNaseI (M0303S, New England BioLabs). After a 20-min incubation at 37°C, an equal volume of 2x protein sample buffer was added, the samples were boiled for 5 minutes, and analyzed by immunoblot as described above.

Real-time quantitative PCR. Media from infected cells was removed at the indicated time points and the cells were incubated overnight at 37°C in SDS-proteinase K buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% SDS (w/v), 1 mg/ml proteinase K) (36). Following phenol-chloroform extraction of the DNA, qPCR was conducted as previously described (41) using 200 ng of total

genomic DNA per reaction. The following primers were used for the hexon region of the genome: 5'-CTT ACC CCC AAC GAG TTT GA and 5'-GGA GTA CAT GCG GTC CTT GT.

Immunoprecipitation. Ten centimeter dishes of A549 cells were infected with AdE1⁺Pol-F or wildtype Ad5 at an MOI of 100, and lysed 24 hours later in modified radio immunoprecipitation assay (RIPA) extraction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% glycerol, 1% NP-40, protease inhibitors). Immunoprecipitation was conducted as previously described (42), using 30 µl Protein G beads (1004D, Dynabeads) for preclearing and 2 µg FLAG antibody or IgG (sc-2025, Santa Cruz) as negative control. Twenty microliters of beads containing the protein-bound antibody was resuspended in 20 µl 2x protein sample buffer before analysis.

Immunofluorescence. A549 cells were seeded on 1 cm rounded coverslips at 1/10th of the confluency density in 12-well tissue-culture plates, and infected as described above with FLAG-tagged Ad constructs at MOIs varying from 10 to 50. Eighteen hours post infection (hpi), media was removed and the coverslips were washed once with cold PBS. The cells were fixed with 4% paraformaldehyde (pH 7.0) in PBS for 15 mins, rinsed with cold PBS, permeabilized with PBS containing 0.25% Triton X-100 (Thermo Fisher Scientific) for 10 min, and then washed three times with PBS for 5 min each. PBST supplemented with 1% BSA was added for 30 min to block nonspecific antibody binding, prior to overnight incubation at 4 °C with the primary antibody diluted in the blocking solution. The next day, the primary antibody was decanted and the coverslips were washed three times as before. The coverslips were incubated for 1 hour at room temperature with secondary antibody, also diluted in the blocking solution. After secondary antibody removal and three washes with PBS in the dark, the cells were incubated with Hoechst

3342 (1 µg/ml, H3570, Life Technologies) for 1 min. Lastly, the coverslips were rinsed with PBS and placed on glass slides using Dako Fluorescent Mounting Medium (Agilent Technologies). The following antibodies were used: mouse monoclonal anti-FLAG antibody (M2, 1/1,000; 200-301-383S, Rockland) and Alexa Fluor 594 anti-mouse IgG (1/5,000; A21203, Thermo Fisher Scientific). Fluorescent images were obtained with a Plan-APOCHROMAT 63x/1.4 oil-immersion Ph3 objective on a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam MRC camera and ZEN 2 software for image processing.

2.4 Results

2.4.1 Insertion of a FLAG-epitope tag into E2-encoded proteins

The N-terminal region (amino acids 1-173) of the Ad5 DBP is relatively weakly conserved, and is dispensable for ssDNA binding activity (43). In contrast, the C-terminal region (amino acids 174-529) is highly conserved and is directly involved in DNA binding (44). Indeed, the cooperative binding of DBP to ssDNA is mostly lost when the very distal C-terminal 17 amino acids of DBP are removed (43). Vos *et al.* used insertion mutagenesis studies to identify regions of the DBP that were tolerant for insertion of small peptides (45). In this approach, small synthetic oligonucleotides (12 bp, corresponding to 4 amino acids) were cloned into various positions in the E2A coding region, and protein function and/or ability to recover the virus was evaluated. If protein function was retained and virus was recovered, the specific region of the protein was tolerant of insertions, suggesting that the region was perhaps not crucial for protein function. However, if no virus was recovered, the region was deemed not tolerant of mutation and likely crucial for function of the protein. Vos *et al.* identified the region between amino acids 39 and 41 of DBP as tolerant for insertion of small peptides with no loss of virus replicative ability (45). Thus, to create our FLAG-tagged DBP construct, we placed the tag following amino acid 39. This virus, designated AdE1⁺DBP-F, was easily rescued and showed similar fitness to wildtype Ad5 (Fig 2.1B and 2.1C). An E1-deleted version was also generated, designated AdE1⁻DBP-F.

Studies by Roovers *et al.* (46) used insertion mutagenesis to identify crucial regions of the E2B-encoded TP and Pol. We used this study to identify regions of the E2B-encoded proteins that would likely accept the FLAG epitope tag. For pTP, Roovers *et al.* identified two insertion sites, ins318 and ins467, that retained full activity during DNA replication (46). We wished to place an

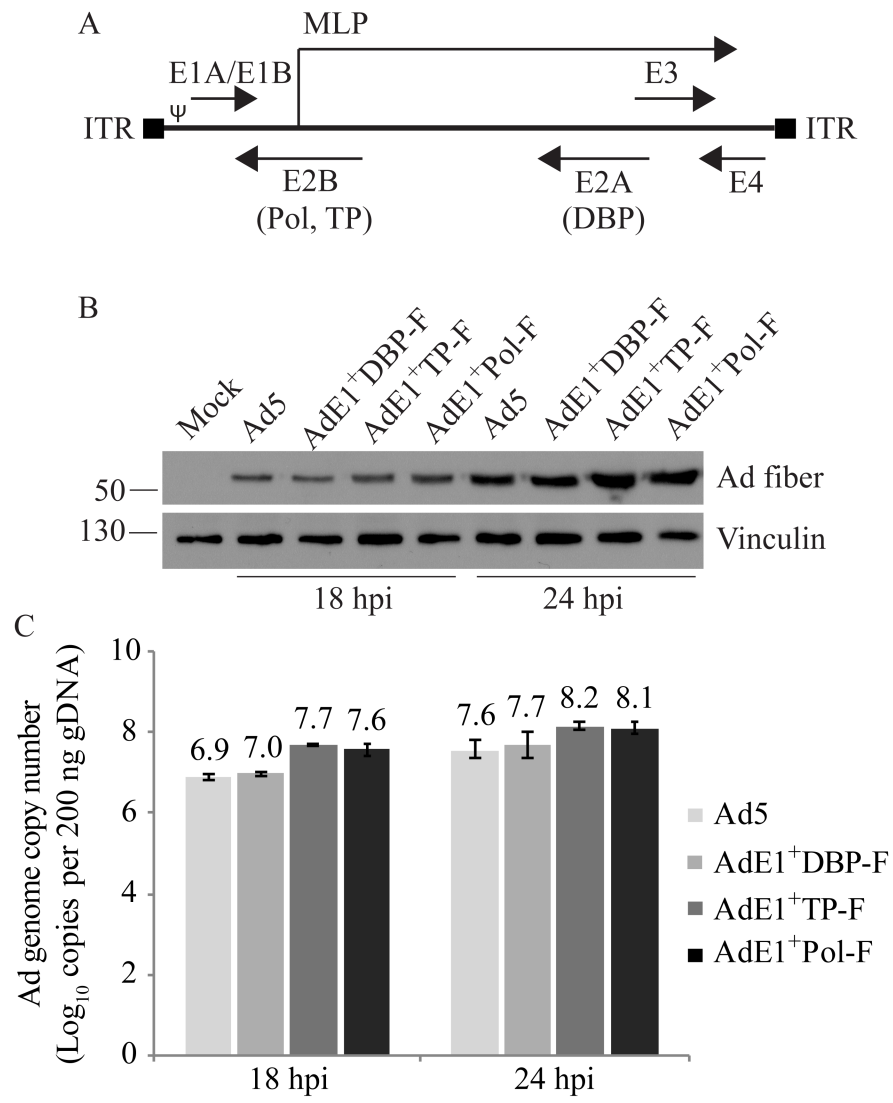


Figure 2.1 Growth of Ad constructs containing FLAG-tags within the E2 proteins is comparable to wildtype Ad5. (Panel A) Schematic diagram of the wildtype Ad genome (not

drawn to scale) showing locations of the early regions E1 to E4. The E2 region consists of E2A and E2B transcription units, which encode for DBP and TP/Pol, respectively. Both E2A and E2B are expressed from a common promoter located upstream of the E2A coding region. MLP is the major late promoter, ITR represents the inverted terminal repeats, and ψ is the packaging element.

(B) A549 cells were infected at a MOI of 10 with Ad5, AdE1⁺DBP-F, AdE1⁺TP-F or AdE1⁺Pol-F and 18 or 24 hours post infection (hpi), the cells were lysed for protein and DNA collection. Immunoblot analysis of fiber expression shows no significant difference between the viruses. Vinculin serves as a loading control. (C) Viral DNA copy number per 200 ng of cellular DNA, as determined by qPCR, are also similar for all four viruses at both 18 and 24 hours. Copy numbers were calculated based on a standard curve obtained by serially diluting a plasmid containing the hexon coding region. The mean of three experiments are shown and the error bars represent standard deviation (SD) of the mean.

epitope tag within pTP in such a way that the tag would be retained in the mature form of the protein after cleavage by the Ad-encoded protease. pTP is initially cleaved by the Ad protease between residues 175-176 or 183-184, generating an intermediate TP (iTP), followed by cleavage between residues 349-350, generating the mature TP (47). TP is covalently attached to the viral DNA at Ser580 (48). Thus, we chose to insert the FLAG-tag after amino acid 466 (see Materials and Methods). This virus, designated AdE1⁺TP-F, was rescued and grew similar to wildtype Ad5 (Fig 2.1B and 2.1C). An E1-deleted version of this virus was also generated: AdE1⁻TP-F.

The insertion mutagenesis studies by Roovers *et al.* also identified two insertion sites within the Ad5 Pol that showed near wildtype polymerase activity: ins130 and ins243. However, only ins130 also retained a significant ability to form the initiation complex through catalyzing the covalent addition of dCMP to pTP (49). Insertion of the FLAG at amino acid position 130 within the Pol gene resulted in a viable virus, designated AdE1⁺Pol-FLAG, that replicated its DNA and expressed the late fiber gene at a level similar to wildtype Ad5 (Fig 2.1B and 2.1C). The various viruses are summarized in Table 2.1.

2.4.2 Virus growth is unaffected by the presence of the FLAG-tag within the E2 region proteins

To determine whether insertion of the FLAG-tag affected DNA replication or late gene expression of the viral constructs, monolayers of A549 cells were infected at an MOI of 10 with wildtype Ad5, AdE1⁺DBP-F, AdE1⁺TP-F or AdE1⁺Pol-F for 18 or 24 hours. The infected cells were then lysed in appropriate buffers for protein and DNA isolation. Analysis of Ad fiber expression following SDS-PAGE and immunoblot analysis showed no significant difference in

Table 2.1 List of Ad constructs used in this study.

Ad construct^a	Modifications to E1 region	Modifications to E2 region	Location of FLAG-tag^b
Ad5	N/A ^c	N/A	N/A
AdE1 ⁺ DBP-F	N/A	DBP-FLAG	39
AdE1 ⁻ DBP-F	Δ E1, CMV-RFP	DBP-FLAG	39
AdE1 ⁺ TP-F	N/A	TP-FLAG	466
AdE1 ⁻ TP-F	Δ E1, CMV-RFP	TP-FLAG	466
AdE1 ⁺ Pol-F	N/A	Pol-FLAG	130
AdE1 ⁻ Pol-F	Δ E1, CMV-RFP	Pol-FLAG	130

^aAd5 is wildtype virus, and retains the native E1 and E3 coding regions. AdE1⁺DBP-F, AdE1⁺TP-F, and AdE1⁺Pol-F retain the native E1 coding region, but lack the E3 coding region. AdE1⁻DBP-F and AdE1⁻TP-F lack the E1A, E1B and the E3 coding regions.

^bThe last column provides the number of amino acid residues after which the FLAG-tag was inserted in the protein sequence.

^cNot applicable (N/A).

late gene expression from the FLAG-tagged viruses compared to Ad5 (Fig 2.1B). Viral DNA copy numbers in the infected cells were also examined by qPCR with primers specific to the Ad hexon region, and DNA replication levels of these viruses appear to be similar to Ad5 as well (Fig 2.1C). All of the FLAG-tagged Ad constructs had production titers above 10^9 pfu/ml (as determined by plaque assay on 293 cell monolayers), suggesting that presence of the FLAG-tags does not impact virus infectivity or growth.

2.4.3 Detection of the FLAG-tagged E2A and E2B proteins

We next determined whether the presence of the FLAG-tag on the various E2-encoded proteins could allow them to be detected by immunoblot and immunofluorescence. To characterize expression of the DBP, A549 cells were infected with AdE1⁺DBP-F at an MOI of 50, and crude protein extracts were prepared at varying times post-infection. As shown in Fig 2.2A, the 72-kDa FLAG-tagged DBP was detected as early as 6 hpi. At late infection times (24 and 30 hpi), the quantity of full-length DBP within the infected cell was very high, and several smaller FLAG-tagged protein species were also present. These smaller species may be the result of premature translation termination, proteolytic degradation, or perhaps alternative splicing events within the E2A transcript (50). Using a lower MOI of 10, the FLAG-tagged DBP was easily detected by immunoblot at a slightly later time point of 8 hpi (data not shown). We next compared the level of DBP expression from AdE1⁺DBP-F with wildtype Ad5 to determine whether presence of the FLAG tag altered expression. A549 cells were infected with the two viruses at an MOI of 50, protein samples were collected 8 and 24 hpi, and DBP expression was evaluated by immunoblot using an antibody to native DBP. As shown in Fig 2.2B, presence of the FLAG-tag did not affect the level of expression of DBP from the virus. The FLAG-tag also allowed us to visualize DBP by

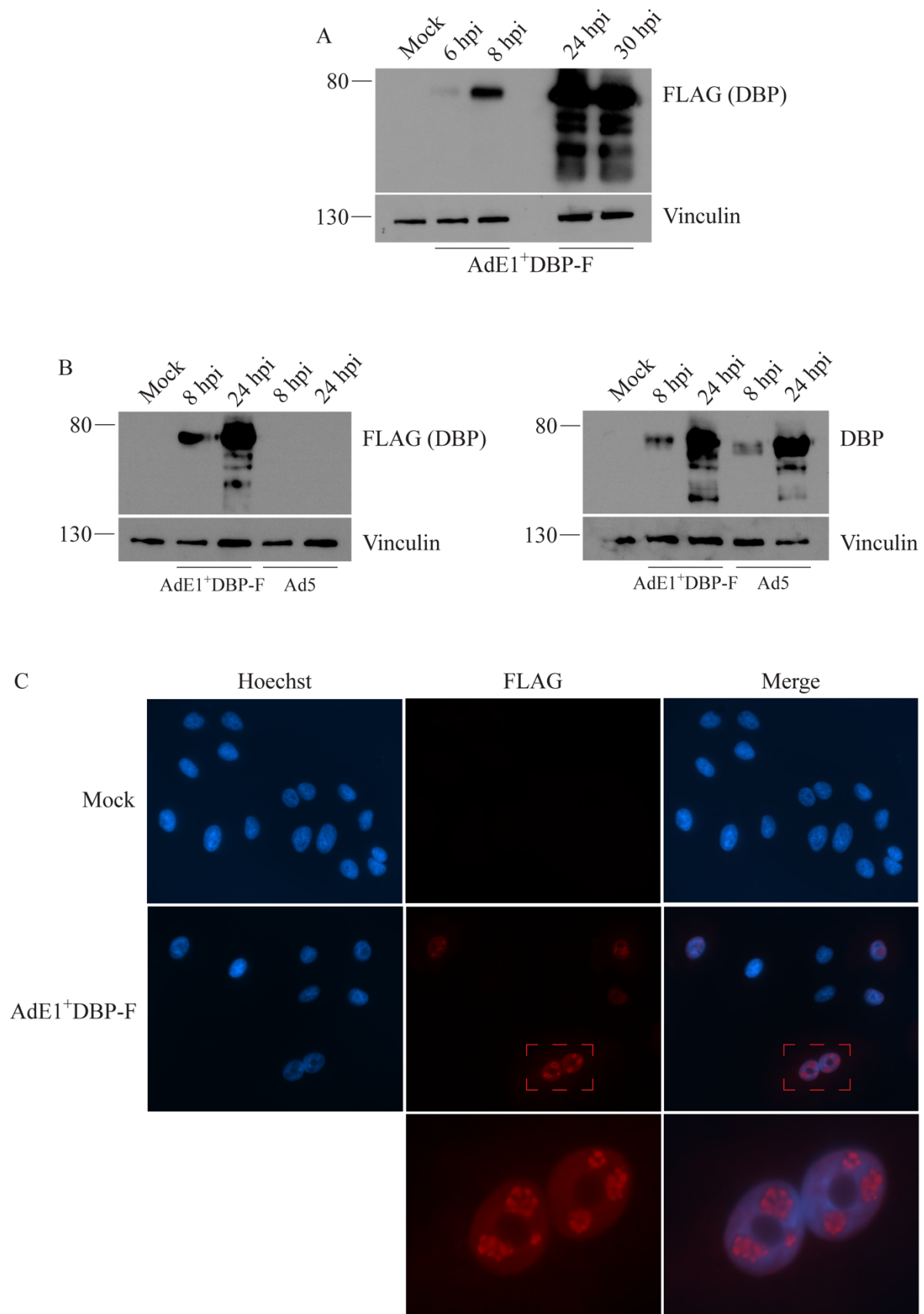


Figure 2.2 Expression of the E2A-encoded, FLAG-tagged DBP from AdE1⁺DBP-F. (A) A549 cells were infected with AdE1⁺DBP-F at an MOI of 50, and crude cell lysates were analyzed for FLAG-tagged DBP expression at varying times post-infection. DBP was readily detected via immunoblotting in cell lysates during both early and late infection. (B) An experiment similar to that in panel A was conducted with Ad5 (MOI 50) in addition to AdE1⁺DBP-F. The lysates were analyzed in duplicate immunoblots to detect DBP using either an anti-FLAG or an anti-DBP antibody. (C) Immunofluorescence analysis of FLAG-tagged DBP expression. A549 cells were plated at 1/10th of the confluency density and infected at an MOI of 10. At 18 hpi, distinct Ad replication foci within the host cell nuclei were visible through immunofluorescence imaging for FLAG-DBP.

immunofluorescence. Ad replication foci within infected cell nuclei were clearly observed at an MOI of 10 using the antibody directed against the FLAG-tag on the DBP (Fig 2.2C). Thus, the FLAG-tagged DBP is readily detectable by immunoblot and immunofluorescence in infected cells.

Unlike the abundantly expressed DBP, biochemical studies on Ad Pol and pTP have been difficult to conduct due to low expression levels of these two proteins, and existence of these proteins as a pTP-Pol complex in infected cells (11, 34). Consistently, we were not able to detect the presence of pTP in cell lysates at 6 or 8 hpi using an MOI of 50 (data not shown), but the FLAG-tagged pTP could be detected at low levels from 9 hpi (Fig 2.3A). Since both DBP and pTP contain an identical epitope tag, and were thus detected using the same antibody against FLAG, our inability to detect pTP at the earlier time points supports the idea that pTP is expressed at a significantly lower level than DBP in the infected cell (with the caveat that the differences in amino acid composition surrounding the FLAG tag in the two proteins may also influence the efficiency of antibody binding). We were also unable to visualize the FLAG-tagged TP by immunofluorescence (data not shown), once again suggesting that the expression levels of pTP from E2B are much lower than DBP from E2A. Only the 80-kDa pTP was observed in infected cells, even at the very late time point of 24 (Fig 2.3B) or 48 hpi (data not shown). We did not observe the mature 55-kDa protein in infected cell lysates, which was somewhat surprising considering that significant numbers of mature virions had likely accumulated in the nucleus of the infected cells by 24 hpi. It is possible that the 55-kDa TP remained attached to the newly-synthesized viral genomes and did not enter the gel, but remained in the sample loading wells instead. Following digestion with DNaseI, the proteolytically-processed 55-kDa mature TP associated with the viral DNA was readily detected by immunoblot analysis of purified virions

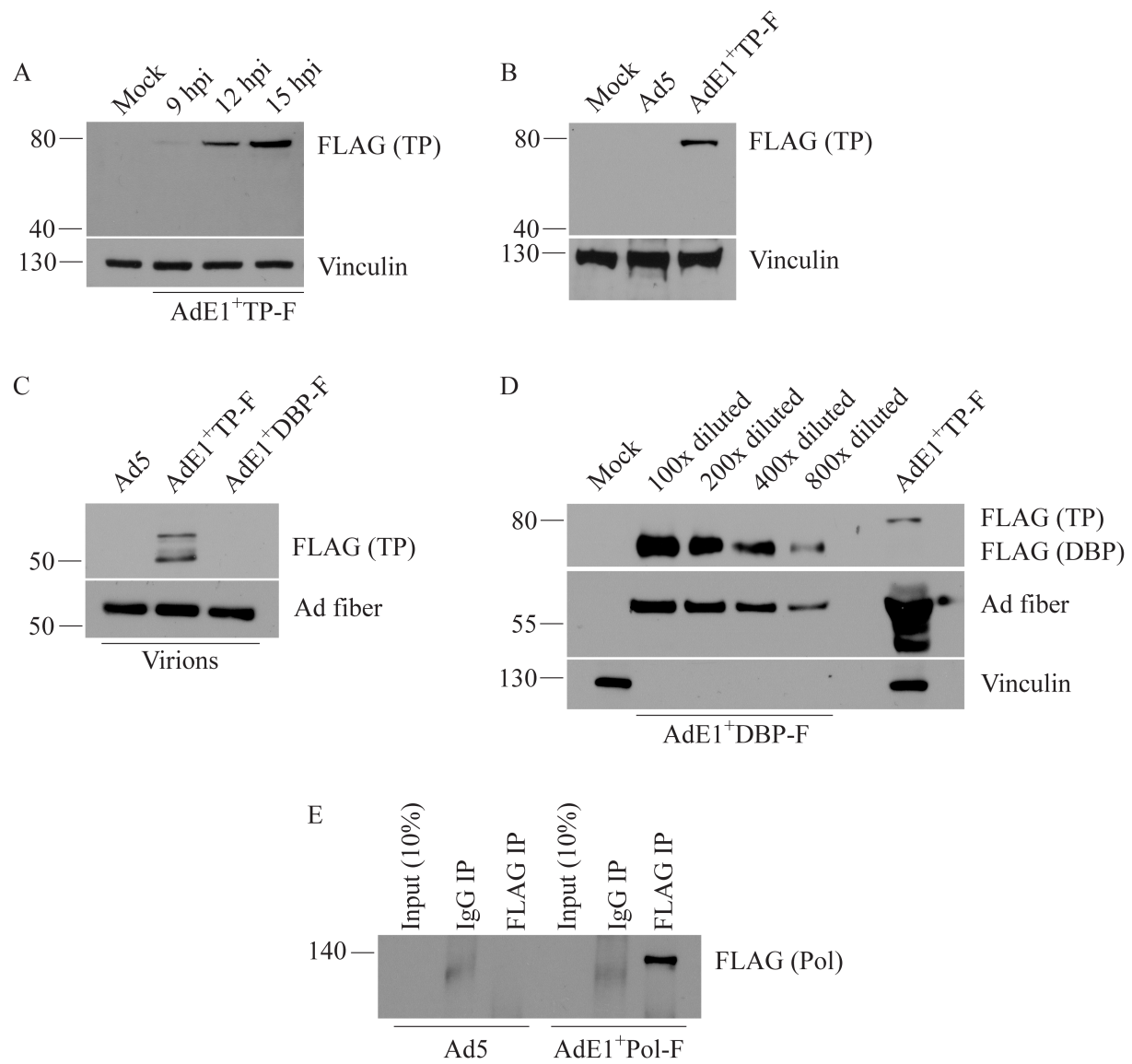


Figure 2.3 Expression of the E2B-encoded, FLAG-tagged TP and Pol. (A) A549 cells were infected with AdE1⁺TP-F at an MOI of 50 and expression of the 80-kDa pTP was observed as early as 9 hpi. (B) Even at a later time point of 24 hpi, only the 80-kDa pTP was detected in infected cell lysates, but not the 55-kDa mature protein. (C) Ad5, AdE1⁺DBP-F or AdE1⁺TP-F (3×10^{10} virus particles) were heat-denatured and DNase-treated. The Ad DNA-associated 55-kDa TP was readily detected by immunoblot. (D) Lysate of cells infected for 24 hrs with AdE1⁺DBP-F was serially diluted 100- to 800-fold. DBP expression in the diluted samples and TP expression in the undiluted AdE1⁺TP-F-infected cell lysate were analyzed by immunoblot to compare expression levels of the two proteins. Undiluted sample showed equal intensity when probed for vinculin (data not shown). (E) A549 cells were infected with Ad5 or AdE1⁺DBP-F at an MOI of 100. Immunoprecipitation was conducted 24 hrs later, followed by SDS-PAGE and immunoblot analysis of the samples. When probed with the anti-FLAG antibody, a single band corresponding to the 140-kDa Ad Pol was clearly visible in lysates of cells infected with AdE1⁺DBP-F.

(Fig 2.3C). Interestingly, we observed two prominent protein bands from the purified virions (Fig 2.3C), which may represent cleavage of the iTP by the Ad protease at positions 317 and 349 (51-53). However, cleavage at position 317 has not been observed experimentally, although there is a type 2 consensus cleavage site for the Ad protease present at that position (51). Alternatively, the two TP species present in mature Ad virions may reflect a difference in post-translational modification of the mature 55-kDa TP (54, 55), or perhaps a small number of viral nucleotides remaining attached to the DNase-treated sample. Regardless, pTP and TP are clearly detectable with the FLAG antibody in protein lysates from infected cells and purified virions, respectively.

The results described above indirectly suggest that pTP is expressed at a much lower level in infected cells compared to DBP. To directly compare the relative level of pTP and DBP expression, we analyzed serially diluted lysates from cells infected with AdE1⁺DBP-F and undiluted lysate from cells infected with AdE1⁺TP-F. Comparison of the signal obtained for pTP with the dilution series of DBP indicates that DBP is expressed at an approximately 800-fold higher level than TP under the conditions examined (A549 cells, MOI of 50, 24 hpi) (Fig 2.3D).

Repeated attempts to detect the FLAG-tagged Ad DNA Pol in crude protein lysates by standard immunoblot, even at as high an MOI as 100, were unsuccessful (data not shown), suggesting that Pol is expressed at very low levels in infected cells. However, using a standard immunoprecipitation assay, we could detect a 140-kDa species representing the full-length Pol (Fig 2.3E). As might be expected, attempts to detect Pol through immunofluorescence of infected cells were unsuccessful (data not shown), once again suggesting that expression of this E2B-encoded Pol protein is extremely low in infected cells.

2.4.4 E2 proteins are expressed at a very low level from E1-deleted Ad vectors

Previous studies have suggested that E1-deleted Ad vectors can replicate in many cell types, particularly transformed cells (26). For example, using an assay in which infecting viral DNA was specifically methylated to prevent cleavage by XhoI restriction endonuclease, and newly-replicated viral DNA became sensitive to cleavage due to absence of this methylation, Nelson and Kay estimated that the genome of an E1-deleted virus at an MOI of 100 underwent replication such that the nascent DNA represented 84% of the total viral genome in A549 cells at 72 hpi (27). Varying levels of replication were observed in almost all cell lines tested in the study, suggesting that E1-deleted viruses could undergo a surprisingly high level of replication in “non-permissive” cells. Since E2 proteins are required for viral DNA replication, and full activation of the E2 promoter requires E1A (30), it has been suggested that certain cellular factors may be able to compensate for lack of E1A in E1-deleted viruses, resulting in expression of the E2 proteins. Several cellular factors have been implicated in complementing the loss of E1A, including in the A549 cell line used in our studies (26, 56). In these studies, viral gene expression and replication of the E1-deleted vectors were enhanced with increasing MOI, suggesting a cooperative effect in which each additional genome contributed replicative proteins to enhance the level of replication. Consequently, there may be a threshold of replicative proteins required to initiate Ad DNA replication (26, 27, 56).

The placement of a FLAG-tag on the E2 proteins provided us with unique reagents to examine the relative level of E2 expression from E1-competent and E1-deficient virus in A549 cells. Given the low expression levels of pTP and Pol within the infected cells, we used the AdE1⁺DBP-F and the AdE1⁻DBP-F constructs to take advantage of the higher DBP expression

levels, and thus greater sensitivity. A549 cells were infected at an MOI of 10 with AdE1⁺DBP-F or AdE1⁻DBP-F and 24 hr later, crude protein extracts were prepared and analyzed for DBP and fiber expression by immunoblot. As shown in Fig 2.4A, we observed a very strong DBP signal for AdE1⁺DBP-F, but the level of DBP expression from AdE1⁻DBP-F was very low and only visible on overexposure of the blot. Similarly, although expression of the late fiber protein, which is only expressed at high levels following DNA replication, was easily detected in cells infected with AdE1⁺DBP-F, no fiber expression was observed in cells infected with AdE1⁻DBP-F. To better gauge the relative level of DBP expression between the two viruses, we analyzed a dilution series of lysate from cells infected with AdE1⁺DBP-F relative to undiluted AdE1⁻DBP-F cell lysate. AdE1⁻DBP-F expressed the E2A-encoded DBP at a level approximately 1/200th that of the E1⁺ virus (Fig 2.4B) under these conditions (MOI of 10, 24 hpi). We also analyzed DNA replication directly by qPCR at 4, 24, 48 and 72 hpi (for AdE1⁺DBP-F, samples were isolated only at 4 and 24 hpi). Although cells infected with both viruses showed comparable levels of viral DNA at 4 hpi (prior to initiation of DNA replication), there was a ~4-log increase in viral genome copy number at 24 hpi for AdE1⁺DBP-F, but no change for AdE1⁻DBP-F (Fig 2.4C). Even at the later time points of 48 and 72 hpi, there was only a very modest increase in viral genome copy number in cells infected with AdE1⁻DBP-F, amounting to a ~5-fold increase in genome copy number within the cells between 24 and 72 hours. Thus, at a relatively low MOI of 10, AdE1⁻DBP-F does express some DBP, but the level is significantly lower than that from the E1⁺ virus, and is not sufficient to allow efficient viral DNA replication and subsequent late gene expression. Similar experiments were conducted in human HepG2 cells and the observations were consistent with those in A549 cells (Fig 2.4D and 2.4E). DBP expression from the AdE1⁻DBP-F was 1/400th of that from AdE1⁺DBP-F at an MOI of 10 for samples isolated at 24 hpi (Fig 2.4D); although viral DNA

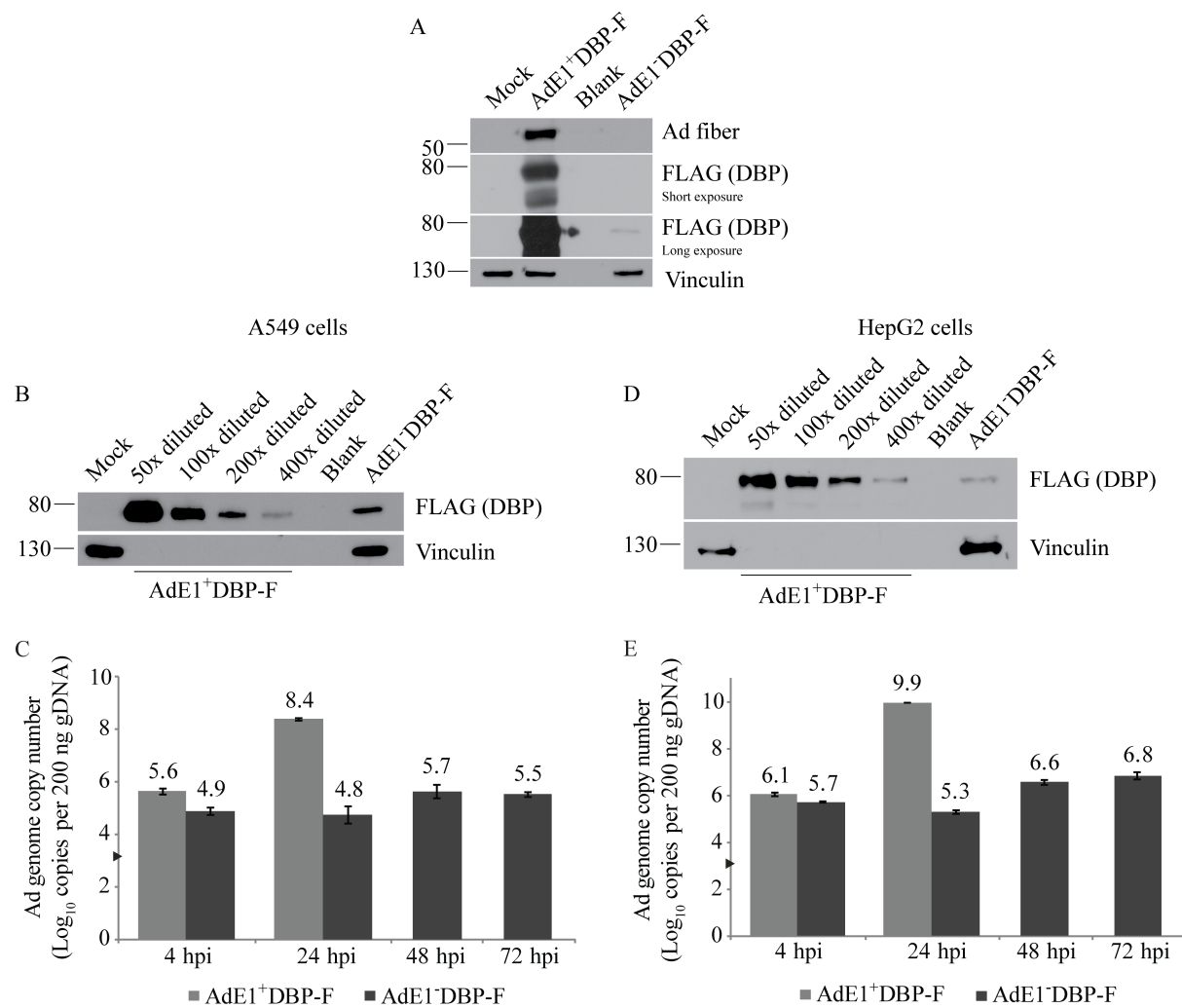


Figure 2.4 Removal of the E1 region results in a dramatic reduction in E2 gene expression and DNA replication in A549 and HepG2 cells. (A) A549 cells were infected at an MOI of 10 with AdE1⁺DBP-F or AdE1⁻DBP-F. Cell lysates isolated at 24 hpi were analyzed for fiber and DBP expression via immunoblot. DBP expressed from the AdE1⁻DBP-F was only observed at high exposure. (B) The AdE1⁺DBP-F-infected sample used in panel A (MOI 10, 24 hpi) was diluted 40- to 500-fold in 2x protein loading buffer. FLAG-DBP expression in undiluted AdE1⁻DBP-F-infected cell lysate and diluted AdE1⁺DBP-F-infected cell lysates were detected by immunoblot to compare expression from the two constructs. (C) A549 cells were infected with AdE1⁺DBP-F for 4-24 hours or AdE1⁻DBP-F for 4-72 hours. DNA was harvested and qPCR was performed as described above to determine the copy numbers of viral DNA per 200 ng DNA isolated from infected cells. The mean of three independent experiments is shown and the error bars represent SD. The arrowhead on the y-axis indicates the limit of detection for the assay, and represents the background copy number calculated in uninfected cells. (D) Lysates of HepG2 cells infected with AdE1⁺DBP-F (MOI 10, 24 hpi) were diluted as described in panel B to compare DBP expression from the E1⁺ and E1⁻ viruses in this cell line. Undiluted sample showed equal intensity when probed for vinculin (data not shown). (E) HepG2 cells were infected and qPCR was conducted as explained for A549 cells in panel C.

replication was slightly higher for both viruses in HepG2 cells compared to A549 cells, only a small increase in genome copy number was observed for AdE1⁻DBP-F between 24 and 72 hours (Fig 2.4E). Taken together, these results suggest that in the absence of E1 proteins, Ad vectors undergo very limited E2 expression and DNA replication in infected cells.

2.4.5 E1-deficient Ad cooperate to enhance virus replication in an additive, but not synergistic, manner

We examined the ability of the E1-deficient virus to synergize in high-MOI infections and enhance virus replication in non-permissive cells (i.e. those not complementing the E1-deficiency). If each virus contributes a small amount of E2 proteins, then with increasing MOI, the cumulative expression could reach the threshold required for efficient replication. Consequently, with increasing MOI, we should observe a non-linear increase in replication and late gene expression. We first examined whether the E1-deleted and the E1⁺ virus had similar abilities to replicate under conditions in which E1 was supplied (i.e. in the E1-complementing 293 cell line). 293 cells were infected at low MOIs with AdE1⁺DBP-F or AdE1⁻DBP-F, crude proteins samples were isolated 24 later, and evaluated for the level of fiber protein (a surrogate marker for viral DNA replication). As shown in Fig 2.5A, both viruses replicated well, and we did not observe a significant difference in fiber expression between the two constructs. Thus, E1-deleted viruses display wildtype levels of gene expression and replication when E1 is provided *in trans*. We next examined replication for the E1-deleted virus in A549 cells as a function of MOI. We saw an approximately linear increase in expression of DBP from AdE1⁻DBP-F between an MOI of 50 to 1000 (Fig 2.5B); however, the level of DBP never reached that produced by the E1⁺ version of the tagged virus applied to cells at an MOI of 1. We could detect a low level of fiber expression from AdE1⁻DBP-F starting at an

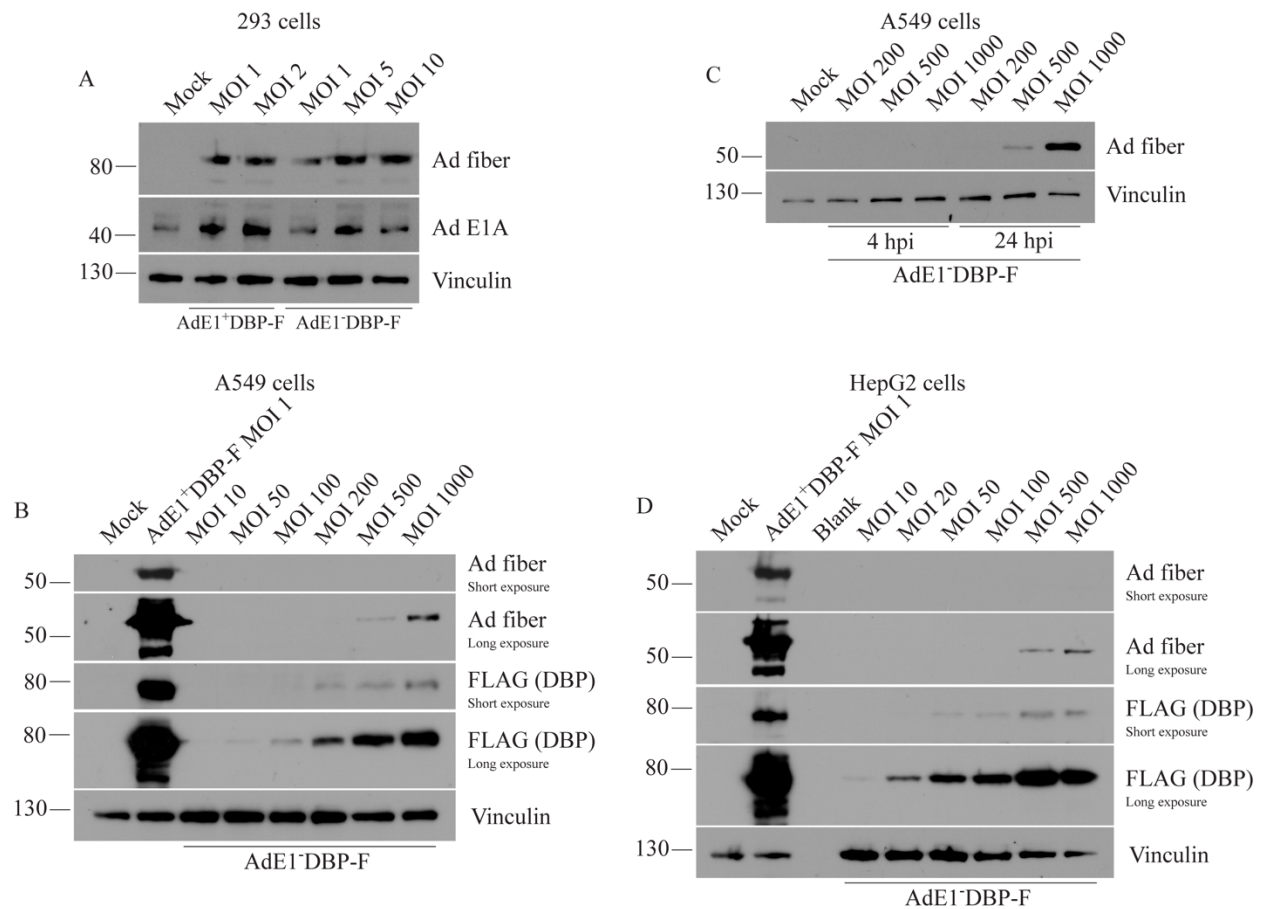


Figure 2.5 Late gene expression from the E1-deficient FLAG-tagged DBP construct is observed at very high MOIs. (A) 293 cells were infected for 24 hours with the indicated MOI of AdE1⁺DBP-F and AdE1⁻DBP-F. Cell lysates were separated by SDS-PAGE and analyzed for fiber expression via immunoblot. (B) A549 cells were infected for 24 hours with AdE1⁺DBP-F at an MOI of 1 or increasing MOI of AdE1⁻DBP-F to determine the level of DBP necessary to achieve fiber expression, which is indicative of AdE1⁻DBP-F replication. Immunoblot analysis of fiber and DBP expression was conducted. (C) Fiber expression from AdE1⁻DBP-F was examined at 4 and 24 hpi in A549 cells. (D) An experiment similar to that in panel A was conducted in the HepG2 cell line.

MOI of 500, suggesting that sufficient DBP, and presumably also the replicative proteins encoded by E2B, was produced to allow for replication of the genome and expression of fiber (Fig 2.5B). Similar to our observation for DBP, even at an MOI as high as 1000, AdE1⁻DBP-F did not express fiber at a level comparable to the low-MOI AdE1⁺DBP-F infection. To confirm that the fiber signal observed for AdE1⁻DBP-F at 24 hpi was due to *de novo* gene expression and not simply from the infecting capsids, we conducted a time-course of expression from AdE1⁻DBP-F. As expected, we did not observe fiber signal at 4 hpi at any MOI, but fiber was detectable at the 24-hour time point for infections using only the very high MOIs (Fig 2.5C). Comparable results were obtained when examining the relationship between MOI, DBP and fiber expression in HepG2 cells (Fig 2.5D). Taken together, these results suggest that E1-deficient viruses can cooperate at high MOI to provide sufficient replicative proteins to promote DNA replication and late gene expression, but this effect does not appear to be synergistic – at least not at the range of MOI we have tested.

2.5 Discussion

In this study, we have created Ad constructs containing a FLAG-tag in each of the three E2 protein coding regions (Table 2.1). Determination of the locations for the FLAG-tag insertion was aided by previous biochemical and linker-insertion mutagenesis studies that identified crucial regions of these proteins, and also regions that were tolerant for insertions of small peptides. We showed that all three proteins could be detected in Ad-infected cells by immunoblot (Fig 2.2 and 2.3); however, the low quantities of Pol necessitated the use of immunoprecipitation to concentrate the protein to a detectable level. The presence of the FLAG at amino acid position 467 of pTP allowed the tag to be retained in both the immature and mature version of the protein. Indeed, we easily observed only the 80-kDa immature protein in the infected cell lysates, and the cleaved 55-kDa version in mature virions (Fig 2.3). Since FLAG-directed reagents are readily commercially available, these E2-tagged viral vectors may be an important resource, permitting renewed investigation of these viral proteins.

Using the FLAG-tagged Ad constructs, we showed that during low MOI infections, the level of DBP expressed from E1-deleted Ad in A549 and HepG2 cells is very low and does not support efficient replication (Fig 2.4). These observations highlight the importance of E1A for full activation of the E2 promoter through both direct and indirect mechanisms. Although the Ad-encoded E4-ORF6/7 fusion protein can activate the E2 promoter to some extent, it is simply not efficient in the absence of E1A (30). Alternatively, since E1A transactivates the E4 promoter, the reduced levels of E4-ORF6/7 may contribute to low E2 promoter activity. In either case, the cellular factors that have been implicated in complementing the loss of E1A (56), leading to activation of the E2 promoter, are likely not as efficient as the native viral protein. Simply put, in

the absence of E1A, insufficient E2 proteins are produced to support efficient viral DNA replication. However, previous studies have reported that under some conditions, E1-deleted viruses can replicate at a significant level in non-permissive cells, but this typically requires a high MOI (26, 27, 56). In our study, we showed that even at an MOI as high as 1000, an E1-deleted vector expressed DBP at a level that was still substantially lower than a comparable E1⁺ virus at an MOI of 1 (Fig 2.5). Nevertheless, we did detect fiber expression from the E1-deficient virus at an MOI of 500 and above, suggesting that viral DNA replication had occurred (Fig 2.5). It is possible that at higher MOIs, the additive effect of each genome enhances the pool of available viral replicative proteins to the point where viral replication can occur. However, aside from expression of the E2 replicative genes, loss of E1A also impacts many other aspects of virus gene expression and replication which likely cumulatively compromise efficient virus replication. For example, since E1A has been shown to interact with several cellular transcription factors (*e.g.* SP1) to directly activate expression from the MLP (57), E1A deletion also directly reduces late gene transcription. Furthermore, E1A interacts with several cellular epigenetic regulatory proteins to cause a global change in epigenetic status of many genes within the host cell, which alters the cellular microenvironment to promote efficient virus replication (58, 59). E1A may also be involved in the epigenetic restructuring of the Ad genome early during infection (60). This wide-ranging epigenetic reprogramming of cellular gene expression would not occur in cells infected with an E1-deleted Ad.

Taken together, our work underscores the crucial role of E1A in orchestrating and modulating various cellular and viral processes to achieve efficient Ad replication. However, the results also clearly show that MOI-dependent effects on replication of E1-deleted Ad is an

important consideration when using Ad vectors for gene delivery. Attempts to increase transgene expression through use of high MOI infections may have the undesired effect of enabling Ad replication, which could ultimately compromise the integrity of the study.

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2.7 References

1. Hoke CH, Jr., Snyder CE, Jr. History of the restoration of adenovirus type 4 and type 7 vaccine, live oral (Adenovirus Vaccine) in the context of the Department of Defense acquisition system. *Vaccine*. 2013;31(12):1623-32.
2. Swaminathan S, Thimmapaya B. Regulation of adenovirus E2 transcription unit. *Current topics in microbiology and immunology*. 1995;199 (Pt 3):177-94.
3. Saha B, Wong CM, Parks RJ. The adenovirus genome contributes to the structural stability of the virion. *Viruses*. 2014;6(9):3563-83.
4. Frisch SM, Mymryk JS. Adenovirus-5 E1A: paradox and paradigm. *Nature reviews Molecular cell biology*. 2002;3(6):441-52.
5. Gonzalez R, Huang W, Finnen R, Bragg C, Flint SJ. Adenovirus E1B 55-Kilodalton Protein Is Required for both Regulation of mRNA Export and Efficient Entry into the Late Phase of Infection in Normal Human Fibroblasts. *J Virol*. 2006;80(2):964-74.
6. Blackford AN, Grand RJA. Adenovirus E1B 55-Kilodalton Protein: Multiple Roles in Viral Infection and Cell Transformation. *J Virol*. 2009;83(9):4000-12.
7. Caravokyri C, Leppard KN. Human adenovirus type 5 variants with sequence alterations flanking the E2A gene: effects on E2 expression and DNA replication. *Virus genes*. 1996;12(1):65-75.
8. de Jong RN, Meijer LAT, van der Vliet PC. DNA binding properties of the adenovirus DNA replication priming protein pTP. *Nucleic acids research*. 2003;31(12):3274-86.
9. Nagata K, Guggenheimer RA, Hurwitz J. Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. *Proceedings of the National Academy of Sciences of the United States of America*. 1983;80(20):6177-81.

10. Parker EJ, Botting CH, Webster A, Hay RT. Adenovirus DNA polymerase: domain organisation and interaction with preterminal protein. *Nucleic acids research*. 1998;26(5):1240-7.
11. Stunnenberg HG, Lange H, Philipson L, van Miltenburg RT, van der Vliet PC. High expression of functional adenovirus DNA polymerase and precursor terminal protein using recombinant vaccinia virus. *Nucleic acids research*. 1988;16(6):2431-44.
12. Chen M, Mermod N, Horwitz MS. Protein-protein interactions between adenovirus DNA polymerase and nuclear factor I mediate formation of the DNA replication preinitiation complex. *The Journal of biological chemistry*. 1990;265(30):18634-42.
13. Horwitz MS. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. *The journal of gene medicine*. 2004;6 Suppl 1:S172-83.
14. Weitzman MD. Functions of the adenovirus E4 proteins and their impact on viral vectors. *Frontiers in bioscience : a journal and virtual library*. 2005;10:1106-17.
15. Sargent KL, Meulenbroek RA, Parks RJ. Activation of Adenoviral Gene Expression by Protein IX Is Not Required for Efficient Virus Replication. *J Virol*. 2004;78(10):5032-7.
16. Parks RJ. Adenovirus protein IX: a new look at an old protein. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2005;11(1):19-25.
17. O'Malley RP, Mariano TM, Siekierka J, Mathews MB. A mechanism for the control of protein synthesis by adenovirus VA RNAI. *Cell*. 1986;44(3):391-400.
18. Aparicio O, Razquin N, Zaratiegui M, Narvaiza I, Fortes P. Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *J Virol*. 2006;80(3):1376-84.

19. Vachon VK, Conn GL. Adenovirus VA RNA: An essential pro-viral non-coding RNA. *Virus research*. 2016;212:39-52.
20. Tollefson AE, Ying B, Doronin K, Sidor PD, Wold WS. Identification of a new human adenovirus protein encoded by a novel late I-strand transcription unit. *J Virol*. 2007;81(23):12918-26.
21. Ying B, Tollefson AE, Wold WSM. Identification of a Previously Unrecognized Promoter That Drives Expression of the UXP Transcription Unit in the Human Adenovirus Type 5 Genome. *J Virol*. 2010;84(21):11470-8.
22. Nash LA, Parks RJ. Adenovirus Biology and Development as a Gene Delivery Vector. In: Ng P, Brunetti-Pierri N, editors. *Therapeutic Applications of Adenoviruses. Gene and Cell Therapy*. Boca Raton, Florida: CRC Press; 2016. p. 1-36.
23. Huang JH, Zhang SN, Choi KJ, Choi IK, Kim JH, Lee MG, et al. Therapeutic and tumor-specific immunity induced by combination of dendritic cells and oncolytic adenovirus expressing IL-12 and 4-1BBL. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2010;18(2):264-74.
24. Bessis N, GarciaCozar FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene therapy*. 2004;11 Suppl 1:S10-7.
25. Nakai M, Komiya K, Murata M, Kimura T, Kanaoka M, Kanegae Y, et al. Expression of pIX gene induced by transgene promoter: possible cause of host immune response in first-generation adenoviral vectors. *Human gene therapy*. 2007;18(10):925-36.

26. Gorziglia MI, Kadan MJ, Yei S, Lim J, Lee GM, Luthra R, et al. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J Virol.* 1996;70(6):4173-8.
27. Nelson JE, Kay MA. Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J Virol.* 1997;71(11):8902-7.
28. Lieber A, He C-y, Kirillova I, Kay MA. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. *Journal of virology.* 1996;70(12):8944-60.
29. Reichel R, Neill SD, Kovesdi I, Simon MC, Raychaudhuri P, Nevins JR. The adenovirus E4 gene, in addition to the E1A gene, is important for trans-activation of E2 transcription and for E2F activation. *J Virol.* 1989;63(9):3643-50.
30. Swaminathan S, Thimmapaya B. Transactivation of adenovirus E2-early promoter by E1A and E4 6/7 in the context of viral chromosome. *Journal of molecular biology.* 1996;258(5):736-46.
31. O'Connor RJ, Hearing P. The E4-6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. *J Virol.* 2000;74(13):5819-24.
32. Reich NC, Sarnow P, Duprey E, Levine AJ. Monoclonal antibodies which recognize native and denatured forms of the adenovirus DNA-binding protein. *Virology.* 1983;128(2):480-4.
33. Schaack J, Guo X, Ho WY, Karlok M, Chen C, Ornelles D. Adenovirus type 5 precursor terminal protein-expressing 293 and HeLa cell lines. *J Virol.* 1995;69(7):4079-85.
34. Friefeld BR, Korn R, de Jong PJ, Sninsky JJ, Horwitz MS. The 140-kDa adenovirus DNA polymerase is recognized by antibodies to Escherichia coli-synthesized determinants

- predicted from an open reading frame on the adenovirus genome. Proceedings of the National Academy of Sciences of the United States of America. 1985;82(9):2652-6.
35. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol*. 1996;70(7):4805-10.
 36. Poulin KL, Tong G, Vorobyova O, Pool M, Kothary R, Parks RJ. Use of Cre/loxP recombination to swap cell binding motifs on the adenoviral capsid protein IX. *Virology*. 2011;420(2):146-55.
 37. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, et al. A monomeric red fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(12):7877-82.
 38. McKinnon RD, Bacchetti S, Graham FL. Tn5 mutagenesis of the transforming genes of human adenovirus type 5. *Gene*. 1982;19(1):33-42.
 39. Bramson JL, Grinshtein N, Meulenbroek RA, Lunde J, Kottachchi D, Lorimer IA, et al. Helper-dependent adenoviral vectors containing modified fiber for improved transduction of developing and mature muscle cells. *Human gene therapy*. 2004;15(2):179-88.
 40. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *The Journal of general virology*. 1977;36(1):59-74.
 41. Ross PJ, Kennedy MA, Christou C, Risco Quiroz M, Poulin KL, Parks RJ. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol*. 2011;85(8):3950-8.

42. Goulet BB, McFall ER, Wong CM, Kothary R, Parks RJ. Supraphysiological expression of survival motor neuron protein from an adenovirus vector does not adversely affect cell function. *Biochemistry and cell biology = Biochimie et biologie cellulaire*. 2013;91(4):252-64.
43. Tucker PA, Tsernoglou D, Tucker AD, Coenjaerts FE, Leenders H, van der Vliet PC. Crystal structure of the adenovirus DNA binding protein reveals a hook-on model for cooperative DNA binding. *The EMBO journal*. 1994;13(13):2994-3002.
44. Tsernoglou D, Tsugita A, Tucker AD, van der Vliet PC. Characterization of the chymotryptic core of the adenovirus DNA-binding protein. *FEBS letters*. 1985;188(2):248-52.
45. Vos HL, Brough DE, Van der Lee FM, Hoeben RC, Verheijden GF, Dooijes D, et al. Characterization of adenovirus type 5 insertion and deletion mutants encoding altered DNA binding proteins. *Virology*. 1989;172(2):634-42.
46. Roovers DJ, Overman PF, Chen XQ, Sussenbach JS. Linker mutation scanning of the genes encoding the adenovirus type 5 terminal protein precursor and DNA polymerase. *Virology*. 1991;180(1):273-84.
47. Mangel WF, San Martin C. Structure, function and dynamics in adenovirus maturation. *Viruses*. 2014;6(11):4536-70.
48. Desiderio SV, Kelly TJ, Jr. Structure of the linkage between adenovirus DNA and the 55,000 molecular weight terminal protein. *Journal of molecular biology*. 1981;145(2):319-37.

49. Roovers DJ, van der Lee FM, van der Wees J, Sussenbach JS. Analysis of the adenovirus type 5 terminal protein precursor and DNA polymerase by linker insertion mutagenesis. *J Virol.* 1993;67(1):265-76.
50. Gattoni R, Keohavong P, Stevenin J. Splicing of the E2A premessenger RNA of adenovirus serotype 2. Multiple pathways in spite of excision of the entire large intron. *Journal of molecular biology.* 1986;187(3):379-97.
51. Ruzindana-Umunyana A, Imbeault L, Weber JM. Substrate specificity of adenovirus protease. *Virus research.* 2002;89(1):41-52.
52. Webster A, Leith IR, Hay RT. Activation of adenovirus-coded protease and processing of preterminal protein. *J Virol.* 1994;68(11):7292-300.
53. Webster A, Leith IR, Nicholson J, Hounsell J, Hay RT. Role of preterminal protein processing in adenovirus replication. *J Virol.* 1997;71(9):6381-9.
54. Bergstrom Lind S, Artemenko KA, Elfineh L, Zhao Y, Bergquist J, Pettersson U. The phosphoproteome of the adenovirus type 2 virion. *Virology.* 2012;433(1):253-61.
55. Kusakawa J, Ramachandra M, Nakano R, Padmanabhan R. Phosphorylation-dependent interaction of adenovirus preterminal protein with the viral origin of DNA replication. *The Journal of biological chemistry.* 1994;269(3):2189-96.
56. Imperiale MJ, Kao HT, Feldman LT, Nevins JR, Strickland S. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Molecular and cellular biology.* 1984;4(5):867-74.
57. Parks CL, Shenk T. Activation of the adenovirus major late promoter by transcription factors MAZ and Sp1. *J Virol.* 1997;71(12):9600-7.

58. Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ, Kurdistani SK. Epigenetic reprogramming by adenovirus e1a. *Science (New York, NY)*. 2008;321(5892):1086-8.
59. Horwitz GA, Zhang K, McBrian MA, Grunstein M, Kurdistani SK, Berk AJ. Adenovirus small e1a alters global patterns of histone modification. *Science (New York, NY)*. 2008;321(5892):1084-5.
60. Giberson AN, Davidson AR, Parks RJ. Chromatin structure of adenovirus DNA throughout infection. *Nucleic acids research*. 2012;40(6):2369-76.

Chapter 3:

Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid Suppresses Human Adenovirus Gene Expression and Replication

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3.1 Abstract

Human adenovirus (HAdV) causes minor illnesses in most patients, but can lead to severe disease and death in pediatric, geriatric and immunocompromised individuals. No approved antiviral therapy currently exists for the treatment of these severe HAdV-induced diseases. In this study, we show that the pan-histone deacetylase (HDAC) inhibitor SAHA reduces HAdV-5 gene expression and DNA replication in tissue culture, ultimately decreasing virus yield from infected cells. Importantly, SAHA also reduced gene expression from more virulent and clinically relevant serotypes including HAdV-4 and HAdV-7. In addition to SAHA, several other HDAC inhibitors (e.g. TSA, apicidin, panobinostat) also affected HAdV gene expression. We determined that loss of class I HDAC activity, mainly HDAC2, impairs efficient expression of viral genes, and that E1A physically interacts with HDAC2. Our results suggest that HDAC activity is necessary for HAdV replication, which may represent a novel pharmacological target in HAdV-induced disease.

3.2 Introduction

Human adenovirus (HAdV) mainly causes mild, self-limiting illnesses in most immunocompetent patients. HAdV-induced diseases vary based on the virus serotype involved, but respiratory tract infections are common and can often lead to bronchitis or pneumonia (1, 2). These minor infections are thought to be underreported, leading to an underestimation of HAdV infection and prevalence (3). HAdV can also cause severe disease, including respiratory failure, disseminated infection, neurologic complications, and even death in pediatric, geriatric and immunocompromised patients (e.g. stem cell or solid organ transplant recipients) (1, 4-6). Recently, several cases of immunocompetent patients succumbing to HAdV-induced respiratory failure was documented (7, 8). No approved antiviral therapy currently exists for the treatment of these severe HAdV infections (9, 10). Although DNA synthesis inhibitors such as cidofovir and ganciclovir are considered to be standard treatments, these drugs are often associated with serious adverse effects (11) and have limited efficacy in systemic HAdV infections (9). Brincidofovir, a phospholipid conjugate of cidofovir, is currently in clinical trial for treatment of HAdV-induced diseases (12, 13). This drug has several advantages over cidofovir (e.g. oral delivery, increased cellular uptake), but it is still associated with gastro-intestinal toxicity in some patients. Thus, novel antiviral therapies are necessary to combat severe HAdV infections.

HAdV, a non-enveloped, double-stranded DNA virus, was first isolated in the 1950's from adenoid tissue (14, 15). Since then, over 60 serotypes of HAdV (categorized into 7 species) have been identified (1), of which species B, C and E are most frequently associated with symptomatic respiratory infections (2). While species C (serotypes 1, 2, 5) mainly causes mild infections in young children, species B (serotypes 3, 7, 14, 21) and E (serotype 4) cause more severe infections

in adults (2). A live oral vaccine against serotypes 4 and 7 was used by the U.S. military from 1971 to 1997 to limit HAdV disease in new recruits (1). The vaccine was later reintroduced in 2011 due to a substantial increase in HAdV infection within military personnel during the intervening period, but it is not available to the general public.

HAdV serotypes 2 and 5 (species C) have been the most extensively studied to gain insights on virus biology, cellular processes and host-pathogen interactions. The 36-Kbp HAdV-5 genome consists of “early” and “late” genes which are expressed before and after viral DNA replication, respectively (16, 17). Of the early genes (E1A, E1B, E2, E3 and E4), the E1A gene product is absolutely necessary for efficient virus replication, and it is the first protein expressed from the infecting virus. E1A transactivates the remaining viral coding regions, interacts with many cellular proteins, and has various other functions that promote a productive infection (18-20). The E2 transcription unit consists of two regions, E2A and E2B. The E2A region encodes the HAdV DNA-binding protein (DBP), while the E2B region codes for the precursor terminal protein (pTP) and the viral DNA polymerase. All three E2 proteins play crucial roles during HAdV DNA replication (17, 21, 22). The E3 and E4 gene products modulate the host immune system and the cellular environment to make it more conducive to virus replication (23, 24). Finally, the late transcription units, L1-L5, are produced from alternative splicing of a common major late transcript containing five different polyadenylation sites which are all used during late infection (25). The major late promoter (MLP) is only strongly active following viral DNA replication (26), and the late regions code for structural and capsid proteins (e.g. fiber, penton, hexon) required for progeny virion formation.

Inside the virion, the virus-encoded, highly basic protein VII (pVII) tightly condenses the HAdV genome into a structure that is refractory to transcription and replication (27). During infection, the pVII-wrapped viral DNA enters the host cell nuclei through the nuclear pores (28). In the early phase of infection, the HAdV DNA dissociates from pVII, associates with cellular proteins including histones, and adopts a nucleoprotein structure similar to the host DNA (29, 30). The nucleosome density on the viral DNA is significantly reduced during late infection and at least some of the newly synthesized viral DNA must associate with pre-pVII for packaging into newly formed capsids (30-32). The cellular proteins involved in mediating these processes and the role of chromatin-modifying enzymes in the epigenetic regulation of the HAdV genome has not yet been fully elucidated (33). A recent study found that the nucleosomes associated with several of the HAdV-5 early promoters and transcription start sites are acetylated at various histone 3 (H3) lysine residues including H3K9, H3K18 and H3K27 (34). The level of acetylation was dynamic throughout infection and required interaction between HAdV-5 E1A and p300/CBP (35). In addition, valproic acid-mediated inhibition of histone deacetylase (HDAC) activity has been shown to inhibit HAdV-5 replication and spread *in vitro* (36), indicating the importance of HDAC activity for optimal virus replication.

In this study, we determined that the pan-HDAC inhibitor SAHA (suberoylanilide hydroxamic acid, also known as vorinostat) suppresses HAdV replication. SAHA treatment reduced viral gene transcription, protein expression and DNA replication, leading to a significant decrease in virus yield from infected cells. Of note, SAHA reduced HAdV yield at nanomolar concentrations, and was effective in decreasing gene expression from virulent serotypes 4 and 7. Trichostatin A (TSA) and several other HDAC inhibitors also exhibited a similar inhibitory activity against HAdV gene

expression. We further determined that class I HDACs, specifically HDAC2, appear to play an important role in the HAdV infection process.

3.3 Materials and Methods

Cell lines and reagents. The 293 cell line was a kind gift from Dr. Frank Graham (Professor Emeritus, McMaster University) (37). The A549-based cell line used in our experiments constitutively express the green fluorescent protein from an integrated lentivirus vector, and has been previously described (38). These cells grow and support HAdV replication identical to the parental A549 cell line (CCL-185, ATCC), and for simplicity, will be referred to as A549 cells in this study. Both 293 and A549 cells were grown in Minimum Essential Medium (MEM, Sigma Aldrich) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Sigma Aldrich), 2 mM GlutaMAX (Invitrogen) and 1x antibiotic-antimycotic (Invitrogen). MRC-5 cells (CCL-171, ATCC) were grown similarly in Dulbecco's Minimum Essential Medium (DMEM, Sigma Aldrich). The following seeding densities were used unless otherwise indicated: 2×10^4 cells per well for a 96-well plate, 4×10^5 cells per well for a 12-well plate, 1.2×10^6 cells per well for a 6-well plate and 8.8×10^6 cells for a 10 cm dish.

SAHA and TSA were purchased from Sigma (SML0061 and T8552) and MC1568 was purchased from Cayman Chemicals (16265). MS-275 was kindly provided by Dr. Jean-Simon Diallo (Ottawa Hospital Research Institute). All compounds were dissolved in DMSO.

Production of HAdV constructs. All HAdV constructs used this study are based on HAdV-5, and were constructed using a combination of conventional cloning and bacterial recombination (39). To generate the Ad-late/RFP virus, the monomeric red fluorescent protein (RFP) (40) was excised from pRP2483 (41) with BamHI and cloned into BamHI-digested pDC5 (41). As such, the RFP gene was placed behind a splice acceptor derived from the HAdV-40 long fiber gene,

generating pRP3085. A PvuI fragment from pRP3085 was cloned into PacI-digested pDC9 (41) placing the RFP cassette within the E3 deletion in an HAdV right-end shuttle plasmid. This construct was designated pRP3087, and then recombined into an HAdV E1/E3-deleted genomic plasmid pRP2468, generating pRP3088. pRP3088 was recombined with pXC1 (42), producing pRP3089 – an HAdV genomic plasmid containing an intact E1 region, with the E3 region replaced by splice acceptor-RFP cassette. pRP3089 was recovered as a virus, and designated Ad-late/RFP for this study. pRP3088 was also recombined with pAVH5 (41) to generate pRP3090, which is an HAdV genomic plasmid containing an enhanced green fluorescent protein under regulation of the human cytomegalovirus (CMV) enhancer/promoter and the bovine hormone polyadenylation sequence replacing the E1 region, as well as the splice acceptor-RFP cassette replacing the E3 region. pRP3090 was recovered as a virus and designated Ad(E1⁻)-late/RFP in this study.

Ad-CMV/E1A (AdRP3169) contains a small murine CMV promoter from pMH4 (43) inserted at 350 bp of the conventional HAdV-5 genome, followed by the E1 region beginning at 420 bp of the HAdV-5 genome. It is deleted of the E3 region. Ad(E1⁻)-CMV/RFP (AdRP2619) and Ad(E1⁺)TP-F (AdRP3000) constructs have been described previously (17, 44, 45). All viruses were propagated in 293 cells and purified by cesium chloride buoyant density centrifugation using standard procedures (46). Viral titers were determined by plaque assay on 293 cells as previously reported (17).

Infections and drug treatment. Unless otherwise indicated, medium was removed from confluent monolayers of A549 cells before infecting with the appropriate HAdV constructs at the indicated multiplicity of infection (MOI). MOIs were calculated as plaque-forming units (PFU) per cell, and

an MOI of 10 was used for most experiments. Virus inoculums were diluted in phosphate-buffered saline (PBS without CaCl_2 and MgCl_2 , Sigma-Aldrich) and added to the cell monolayer for 1 hour at 37°C with periodic rocking. Medium containing either vehicle or drug was then added to the cells, and incubated in a humidified CO_2 incubator at 37°C until the indicated time points. This medium (with vehicle or drug) was replaced every 24 hours of infection.

For synchronized or cold infections, the cells were incubated with the virus inoculum for 30 min on ice, allowing the viruses to attach but not enter the cells. Infection started when warm media (containing vehicle or drug) was added and the cells were placed at 37°C .

Immunoblot analysis. Protein samples were prepared in 2x Laemmli buffer, subjected to SDS-PAGE and transferred on a PVDF membrane (Millipore) as previously described (17). The membrane was blocked in 5% milk prepared in tris-buffered saline containing 0.2% Tween 20 (Thermo Fisher Scientific), and probed with antibodies diluted in the 5% milk solution. The following primary antibodies were used: HAdV-5 fiber (1/10,000 dilution; MS-1027-P0, Neomarkers), anti-HAdV-5 (1/10,000; ab6982, Abcam), HAdV-5 E1A (1/5,000; ab31686, Abcam or MA5-13643, Invitrogen), HDAC1 (1/2,000; 5356P, Cell Signalling), HDAC2 (1/5,000; ab16032, Abcam), HDAC3 (1/2,000; 3949S, Cell Signalling), acetyl-H3 (1/2,000; 06-599, Millipore), vinculin (1/10,000; ab129002, Abcam), tubulin (1/5,000; CP06, Calbiochem), actin (1/10,000; A1978, Sigma Aldrich), Rb (1/5,000; 554136, BD Pharmingen), FLAG (M2, 1/5,000; 200-301-383S, Rockland) and RFP (1/5,000; ab62341, Abcam). The p21 antibody (1/1,000; 2947, New England Biolabs) was kindly provided by Dr. Lynn Megeney (Ottawa Hospital Research Institute). The membranes were then washed twice in TBST and incubated with the appropriate

secondary antibodies conjugated to horseradish peroxidase (HRP). Blots were developed using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) or the Immobilon Classico Western HRP Substrate (Millipore). All immunoblot data are representative of two or more independent experiments.

Quantitative real-time PCR (qPCR). Medium was removed from infected cells at the indicated time points and the cells were incubated overnight at 37°C in SDS-proteinase K buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% w/v SDS, 1 mg/ml proteinase K). DNA was extracted from the cell lysates using the standard phenol-chloroform method and ethanol precipitation, then dissolved in 1x Tris-EDTA (TE) buffer. qPCR was conducted as previously described (45) using 200 ng of genomic DNA per reaction. The following primer sets were used: 5'-CTT ACC CCC AAC GAG TTT GA and 5'-GGA GTA CAT GCG GTC CTT GT for HAdV hexon; 5'-CCA TTA AAC CAG TTG CCG TGA GAG and 5'-GGC GTT TAC AGC TCA AGT CCA AAG for HAdV E1A; 5'-GGC CGG TGC TGA GTA TGT CG and 5'-TTC AAG TGG GCC CCG GCC TT for cellular *GAPDH*. Viral genome copy numbers were calculated from the Ct values using a standard curve obtained from serial dilutions of the pRP3089 plasmid.

RNA extraction and reverse transcription qPCR. Medium was removed from infected cells and total cellular RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) and the PureLink RNA Mini Kit (Ambion) according to the manufacturer's protocol. To synthesize cDNA from the extracted RNA by reverse transcription (RT), a mixture of random hexamers (New England Biolabs) and the M-MuLV Reverse Transcriptase (New England Biolabs) were used

according to the manufacturer's protocol. 800 ng of RNA was used per RT reaction. qPCR was then conducted as described above using 2 µl of the cDNA mixture.

Plaque assay to determine virus yield. Monolayers of A549 cells were infected with Ad-late/RFP or HAdV-5 (MOI 10) and the virus inoculum was removed after 1 hour of infection. The cells were washed with PBS, and fresh medium containing vehicle or various concentrations of SAHA was added. After 24-48 hours of infection, the cells were collected by scraping into the medium, sucrose (diluted in 10 mM Tris) was added to a final concentration of 4% v/v, and the samples were flash-frozen in liquid nitrogen. For the plaque assay, monolayers of A549 cells were infected with serial dilutions of this cell lysate. After 1 hour of infection at 37°C, the cells were overlaid with medium containing agarose (50% v/v of a 1% w/v agarose solution, 43% clear 2x MEM, 5% FBS, 1% GlutaMAX and 1% antibiotic-antimycotic). Plaques were counted 8-10 days later.

Fluorescence microscopy. Live imaging of infected A549 cells was performed 24 hours after infection using a 10x objective on a Zeiss Axio Observer.Z1 microscope equipped with an Axiocam MRC camera and ZEN 2 software for image processing.

Co-immunoprecipitation (co-IP). Ten-centimeter dishes of A549 cells were infected with Ad-late/RFP (MOI 10) and treated with vehicle or drug. 24 hours later, medium was removed and the cells were collected in 500 µl cold PBS. Following centrifugation at 1500 g, the volumes of the cell pellets were estimated, and the pellets were resuspended in twice the volume of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.5% Triton X-100, and protease inhibitors) for 30 min on ice. Cell lysates were cleared by centrifugation and diluted to 1

ml with additional lysis buffer. Following ~1.5 hour of pre-clearing with Protein G beads (1004D, Dynabeads), equal volumes of lysate were added to 30 μ l protein G beads that had been pre-incubated for 5 hours with 4 μ g of E1A antibody (ab31686, Abcam) or IgG (5415S, Cell Signalling) as negative control. 10% of the IP volume was retained as input sample. The antibody-bead complex was incubated with the cell lysate overnight at 4°C. The protein-bound beads were washed twice with lysis buffer and eluted with 2x Laemmli buffer for 15 minutes at 65°C. β -mercaptoethanol was added to a final concentration of 0.05% prior to subjecting the samples to SDS-PAGE and immunoblotting with the indicated antibodies.

Chromatin-immunoprecipitation (ChIP). Ten-centimeter dishes of A549 cells were infected with Ad-late/RFP (MOI 10) using the synchronized infection method and treated with vehicle or drug. After 6 hours of infection, the medium was removed and the cells were washed in cold PBS. 8-10 aliquots of roughly 500,000 cells were suspended in 1 ml media each, and 100 μ l formaldehyde buffer (50 mM HEPES-KOH, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% formaldehyde) was added to allow protein-DNA crosslinking. After 10 min of incubation on a rotating platform, the cells were treated with 50 μ l of 2.5 M glycine for 5 min and washed 3 times in cold PBS (containing calcium and magnesium) with centrifugation at 1350g for 5 min at 4°C. The PBS was discarded, and the cell pellets were flash frozen and stored at -80°C overnight.

To prepare the antibody-bead complex for IP, 50 μ l magnetic Protein G beads (1004D, Dynabeads) were washed in blocking solution (5% bovine serum albumin in PBS) three times, and incubated in 250 μ l blocking buffer containing 10 μ g IgG (SC-2027X, Santa Cruz), histone H3 (ab1791, Abcam), acetyl-H3 (ab47915, Abcam) or acetyl-H4 (06-598, Millipore) antibody overnight at 4°C

to allow antibody-bead complex formation. The unbound antibody was removed from the beads prior to addition of sheared DNA for ChIP (see below).

The frozen cell pellets were resuspended in 140 μ l lysis buffer (0.1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8, 1x protease inhibitor) and sonicated for 10 min at 4°C using the Covaris S220 sonicator to 500-1000 bp fragments. The sheared DNA samples were then diluted to 0.1 μ g/ μ l in lysis buffer. Equal amounts of DNA were added to 100 μ l of the various antibody-bead complexes and incubated overnight at 4°C with rotation. The next day, the beads (now complexed with DNA) were washed for 5 min each in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl pH 8, 500 mM NaCl), wash buffer (0.25 M LiCl, 1% GEPAL, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris pH 8) and 1x Tris-EDTA (TE) buffer. The DNA was eluted from the beads in 200 μ l elution buffer (50 mM Tris-HCl pH 8, 10 mM, 1% SDS) using a thermo-mixer at 65°C for 15 min. The eluate was separated from the beads and incubated overnight at 65°C with 20 μ g proteinase K (Sigma-Aldrich) to reverse DNA-protein crosslink and digest the proteins. The next day, the DNA samples were treated with 20 μ g RNase (Sigma-Aldrich) for 30 min at 37°C. One volume of phenol-chloroform was added to each sample, which were vortexed briefly and centrifuged at 15,000g for 15 min 4°C. The aqueous phase was collected and the following were added prior to overnight incubation at -80°C: 1/10th volume of 3 M sodium acetate, 5 μ g of glycogen (Roche), 5 μ g of linear acrylamide (Thermo Fisher Scientific) and 2.5 volumes of 99% ethanol. On the next day, all samples were centrifuged for 30 min at 4°C to obtain a DNA pellet, which was washed in 70% ethanol, air-dried and dissolved in 20 μ l 1x TE buffer. qPCR was performed as described above using 2 μ l of this ChIP DNA.

Quantification of RFP intensity. 96-well plates of A549 cells were infected with Ad-late/RFP (MOI 10) and treated with vehicle or HDAC inhibitors. Virus inoculum and medium containing drug were added to the existing cell medium simultaneously. 24 hours later, medium was removed, the cells were washed with PBS and fixed with 4% paraformaldehyde (pH 7). The cell nuclei were stained with 10 µg/ml Hoechst 33342 (Life Technologies) for ~20 min before RFP quantification using the Cellomics High Content Screening (HCS) Platform and the Cellomics navigator version of the HCS Studio software (Thermo Fisher Scientific). The 10x objective and the following filters were used: 386_{excitation (ex)} nm for Hoechst and 549_{ex} nm for RFP. Roughly 1,000 cells (Hoechst-stained intact nuclei) were analyzed per well and their total RFP intensity was measured. Further information on the quantification parameters is available upon request.

siRNA-mediated knockdown. Pooled siRNA targeting human HDAC1 (M-003493-02), HDAC2 (M-003495-02), HDAC3 (M-003496-02), p21 (M-003471-00) and siGenome Non-Targeting Control siRNA were obtained from Dharmacon. 5×10^4 A549 cells were plated in 24-well plates and transfected with 100 nM siRNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions for A549 cells. Transfection medium was replaced with regular medium after 5 hours of treatment. Knockdown was confirmed 48 hours post transfection via immunoblot analysis and the knockdown cells were infected at this point.

MTS metabolic activity assays. 96-well plates of A549 cells were prepared and infected/drug-treated as described above for RFP quantification. 24 hours post infection, the relative metabolic activity was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay

(Promega) according to the manufacturer's instructions. Cells were incubated for 45 mins at 37°C with 20 µl of the MTS substrate, and absorbance readings were obtained at 490 nm using the SpectraMax 190 plate spectrophotometer.

3.4 Results

3.4.1 RFP expression from a novel HAdV construct serves as an efficient tool to monitor virus replication

We generated an E1⁺ wildtype-like HAdV construct containing the RFP gene with an upstream splice acceptor site replacing the viral E3 region, thus placing RFP expression under the control of the MLP, which is only active following initiation of viral DNA replication (16, 47). This replication-competent construct was designated Ad-late/RFP (figure 3.1A). An E1-deleted, replication-deficient version of this virus, Ad(E1⁻)-late/RFP, and a control virus with RFP under the regulation of the ubiquitously active CMV enhancer/promoter, Ad(E1⁻)-CMV/RFP, were also created. Absence of the E1 region renders both of these latter viruses replication-defective, allowing them to only replicate in E1-complementing cells, such as 293 cells.

We first verified that RFP expression from the late/RFP constructs coincided with virus replication by examining late gene expression from the E1-deleted Ad(E1⁻)-late/RFP and Ad(E1⁻)-CMV/RFP in A549 and 293 cells. The late HAdV fiber (a capsid protein) is only expressed at appreciable levels after active virus DNA replication, which can occur in the E1-complementing 293 cell line for these viruses (figure 3.1B). As expected, RFP from the Ad(E1⁻)-late/RFP was also only detected in 293 cells, whereas RFP production from the Ad(E1⁻)-CMV/RFP was independent of virus replication and occurred in both 293 and A549 cells (figure 3.1B). Similar results were observed by fluorescence microscopy of cells infected with these viruses (figure 3.1C). These observations confirm that RFP expression can be used to monitor active virus replication, and can serve as an effective surrogate marker to quantify drug-induced changes in viral gene expression and replication. As such, the Ad-late/RFP construct is also ideal for conducting small-molecule

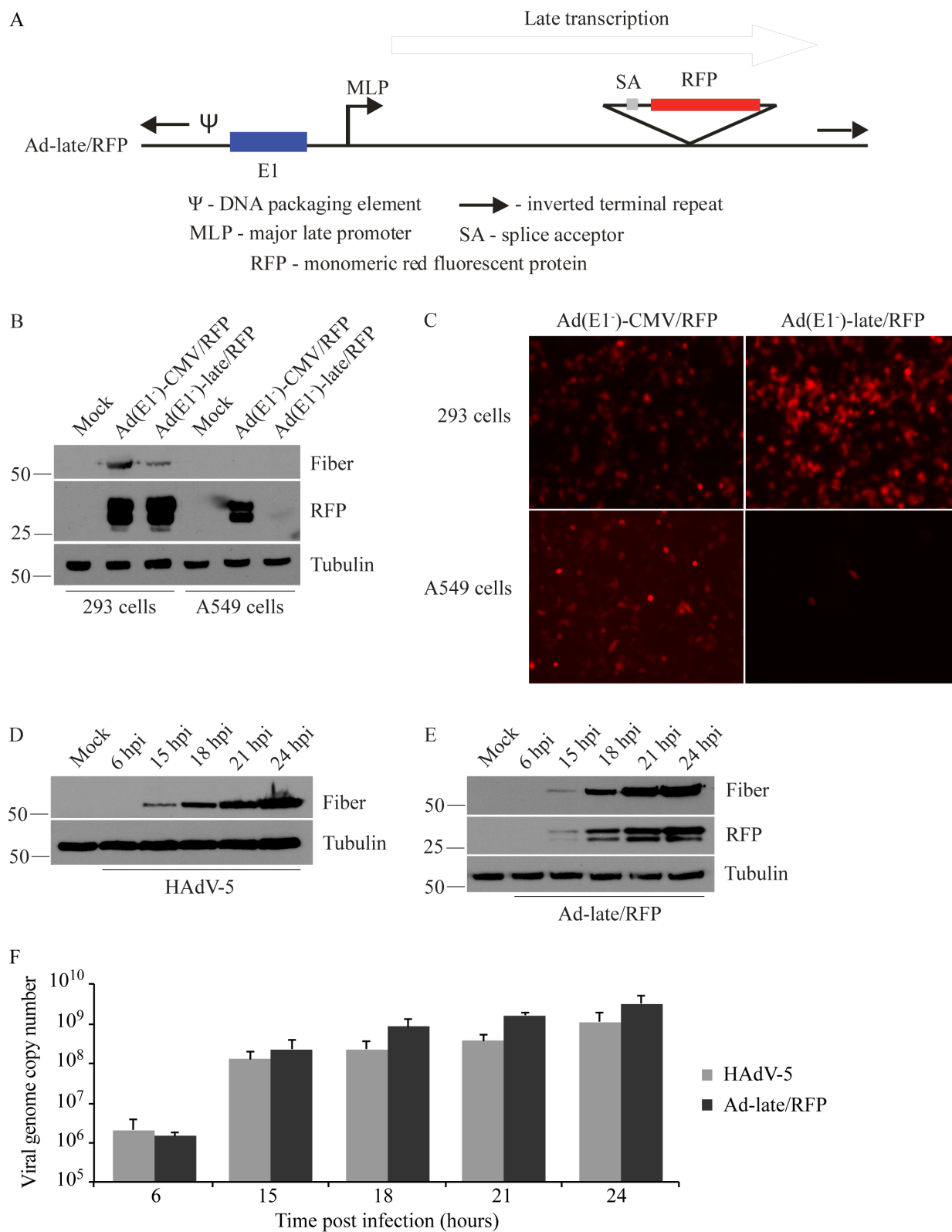


Figure 3.1 Validation of the Ad-late/RFP construct. (A) Schematic diagram of Ad-late/RFP (not drawn to scale). The red fluorescent protein (RFP) cDNA is under the control of the HAdV major late promoter (MLP) and the E1 region is present. An E1-deleted, replication-defective version of the Ad-late/RFP (referred to as Ad(E1⁻)-late/RFP) was also generated, which is able to replicate only in E1-complementing cells. (B) 293 and A549 cells were infected with Ad(E1⁻)-late/RFP or Ad(E1⁻)-CMV/RFP at a multiplicity of infection (MOI) of 1. The latter is a control virus with the ubiquitously expressed cytomegalovirus enhancer/promoter driving RFP expression. Whole cell lysates were collected at 24 hours post infection (hpi) for the detection of RFP, fiber (positive control for HAdV replication) and tubulin (loading control). RFP from the Ad(E1⁻)-CMV/RFP is present in both 293 and A549 cell lysates independent of virus replication. However, RFP from Ad(E1⁻)-late/RFP is only present in 293 cells. (C) Cells were infected as in panel B and fluorescence microscopy results at 24 hpi corroborate the selective expression of RFP from Ad(E1⁻)-late/RFP. (D-F) A549 cells were infected with HAdV-5 or Ad-late/RFP at an MOI of 10 for 6-24 hours. (D, E) Fiber and RFP were detected by immunoblot of cell lysates. (F) qPCR was performed on genomic DNA isolated from infected cells, using oligonucleotide primers specific for the gene encoding capsid protein hexon (error bars represent the range of 3 independent experiments). As indicated by viral protein and DNA levels, Ad-late/RFP is able to grow as well as wildtype HAdV.

screens to identify novel inhibitors of HAdV.

The E1⁺ replication-competent Ad-late/RFP (figure 3.1A) is used in all subsequent experiments as it is more characteristic of the wildtype HAdV-5. Comparison of protein production and DNA replication revealed that Ad-late/RFP grows similar to HAdV-5 (figures 3.1D-F). A time course of fiber expression for the two viruses show a similar extent of accumulation within the cell (figures 3.1D and 3.1E), and the kinetics of DNA replication was comparable as well (figure 3.1F). RFP expression correlates with fiber protein expression (figure 3.1E), and appears to reflect the degree of viral DNA replication. Importantly, the presence of the RFP gene in E3 does not impair Ad-late/RFP replication.

3.4.2 SAHA has anti-HAdV activity

In the infected cell nucleus, HAdV associates with nucleosomes, suggesting that viral gene expression should be influenced by cellular epigenetic regulator proteins, such as histone acetyltransferases (HATs) and HDACs. Several studies have shown that HDAC inhibitors can enhance expression of reporter genes regulated by heterologous promoters within HAdV vectors (48, 49), whereas replication of the wildtype virus is inhibited (36). To test the effects of HDAC inhibitors in our system, we examined RFP expression from Ad-late/RFP in cells treated with pan-HDAC inhibitors TSA and SAHA at a concentration of 10 μ M. As shown in figure 3.2A, both compounds inhibited RFP expression at 24 hours post infection (hpi). This observation was corroborated by the absence of both fiber and RFP in cell lysates following immunoblot analysis (figure 3.2B). We next examined the effects of SAHA over a longer time course of infection. As shown in figure 3.2C, in vehicle-treated cells, hexon, penton, fiber and RFP expression were

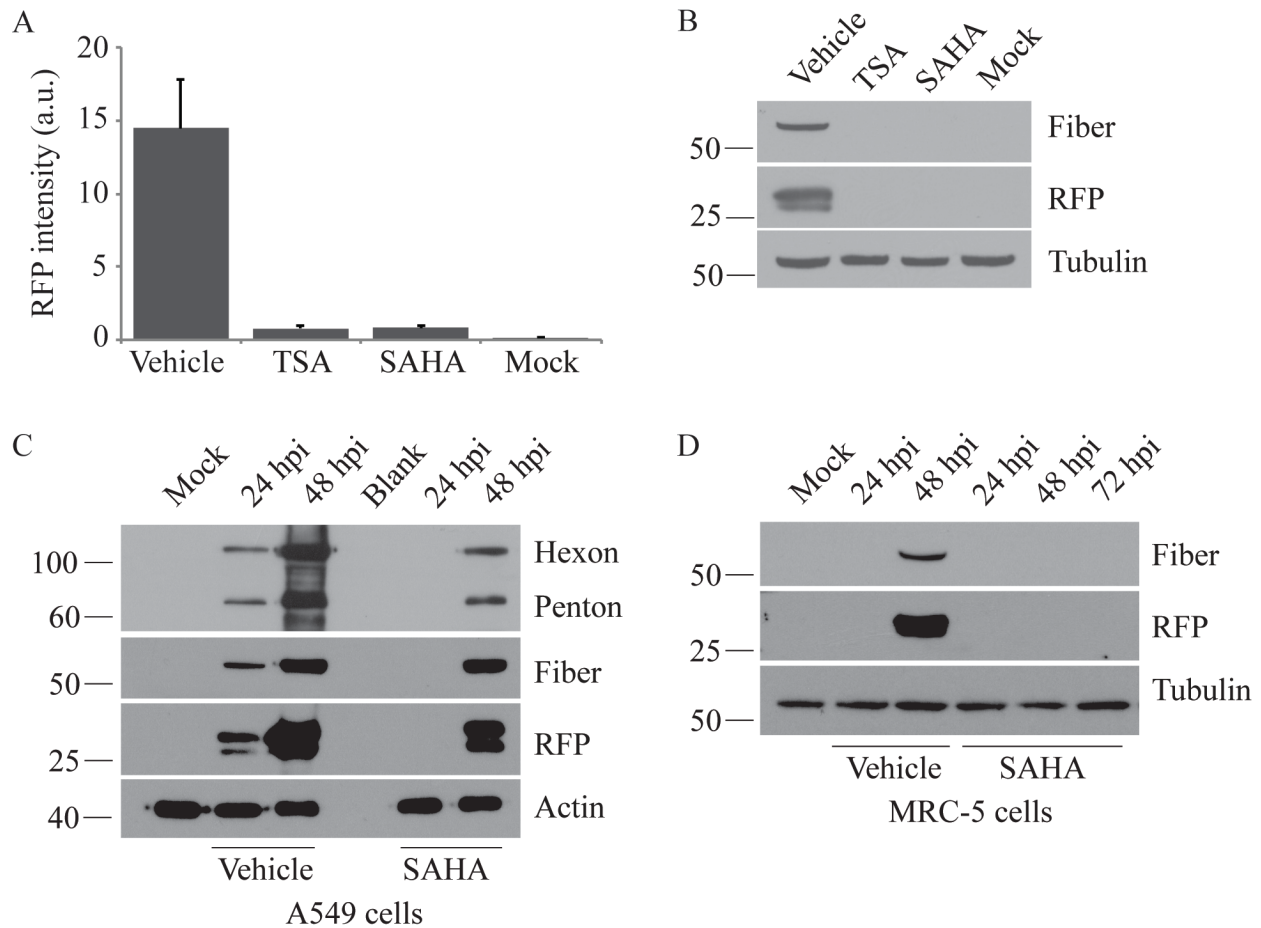


Figure 3.2 Pan-HDAC inhibitor SAHA suppresses HAdV late gene expression. A549 cells were infected with Ad-late/RFP (MOI 10) and treated with vehicle, 10 μ M TSA or 10 μ M SAHA. (A) Cells were fixed at 24 hpi, and the RFP fluorescence intensities were quantified using the Cellomics HCS Platform. a.u. indicates arbitrary units. Error bars represent standard deviation (SD) of analytical replicates (n=16). (B) Immunoblot analysis of cell lysates collected at 24 hpi confirmed the results in panel A. Both TSA and SAHA inhibited RFP production at 24 hpi. (C) A549 cells and (D) MRC-5 cells were infected with Ad-late/RFP and treated with 10 μ M SAHA. Expression of late proteins were analyzed by immunoblot of cell lysates collected at the indicated times. SAHA significantly reduces late gene expression in both cell lines.

readily observed by 24 hpi. In contrast, late gene expression was undetectable at 24 hpi, but could be detected at lower levels at 48 hpi in cells treated with SAHA (figure 3.2C). This is unlikely due to drug degradation over time as fresh medium containing drug was applied every 24 hours in this experiment. Although we were unable to find accurate published data on the half-life of SAHA in A549 cell culture, we obtained the same results with MS-275, also known as entinostat (data not shown), which we expect to have a longer half-life than SAHA *in vitro* based on its significantly longer half-life (over 30 hours) *in vivo* (50, 51). We conducted a similar experiment in the MRC-5 cell line, which is derived from normal lung tissue, to confirm that these results were not specific to the transformed A549 cells. The virus lifecycle is prolonged in MRC-5 cells, and we detected fiber and RFP expression from Ad-late/RFP at 48 hpi with vehicle treatment (figure 3.2D). However, neither fiber nor RFP was observed in SAHA-treated MRC-5 cells at 48 or 72 hpi. Taken together, these data illustrate that HDAC inhibitors repress expression of HAdV late genes.

3.4.3 SAHA reduces late gene expression from clinically relevant HAdV serotypes, and at low concentrations

Ad-late/RFP was constructed based on HAdV-5, but it does not encode all of the wildtype HAdV genes (i.e. RFP replaces the viral E3 region). Therefore, we evaluated the efficacy of SAHA against more virulent and clinically relevant HAdV serotypes 4, 5 and 7 in A549 cells (1, 2). For HAdV-4, fiber gene expression was only detectable at 48 hpi, and was significantly reduced in cells treated with 10 μ M SAHA compared to vehicle (figure 3.3A). Similar results were obtained with HAdV-5 at 24 hpi – fiber protein decreased in a dose-dependent manner (figure 3.3B). Reduced fiber was also observed at 48 hpi in the presence of 10 μ M SAHA (figure 3.3C). Lastly, a modest dose-dependent decrease in fiber protein was observed for HAdV-7 at both 24 and 48

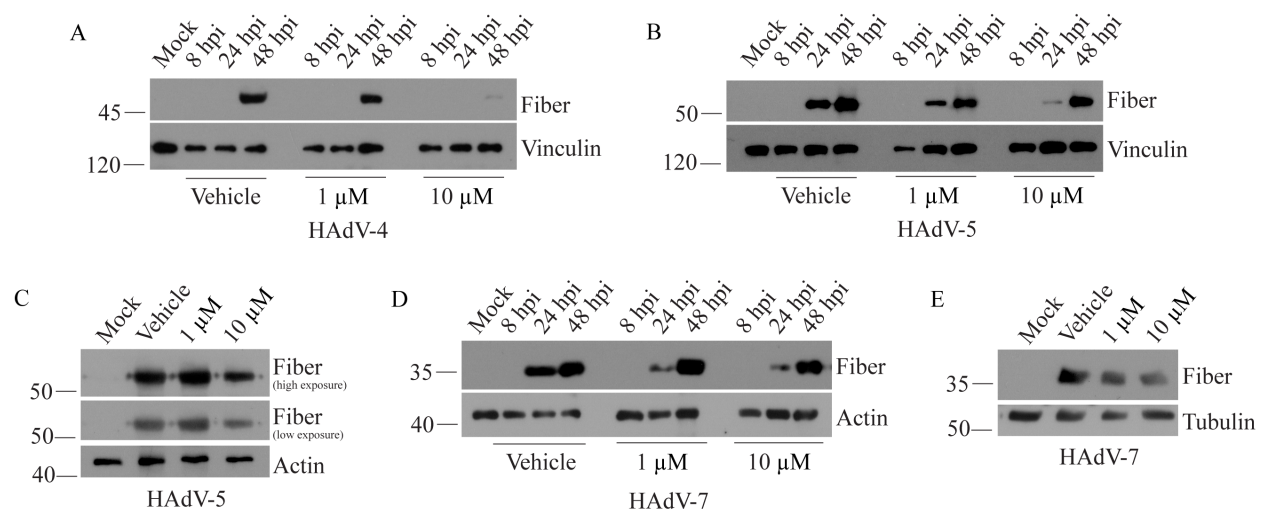


Figure 3.3 SAHA decreases late protein expression from clinically relevant HAdV serotypes.

A549 cells were infected with HAdV-4 (A), HAdV-5 (B) or HAdV-7 (D) at an MOI of 10 and treated with the indicated concentrations of SAHA for 8-48 hours. Fiber protein was detected in cell lysates by immunoblot. The 48 hpi samples from panels B and D were re-analyzed using shorter exposures in panels C and E, respectively, to illustrate the decrease in late gene expression more clearly.

hpi (figures 3.3D and 3.3E). As with Ad-late/RFP in MRC-5 cells in figure 3.2D, late gene expression from wildtype HAdV-5 was suppressed for at least 24 hours in primary mouse embryonic fibroblasts (data not shown). Thus, SAHA is inhibitory towards multiple HAdV serotypes. Our findings are consistent with that of Höti *et al.*, who showed that HAdV-5 replication and spread were substantially reduced when treated with a different HDAC inhibitor, valproic acid (36).

To examine the efficacy of SAHA at low concentrations, we conducted a dose-response assay. SAHA reduced RFP expression considerably at concentrations below 1 μ M (figure 3.4A). For example, compared to vehicle-treatment, RFP was reduced by more than 50% with 0.25 μ M SAHA. Importantly, this effect was not due to compromised cell viability as the cells retained high metabolic activity (>80% at 24 hpi and >70% at 48 hpi) at all concentrations (figure 3.4B). Furthermore, the cells did not exhibit any irregular cell morphology or significant detachment from culture dishes (assessed by light microscopy and visual inspection), and minimal cell death (less than 3%) was detected with 0.25-10 μ M SAHA via Trypan Blue exclusion assay (data not shown). These observations are consistent with previous reports showing that treatment with SAHA for 24 hours has a minor effect on A549 cell density and viability (52). The decrease in RFP expression was concomitant with a similar decrease in virus yield (~100-fold or more) for Ad-late/RFP (figures 3.4C and 3.4D) and HAdV-5 (figure 3.4E) in the presence of 10 μ M SAHA. In addition to valproic acid, SAHA, TSA and MS-275, we tested seventeen additional HDAC inhibitors, eleven of which were found to reduce RFP expression from the Ad-late/RFP by at least 50% at a concentration of 1 μ M (table 3.1). Overall, these results highlight the potential of SAHA and other pan-HDAC inhibitors as anti-HAdV agents.

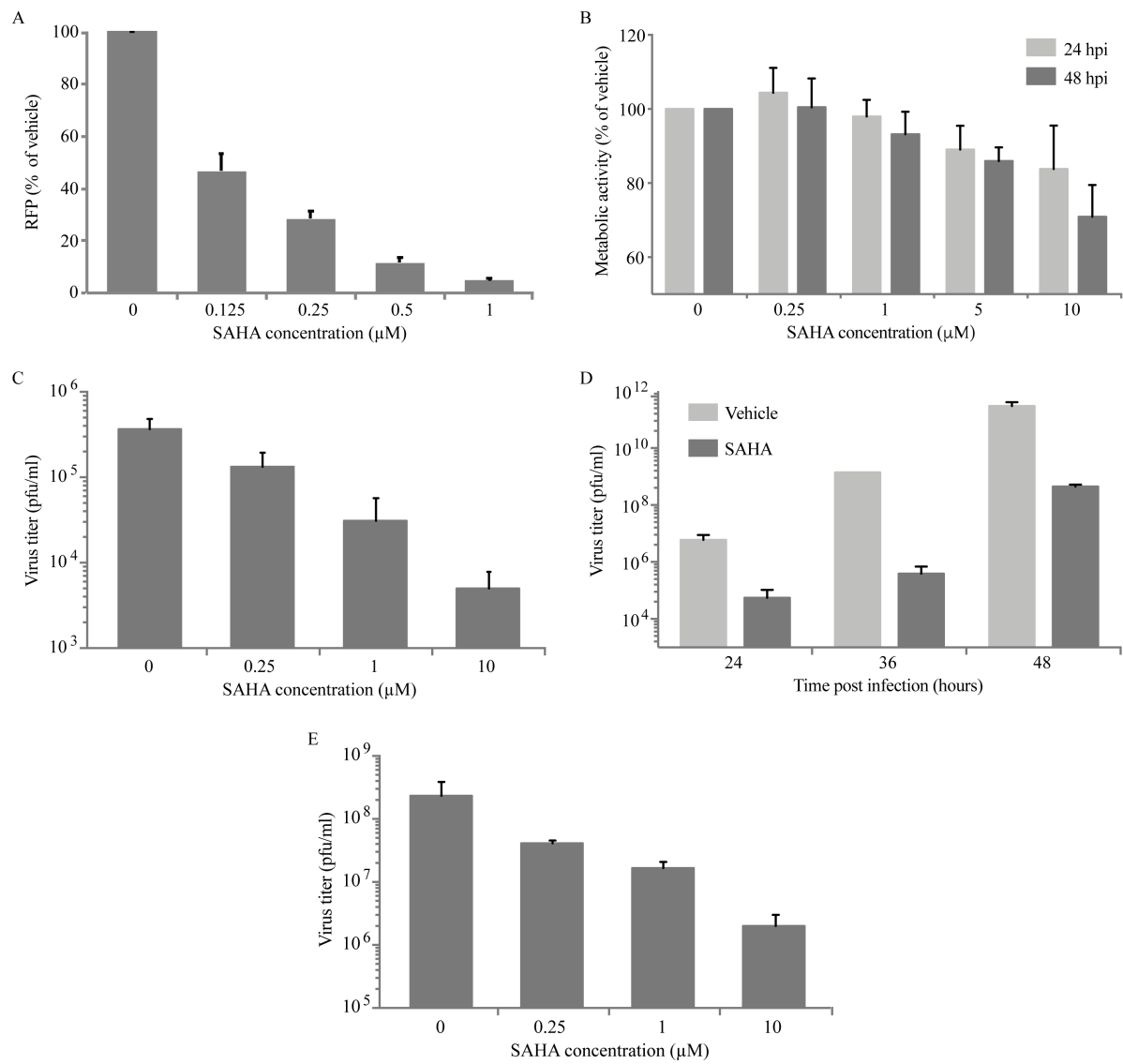


Figure 3.4 SAHA reduces RFP expression and virus yield at low concentrations. A549 cells were infected with Ad-late/RFP (MOI 10) and treated with vehicle or the indicated concentrations of SAHA for 24 hours. (A) Cells were fixed and RFP levels were quantified using the Cellomics HCS Platform. (B) Cellular metabolic activity was determined by MTS assay in live cells. In both panels, changes in SAHA-treated cells were plotted relative to vehicle treatment. (C, D) Infected A549 cells were treated with various concentrations of SAHA for 24 hours (C), or with 10 μ M SAHA for 24 to 48 hours (D). The cell lysates were subjected to plaque assay for determination of virus yield. (E) Plaque assay was conducted as in panel C with wildtype HAdV-5. Error bars represent SD (n=3).

3.4.4 SAHA treatment affects HAdV early/late gene transcription, protein expression, and DNA replication

SAHA reduced viral late protein expression from Ad-late/RFP (figure 3.2), and also from HAdV-4, -5 and -7 (figure 3.3). However, this effect could be due to action of the drug on preceding stages of the virus lifecycle, including virus attachment/internalization, viral genome chromatinization, early gene expression, DNA replication or transcription from the MLP. Thus, we performed additional studies to determine at which stage of the virus lifecycle SAHA exerted its effect.

A preliminary study assessing hexon, penton and fiber protein levels from the capsids of cell surface-attached and internalized virions indicated that the drug does not adversely affect virus attachment (data not shown), which is consistent with studies reporting that HDAC inhibitors do not downregulate cell-surface expression of the coxsackie-adenovirus receptor (49). HAdV DNA associates with nucleosomes soon after reaching the host cell nucleus (29, 30), so we next examined the impact of SAHA on Ad-late/RFP genome “chromatinization” by ChIP assay at 6 hours following infection and drug treatment. Consistent with previous studies (29, 30), we found that the early E1A region associated with H3 (figure 3.5A). Although SAHA seems to reduce H3 association with this region (figure 3.5A), this did not reach statistical significance with four experimental replicates. HDACs are epigenetic modifiers which remove acetyl groups that neutralize the positive charge on the lysine residues of histones, thereby regulating accessibility of specific genomic regions to transcription factor binding (53). Thus, treatment of infected cells with an HDAC inhibitor could alter the post-translational modifications present on the viral genome-associated histones. To test this possibility, we examined the acetylation levels of H3/H4

Table 3.1 HDAC inhibitors from the Cayman Epigenetics Screening Library that reduced RFP from Ad-late/RFP.

HDAC inhibitor	% RFP^a
1) Apicidin	12
2) HC Toxin	13
3) Panobinostat	14
4) Givinostat	17
5) Oxamflatin	17
6) Pracinostat	20
7) CAY10603	34
8) M344	41
9) 4-iodo-SAHA	45
10) CBHA	47
11) Scriptaid	49

^a The percentage values indicate the RFP intensities in cells treated with 1 μ M drug relative to vehicle-treated cells at 24 hpi.

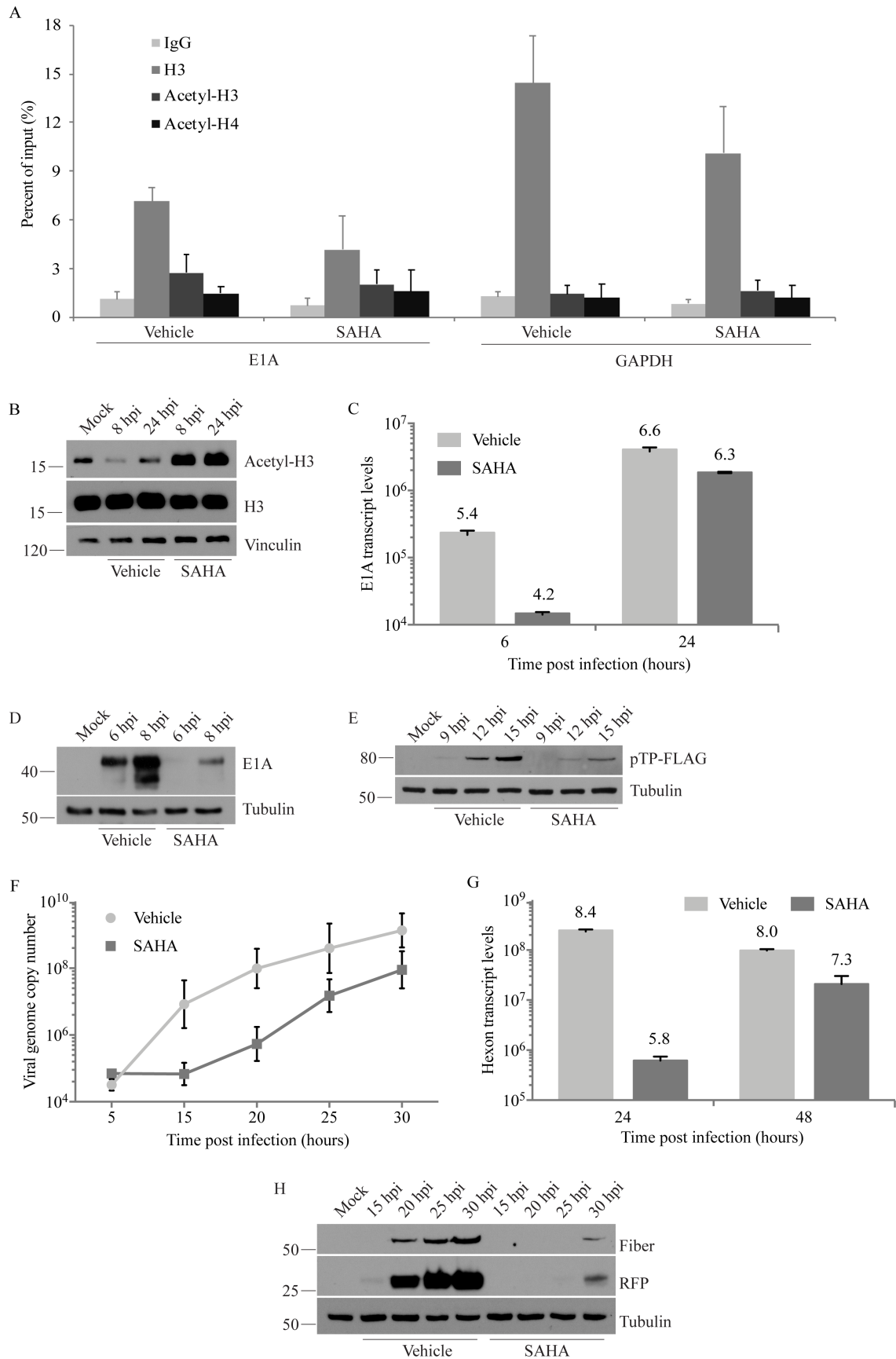


Figure 3.5 SAHA impacts multiple stages of HAdV lifecycle. A549 cells and 10 μ M SAHA were used in all experiments. (A) Ad-late/RFP (MOI 10) was used in a “cold infection” to synchronize virus entry into cells. The cells were treated with vehicle or SAHA and subjected to ChIP at 6 hpi with the indicated antibodies, followed by qPCR with primers specific to HAdV E1A or cellular GAPDH regions. (B) Lysates of cells infected with Ad-late/RFP (MOI 10) and treated with SAHA were collected at 8 or 24 hpi for immunoblot analysis. (C, G) Following infection and drug treatment as in panel B, total cellular RNA was extracted at the indicated times and cDNA was generated by reverse transcription. qPCR analysis was conducted using primers to the HAdV E1A (C) or hexon (G) regions. (D, H) Infected, SAHA-treated cell lysates were collected at the indicated times for immunoblot analysis to detect viral early (D) and late (H) proteins. (E) Replication-competent Ad(E1⁺)TP-F was used at MOI of 50 for infection to assess expression from the E2 region in the presence and absence of SAHA. (F) Infection and drug treatment were carried out as in panel B, and genomic DNA was extracted at the indicated times for qPCR using primers specific to hexon. SAHA did not affect HAdV entry, DNA association with H3 or acetyl-H3/acetyl-H4 levels, but inhibited expression of early and late genes and viral DNA replication. All error bars represent SD (n=4 for ChIP and n=2 for all other experiments).

associated with the E1A region using pan-acetyl antibodies. We observed no difference in association of this region with acetylated H3 or H4 (figure 3.5A), even though overall cellular H3 acetylation was increased by SAHA as early as 8 hpi in cells infected with Ad-late/RFP (figure 3.5B). Similar results were obtained when examining SAHA-induced changes in histone acetylation and association with the late hexon region (data not shown). The cellular GAPDH region was used as a positive control for H3 association. HDAC inhibition did not affect H3 association or acetylation in this region either (figure 3.5A), which has also been observed by others (54-58). Since housekeeping genes are constitutively active, it is possible that the histones associated with these genes are already maximally acetylated, and therefore, are not influenced by SAHA-treatment.

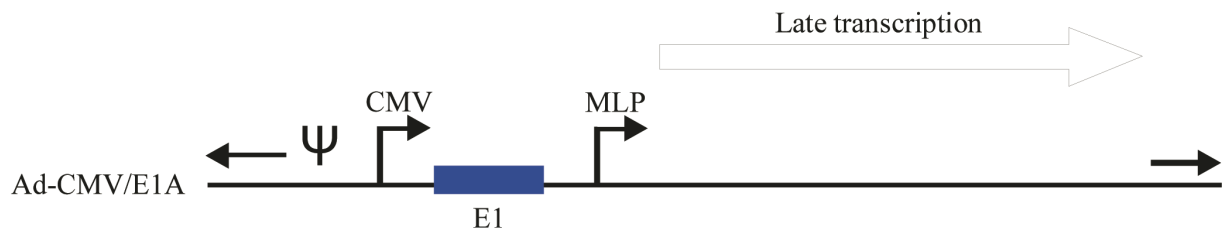
In the HAdV lifecycle, the E1 region is the first to be transcribed. We performed RT-qPCR to analyze E1A transcript levels in infected, drug-treated cells over a period of 24 hours. Treatment with SAHA caused over 10-fold (1.2-log) reduction in E1A transcript levels at 6 hpi (figure 3.5C), and this was concomitant with a reduction in E1A protein levels (figure 3.5D). Expression of the pTP and DBP from the E2 region was also decreased (figures 3.5E and S1). We next examined viral DNA replication through analysis of genome copy numbers obtained by qPCR. The genome copy numbers at 5 hpi (before the onset of viral DNA replication) was identical in cells treated with SAHA or vehicle (figure 3.5F), indicating that the compound did not impact the ability of the genomes from infecting virions to reach the nucleus. Thus, viral attachment and internalization are not affected by SAHA. Viral DNA replication, however, was decreased compared to vehicle over a 30-hour time course of infection – especially at its onset at 15-20 hpi (figure 3.5F). Even at 48 hpi, Ad-late/RFP genome copy numbers were 3-fold lower in SAHA-treated cells (data not

shown). As expected, late gene transcription was inhibited at 24 hpi with a ~400-fold (2.6-log) decrease in hexon transcripts (figure 3.5G). Lastly, analysis of protein expression from 15 to 30 hpi revealed that SAHA treatment delays detectable late protein expression by about 10-15 hours in A549 cells (figure 3.5H). Thus, SAHA affects multiple stages of the HAdV lifecycle, ultimately leading to a reduction in virus yield.

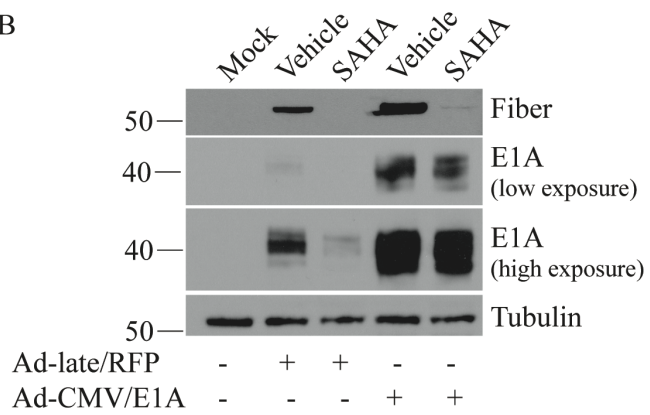
3.4.5 SAHA impacts HAdV lifecycle through mechanisms independent of E1A protein levels

The E1A protein is required for the transcriptional activation of all other HAdV genes and for making the cellular microenvironment more conducive to virus replication (20). Since SAHA reduced E1A expression in early infection, we considered the possibility that the loss of E1A could be adversely impacting the subsequent stages of the virus lifecycle (i.e. treatment with SAHA reduces E1A expression, and reduced E1A protein levels cause the decrease in expression of other viral genes). To investigate this, an Ad-CMV/E1A construct was created, where the E1 promoter was replaced with the CMV promoter (figure 3.6A). The purpose of this construct was to overexpress E1A even in the presence of SAHA. A549 cells were infected with Ad-late/RFP or Ad-CMV/E1A, treated with vehicle or SAHA, and examined for early/late gene expression at 24 hpi. Ad-CMV/E1A indeed produced very high amounts of E1A compared to Ad-late/RFP under all conditions (figure 3.6B). In vehicle-treated cells, the enhanced E1A expression from Ad-CMV/E1A was associated with a higher level of fiber expression compared to Ad-late/RFP – likely due to accelerated growth kinetics of Ad-CMV/E1A as a direct consequence of increased E1A expression. However, late gene expression was not fully rescued in SAHA-treated cells (figure 3.6B), or when 293 cells that constitutively express E1A were infected with Ad-late/RFP and Ad(E1⁻)-late/RFP (figures 3.6C and 3.6D). These results suggest that SAHA affects stages of the

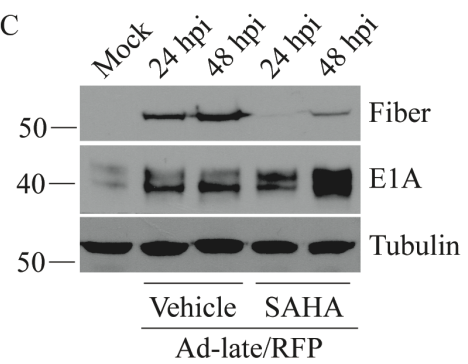
A



B



C



D

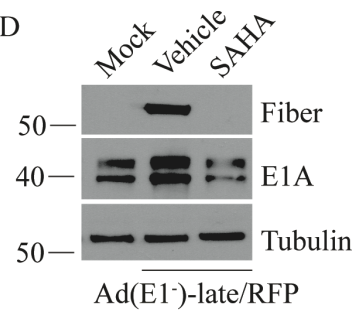


Figure 3.6 SAHA affects late gene expression through E1A-independent mechanisms. (A) Schematic diagram of the Ad-CMV/E1A construct (not drawn to scale). The E1 promoter was replaced with the CMV promoter to allow high level expression of the E1 proteins even in the presence of SAHA. (B) A549 cells were infected with Ad-late/RFP or Ad-CMV/E1A (MOI 10) and treated with vehicle or 10 μ M SAHA. Cell lysates were collected for immunoblot analysis of E1A and fiber at 24 hours post infection. (C) 293 cells were infected with Ad-late/RFP (MOI 10) and treated with 10 μ M SAHA for the indicated time points. (D) 293 cells were infected with Ad(E1⁻)-late/RFP (MOI 10) and treated with SAHA for 24 hours. Neither forced expression of E1A, nor infection in the presence of existing E1A from 293 cells, fully rescued fiber expression in SAHA-treated cells.

HAdV lifecycle through other E1A-independent mechanisms.

3.4.6 SAHA-induced increase in p21 is not responsible for reduction of HAdV replication

HAdV early proteins naturally induce a transition of the infected cell into S phase in order to provide an optimal environment for virus replication (59). Inactivation of cyclin-dependent kinase inhibitor p21 by the E1A protein is one of the main mechanisms by which this transition occurs (60). SAHA is an FDA-approved treatment for T-cell lymphoma, and its anticancer properties include the ability to induce cell cycle arrest and prevent progression to S phase through upregulation of p21 (61). Since HDACs typically repress p21 expression (62), Höti *et al.* speculated that inhibition of HAdV-5 replication by valproic acid was dependent on the compound's effects on HDAC activity, leading to p21-mediated cell cycle arrest (36). However, a direct relationship between valproic acid-induced increase in p21 and suppression of HAdV replication has not been shown. Thus, we assessed the correlation between cellular p21 levels and Ad-late/RFP replication in our system. SAHA indeed enhanced p21 protein levels in both infected and uninfected A549 cells (figure 3.7A). Consistent with literature (63), Ad-late/RFP infection caused a reduction in the cellular levels of p21 (figure 3.7A). Treatment with SAHA prevented the loss of p21 in the infected cells, but siRNA-mediated knockdown of p21 (figure 3.7B) did not rescue Ad-late/RFP late gene expression in SAHA-treated cells (figure 3.7C). These results indicate that SAHA's inhibitory effects on HAdV gene expression and replication was not due to increased p21, and that SAHA acts through other viral (and/or cellular) processes to impair virus replication.

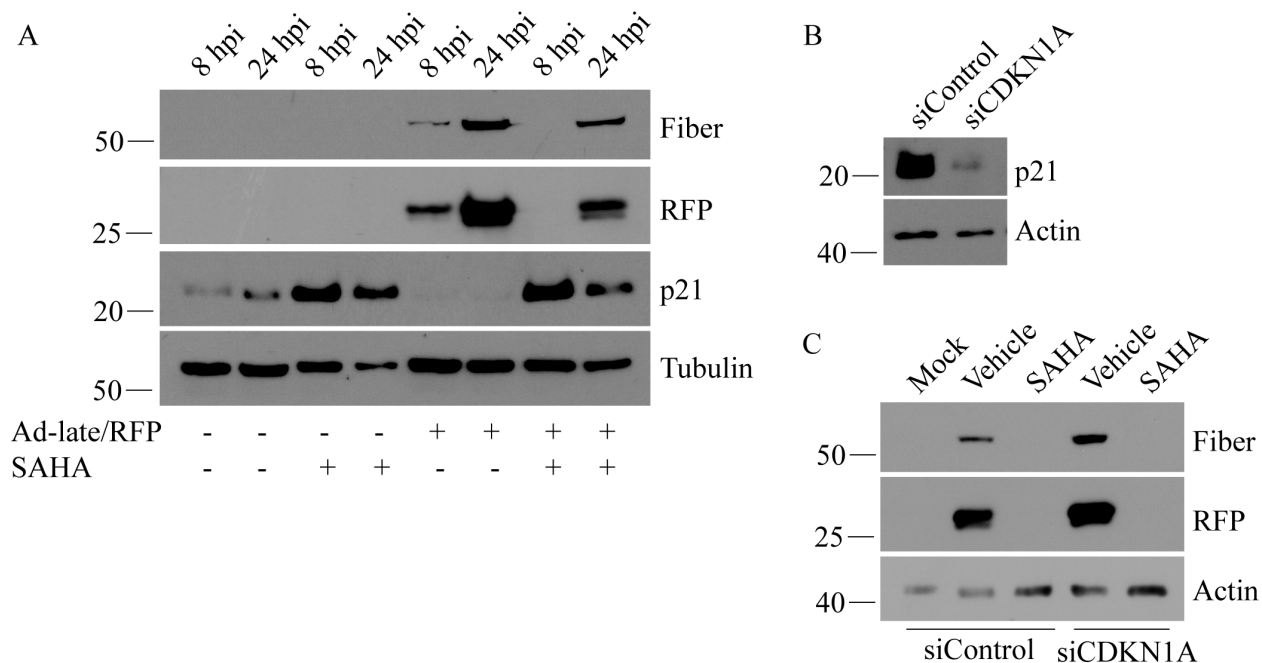


Figure 3.7 SAHA-induced increase in p21 is not responsible for the impairment of HAdV replication. (A) p21 levels were detected by immunoblot analysis in both infected (Ad-late/RFP, MOI 10) and uninfected A549 cells treated with vehicle or 10 μ M SAHA. SAHA treatment increases p21 expression in both infected and uninfected cells, while infection with Ad-late/RFP alone decreases expression. (B) Cells were transfected with control siRNA or siRNA specific to the CDKN1A transcript for 48 hours to transiently knockdown cellular p21. (C) These cells were infected with Ad-late/RFP and treated with vehicle or SAHA. Loss of p21 did not rescue late gene expression at 24 hpi following SAHA treatment.

3.4.7 Class I HDAC activity is necessary for efficient HAdV gene expression

SAHA inhibits both class I and II HDACs, which are comprised of a total of ten HDAC proteins (64, 65). To determine whether suppression of HAdV was due to the inhibition of a specific class of HDAC, we first conducted class-specific HDAC inhibition studies using MS-275 (preferentially inhibits class I HDACs 1, 2 and 3) and MC1568 (mainly inhibits class II HDACs 4 and 5) at 10 μ M (66). When Ad-late/RFP-infected cells were treated with each drug for 24 hours, we observed a reduction in fiber and RFP levels with MS-275 (figure 3.8A), suggesting that inhibition of class I HDAC activity prevents efficient expression of HAdV late genes. Although treatment with MC1568 increased overall H3 acetylation (figure S2A), it had no detectable effect on viral gene expression (figure 3.8A). We then knocked down class I HDACs 1, 2 and 3 with siRNAs (figure 3.8B), and found that fiber and RFP decreased only when HDAC2 activity was lost (figure 3.8C). We also investigated whether knocking down of HDAC2 alters Ad-late/RFP DNA association with histones by ChIP and DNA replication by qPCR, but did not observe any discernible differences in either (figures S2B and S2C). These findings suggest that HDAC2 is necessary for efficient HAdV gene expression, but the inhibition of HDAC2 activity alone does not fully account for SAHA's anti-HAdV effects. Moreover, the HDACs we did not specifically inhibit or individually knockdown may also be involved.

HAdV E1A interacts with a variety of cellular proteins including transcription factors and epigenetic regulators, such as p300/CBP and E2F1, amongst many others (35, 67, 68). E1A protein can alter both cellular and viral gene transcription directly by recruiting chromatin modulating proteins to specific promoters, or indirectly by interacting with other components of chromatin remodelling complexes (69). We considered the possibility of a physical interaction between E1A

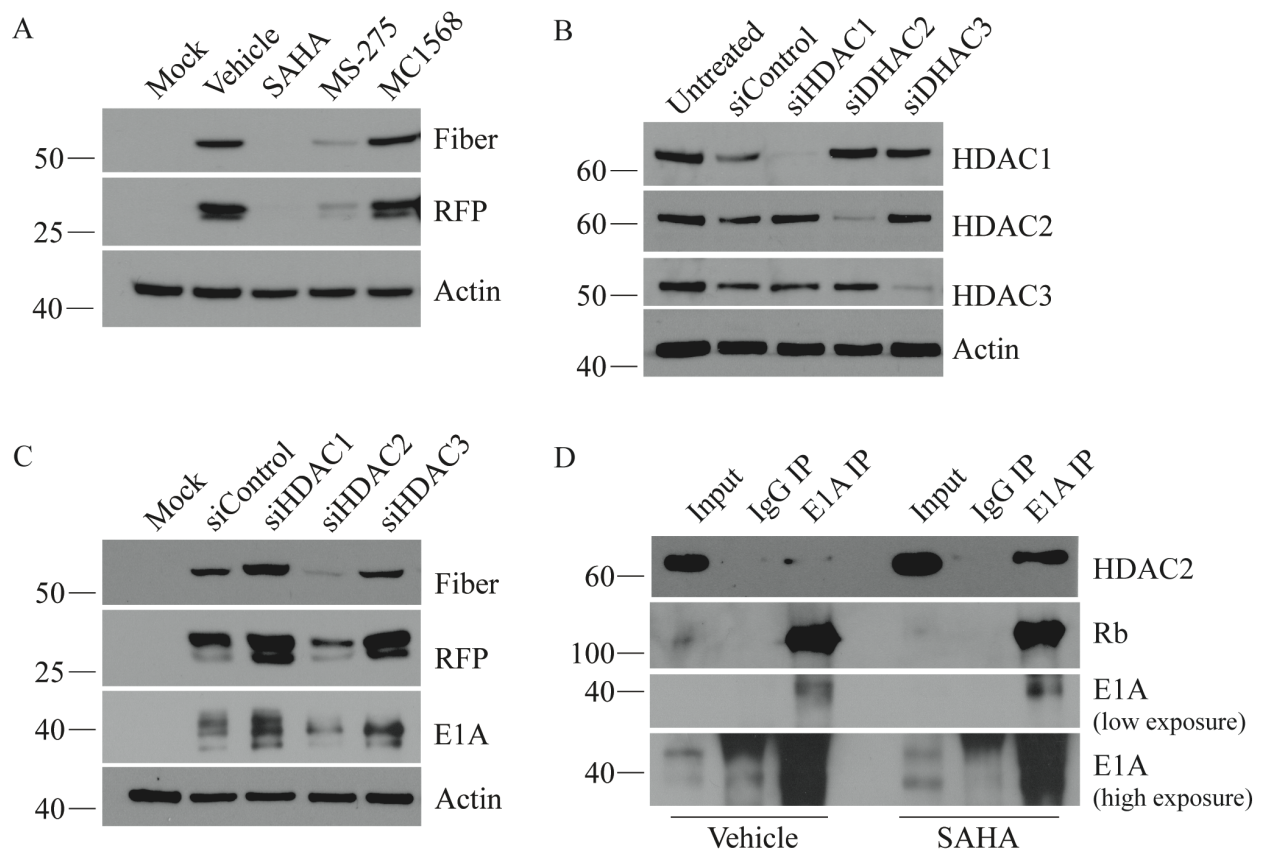


Figure 3.8 HDAC2 activity is necessary for efficient expression of HAdV late genes. (A) A549 cells were infected with Ad-late/RFP (MOI 10) and treated with vehicle, SAHA, MS-275 or MC1568 (10 μ M of each) for 24 hours. Late gene expression decreased with MS-275, but not MC1568. (B) A549 cells were transfected with 100 nM control, HDAC1, HDAC2 or HDAC3 siRNA for 48 hours. (C) Cells knocked-down for each HDAC were then infected with Ad-late/RFP (MOI 10) for 24 hours to analyze fiber and RFP protein expression. (D) co-IP was performed with IgG (negative control) or E1A antibody on cells infected and drug-treated as in panel A. Input was 10% of the IP volume. HDAC2 knockdown decreased fiber and RFP expression, and the protein was found to interact with E1A.

and HDAC2 (either directly or through interaction with chromatin remodelling complexes). HDAC2 was indeed found to interact with E1A in co-immunoprecipitation experiments (figure 3.8D). Interestingly, treatment with SAHA appeared to enhance the interaction of HDAC2 with E1A protein (figure 3.8D) in a dose-dependent manner (figure S2D). Rb (positive control for interaction with E1A) was also present in the E1A co-IP samples (figure 3.8D). We did not detect an interaction between HDAC1 and E1A (data not shown).

3.5 Discussion

Like several other DNA viruses and retroviruses (e.g. herpes simplex virus, simian virus 40, murine leukemia virus), the HAdV DNA associates with cellular histones during specific stages of the virus lifecycle (70-72). Modifications of these histones has very recently been shown to modulate HAdV gene expression (34), but many questions regarding the “chromatinization” and epigenetic regulation of the HAdV genome still remain unanswered. The cellular proteins and chromatin-remodelling enzymes involved in mediating these processes have yet to be identified. In this study, we have used an E1⁺, RFP-expressing HAdV reporter virus (figure 3.1) to determine that the HDAC inhibitor SAHA exhibits anti-HAdV activity (figure 3.2). Given that HDACs are naturally associated with decreases in gene repression, we had expected HDAC inhibition to increase HAdV DNA-associated histone acetylation and consequently, viral gene expression. Surprisingly, SAHA reduced late gene expression from several serotypes of HAdV (figure 3.3) and was effective in reducing virus yield even at low concentrations (figures 3.4). Further investigation led us to determine that SAHA negatively impacts various steps in the HAdV lifecycle throughout infection, including viral gene expression and DNA replication (figure 3.5), and that this was independent of reduced E1A protein levels (figure 3.6). However, it is unclear whether this effect is due to a complete but transient inhibition, which delayed the onset of viral gene expression and replication, or whether SAHA decreased expression throughout infection leading to a slow accumulation of transcripts/proteins/DNA over time. Furthermore, experiments conducted in MRC-5 cells (figure 3.2) indicate that SAHA’s effects last longer in primary cells. Taken together, these results highlight the potential of HDAC inhibitors as anti-HAdV compounds.

Our data suggest a role of HDACs, specifically class I HDACs, in modulating HAdV gene expression (figure 3.8). Knocking down HDAC2 reduced HAdV late gene expression, and we further showed that HDAC2 interacts with the early protein E1A (figure 3.8). E1A could be involved in redirecting or recruiting HDAC2 to protein substrates that are important for viral replication. HDAC2 is well known for its ability to modify histones, and has specific targets on H3 and H4 (e.g. H3K9, H4K16). Although SAHA treatment did not appear to cause a change in acetylation status of H3 or H4 in the E1A/hexon coding regions we examined (figure 3.5), loss of HDAC2 activity could affect acetylation at the promoter regions or transcriptional start sites of the early and late genes, since H3 acetylation was observed at HAdV-5 early promoters (34). Interestingly, knockdown of the histone acetyltransferase Tip60 and reduced H4 acetylation actually resulted in increased HAdV-5 E1A promoter activity (73). Thus, HAdV may have a complex or temporal requirement for histone acetylation for proper promoter function. Alternatively, HDAC2 may even target specific lysine acetyl groups on the E1A protein itself to regulate its function (74, 75). However, HDAC2-E1A association is likely not restricted to the enzyme's active site as association increases in the presence of SAHA – a competitive inhibitor which occupies the active site of HDAC2 and prevents substrate binding (figure 3.8). It is also possible that HDAC2 binding interferes with other E1A functions (e.g. interaction with other proteins), or that the interaction opposes the beneficial functions of HDAC2, contributing to the impairment of virus replication observed with SAHA.

Our studies have uncovered a specific role for HDAC2 in activating HAdV gene expression, which cannot be compensated for by HDAC1 or HDAC3. Knockdown of HDAC1 and HDAC3 slightly enhanced expression of HAdV genes (figure 3.8). This observation is similar to

that of Wang *et al.* for yeast HDACs Rpd3 and Hos2 (76). Although both are class I HDACs, Rpd3 represses the yeast *GALI* gene, while Hos2 activates *GALI*. It is also intriguing that HDAC1 cannot fully compensate for the loss of HDAC2 activity in our system. The two enzymes co-exist in several co-repressor complexes and while they are generally known to be functionally redundant and compensatory, some studies report that HDAC2 has specific targets and plays distinct roles in certain cellular processes (77, 78). Feng *et al.* found that the respiratory syncytial virus upregulates HDAC2 expression in primary airway epithelial cells (79). However, we did not observe any such changes in HDAC2 levels by immunoblot over a 48-hour time course of HAdV-5 infection in A549 cells (figure S2E).

It is important to note that HDAC2 knockdown alone did not recapitulate the full extent of SAHA-mediated inhibition (compare figures 3.8A and 3.8C). While this may reflect the relative efficiency of drug treatment versus siRNA-mediated knockdown, it may also suggest that other cellular pathways or processes affected by SAHA, besides those regulated through HDAC2, are important for HAdV function during a productive infection. In addition to modifying histone acetylation status, HDACs have numerous other non-histone targets including proteins involved in transcription regulation, cell proliferation, metabolism, apoptosis and immune response (80). HDAC inhibitors have been shown to alter the expression level and function of cellular proteins p21, E2F1 and SP1 (81-84), all of which can impact upon optimal viral gene expression and growth. The transcription factors E2F1 and SP1 both play a role in transcriptional control of E1A (85, 86). E2F1 and SP1 also bind to the E2 promoter and the MLP, respectively, and binding is required for the maximum expression of proteins from those regions (17, 68, 87-89). Although we were unable to rescue HAdV gene expression via knockdown of p21 (figure 3.7) or overexpression

of E2F1 or SP1 (data not shown), a more sensitive experimental design may be required to conclusively exclude involvement of these proteins. SAHA's effects on HAdV may also be a cumulative consequence of several factors, as opposed to changes in a single pathway or protein. While further investigation is required to fully understand the underlying molecular mechanism, our findings enhance the current knowledge of the HAdV infection process and elucidate the need of HDAC2 activity for optimal viral gene expression and replication. Future studies should also focus on determining the *in vivo* efficacy of SAHA against HAdV in the Syrian hamster or the cotton rat model, both of which are permissive for HAdV infection.

The costs associated with HAdV-induced disease are significant in terms of medical expenses, lost work hours and loss of life in some populations. Thus, development of novel and effective antiviral therapies is necessary to reduce disease pathogenesis and increase survival rates in severe infections. Our results show that SAHA led to a significant reduction in virus yield at low concentrations and was effective in reducing gene expression from virulent serotypes 4 and 7. Understanding the molecular mechanism underlying SAHA's inhibitory effects on HAdV may make it possible to design better compounds with longer-lasting effects and lower toxicity for the treatment of HAdV infections. As HDAC inhibitors have been reported to exhibit antiviral activity against other viruses including RSV and HCV (79, 90), these novel compounds may be useful in treating a variety of viral infections.

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3.7 References

1. Binder AM, Biggs HM, Haynes AK, Chommanard C, Lu X, Erdman DD, Watson JT, Gerber SI. 2017. Human Adenovirus Surveillance - United States, 2003-2016. *MMWR Morb Mortal Wkly Rep* 66:1039-1042.
2. Scott MK, Chommanard C, Lu X, Appelgate D, Grenz L, Schneider E, Gerber SI, Erdman DD, Thomas A. 2016. Human Adenovirus Associated with Severe Respiratory Infection, Oregon, USA, 2013-2014. *Emerg Infect Dis* 22:1044-51.
3. Kajon AE, Lamson DM, Bair CR, Lu X, Landry ML, Menegus M, Erdman DD, St George K. 2018. Adenovirus Type 4 Respiratory Infections among Civilian Adults, Northeastern United States, 2011-2015(1). *Emerg Infect Dis* 24:201-209.
4. Kojaoghlanian T, Flomenberg P, Horwitz MS. 2003. The impact of adenovirus infection on the immunocompromised host. *Rev Med Virol* 13:155-71.
5. Ying B, Tollefson AE, Spencer JF, Balakrishnan L, Dewhurst S, Capella C, Buller RM, Toth K, Wold WS. 2014. Ganciclovir inhibits human adenovirus replication and pathogenicity in permissive immunosuppressed Syrian hamsters. *Antimicrob Agents Chemother* 58:7171-81.
6. Caravokyri C, Leppard KN. 1996. Human adenovirus type 5 variants with sequence alterations flanking the E2A gene: effects on E2 expression and DNA replication. *Virus Genes* 12:65-75.
7. Bhatti Z, Dhamoon A. 2017. Fatal adenovirus infection in an immunocompetent host. *Am J Emerg Med* 35:1034.e1-1034.e2.
8. Adriana EK, Michael GI. 2016. Severe Infections with Human Adenovirus 7d in 2 Adults in Family, Illinois, USA, 2014. *Emerging Infectious Disease journal* 22:730.

9. Ronchi A, Doern C, Brock E, Pagni L, Sanchez PJ. 2014. Neonatal adenoviral infection: a seventeen year experience and review of the literature. *J Pediatr* 164:529-35.e1-4.
10. Lenaerts L, De Clercq E, Naesens L. 2008. Clinical features and treatment of adenovirus infections. *Rev Med Virol* 18:357-74.
11. Keyes A, Mathias M, Boulad F, Lee YJ, Marchetti MA, Scaradavou A, Spitzer B, Papanicolaou GA, Wiecek I, Busam KJ. 2016. Cutaneous involvement of disseminated adenovirus infection in an allogeneic stem cell transplant recipient. *Br J Dermatol* 174:885-888.
12. Detweiler CJ, Mueller SB, Sung AD, Saullo JL, Prasad VK, Cardona DM. 2018. Brincidofovir (CMX001) Toxicity Associated With Epithelial Apoptosis and Crypt Drop Out in a Hematopoietic Cell Transplant Patient: Challenges in Distinguishing Drug Toxicity From GVHD. *J Pediatr Hematol Oncol* 40:e364-368.
13. Ramsay ID, Attwood C, Irish D, Griffiths PD, Kyriakou C, Lowe DM. 2017. Disseminated adenovirus infection after allogeneic stem cell transplant and the potential role of brincidofovir - Case series and 10 year experience of management in an adult transplant cohort. *J Clin Virol* 96:73-79.
14. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* 84:570-3.
15. Hilleman MR, Werner JH. 1954. Recovery of new agent from patients with acute respiratory illness. *Proc Soc Exp Biol Med* 85:183-8.
16. Thomas GP, Mathews MB. 1980. DNA replication and the early to late transition in adenovirus infection. *Cell* 22:523-33.

17. Saha B, Parks RJ. 2017. Human adenovirus type 5 vectors deleted of early region 1 (E1) undergo limited expression of early replicative E2 proteins and DNA replication in non-permissive cells. *PLoS One* 12:e0181012.
18. Flint J. 1999. p 17-30, *Adenoviruses: Basic Biology to Gene Therapy*. Landes Company
19. Frisch SM, Mymryk JS. 2002. Adenovirus-5 E1A: paradox and paradigm. *Nat Rev Mol Cell Biol* 3:441-52.
20. Berk AJ. 1986. Adenovirus promoters and E1A transactivation. *Annu Rev Genet* 20:45-79.
21. de Jong RN, Meijer LAT, van der Vliet PC. 2003. DNA binding properties of the adenovirus DNA replication priming protein pTP. *Nucleic Acids Res* 31:3274-86.
22. Parker EJ, Botting CH, Webster A, Hay RT. 1998. Adenovirus DNA polymerase: domain organisation and interaction with preterminal protein. *Nucleic Acids Res* 26:1240-7.
23. Horwitz MS. 2004. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. *J Gene Med* 6 Suppl 1:S172-83.
24. Weitzman MD. 2005. Functions of the adenovirus E4 proteins and their impact on viral vectors. *Front Biosci* 10:1106-17.
25. Prescott JC, Falck-Pedersen E. 1992. Varied poly(A) site efficiency in the adenovirus major late transcription unit. *J Biol Chem* 267:8175-81.
26. Young CS. 2003. The structure and function of the adenovirus major late promoter. *Curr Top Microbiol Immunol* 272:213-49.
27. Korn R, Horwitz MS. 1986. Adenovirus DNA synthesis in vitro is inhibited by the virus-coded major core protein. *Virology* 150:342-51.

28. Strunze S, Engelke MF, Wang IH, Puntener D, Boucke K, Schleich S, Way M, Schoenenberger P, Burckhardt CJ, Greber UF. 2011. Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. *Cell Host Microbe* 10:210-23.
29. Komatsu T, Nagata K. 2012. Replication-uncoupled histone deposition during adenovirus DNA replication. *J Virol* 86:6701-11.
30. Giberson A, Saha B, Campbell K, Christou C, Poulin KL, Parks RJ. 2018. Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication. *Biochemistry and Cell Biology* doi:10.1139/bcb-2018-0117.
31. Daniell E, Groff DE, Fedor MJ. 1981. Adenovirus chromatin structure at different stages of infection. *Mol Cell Biol* 1:1094-105.
32. Beyer AL, Bouton AH, Hodge LD, Miller OL, Jr. 1981. Visualization of the major late R strand transcription unit of adenovirus serotype 2. *J Mol Biol* 147:269-95.
33. Giberson AN, Davidson AR, Parks RJ. 2012. Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res* 40:2369-76.
34. Hsu E, Pennella MA, Zemke NR, Eng C, Berk AJ. 2018. Adenovirus E1A Activation Domain Regulates H3 Acetylation Affecting Varied Steps in Transcription at Different Viral Promoters. *J Virol* doi:10.1128/jvi.00805-18.
35. Pelka P, Ablack JN, Torchia J, Turnell AS, Grand RJ, Mymryk JS. 2009. Transcriptional control by adenovirus E1A conserved region 3 via p300/CBP. *Nucleic Acids Res* 37:1095-106.

36. Hoti N, Chowdhury W, Hsieh JT, Sachs MD, Lupold SE, Rodriguez R. 2006. Valproic acid, a histone deacetylase inhibitor, is an antagonist for oncolytic adenoviral gene therapy. *Mol Ther* 14:768-78.
37. Graham FL, Smiley J, Russell WC, Nairn R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36:59-74.
38. Wong CM, Poulin KL, Tong G, Christou C, Kennedy MA, Falls T, Bell JC, Parks RJ. 2016. Adenovirus-Mediated Expression of the p14 Fusion-Associated Small Transmembrane Protein Promotes Cancer Cell Fusion and Apoptosis In Vitro but Does Not Provide Therapeutic Efficacy in a Xenograft Mouse Model of Cancer. *PLoS One* 11:e0151516.
39. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol* 70:4805-10.
40. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY. 2002. A monomeric red fluorescent protein. *Proc Natl Acad Sci U S A* 99:7877-82.
41. Poulin KL, Lanthier RM, Smith AC, Christou C, Risco Quiroz M, Powell KL, O'Meara RW, Kothary R, Lorimer IA, Parks RJ. 2010. Retargeting of Adenovirus Vectors through Genetic Fusion of a Single-Chain or Single-Domain Antibody to Capsid Protein IX. *Journal of Virology* 84:10074-10086.
42. McKinnon RD, Bacchetti S, Graham FL. 1982. Tn5 mutagenesis of the transforming genes of human adenovirus type 5. *Gene* 19:33-42.

43. Addison CL, Hitt M, Kunsken D, Graham FL. 1997. Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. *J Gen Virol* 78 (Pt 7):1653-61.
44. Wong CM, Nash LA, Del Papa J, Poulin KL, Falls T, Bell JC, Parks RJ. 2016. Expression of the fusogenic p14 FAST protein from a replication-defective adenovirus vector does not provide a therapeutic benefit in an immunocompetent mouse model of cancer. *Cancer Gene Ther* 23:355-364.
45. Ross PJ, Kennedy MA, Christou C, Risco Quiroz M, Poulin KL, Parks RJ. 2011. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol* 85:3950-8.
46. Ross PJ, Parks RJ. 2009. Construction and characterization of adenovirus vectors. *Cold Spring Harb Protoc* 2009:pdb.prot5011.
47. Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS. 1996. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* 70:2296-306.
48. Ross PJ, Kennedy MA, Parks RJ. 2009. Host cell detection of noncoding stuffer DNA contained in helper-dependent adenovirus vectors leads to epigenetic repression of transgene expression. *J Virol* 83:8409-17.
49. Goldsmith ME, Aguila A, Steadman K, Martinez A, Steinberg SM, Alley MC, Waud WR, Bates SE, Fojo T. 2007. The histone deacetylase inhibitor FK228 given prior to adenovirus infection can boost infection in melanoma xenograft model systems. *Mol Cancer Ther* 6:496-505.

50. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, MacGregore-Cortelli B, Tong W, Secrist JP, Schwartz L, Richardson S, Chu E, Olgac S, Marks PA, Scher H, Richon VM. 2005. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J Clin Oncol* 23:3923-31.
51. Ryan QC, Headlee D, Acharya M, Sparreboom A, Trepel JB, Ye J, Figg WD, Hwang K, Chung EJ, Murgo A, Melillo G, Elsayed Y, Monga M, Kalnitskiy M, Zwiebel J, Sausville EA. 2005. Phase I and pharmacokinetic study of MS-275, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumors or lymphoma. *J Clin Oncol* 23:3912-22.
52. Lee JH, Choy ML, Ngo L, Foster SS, Marks PA. 2010. Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair. *Proc Natl Acad Sci U S A* 107:14639-44.
53. Luo RX, Dean DC. 1999. Chromatin remodeling and transcriptional regulation. *J Natl Cancer Inst* 91:1288-94.
54. Kumari A, Cacan E, Greer SF, Garnett-Benson C. 2013. Turning T cells on: epigenetically enhanced expression of effector T-cell costimulatory molecules on irradiated human tumor cells. *J Immunother Cancer* 1:17.
55. Gavin DP, Kartan S, Chase K, Jayaraman S, Sharma RP. 2009. Histone deacetylase inhibitors and candidate gene expression: An in vivo and in vitro approach to studying chromatin remodeling in a clinical population. *J Psychiatr Res* 43:870-6.
56. To KK, Polgar O, Huff LM, Morisaki K, Bates SE. 2008. Histone modifications at the ABCG2 promoter following treatment with histone deacetylase inhibitor mirror those in multidrug-resistant cells. *Mol Cancer Res* 6:151-64.

57. Sambucetti LC, Fischer DD, Zabudoff S, Kwon PO, Chamberlin H, Trogani N, Xu H, Cohen D. 1999. Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. *J Biol Chem* 274:34940-7.
58. Hu QP, Mao DA. 2016. Histone deacetylase inhibitor SAHA attenuates post-seizure hippocampal microglia TLR4/MYD88 signaling and inhibits TLR4 gene expression via histone acetylation. *BMC Neurosci* 17:22.
59. Ben-Israel H, Kleinberger T. 2002. Adenovirus and cell cycle control. *Front Biosci* 7:d1369-95.
60. Chattopadhyay D, Ghosh MK, Mal A, Harter ML. 2001. Inactivation of p21 by E1A leads to the induction of apoptosis in DNA-damaged cells. *J Virol* 75:9844-56.
61. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. 2000. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci U S A* 97:10014-9.
62. Wilson AJ, Byun DS, Popova N, Murray LB, L'Italien K, Sowa Y, Arango D, Velcich A, Augenlicht LH, Mariadason JM. 2006. Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem* 281:13548-58.
63. Flak MB, Connell CM, Chelala C, Archibald K, Salako MA, Pirlo KJ, Lockley M, Wheatley SP, Balkwill FR, McNeish IA. 2010. p21 Promotes oncolytic adenoviral activity in ovarian cancer and is a potential biomarker. *Mol Cancer* 9:175.
64. Dokmanovic M, Clarke C, Marks PA. 2007. Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* 5:981-9.

65. Butler LM, Zhou X, Xu WS, Scher HI, Rifkind RA, Marks PA, Richon VM. 2002. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. *Proc Natl Acad Sci U S A* 99:11700-5.
66. Scognamiglio A, Nebbioso A, Manzo F, Valente S, Mai A, Altucci L. 2008. HDAC-class II specific inhibition involves HDAC proteasome-dependent degradation mediated by RANBP2. *Biochim Biophys Acta* 1783:2030-8.
67. Swaminathan S, Thimmapaya B. 1996. Transactivation of adenovirus E2-early promoter by E1A and E4 6/7 in the context of viral chromosome. *J Mol Biol* 258:736-46.
68. O'Connor RJ, Hearing P. 2000. The E4-6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. *J Virol* 74:5819-24.
69. Gallimore PH, Turnell AS. 2001. Adenovirus E1A: remodelling the host cell, a life or death experience. *Oncogene* 20:7824-35.
70. Wang GZ, Wang Y, Goff SP. 2016. Histones Are Rapidly Loaded onto Unintegrated Retroviral DNAs Soon after Nuclear Entry. *Cell Host Microbe* 20:798-809.
71. Milavetz BI, Balakrishnan L. 2015. Viral epigenetics. *Methods Mol Biol* 1238:569-96.
72. Knipe DM, Lieberman PM, Jung JU, McBride AA, Morris KV, Ott M, Margolis D, Nieto A, Nevels M, Parks RJ, Kristie TM. 2013. Snapshots: Chromatin Control of Viral Infection. *Virology* 435:141-56.
73. Gupta A, Jha S, Engel DA, Ornelles DA, Dutta A. 2012. Tip60 degradation by adenovirus relieves transcriptional repression of viral transcriptional activator E1A. *Oncogene* 32:5017.
74. Zhang Q, Yao H, Vo N, Goodman RH. 2000. Acetylation of adenovirus E1A regulates binding of the transcriptional corepressor CtBP. *Proc Natl Acad Sci U S A* 97:14323-8.

75. Madison DL, Yaciuk P, Kwok RP, Lundblad JR. 2002. Acetylation of the adenovirus-transforming protein E1A determines nuclear localization by disrupting association with importin-alpha. *J Biol Chem* 277:38755-63.
76. Wang A, Kurdistani SK, Grunstein M. 2002. Requirement of Hos2 histone deacetylase for gene activity in yeast. *Science* 298:1412-4.
77. Somanath P, Herndon Klein R, Knoepfler PS. 2017. CRISPR-mediated HDAC2 disruption identifies two distinct classes of target genes in human cells. *PLOS ONE* 12:e0185627.
78. Lai Q, Du W, Wu J, Wang X, Li X, Qu X, Wu X, Dong F, Yao R, Fan H. 2017. H3K9ac and HDAC2 Activity Are Involved in the Expression of Monocarboxylate Transporter 1 in Oligodendrocyte. *Frontiers in Molecular Neuroscience* 10:376.
79. Feng Q, Su Z, Song S, Chiu H, Zhang B, Yi L, Tian M, Wang H. 2016. Histone deacetylase inhibitors suppress RSV infection and alleviate virus-induced airway inflammation. *Int J Mol Med* 38:812-22.
80. Marks PA. 2007. Discovery and development of SAHA as an anticancer agent. *Oncogene* 26:1351-6.
81. Wu Y, Ma S, Xia Y, Lu Y, Xiao S, Cao Y, Zhuang S, Tan X, Fu Q, Xie L, Li Z, Yuan Z. 2017. Loss of GCN5 leads to increased neuronal apoptosis by upregulating E2F1- and Egr-1-dependent BH3-only protein Bim. *Cell Death & Disease* 8:e2570.
82. Abramova MV, Pospelova TV, Nikulenkov FP, Hollander CM, Fornace AJ, Jr., Pospelov VA. 2006. G1/S arrest induced by histone deacetylase inhibitor sodium butyrate in E1A + Ras-transformed cells is mediated through down-regulation of E2F activity and stabilization of beta-catenin. *J Biol Chem* 281:21040-51.

83. Jeon YJ, Ko SM, Cho JH, Chae JI, Shim JH. 2013. The HDAC inhibitor, panobinostat, induces apoptosis by suppressing the expression of specificity protein 1 in oral squamous cell carcinoma. *Int J Mol Med* 32:860-6.
84. Hedrick E, Crose L, Linardic CM, Safe S. 2015. Histone Deacetylase Inhibitors Inhibit Rhabdomyosarcoma by Reactive Oxygen Species–Dependent Targeting of Specificity Protein Transcription Factors. *Molecular Cancer Therapeutics* 14:2143.
85. Rojas JJ, Guedan S, Searle PF, Martinez-Quintanilla J, Gil-Hoyos R, Alcayaga-Miranda F, Cascallo M, Alemany R. 2010. Minimal RB-responsive E1A Promoter Modification to Attain Potency, Selectivity, and Transgene-arming Capacity in Oncolytic Adenoviruses. *Mol Ther* 18:1960-71.
86. Hedjran F, Shantanu K, Tony R. 2011. Deletion analysis of Ad5 E1a transcriptional control region: impact on tumor-selective expression of E1a and E1b. *Cancer Gene Ther* 18:717-23.
87. Reichel R, Neill SD, Kovesdi I, Simon MC, Raychaudhuri P, Nevins JR. 1989. The adenovirus E4 gene, in addition to the E1A gene, is important for trans-activation of E2 transcription and for E2F activation. *J Virol* 63:3643-50.
88. Parks CL, Shenk T. 1997. Activation of the adenovirus major late promoter by transcription factors MAZ and Sp1. *J Virol* 71:9600-7.
89. Swaminathan S, Thimmapaya B. 1995. Regulation of adenovirus E2 transcription unit. *Curr Top Microbiol Immunol* 199 (Pt 3):177-94.
90. Ai T, Xu Y, Qiu L, Geraghty RJ, Chen L. 2015. Hydroxamic acids block replication of hepatitis C virus. *J Med Chem* 58:785-800.

Chapter 4:

Development of a Novel Screening Platform for the Identification of Small Molecule Inhibitors of Human Adenovirus

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4.1 Abstract

Human adenovirus (HAdV) can cause severe disease and death in both immunocompromised and immunocompetent patients. The current standards of treatment are often ineffective, and no approved antiviral therapy against HAdV exists. We report here the design and validation of a fluorescence-based high-content screening platform for the identification of novel anti-HAdV compounds. The screen was conducted using a wildtype-like virus containing the red fluorescent protein (RFP) gene under the regulation of the HAdV major late promoter. Thus, RFP expression allows monitoring of viral late gene expression (a surrogate marker for virus replication), and compounds affecting virus growth can be easily discovered by quantifying RFP intensity. We used our platform to screen ~1200 FDA-approved small molecules, and identified several cardiotonic steroids, corticosteroids and chemotherapeutic agents as anti-HAdV compounds. Our screening platform provides the stringency necessary to detect compounds with varying degrees of antiviral activity, and facilitates drug discovery/repurposing to combat HAdV infections.

4.2 Introduction

The human adenovirus (HAdV) was first isolated in the 1950's from adenoid tissue (1, 2), and over 80 types (categorized into 7 species) have been identified to date (3, 4). HAdV mainly causes minor illnesses in most individuals, and the type of disease and tissue tropism varies depending on the type involved. For example, HAdV species B and C are mainly responsible for respiratory infections, while species D is associated with conjunctivitis, and species F with the gastroenteritis (5). HAdV can also cause severe disease (including systemic infection and respiratory failure) and death in pediatric, geriatric, immunocompromised, and more rarely, in immunocompetent patients (3, 5-9). In the clinic, antivirals typically used for other viral infections, such as cidofovir, ganciclovir and ribavirin, are used as standard treatments for HAdV-induced disease (10-12). However, these drugs often have low efficacy against HAdV and can have serious side effects such as nephrotoxicity (10, 12, 13). Thus, a more effective anti-HAdV therapy is necessary. Brincidofovir, a phospholipid conjugate of cidofovir, recently received Fast Track Designation from the FDA for treatment of HAdV infections (14, 15), and clinical trials assessing virus-specific T-cells are currently ongoing (16, 17). A live oral vaccine against types 4 and 7 was used by the U.S. military from 1971 to 1997, and then reintroduced in 2011 to limit HAdV infection in recruits (3). This vaccine is not available to the general population.

HAdV is a nuclear virus, with replication and progeny virion formation occurring within the nucleus of the infected cell (18). Proteins from the early viral transcription units (E1A, E1B, E2, E3 and E4) modulate cellular pathways to make the environment more conducive to viral gene expression and replication, or are necessary for the replication of the viral genome (19-22). In particular, the early region 1A (E1A) proteins are required for efficient virus replication as they

transactivate expression of other HAdV genes, and the E2 proteins (DNA-binding protein or DBP, precursor terminal protein, viral DNA polymerase) are directly involved in synthesizing the viral DNA (23-26). The HAdV major late promoter (MLP) is only fully activated following initiation of viral genome replication, and it regulates expression of the late regions L1-L5 which mainly encode structural proteins (e.g. fiber, penton, hexon) (27-29). As with all viruses, HAdV is reliant on the cellular machinery to assist in virus entry, viral gene expression, genome replication and production of progeny virions. For example, E1A interacts with a variety of cellular proteins, altering their regular functions and modifying the gene expression profile within the infected cell in order to provide an environment that promotes virus replication (30, 31). These cellular proteins include epigenetic modifiers, cell cycle regulators and transcription factors such as p300/CBP, p21, pRb, E2F1, TFIID and CtBP, amongst others (22, 32). In addition to E1A, several other HAdV-encoded proteins (e.g. E1B, E3, E4, pVII) also alter host protein functions and gene expression throughout the virus lifecycle (19, 20, 33, 34). Small molecules that disrupt these interactions or pathways, without severely affecting cell health, may serve as effective anti-HAdV compounds. Indeed, changes in the localization of cellular RNA splicing proteins by cardiotonic steroids have been shown to alter HAdV-5 RNA processing, leading to drastic reductions in virus yield (35). Tazarotene, a selective agonist of the retinoic acid receptor β , prevented virus replication in A549 cells (36). Identification of such compounds may eventually uncover effective therapeutic agents to treat HAdV-induced disease.

Screening of small molecules is a widely used, cost-effective drug-discovery approach which has led to the discovery of compounds against previously unexploited viral and host targets to combat a variety of viral infections (37-40). The majority of published studies on new HAdV

inhibitors have evaluated only a small number of select compounds (35, 36, 41, 42), and very few drug library screens have been performed (43, 44). A fluorescence-based high throughput screen (HTS) was designed by Duffy *et al.* to specifically identify small molecules that inhibited coagulation factor X-mediated entry of HAdV-5 vectors by cells (44). Another HTS conducted to discover novel HAdV inhibitors utilized E1-deficient, non-replicating viruses encoding GFP (under the control of the CMV promoter) to measure viral gene expression following infection and drug treatment (45). More recently, Hartline *et al.* described an approach using luminescence-based detection of drug-induced changes in viral cytopathic effects (CPE), which allowed broad-spectrum screening for antiviral compounds against various DNA viruses including HAdV (43). Although the two latter studies identified several compounds with previously unreported anti-HAdV activity (FCV, 4-thio-IDU and a piperazin-2-one derivative), both screening systems relied on more indirect measurements of viral gene expression/replication, which may limit the ability of the screens to detect compounds with modest effects on virus replication.

To facilitate antiviral discovery and drug repurposing for ameliorating HAdV-induced disease, we designed an efficient high-content screening method using a wildtype-like HAdV-5 reporter construct, Ad-late/RFP. This virus contains the red fluorescent protein (RFP) gene under the regulation of the HAdV MLP and consequently, RFP expression is only detected upon full activation of the MLP following active viral DNA replication (46). As RFP levels reflect the degree of viral late gene expression and DNA replication (46), quantification of fluorescence intensity allows us to easily identify compounds that affect these processes. In this study, we have used our platform to screen ~1200 FDA-approved compounds. We validated the top positive hits by investigating their impact on cell health, viral gene expression, DNA replication and yield of

infectious virions. We found that cardiotonic steroids suppressed multiple stages of the virus lifecycle and corticosteroids reduced early/late protein levels, resulting in decreased virus yield in both cases. Thus, our screening platform offers the sensitivity required to detect compounds that lead to even modest or transient reductions in viral gene expression/replication, as well as those with more robust antiviral activity.

4.3 Materials and Methods

Cell lines and reagents. Most experiments were conducted in the human lung adenocarcinoma-derived A549 cell line (CCL-185, ATCC), unless otherwise indicated. To monitor significant changes in cell viability and density in the small molecule screen and dose-response assays, A549 cells were transduced with a lentiviral vector to constitutively express green fluorescent protein (GFP) (47). The A549-GFP cell line grows and supports HAdV replication as well as the parental A549 cell line, and has been referred to as “GFP-expressing A549 cells” where applicable in this study. Both cell lines were cultured in Minimum Essential Medium (MEM, Sigma-Aldrich) containing 10% (v/v) Fetal Bovine Serum (FBS, Sigma Aldrich), 2 mM GlutaMAX (Invitrogen) and 1x antibiotic-antimycotic (Invitrogen). MRC-5 cells (CCL-171, ATCC) were cultured similarly in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich).

The Prestwick Chemical Library was obtained from Prestwick Chemical. For validation and follow-up experiments, SAHA (SML0061; $\geq 98\%$ purity by HPLC), digoxin (D6003; $\geq 95\%$) and lanatoside C (L2261; $\geq 95\%$) were purchased from Sigma; cytarabine (16069; $\geq 95\%$), methotrexate (13960; $\geq 98\%$) and digitoxigenin (18229; $\geq 98\%$) were purchased from Cayman Chemical; dexamethasone acetate (SC-204714; $\geq 99\%$) and flunisolide (SC-215039; $\geq 98\%$) were purchased from Santa Cruz Biotechnology. All compounds were dissolved in DMSO to prepare stock solutions and diluted in cell media for treatments.

HAdV constructs. Construction of the Ad-late/RFP has been described in detail previously (46). Briefly, the monomeric RFP coding sequence with an upstream splice acceptor site replaces the viral E3 region, which places RFP expression under the control of the viral MLP (48). This

construct was generated based on HAdV-5, using a combination of conventional cloning and bacterial recombination (49). The virus was propagated in 293 cells and purified by cesium chloride buoyant density centrifugation using standard procedures (50). Viral titer (plaque forming units) was determined by plaque assay on 293 cells as previously reported (51).

Infection and drug treatment. Unless otherwise indicated, medium was removed from confluent monolayers of A549 cells before infecting with the Ad-late/RFP. Multiplicity of infection (MOI) was calculated as plaque-forming units (PFU) per cell. Virus inoculums were diluted in phosphate-buffered saline (PBS, Sigma-Aldrich) and added to the cells for 1 hour at 37°C with periodic rocking. Medium containing either vehicle or drug was then added to the cells, followed by incubation in a humidified CO₂ incubator at 37°C until the indicated time points.

For experiments involving pretreatment with compounds, the cells were incubated with vehicle/drug-containing medium for 2 hours at 37°C before infection. Drug treatment was maintained throughout the course of infection and the treatment medium was replaced every 24 hours for experiments that extended beyond one day.

Fluorescence microscopy. Live cells were imaged 18-24 hours after infection using a 10x objective on a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam MRC camera and ZEN 2 software for image processing.

Immunoblot analysis. Cells were lysed in 2x Laemmli buffer containing 5% β -mercaptoethanol, subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) as previously described (51). The membrane was blocked with 5% milk in tris-buffered saline containing 0.2% Tween 20 (Thermo Fisher Scientific), and probed with antibodies diluted

in the 5% milk solution. The following primary antibodies were used: HAdV-5 fiber (1/10,000 dilution; MS-1027-P0, Neomarkers), HAdV-5 E1A (1/5,000; MA5-13643, Invitrogen), vinculin (1/10,000; ab129002, Abcam), tubulin (1/5,000; CP06, Calbiochem), actin (1/10,000; A1978, Sigma Aldrich), and RFP (1/5,000; ab62341, Abcam). The HAdV-5 fiber antibody from Neomarkers was discontinued during the course of this study. Therefore, hexon was detected instead using the HAdV-5 antibody from Abcam (1/10,000; ab6982) to examine late gene expression in some of the immunoblots. This antibody detects all of the major HAdV-5 structural proteins, and also cross-reacts with some of the HAdV-4 and HAdV-7 capsid proteins. The monoclonal antibody against HAdV-5 DBP (antibody 72K-B6, 1/5,000 dilution) was a kind gift from Dr. Arnold Levine (Institute for Advanced Study, Princeton, New Jersey) (52). The membranes were then washed three times in TBST and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). Blots were developed using the Immobilon Classico Western HRP Substrate (Millipore). All immunoblot data are representative of two or more independent experiments.

Small molecule screen and RFP quantification assays. GFP-expressing A549 cells were seeded in 96-well plates at a density of 20,000 cells/well using the MicroFlo Select Dispenser (BioTek). Compounds from the library plates were diluted to a working concentration in PBS containing 10% DMSO, and vehicle/compounds were added to confluent monolayers of cells for pretreatment (final concentration of 250 nM in culture media) using the Precision Microplate Pipetting System (BioTek). After 2 hours of pretreatment, these cells were infected with Ad-late/RFP (MOI 10) in the presence of the compounds. At 24 hpi, the cell media was aspirated and the cell monolayers were washed once in PBS. The cells were fixed with freshly made 4% w/v paraformaldehyde (pH

7) for 15 minutes. The fixed cells were stored in PBS overnight at 4°C. The following day, the PBS was removed and the cell nuclei were stained for 15-20 min with 10 µg/ml Hoechst 33342 (Life Technologies) diluted in PBS. New PBS was placed back on the stained cells before RFP quantification using the Cellomics High Content Screening (HCS) Platform and the Cellomics navigator version of the HCS Studio software (Thermo Fisher Scientific). The 10x objective and the following filters were used: 386_{excitation (ex)} nm for Hoechst, 549_{ex} nm for RFP and 485_{ex} nm for GFP. Roughly 1,000 cells (Hoechst-stained intact nuclei) were analyzed per well and their total RFP intensity was measured. Since each library plate was treated and screened in triplicate, the mean data from the three replicate plates was used for analysis. The mean RFP intensities in drug-treated wells were calculated as a percentage of the mean intensities in vehicle-treated wells. Further information on screen parameters and analysis is available upon request.

For the dose-response assays, drug treatment, infection, cell fixation and RFP quantification were carried out as described above for the screen.

Quantitative real-time PCR (qPCR). A549 cells were pretreated with compounds and infected as described above. Medium was removed from the infected cells at the indicated time points and the cells were incubated overnight in SDS-proteinase K buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% w/v SDS, 1 mg/ml proteinase K) at 37°C. DNA was extracted from the cell lysates using the standard phenol-chloroform method, precipitated with ethanol and NaCl, and the resulting DNA pellet was dissolved in 1x Tris-EDTA (TE) buffer. qPCR was conducted as previously described (53) using 200 ng of genomic DNA per reaction. The following primers were used: 5'-CTT ACC CCC AAC GAG TTT GA and 5'-GGA GTA CAT GCG GTC CTT GT for HAdV hexon; 5'-CCA TTA AAC CAG TTG CCG TGA GAG and 5'-GGC GTT TAC AGC TCA

AGT CCA AAG for HAdV E1A. Viral genome copy numbers were calculated from the Ct values using a standard curve obtained using serial dilutions of the pRP3089, the bacterial plasmid used to generate Ad-late/RFP.

RNA extraction and reverse transcription qPCR. A549 cells were pretreated with compounds and infected as described above. Medium was removed from the infected cells and total cellular RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) and the PureLink RNA Mini Kit (Ambion) according to the manufacturer's protocol. To synthesize cDNA from the extracted RNA by reverse transcription (RT), the M-MuLV Reverse Transcriptase was used (New England Biolabs) along with a mixture of random hexamers (New England Biolabs), according to the manufacturer's protocol. 800 ng of RNA was used per RT reaction. qPCR was then conducted as described above using 2 µl of the cDNA mixture.

Plaque assay for virus yield. Monolayers of A549 cells were pretreated with each compound, and infected with Ad-late/RFP (MOI 10). The virus inoculum was removed after 1 hour of infection, the cells were washed with PBS, and fresh medium containing vehicle or compounds was added. After 24 hours of infection, the cells were collected by scraping into the medium, 40% w/v sucrose (diluted in 10 mM Tris) was added to a final concentration of 4% v/v, and the samples were flash-frozen in ethanol and dry ice. For the plaque assay, monolayers of A549 cells were infected with dilutions of this cell lysate. After 1 hour of infection, the cells were overlaid with medium containing agarose (50% v/v of a 1% w/v agarose solution, 43% clear 2x MEM, 5% FBS, 1% GlutaMAX and 1% antibiotic-antimycotic) and placed in a 37°C incubator. Plaques were counted 8-10 days later.

MTS metabolic activity assays. 96-well plates of A549 cells were pretreated with compounds and infected with Ad-late/RFP (MOI 10). 24 hours post infection, the metabolic activity was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Briefly, cells were incubated for 1 hour at 37°C with 20 µl of the MTS substrate, and absorbance readings were obtained at 490 nm using the SpectraMax 190 plate spectrophotometer (Molecular Devices).

4.4 Results

4.4.1 Design and optimization of the small molecule screen

We generated a replication-competent (E1⁺), wildtype-like HAdV construct, designated Ad-late/RFP, which encodes the RFP gene under the regulation of the viral MLP (46). Since the MLP is only fully active following viral DNA replication (48, 54), RFP expression from Ad-late/RFP increases with time during late infection (figure 4.1A). In a previous study, we confirmed that Ad-late/RFP grows similar to the wildtype HAdV-5 in terms of protein expression and DNA replication, and that RFP is produced only when virus replication occurs (46). Thus, RFP fluorescence can be used as a surrogate marker to efficiently monitor any changes in HAdV DNA and late protein levels induced by small molecules that affect the earlier stages of the virus lifecycle, or the stability of viral RNA/proteins.

To detect real-time changes in RFP intensity induced by small molecules during a screen, we first attempted to measure the intensities over a time course of infection in live (i.e. non-fixed) GFP-expressing A549 cells. We designed a live-cell screen protocol using the Ad-late/RFP and the Cellomics ArrayScan VTI HCS Platform connected to a Live Cell Module, which controls temperature and CO₂ in the imaging chamber. Infected cells were placed in the Live Cell Chamber over a 24-hour time course of infection to periodically quantify RFP fluorescence. RFP increased 10- to 30-fold over time and as expected, the extent of increase positively correlated with the MOI of virus (figure 4.1B). However, with the live-cell imaging approach, high variability was observed within experimental replicates at each time point (the 24-hour time points with Ad-late/RFP at MOI 10 are shown in figure 4.1B; mean of 42±40 a.u.). Furthermore, in many of the preliminary test experiments we conducted with live-cell imaging (similar to that in figure 4.1B), the virus

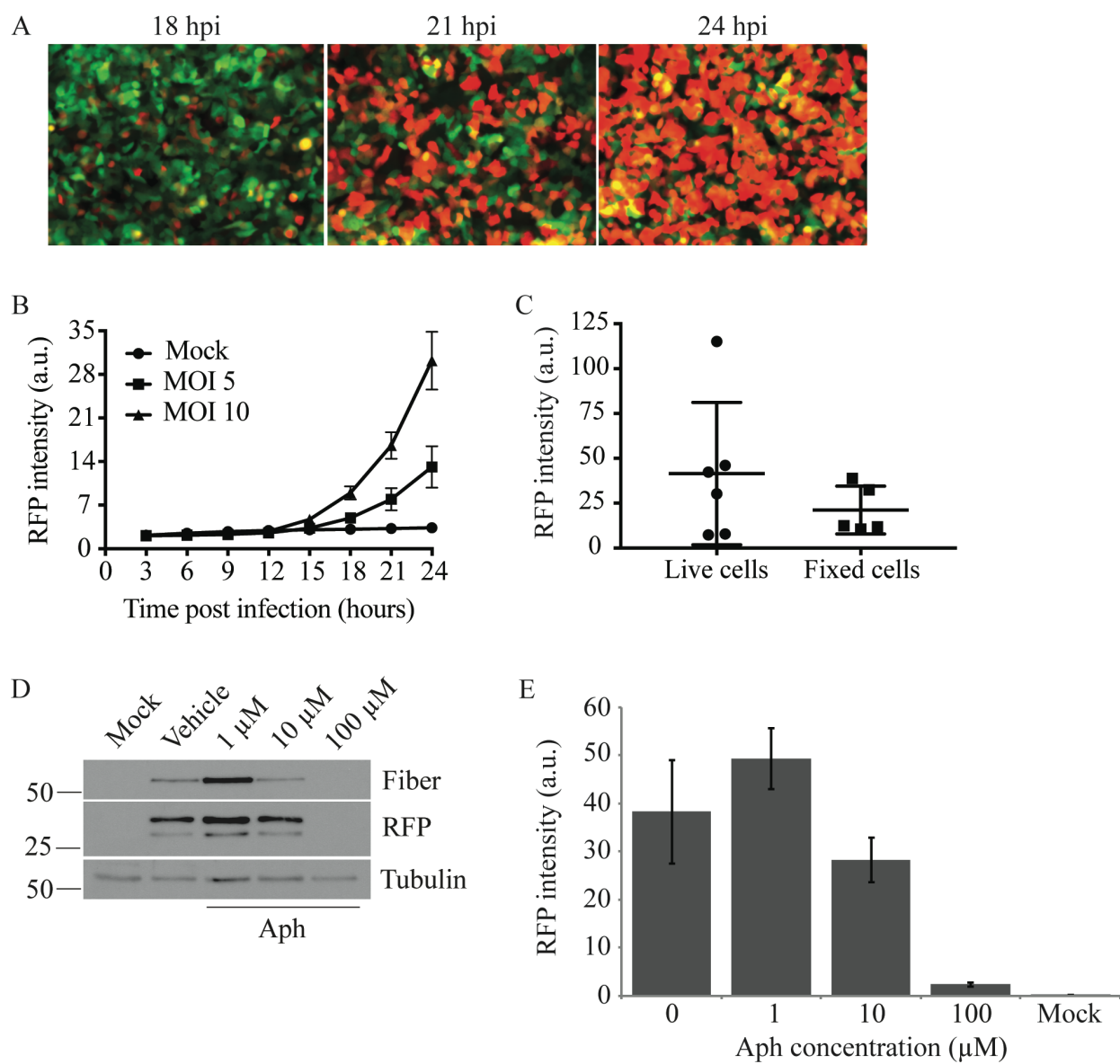


Figure 4.1 Optimization and validation of a small molecule screen for anti-HAdV compounds. GFP-expressing A549 cells were infected with Ad-late/RFP at an MOI of 10, unless indicated otherwise. (A) Infected, live cells were visualized after the onset of viral DNA replication, at the indicated times post infection. RFP expression from Ad-late/RFP increases along with virus replication over time. (B) RFP intensities within infected cells (MOI 5 or 10 of Ad-late/RFP) were quantified every 3 hours over 24 hours post infection (hpi) in live cells using the Cellomics ArrayScan VTI High Content Screening Platform. a.u. represents arbitrary units. Error bars represent standard deviation (SD) of analytical replicates (n=14). (C) RFP fluorescence in live or fixed cells was quantified as in panel B. Intensities were less variable between five independent experiments in the fixed-cell screen. Error bars represent SD of experimental replicates (n=5 or 6). (D) Infected cells were treated with vehicle or 1-100 μ M aphidicolin (Aph). Cell lysates were collected at 24 hours after infection and drug treatment for analysis of viral late gene expression by immunoblot. Aph decreases fiber and RFP production in a dose-dependent manner. (E) Infected, Aph-treated cells were fixed at 24 hpi and the RFP intensity was quantified as in panel B. The results corroborate observations from the immunoblot. Error bars represent SD of analytical replicates (n=16).

simply failed to propagate, perhaps due to the non-optimal cell growth conditions within the Live Cell Chamber (as explained in the Discussion section below). This issue was resolved and the experimental variability was significantly reduced (22 ± 13 a.u.) when RFP intensity was quantified following cell fixation at 24 hpi (figure 4.1C). As such, all subsequent RFP quantification assays and the screen were conducted on drug-treated and infected GFP-expressing cells that were fixed at 24 hpi as described in the Materials and Methods.

To test the reliability of the fixed-cell screen protocol, we conducted a dose-response assay with aphidicolin (Aph), a DNA polymerase inhibitor that prevents HAdV replication by interfering with dCTP incorporation during DNA synthesis (55). Immunoblot analysis of Ad-late/RFP-infected, Aph-treated cells showed that the drug reduced protein levels of both fiber and RFP in a dose-dependent manner (figure 4.1D). These results were corroborated by the RFP intensities quantified using the Cellomics ArrayScan in infected, drug-treated cells at 24 hpi (figure 4.1E), which confirmed the reliability of the screen protocol.

4.4.2 Identification and validation of HAdV inhibitors from the Prestwick Chemical Library

We next used our platform to screen ~1200 chemically and pharmacologically diverse compounds in the Prestwick Chemical Library to identify novel compounds with anti-HAdV activity. Although the screened compounds are likely optimally active at different concentrations *in vivo*, we used a single concentration in our initial pilot screen and subsequently tested the positive hits at varying concentrations in our validation experiments. In a recent study, we showed that the pan-histone deacetylase (HDAC) inhibitor SAHA reduces RFP expression from Ad-late/RFP, as well as virus yield, in a dose-dependent manner (46). SAHA induced more than a

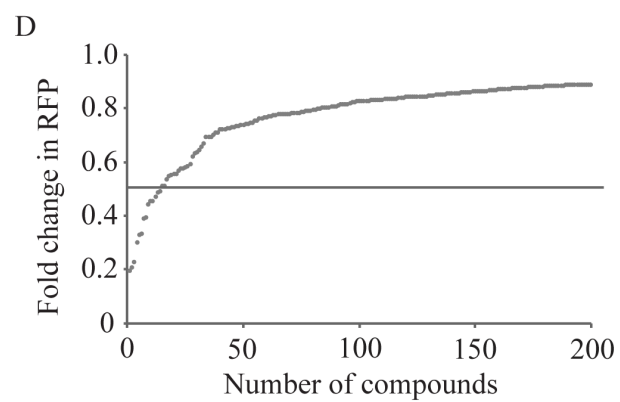
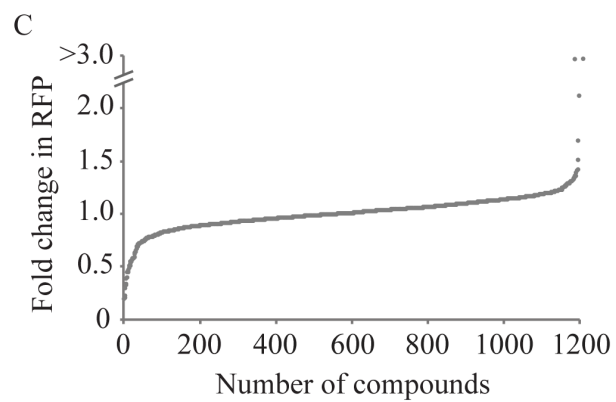
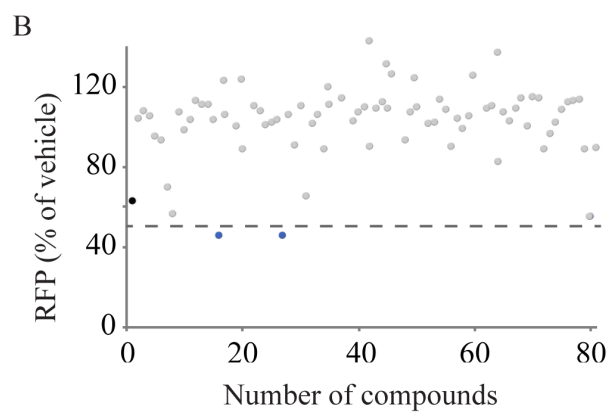
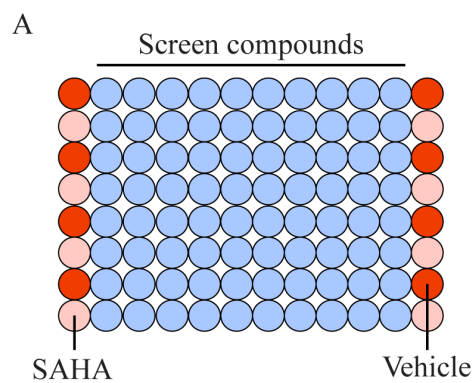


Figure 4.2 Screen of the Prestwick Chemical Library. (A) Layout of 96-well plates in the screen. Test compounds were added to 80 wells (blue). Vehicle (negative control) and SAHA (positive control) were added in alternate wells of the first and last columns. Cells were pretreated with vehicle or compounds at a concentration of 0.25 μ M for 2 hours prior to infection with Ad-late/RFP (MOI 10). Cells were fixed at 24 hpi and RFP intensities were measured using the Cellomics ArrayScan. (B) Representative data from a single screen plate (80 test compounds). Changes in RFP levels were calculated as a percentage of vehicle treatment. The black data point represents SAHA, while the blue data points represent the compounds that reduced RFP expression by more than 50%. Each set of compounds was screened in three independent assays and the data points represent the mean. (C) The average RFP intensities in drug-treated cells were calculated as fold change relative to vehicle-treated cells. Fold changes for all 1200 compounds were plotted in order of increasing magnitude. While most compounds did not alter RFP expression from Ad-late/RFP, several compounds were found to either significantly reduce or moderately enhance RFP levels. (D) A magnified view of panel C highlighting fourteen compounds that reduced RFP intensities to half that in vehicle-treated cells. Error bars have been omitted for clarity.

50% decrease in RFP intensity at a concentration of 250 nM. Thus, SAHA was used as a positive control for reduction of RFP levels, while vehicle treatment served as the negative control, on each screen plate of 80 test compounds from the library (figure 4.2A). We also selected 250 nM as the screen concentration because it is relatively low and would allow detection of compounds with higher efficacy against Ad-late/RFP. Each library plate was screened in triplicate, and the mean data for one representative plate is shown in figure 4.2B. While the majority of the small molecules screened did not affect RFP compared to vehicle treatment, we identified a subset of compounds that either decreased or increased RFP intensities significantly (figures 4.2C and 4.2D). To limit the number of compounds considered for further investigation, we selected fourteen that decreased RFP by at least 50% (figure 4.2D) and conducted subsequent dose-response assays to confirm the positive hits (data not shown). Of those, eleven compounds were confirmed to indeed reduce RFP from Ad-late/RFP at nanomolar concentrations (table 1).

The most effective inhibitors of RFP expression identified from the screen were digoxin, digitoxigenin and lanatoside C (table 4.1). The anti-HAdV activity of cardiotonic steroids was recently reported in another study (35), which further supports the reliability of our screen results. To validate these compounds in our system, we first conducted a dose-response assay to determine whether these compounds were effective in reducing RFP intensities at low concentrations. As in the screen, GFP-expressing A549 cells were pretreated with the compounds (~15 nM to 1 μ M), and infected for 24 hours prior to RFP quantification in fixed cells. All three compounds were more effective than SAHA in decreasing RFP at the concentrations tested (figure 4.3A). The IC_{50} values (with regards to RFP levels) were determined to be approximately 240 nM, 20 nM, 64 nM and 32 nM for SAHA, digoxin, digitoxigenin and lanatoside C, respectively. This inhibitory

Table 4.1 Validated positive hits from the Prestwick Chemical Library. Of the fourteen compounds that reduced RFP levels by at least 50% in the initial screen, eleven were confirmed to decrease RFP from the Ad-late/RFP in subsequent assays. The percentage values indicate the RFP intensity in cells treated with 0.25 μ M drug relative to vehicle-treated cells (100% RFP) in the screen.

Cardenolides	Corticosteroids	Others
1) Lanatoside C (19%)	4) Dexamethasone acetate (39%)	9) Cytarabine (30%)
2) Digoxin (21%)	5) Flumethasone (46%)	10) Methotrexate (33%)
3) Digitoxigenin (23%)	6) Flurandrenolide (47%)	11) Podophyllotoxin (45%)
	7) Diflorasone diacetate (49%)	
	8) Flunisolide (49%)	

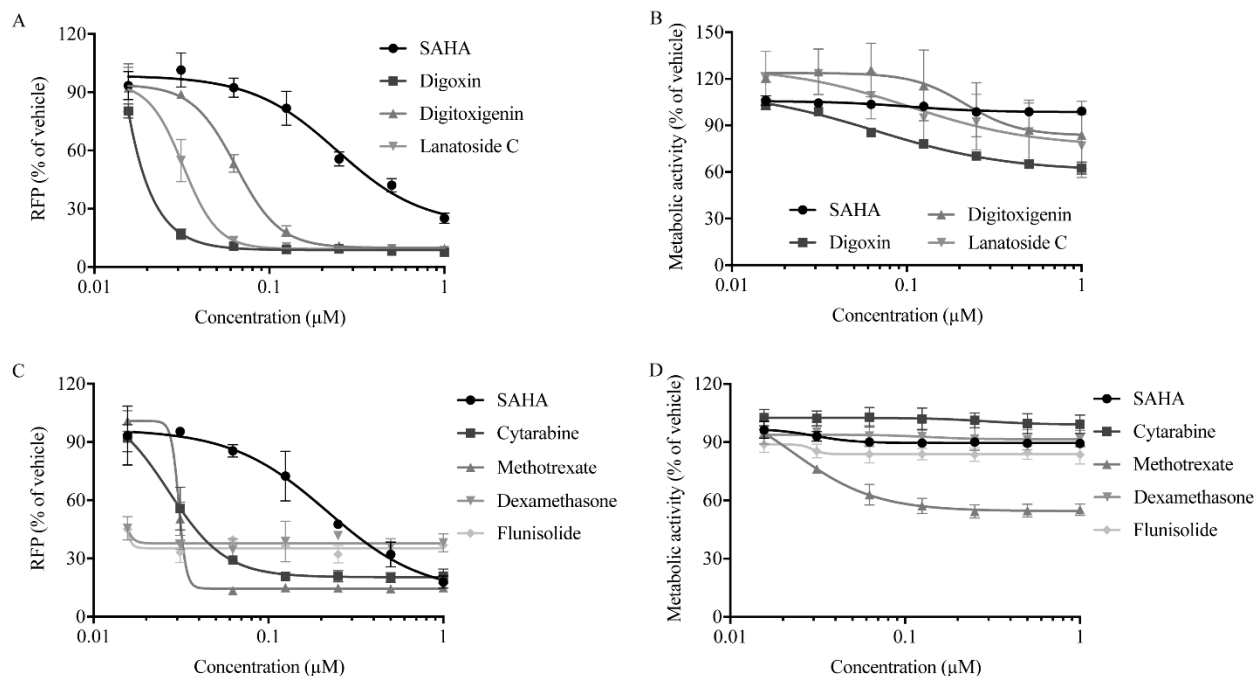


Figure 4.3 Dose-dependent effects of the selected positive hits on RFP and cellular metabolic activity. GFP-expressing A549 cells were treated for 2 hours with each compound at the indicated concentrations, then infected with Ad-late/RFP (MOI 10) for 24 hours. (A, C) Infected, drug-treated cells were fixed and RFP intensities were quantified using the Cellomics ArrayScan. (B, D) Cellular metabolic activity was determined by MTS assay. Both RFP expression and metabolic activity were calculated as a percentage of vehicle treatment, and the data was fitted to a sigmoidal or dose-response curve in GraphPad Prism. Error bars represent SD of experimental replicates ($n=3$).

effect on RFP was not due to compromised cell health as metabolic activity was 80% or above for all three compounds compared to vehicle, at least at concentrations up to ~100 nM (figure 4.3B), and endogenous GFP fluorescence from these cells was not reduced (data not shown). Our results are consistent with the findings of Grosso *et al.*, who also found that cardiotonic steroids suppress HAdV replication, but not cell viability, at similar concentrations (35).

We next conducted dose-response and MTS assays with three additional compounds which were selected based on their effectiveness (table 1): chemotherapy agents cytarabine and methotrexate, and the anti-inflammatory corticosteroid dexamethasone. We included flunisolide for validation as it is also a corticosteroid, and would be expected to have similar efficacy as dexamethasone. These two classes of drugs target different cellular proteins and pathways than the cardiotonic steroids, and likely inhibit Ad-late/RFP gene expression by other mechanisms. Both cytarabine and methotrexate significantly reduced RFP fluorescence at low concentrations, while the corticosteroids reduced RFP to 30-40% of vehicle treatment at all concentrations examined (figure 4.3C). With the exception of methotrexate, these compounds also did not affect cellular metabolic activity or GFP intensity (figure 4.3D and data not shown). The effect of methotrexate on RFP may be (at least partially) due to drug-induced cytotoxicity, and it was excluded from further investigation. Overall, these results suggest that the screening platform we developed provides the sensitivity and stringency necessary to detect and quantify varying degrees of small molecule-induced changes on HAdV gene expression.

4.4.3 Cardiotonic steroids are potent inhibitors of HAdV

Since digoxin, digitoxigenin and lanatoside C were very effective in reducing RFP levels at low concentrations in the validation assays (figure 4.3), we conducted further studies to determine whether other stages of the virus lifecycle are impacted by these drugs (e.g. early/late gene transcription, protein expression, DNA replication). Cells were pretreated with vehicle or each compound at 100 nM for 2 hours, and infected with Ad-late/RFP in the presence of the compounds. Although analysis of E1A transcript levels at 6 hpi showed no difference between vehicle- and drug-treated cells (figure 4.4A), early proteins E1A and DBP were both decreased substantially at 8 hpi (figure 4.4B). E1A transcript levels also remained unchanged between the various treatments at 24 hpi (figure 4.4A). E1A and DBP proteins were easily detected at this later time point, but the overall amount was reduced relative to vehicle-treated cells (figure 4.4B). These observations suggest that the cardiotonic steroids do not affect E1A transcription or mRNA stability, but may affect E1A protein levels by altering protein translation or stability.

In the HAdV lifecycle, early gene expression is followed by viral DNA replication. Therefore, we next examined viral genome copy numbers at early and late times of infection in cells treated with the cardiotonic steroids. qPCR with primers specific to the viral hexon region showed that the Ad-late/RFP genome copy number at 8 hpi (before the onset of viral DNA replication) were similar in vehicle- and drug-treated cells (figure 4.4C). Digoxin was a potent inhibitor of Ad-late/RFP DNA replication as it led to a 1,000-fold decrease in Ad-late/RFP genome copy numbers at 24 hpi, and a continued trend towards decreased genome copies was observed at 48 hpi (figure 4.4C). Although less effective than digoxin, reduced DNA replication was also evident at 24 hpi with digitoxigenin and lanatoside C, but there were no significant differences

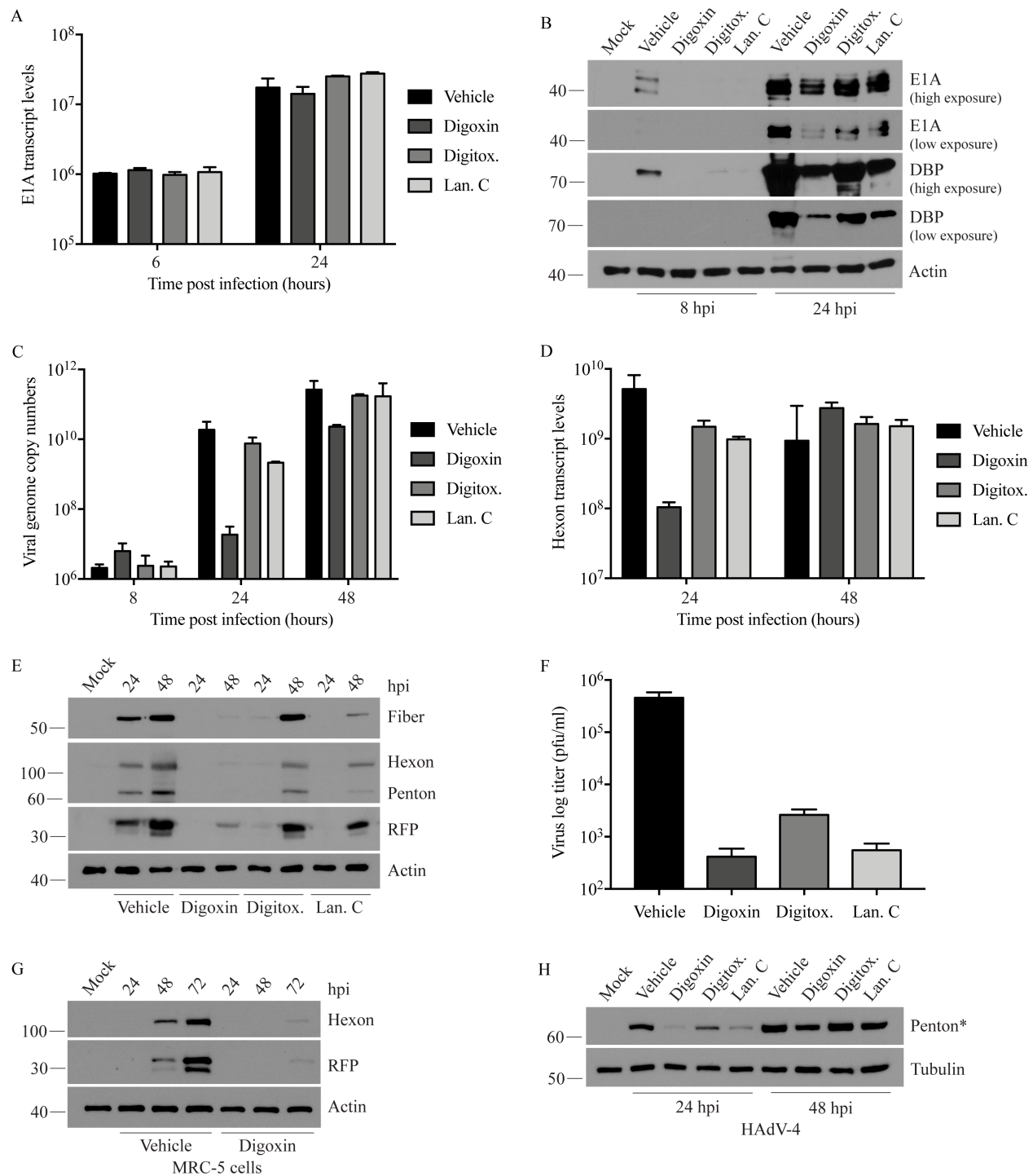


Figure 4.4 Cardiotonic steroids reduce virus gene expression, DNA replication and yield. In

all experiments, cells were pretreated with vehicle, digoxin, digitoxigenin or lanatoside C at 100 nM for 2 hours. Treated cells were infected at an MOI of 10 in the presence of the compounds, and cell lysates were collected at the indicated times for analysis of viral proteins/DNA/RNA. (A, D) Total RNA was extracted from Ad-late/RFP-infected A549 cells and cDNA was generated by reverse transcription. qPCR analysis was conducted using primers binding to the HAdV E1A (A) or hexon (D) regions. (B, E) A549 cell lysates were subjected to immunoblot analysis for detection of early (E1A, DBP) and late (fiber, RFP) proteins from Ad-late/RFP. (C) Genomic DNA (gDNA) was extracted from infected A549 cells at the indicated times for qPCR using primers specific to the hexon gene. Ad-late/RFP genome copy numbers per 200 ng of total gDNA are reported. (F) Drug-treated A549 cells were infected with Ad-late/RFP and collected at 24 hpi to determine virus yield by plaque assay. (G) Pretreated MRC-5 cells were infected with Ad-late/RFP and cell lysates were collected for immunoblotting. (H) A549 cells were pre-treated with vehicle or compounds, and infected with HAdV-4. *The protein (detected using an anti-HAdV-5 antibody that cross-reacts with some of the HAdV-4 and HAdV-7 capsid proteins) is most likely penton based on its size of ~60 kDa. Error bars represent SD (n=2 for panels A and D; n=3 for panels C and F).

with these compounds later on (figure 4.4C). This is unlikely due to drug degradation over the time course of infection as all three compounds have fairly long half-lives (at least 30 hours *in vivo*) (56), and the cell medium containing drug was replaced every 24 h in our experiments. As expected, all three compounds affected late gene transcription at 24 hpi (figure 4.4D), which was concomitant with a substantial decrease in viral late proteins (fiber, hexon, penton) and RFP levels (figure 4.4E). Late protein expression remained low with digoxin and lanatoside C at 48 hpi (figure 4.4E), but late transcript levels were comparable to vehicle treatment at that time point (figure 4.4D), further supporting the possibility that these compounds may act by altering protein production and/or stability. Inhibition of multiple stages of the HAdV lifecycle by the cardiotonic steroids consequently led to a 100- to 1000-fold reduction in Ad-late/RFP yield as determined by plaque assay of virus recovered from treated cells (figure 4.4F). The antiviral activity of the cardiotonic steroids was also observed against Ad-late/RFP in primary MRC-5 cells (figures 4.4G and S3A), and against clinically relevant types 4 and 7 (figures 4.4H and S3B). Our results, combined with that obtained by Grosso *et al.* (35), confirm that digoxin, digitoxigenin and lanatoside C are effective inhibitors of HAdV, and these compounds were successfully detected by our screen methodology.

4.4.4 Cytarabine, dexamethasone and flunisolide have modest anti-HAdV activity

We next examined how cytarabine, dexamethasone and flunisolide affect the HAdV lifecycle. Each compound was used at 200 nM, and drug treatment and infection were carried out as described above for the cardiotonic steroids. Interestingly, cytarabine did not decrease the transcript levels of E1A or expression of the early genes (figures 4.5A and 4.5B). Although a trend towards reduced E1A transcript was observed with dexamethasone and flunisolide at 6 hpi, this

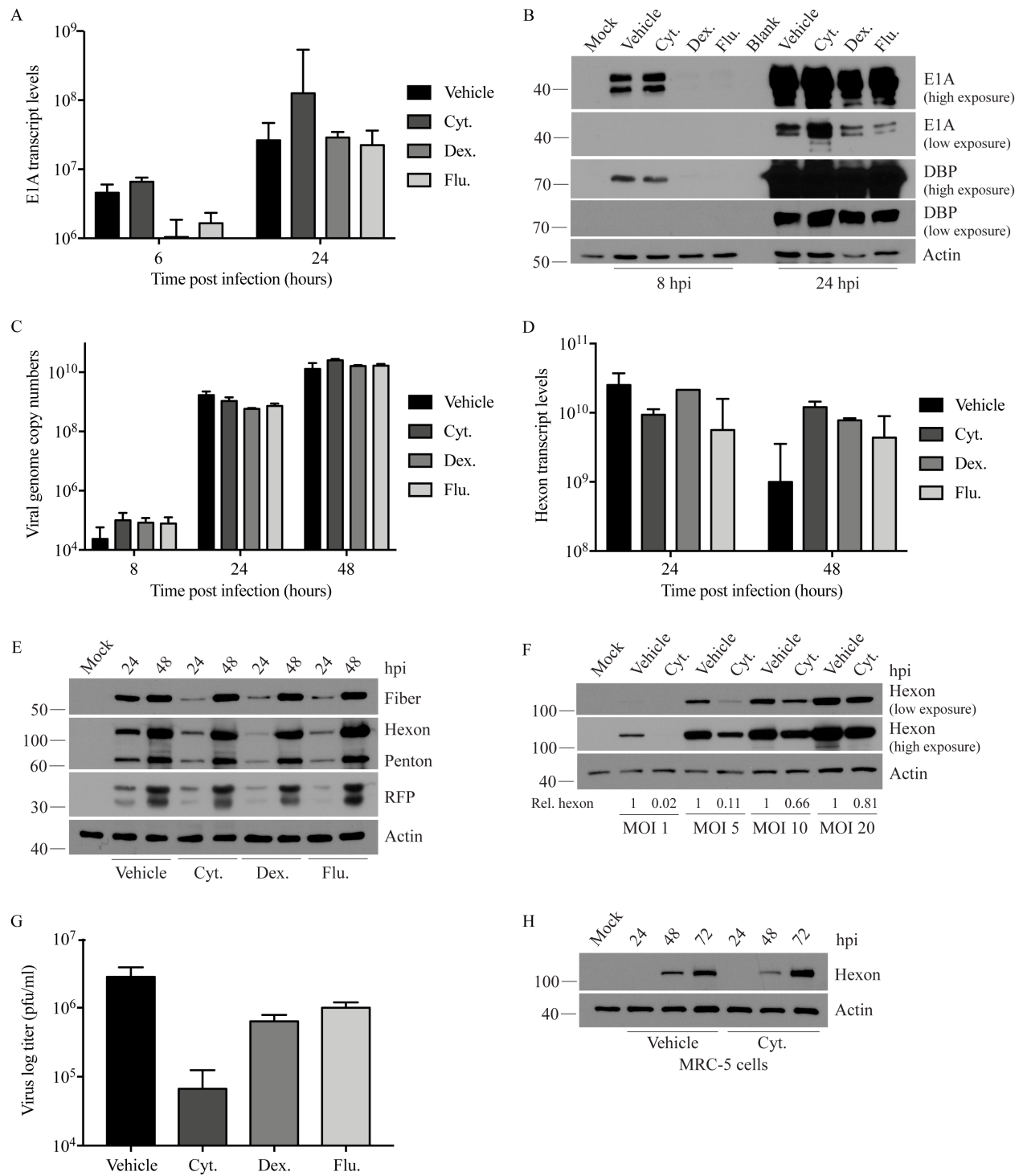


Figure 4.5 Cytarabine, dexamethasone and flunisolide reduce HAdV gene expression and yield. All cells were pretreated with vehicle, cytarabine, dexamethasone or flunisolide at 200 nM for 2 hours, and then infected with Ad-late/RFP (MOI 10, unless otherwise indicated) over the indicated time periods. (A, D) RNA was extracted from infected A549 cells and reverse transcribed to cDNA. qPCR analysis was conducted using primers to the HAdV E1A (A) or hexon (D) regions. (B, E) A549 cell lysates were examined for viral early (B) and late (E) protein expression by immunoblot. (C) gDNA was extracted from infected A549 cells at the indicated times for qPCR using primers specific to hexon. Viral genome copy numbers per 200 ng of total gDNA are reported. (F) Following pretreatment, A549 cells were infected at various MOIs for 24 hours, and crude protein extracts from the cells were used for immunoblot analysis. Relative hexon protein levels were determined using the Image J software. Hexon bands were first normalized to actin (loading control), followed by calculation of drug-treated band intensities relative to vehicle-treated bands at each specific MOI. (G) Infected, drug-treated A549 cells were collected at 24 hpi and subjected to plaque assay for quantification of virus yield. (H) MRC-5 cells were treated with cytarabine and infected with Ad-late/RFP as described above. Error bars represent SD (n=2 for panels A and D; n=3 for panels C and G).

was not statistically significant (figure 4.5A). However, both E1A and DBP expression was reduced at 8 hpi (figure 4.5B). By 24 hpi, transcript levels of E1A were all comparable to vehicle treatment (figure 4.5A), and a small decrease in E1A protein was still evident with the corticosteroids (figure 4.5B). Somewhat surprisingly, we did not detect any differences in Ad-late/RFP genome copy numbers with any of the compounds, at least at the time points examined (figure 4.5C). This observation may mimic our results with SAHA, where inhibition of viral DNA replication was greater earlier on during the replicative phase of infection, and was not as pronounced at later time points (46). Alternatively, early proteins may increase over time to sufficient amounts that permit nearly normal levels of DNA replication in late infection.

As with early transcripts, there were no significant changes in late transcript levels with cytarabine and the corticosteroids (figure 4.5D), but we did detect a decrease in late proteins at 24 hpi (figure 4.5E). For all three compounds, the extent of late protein reduction was more pronounced at lower MOIs (figures 4.5F, S4A and S4B) which are more relevant to clinical settings. Treatment with cytarabine and the corticosteroids also reduced virus yield (~3- to ~30-fold) from infected cells (figure 4.5G). Thus, these compounds all act as antivirals mainly through effects on HAdV protein expression, ultimately lowering virus yield. In MRC-5 cells, cytarabine reduced hexon expression to a similar extent as in A549s (figure 4.5H), but the corticosteroids were ineffective (data not shown). Taken together, our results illustrate that the screening method we developed provides the necessary sensitivity to detect even modest or transient changes in viral gene expression, which can impact upon virus growth.

4.5 Discussion and Conclusions

Development of various high-throughput screening platforms have allowed the identification of novel antiviral compounds, and highlighted the potential of repurposing existing drugs as antivirals for several viruses including Ebola, hepatitis B virus and herpes simplex virus (37-40, 57, 58). To date, no specific screens have been developed or conducted for the discovery of anti-HAdV compounds despite the need for better antiviral therapies to combat serious HAdV infections. To identify novel small molecule inhibitors of HAdV, we developed an efficient screening platform using a HAdV reporter construct, designated Ad-late/RFP (46), which encodes the RFP gene under regulation of the viral MLP. As such, RFP expression coincides with the expression of the endogenous viral late genes and reflects the extent of DNA replication (46). Thus, virus replication can be monitored by RFP fluorescence (figure 4.1), which is sufficiently sensitive to detect small changes in virus growth. Although this virus can be used for both live-cell and fixed-cell imaging, we observed higher experimental variability in preliminary screens using live-cell imaging (figure 4.1). Cell fixation reduced this variability (figure 4.1), and overcame several other drawbacks associated with screening live cells, including i) impacts of temperature and CO₂ fluctuations in the live-cell imaging chamber on cell health and virus growth; ii) poor microscope focus for the detection and quantification of RFP due to presence of cell media (compared to PBS for the fixed-cell method); and iii) 20-30 minutes time difference between reading the first and last wells of the 96-well screen plates, which can contribute to experimental variability. Consequently, we demonstrated that a fixed-cell screening method can be used to perform small molecule screens to identify novel HAdV inhibitors, as well as enhancers which can potentially be used to increase the expression level of therapeutic transgenes encoded within

HAdV-based gene therapy vectors. Importantly, our screening procedure can be easily adapted for screening larger drug libraries in a high-throughput manner.

Using the screening platform we developed, we screened the Prestwick Chemical Library containing ~1200 chemically and pharmacologically diverse compounds (figure 4.2). Although we chose to use one cell line, a single compound concentration and a pretreatment strategy in this proof-of-concept study to test the functionality of the screen methodology, these conditions can be easily modified to discover compounds that impact different aspects of virus biology. For example, varying treatment strategies may allow the identification of positive hits that can interfere at specific stages of an ongoing infection, while employing several compound concentrations and multiple cell lines (e.g. primary or transformed) could provide data on the general efficacy of the positive hits prior to further validation. One of the main advantages of the Prestwick library is that most of the compounds in it are FDA-approved, which could make the transitioning of positive hits from *in vitro* to *in vivo* models (and ultimately to humans) easier due to the availability of information on dosing, kinetics, bioavailability and safety. Of the eleven confirmed positive hits from the screen (table 4.1), we conducted further assays on six (digoxin, digitoxigenin, lanatoside C, cytarabine, dexamethasone acetate and flunisolide) to characterize their impact on the virus lifecycle. Digoxin, digitoxigenin and lanatoside C are cardiac glycosides that function by inhibiting the activity of Na⁺/K⁺-ATPases (59, 60). These compounds reduced HAdV early/late gene expression, DNA replication and virus yield at nanomolar concentrations (figures 4.3 and 4.4), and the results are consistent with a previous report showing that cardiotonic steroids suppress HAdV replication (35). The antiviral effects were not specific to A549 cells and the Ad-late/RFP, as late gene expression was decreased to a similar extent in MRC-5 cells, and for HAdV-4 and

HAdV-7 (figures 4.4 and S3). It is possible that reduced viral DNA replication and late gene expression is a direct consequence of low E1A levels, as E1A is required for the transcriptional activation of the other viral genes (61). Differential splicing of the primary transcript produced from the E1A region gives rise to five different transcripts, of which, the 13S and 12S are the most abundant in early infection and the 9S is the major form in late infection (62). Grosso *et al.* found that cardiotonic steroids alter the localization of cellular SR proteins, leading to changes in viral early RNA splicing and in the relative levels of the transcripts such that the 13S increased in early infection and 12S increased in late infection, while 9S decreased to a similar extent at both times (35). In our RT-qPCR experiments, we used primers which bind to cDNA generated from both the 13S and 12S RNAs. However, despite the marked decrease in overall E1A protein amount, we did not detect any obvious alterations in the expressed isoforms of E1A protein or the quantity of E1A transcripts (figure 4.4), suggesting that other mechanisms may also be involved. Cardiotonic steroids have shown antiviral activity against other DNA viruses (e.g. CMV and HSV) and in these cases, virus inhibition was due to impaired viral gene expression, protein translation and virus release (63-66). Future studies should involve investigation of whether these mechanisms also play a role in the abrogation of HAdV by cardiotonic steroids.

Cytarabine, another positive hit from our screen, is a chemotherapeutic agent used in the treatment of acute myeloid leukemia – a deoxycytidine analogue that interferes with DNA and RNA synthesis (67). This compound has demonstrated antiviral activity against both DNA and RNA viruses (68, 69), and is thought to act through the inhibition of viral DNA polymerases. Although we did not detect any obvious changes in viral DNA/RNA levels with cytarabine in our assays/time points, it indeed reduced late gene expression from Ad-late/RFP (in both transformed

and primary cells) and virus yield at low concentrations (figures 4.3 and 4.5). Since higher plasma levels of cytarabine are frequently observed during treatment (70, 71), higher concentrations of the drug may be used to obtain a more pronounced difference in HAdV yield, gene expression and DNA/RNA synthesis. Infection of cells treated with glucocorticoid receptor agonists dexamethasone and flunisolide also reduced viral early/late gene expression and yield (figure 4.5). These compounds modulate a multitude of cellular pathways and have an overall anti-inflammatory, immunosuppressive and anti-proliferative effects on cells (72). Thus, their anti-HAdV activity may be a consequence of their ability to alter cell signaling and homeostasis.

With digitoxigenin, cytarabine and the corticosteroids, we observed a lower impact on viral gene expression and replication at 48 hpi. Based on these results alone, we cannot conclude whether this is due to a complete but transient inhibition, which delays the onset of viral gene expression and replication, or whether the compounds allowed limited expression throughout infection, leading to a slow accumulation of viral transcripts/proteins/DNA at 48 hpi. We also note that the corticosteroids did not exhibit antiviral activity in MRC-5 cells, or against HAdV-4 and HAdV-7 (data not shown), which emphasizes the importance of validating positive hits from small molecule screens using multiple cell lines and virus types. However, further investigation of cytarabine, dexamethasone and flunisolide as anti-HAdV-5 agents is warranted since all three compounds displayed higher efficacy with lower MOIs of Ad-late/RFP (figures 4.5 and S4). In clinical settings, where infection generally occurs at low MOIs, these compounds may reduce virus replication sufficiently to allow host immune system a greater opportunity to combat the infection.

In conclusion, the small molecule screening platform we have developed has the required sensitivity to detect varying degrees of drug-induced changes in HAdV gene expression or replication, and therefore, can be used to efficiently conduct high-throughput screens to identify modulators of virus growth. This screening system permits the discovery of new HAdV inhibitors with different efficacies, including pre-existing drugs which have already been evaluated for *in vivo* safety and pharmacokinetics. Elucidating the mechanism by which these anti-HAdV compounds (e.g. the cardiotonic steroids, corticosteroids) exert their effects will not only enhance our knowledge of cellular pathways that play a role in HAdV lytic infection, but will also enable the identification of novel pharmacological targets and the development of more effective antivirals against HAdV.

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4.7 Author Contributions

Bratati Saha was responsible for conceiving, designing and conducting experiments, analyzing data, generating figures, as well as writing, reviewing and editing the manuscript. Dr. Robin Parks was responsible for conceiving experiments, providing funding and resources, and reviewing and editing the manuscript. Dr. Jean-Simon Diallo and Dr. William Stanford provided expert advices, reagents and access to equipment necessary for the design and execution of the small molecule screens. Oliver Varette provided technical assistance with the Prestwick Chemical Library screen.

4.8 References

1. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* 84:570-3.
2. Hilleman MR, Werner JH. 1954. Recovery of new agent from patients with acute respiratory illness. *Proc Soc Exp Biol Med* 85:183-8.
3. Binder AM, Biggs HM, Haynes AK, Chommanard C, Lu X, Erdman DD, Watson JT, Gerber SI. 2017. Human Adenovirus Surveillance - United States, 2003-2016. *MMWR Morb Mortal Wkly Rep* 66:1039-1042.
4. Dhingra A, Hage E, Ganzenmueller T, Bottcher S, Hofmann J, Hamprecht K, Obermeier P, Rath B, Hausmann F, Dobner T, Heim A. 2019. Molecular Evolution of Human Adenovirus (HAdV) Species C. *Sci Rep* 9:1039.
5. Lion T. 2014. Adenovirus Infections in Immunocompetent and Immunocompromised Patients. *Clin Microbiol Rev* 27:441-62.
6. Kojaoghlanian T, Flomenberg P, Horwitz MS. 2003. The impact of adenovirus infection on the immunocompromised host. *Rev Med Virol* 13:155-71.
7. Ying B, Tollefson AE, Spencer JF, Balakrishnan L, Dewhurst S, Capella C, Buller RM, Toth K, Wold WS. 2014. Ganciclovir inhibits human adenovirus replication and pathogenicity in permissive immunosuppressed Syrian hamsters. *Antimicrob Agents Chemother* 58:7171-81.
8. Kajon AE, Ison MG. 2016. Severe Infections with Human Adenovirus 7d in 2 Adults in Family, Illinois, USA, 2014. *Emerging Infectious Disease journal* 22:730.

9. Bhatti Z, Dhamoon A. 2017. Fatal adenovirus infection in an immunocompetent host. *Am J Emerg Med* 35:1034.e1-1034.e2.
10. Ronchi A, Doern C, Brock E, Pugni L, Sanchez PJ. 2014. Neonatal adenoviral infection: a seventeen year experience and review of the literature. *J Pediatr* 164:529-35.e1-4.
11. Lenaerts L, De Clercq E, Naesens L. 2008. Clinical features and treatment of adenovirus infections. *Rev Med Virol* 18:357-74.
12. Keyes A, Mathias M, Boulad F, Lee YJ, Marchetti MA, Scaradavou A, Spitzer B, Papanicolaou GA, Wiecek I, Busam KJ. 2016. Cutaneous involvement of disseminated adenovirus infection in an allogeneic stem cell transplant recipient. *Br J Dermatol* 174:885-888.
13. Caruso Brown AE, Cohen MN, Tong S, Braverman RS, Rooney JF, Giller R, Levin MJ. 2015. Pharmacokinetics and safety of intravenous cidofovir for life-threatening viral infections in pediatric hematopoietic stem cell transplant recipients. *Antimicrob Agents Chemother* 59:3718-25.
14. Detweiler CJ, Mueller SB, Sung AD, Saullo JL, Prasad VK, Cardona DM. 2018. Brincidofovir (CMX001) Toxicity Associated With Epithelial Apoptosis and Crypt Drop Out in a Hematopoietic Cell Transplant Patient: Challenges in Distinguishing Drug Toxicity From GVHD. *J Pediatr Hematol Oncol* 40:e364-e368.
15. Ramsay ID, Attwood C, Irish D, Griffiths PD, Kyriakou C, Lowe DM. 2017. Disseminated adenovirus infection after allogeneic stem cell transplant and the potential role of brincidofovir - Case series and 10 year experience of management in an adult transplant cohort. *J Clin Virol* 96:73-79.

16. ClinicalTrials.gov. Clinical Grade Adenovirus Specific T Cells for Immunotherapy After Allogeneic Stem Cell Transplantation (NCT02851576). <https://www.clinicaltrials.gov/>. Accessed December 6, 2018.
17. ClinicalTrials.gov. Administration of Virus-Specific Cytotoxic T-Lymphocytes (NCT00590083). <https://www.clinicaltrials.gov/>. Accessed December 6, 2018.
18. Berk AJ. 2007. Adenoviridae: The Viruses and Their Replication. , p 2355–2394. *In* Knipe DM, Howley PM (ed), Fields Virology 5ed. Lippincott Williams & Wilkins, Philadelphia, PA, USA.
19. Horwitz MS. 2004. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. *J Gene Med* 6 Suppl 1:S172-83.
20. Weitzman MD. 2005. Functions of the adenovirus E4 proteins and their impact on viral vectors. *Front Biosci* 10:1106-17.
21. Saha B, Wong CM, Parks RJ. 2014. The adenovirus genome contributes to the structural stability of the virion. *Viruses* 6:3563-83.
22. King CR, Zhang A, Tessier TM, Gameiro SF, Mymryk JS. 2018. Hacking the Cell: Network Intrusion and Exploitation by Adenovirus E1A. *mBio* 9:e00390-18.
23. Flint J. 1999. p 17-30, *Adenoviruses: Basic Biology to Gene Therapy*. Landes Company
24. Frisch SM, Mymryk JS. 2002. Adenovirus-5 E1A: paradox and paradigm. *Nat Rev Mol Cell Biol* 3:441-52.
25. de Jong RN, Meijer LAT, van der Vliet PC. 2003. DNA binding properties of the adenovirus DNA replication priming protein pTP. *Nucleic Acids Res* 31:3274-86.
26. Parker EJ, Botting CH, Webster A, Hay RT. 1998. Adenovirus DNA polymerase: domain organisation and interaction with preterminal protein. *Nucleic Acids Res* 26:1240-7.

27. Young CS. 2003. The structure and function of the adenovirus major late promoter. *Curr Top Microbiol Immunol* 272:213-49.
28. Wodrich H, Guan T, Cingolani G, Von Seggern D, Nemerow G, Gerace L. 2003. Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *Embo j* 22:6245-55.
29. Ahi YS, Mittal SK. 2016. Components of Adenovirus Genome Packaging. *Frontiers in microbiology* 7:1503.
30. Zhao H, Granberg F, Elfineh L, Pettersson U, Svensson C. 2003. Strategic Attack on Host Cell Gene Expression during Adenovirus Infection. *J Virol* 77:11006-15.
31. Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ, Kurdistani SK. 2008. Epigenetic reprogramming by adenovirus e1a. *Science* 321:1086-8.
32. Horwitz GA, Zhang K, McBrien MA, Grunstein M, Kurdistani SK, Berk AJ. 2008. Adenovirus small e1a alters global patterns of histone modification. *Science* 321:1084-5.
33. Blackford AN, Grand RJA. 2009. Adenovirus E1B 55-Kilodalton Protein: Multiple Roles in Viral Infection and Cell Transformation. *J Virol* 83:4000-12.
34. Avgousti DC, Della Fera AN, Otter CJ, Herrmann C, Pancholi NJ, Weitzman MD. 2017. Adenovirus Core Protein VII Downregulates the DNA Damage Response on the Host Genome. *Journal of virology* 91:e01089-17.
35. Grosso F, Stoilov P, Lingwood C, Brown M, Cochrane A. 2017. Suppression of Adenovirus Replication by Cardiotonic Steroids. *Journal of virology* 91:e01623-16.
36. Wang X, Zhang Q, Zhou Z, Liu M, Chen Y, Li J, Xu L, Guo J, Li Q, Yang J, Wang S. 2018. Retinoic acid receptor beta, a potential therapeutic target in the inhibition of adenovirus replication. *Antiviral Res* 152:84-93.

37. Stray SJ, Johnson JM, Kopek BG, Zlotnick A. 2006. An in vitro fluorescence screen to identify antivirals that disrupt hepatitis B virus capsid assembly. *Nat Biotechnol* 24:358-62.
38. Nun TK, Kroll DJ, Oberlies NH, Soejarto DD, Case RJ, Piskaut P, Maitinaho T, Hilscher C, Wang L, Dittmer DP, Gao S-J, Damania B. 2007. Development of a fluorescence-based assay to screen antiviral drugs against Kaposi's sarcoma associated herpesvirus. *Molecular cancer therapeutics* 6:2360-2370.
39. Luthra P, Liang J, Pietzsch CA, Khadka S, Edwards MR, Wei S, De S, Posner B, Bukreyev A, Ready JM, Basler CF. 2018. A high throughput screen identifies benzoquinoline compounds as inhibitors of Ebola virus replication. *Antiviral Research* 150:193-201.
40. Johansen LM, Brannan JM, Delos SE, Shoemaker CJ, Stossel A, Lear C, Hoffstrom BG, Dewald LE, Schornberg KL, Scully C, Lehár J, Hensley LE, White JM, Olinger GG. 2013. FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. *Sci Transl Med* 5:190ra79.
41. Marrugal-Lorenzo JA, Serna-Gallego A, Gonzalez-Gonzalez L, Bunuales M, Poutou J, Pachon J, Gonzalez-Aparicio M, Hernandez-Alcoceba R, Sanchez-Cespedes J. 2018. Inhibition of adenovirus infection by mifepristone. *Antiviral Res* 159:77-83.
42. Sanchez-Cespedes J, Martinez-Aguado P, Vega-Holm M, Serna-Gallego A, Candela JJ, Marrugal-Lorenzo JA, Pachon J, Iglesias-Guerra F, Vega-Perez JM. 2016. New 4-Acyl-1-phenylaminocarbonyl-2-phenylpiperazine Derivatives as Potential Inhibitors of Adenovirus Infection. Synthesis, Biological Evaluation, and Structure-activity Relationships. *J Med Chem* 59:5432-48.

43. Hartline CB, Keith KA, Eagar J, Harden EA, Bowlin TL, Prichard MN. 2018. A standardized approach to the evaluation of antivirals against DNA viruses: Orthopox-, adeno-, and herpesviruses. *Antiviral Res* 159:104-112.
44. Duffy MR, Parker AL, Kalkman ER, White K, Kovalskyy D, Kelly SM, Baker AH. 2013. Identification of novel small molecule inhibitors of adenovirus gene transfer using a high throughput screening approach. *J Control Release* 170:132-40.
45. Sanchez-Céspedes J, Moyer CL, Whitby LR, Boger DL, Nemerow GR. 2014. Inhibition of adenovirus replication by a trisubstituted piperazin-2-one derivative. *Antiviral Res* 108:65-73.
46. Saha B, Parks RJ. 2019. Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid Suppresses Human Adenovirus Gene Expression and Replication. *Journal of Virology* 93:e00088-19.
47. Wong CM, Poulin KL, Tong G, Christou C, Kennedy MA, Falls T, Bell JC, Parks RJ. 2016. Adenovirus-Mediated Expression of the p14 Fusion-Associated Small Transmembrane Protein Promotes Cancer Cell Fusion and Apoptosis In Vitro but Does Not Provide Therapeutic Efficacy in a Xenograft Mouse Model of Cancer. *PloS one* 11:e0151516-e0151516.
48. Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS. 1996. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* 70:2296-306.
49. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol* 70:4805-10.

50. Ross PJ, Parks RJ. 2009. Construction and characterization of adenovirus vectors. *Cold Spring Harb Protoc* 2009:pdb.prot5011.
51. Saha B, Parks RJ. 2017. Human adenovirus type 5 vectors deleted of early region 1 (E1) undergo limited expression of early replicative E2 proteins and DNA replication in non-permissive cells. *PLoS One* 12:e0181012.
52. Reich NC, Sarnow P, Duprey E, Levine AJ. 1983. Monoclonal antibodies which recognize native and denatured forms of the adenovirus DNA-binding protein. *Virology* 128:480-4.
53. Ross PJ, Kennedy MA, Christou C, Risco Quiroz M, Poulin KL, Parks RJ. 2011. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol* 85:3950-8.
54. Thomas GP, Mathews MB. 1980. DNA replication and the early to late transition in adenovirus infection. *Cell* 22:523-33.
55. Krokan H, Wist E, Krokan RH. 1981. Aphidicolin inhibits DNA synthesis by DNA polymerase alpha and isolated nuclei by a similar mechanism. *Nucleic Acids Res* 9:4709-19.
56. Doherty JE, Kane JJ. 1973. Clinical pharmacology and therapeutics use of digitalis glycosides. *Drugs* 6:182-221.
57. Pilger BD, Cui C, Coen DM. 2004. Identification of a Small Molecule that Inhibits Herpes Simplex Virus DNA Polymerase Subunit Interactions and Viral Replication. *Chemistry & Biology* 11:647-654.
58. de Wispelaere M, Carocci M, Liang Y, Liu Q, Sun E, Vetter ML, Wang J, Gray NS, Yang PL. 2017. Discovery of host-targeted covalent inhibitors of dengue virus. *Antiviral Res* 139:171-179.

59. Fozzard HA, Sheets MF. 1985. Cellular mechanism of action of cardiac glycosides. *J Am Coll Cardiol* 5:10a-15a.
60. Prassas I, Diamandis EP. 2008. Novel therapeutic applications of cardiac glycosides. *Nature Reviews Drug Discovery* 7:926.
61. Berk AJ. 1986. Adenovirus promoters and E1A transactivation. *Annu Rev Genet* 20:45-79.
62. Stephens C, Harlow E. 1987. Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. *Embo j* 6:2027-35.
63. Su CT, Hsu JT, Hsieh HP, Lin PH, Chen TC, Kao CL, Lee CN, Chang SY. 2008. Anti-HSV activity of digitoxin and its possible mechanisms. *Antiviral Res* 79:62-70.
64. Dodson AW, Taylor TJ, Knipe DM, Coen DM. 2007. Inhibitors of the sodium potassium ATPase that impair herpes simplex virus replication identified via a chemical screening approach. *Virology* 366:340-8.
65. Cohen T, Williams JD, Opperman TJ, Sanchez R, Lurain NS, Tortorella D. 2016. Convallatoxin-Induced Reduction of Methionine Import Effectively Inhibits Human Cytomegalovirus Infection and Replication. *J Virol* 90:10715-10727.
66. Amarelle L, Lecuona E. 2018. The Antiviral Effects of Na,K-ATPase Inhibition: A Minireview. *International journal of molecular sciences* 19:2154.
67. Lamba JK. 2009. Genetic factors influencing cytarabine therapy. *Pharmacogenomics* 10:1657-1674.
68. Hryniuk W, Foerster J, Shojania M, Chow A. 1972. Cytarabine for herpesvirus infections. *JAMA* 219:715-718.

69. Hawley T, Spear M, Guo J, Wu Y. 2013. Inhibition of HIV replication in vitro by clinical immunosuppressants and chemotherapeutic agents. *Cell Biosci* 3:22.
70. Momparler RL. 2013. Optimization of cytarabine (ARA-C) therapy for acute myeloid leukemia. *Exp Hematol Oncol* 2:20.
71. Ersvaer E, Brenner AK, Vetås K, Reikvam H, Bruserud Ø. 2015. Effects of cytarabine on activation of human T cells - cytarabine has concentration-dependent effects that are modulated both by valproic acid and all-trans retinoic acid. *BMC Pharmacol Toxicol* 16:12.
72. Almawi WY, Melemedjian OK. 2002. Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. *J Leukoc Biol* 71:9-15.

Chapter 5:

General Discussion and Conclusions

HAdV is a common pathogen that causes minor diseases in most individuals, but these infections can be fatal in immunocompromised patients, the very young or the very old. No effective antivirals against HAdV exist and the need for new treatment options have been highlighted by many case reports (130, 132, 135-141). Since HAdV is reliant on cellular proteins and processes for nearly all steps of the virus lifecycle, small molecules targeting certain proteins/pathways (without having detrimental effects on cell biology) may serve as effective antivirals. Thus, the overall purpose of this work was to assess the efficacy of HDAC inhibitors as antivirals in infected cells; investigate the role of HDACs in HAdV infection; and develop a small molecule screening protocol that allows identification of novel HAdV inhibitors (and their pharmacological targets) for therapeutic intervention. The results obtained, their implications and potential future directions are discussed below.

5.1 Utility of the FLAG-tagged and RFP-expressing HAdV constructs

As explained in chapters 1 and 2, the HAdV E2-encoded proteins (DBP, TP and Pol) are crucial for virus DNA replication. However, there are no commercially available antibodies or tools to detect their expression in infected cells. To facilitate investigations of E2 proteins and determine how HAdV inhibitors in our study affect their expression, we generated HAdV-5-based constructs containing a FLAG-tag in the coding regions of each protein (table 2.1). Characterization of these constructs revealed that the FLAG-tagged protein expressed from each construct can be easily detected using antibodies against the tag (figures 2.2 and 2.3), and that expression levels are inversely proportional to the distance of the coding region from the E2 promoter (figures 2.2 and 2.3). We also used these HAdV constructs to study the relationship between E2 protein expression and DNA replication from E1-deleted fgAd vectors. We found that

the expression of these proteins is substantially reduced in the absence of E1A at all MOIs, but low-level DNA replication does occur at very high MOIs (figures 2.4 and 2.5). Our results confirm that other viral/cellular proteins are unable to fully activate the E2 promoter and allow DNA replication without E1A, and simultaneously emphasize the importance of considering vector dose-dependent effects in gene therapy studies.

AdE1⁺DBP-F and AdE1⁺TP-F served as efficient tools to examine changes in DBP and TP levels upon treatment with HDAC inhibitors and the positive hits from the small molecule screen (figures 3.5, 4.4 and 4.5). Overall, these constructs have a broad range of utility and applicability in studies on HAdV-5 infection. For example, the presence of (previously unidentified) cellular proteins at the virus replication foci within the cell nuclei can be detected by immunofluorescence with AdE1⁺DBP-F, while nascent viral DNA in infected cells can be pulled down using AdE1⁺TP-F for the identification/characterization of proteins that interact with the HAdV genome. AdE1⁺Pol-F can be used to sufficiently concentrate Pol for structural investigations, which may enable the design of specific Pol inhibitors. Such studies would further elucidate virus biology and host interactions, and contribute to the development of novel antiviral therapies.

Lastly, in order to monitor HAdV gene expression and replication effectively and conveniently in small molecule screens, we designed another E1⁺ construct containing the RFP gene within the E3 region, under the control of the MLP. This strategic placement of the RFP gene did not compromise virus growth (compared to wildtype HAdV-5) and allowed RFP to be expressed concomitantly with endogenous late gene expression (figure 3.1). It also eliminated exogenous promoter-dependent effects on RFP expression – for example, if RFP had been

expressed from a heterologous promoter, the “hits” in our screen may have been small molecules that up- or down-regulated expression from that promoter, rather than affecting the HAdV directly. The Ad-late/RFP reporter virus was used in small molecule screens and other assays in our study as a surrogate marker of virus replication (chapters 3 and 4), and it can serve as a useful tool to detect the presence of virus in experimental samples and to track viral gene expression/protein levels/replication kinetics by fluorescence microscopy *in vitro* and *in vivo*.

5.2 Therapeutic potential of compounds inhibiting HDACs and other epigenetic regulators in HAdV infection

Within the first few hours of entry into the host cell nucleus, the wildtype HAdV DNA associates with cellular nucleosomes mainly containing the H3 variant H3.3 (88-90, 94). Although it is generally assumed that the histones act to protect the HAdV DNA from immune/DNA damage responses and promote viral gene expression, contradicting evidence also exists (108). The exact purpose of nucleosome deposition and the chaperones responsible have not yet been determined. Histone modifications, specifically H3 and H4 acetylation, have been observed at the highly active HAdV promoters in several studies (88, 111-113). The level of acetylation was dynamic throughout infection, and the implications on gene transcription were often variable depending on location, suggesting a high degree of regulation. These studies have also shed some light on the involvement of chromatin modifying enzymes (e.g. p300/CBP and Tip60) in the HAdV lifecycle, but their exact function in regulating HAdV gene expression, the consequences of their modifications, and the involvement of specific chromatin remodeling complexes remain to be elucidated. Since remodeling of the HAdV genome nucleoprotein structure is one of the earliest steps of the virus lifecycle, preventing this and appropriately modulating epigenetic regulation of

HAdV gene expression by small molecules may be an effective strategy to limit HAdV infection.

In chapter 3, we thoroughly investigated the efficacy of SAHA against HAdV in cell culture. This FDA-approved anticancer drug impaired stages of the virus lifecycle following attachment/entry – it reduced gene expression from the Ad-late/RFP, HAdV-4, HAdV-5 and HAdV-7 (figures 3.2 and 3.3), as well as virus yield from infected cells in a dose-dependent manner (figure 3.4). These results are consistent with the findings of Höti *et al.* on the anti-HAdV properties of HDAC inhibitor VPA, and that of Gupta *et al.* on the repression of E1A expression and HAdV replication as a consequence of H4 acetylation by Tip60 (112, 114). SAHA-induced reduction of early/late gene expression and DNA replication (figure 3.5) was independent of the changes in viral E1A and cellular p21 protein levels caused by SAHA treatment (figures 3.6 and 3.7). However, it is possible that HDAC inhibition alters E1A post-translational modifications (e.g. increased acetylation), which could interfere with its function and interaction with other proteins (182). Our data further suggests that HDAC inhibition decreases HAdV proteins through effects on transcription, RNA stability and/or translation, but not protein stability, as treatment with the proteasomal degradation inhibitor MG132 did not rescue early/late protein expression in SAHA-treated cells (data not shown). Although SAHA did not impact pVII loss or histone association or H3/H4 acetylation within the selected E1A and hexon coding regions in our study (figures S5 and 3.5), it is possible that histone association/acetylation in the promoter and TSS regions could be altered, leading to transcriptional repression of some of the early genes (111, 113). In addition, comparison of the cellular gene expression profile in infected, SAHA- and vehicle-treated cells (e.g. by RNAseq) may point to other non-histone proteins and pathways that contribute to the inhibitory effects of SAHA. For example, pan-HDAC inhibitors have previously been shown to

limit virus replication through upregulation of interferon-mediated antiviral responses (183). Lastly, the therapeutic efficacy of SAHA against HAdV must be evaluated in appropriate animal models, such as the cotton rat or the Syrian hamster. The latter can be infected intranasally or intravenously, and presents symptoms comparable to human disease upon infection with species C HAdV (184). Given that antiviral activity persisted longer in primary cells (figure 3.2), SAHA may provide a more significant benefit *in vivo* (where infection would occur at lower MOIs) by delaying/reducing virus replication sufficiently for the host immune system to counter the infection.

We examined the previously unidentified role of HDACs in HAdV infection, and determined that class I HDACs, mainly HDAC2, promote viral late gene expression (figure 3.8). Since HDACs normally function to repress transcription, this observation is surprising. It is also intriguing that HDAC1 was unable to compensate for the loss of HDAC2 activity in our single and combination knockdown experiments (figure 3.8 and data not shown). Although HDAC2 is very similar to HDAC1 in structure, function and localization, a few studies have investigated the specific, non-redundant roles of the protein. By knocking out HDAC2 in human HEK293FT cells, Somanath *et al.* identified a subset of cellular genes that were repressed in HDAC2-null cells, even though H3 acetylation at their promoters increased (185). Similar assessments of viral gene expression and histone acetylation at viral promoters in HDAC1- and HDAC2-deficient cells may provide additional information on the requirement of HDAC2 during the HAdV lifecycle. We showed that only HDAC2 interacts with E1A and that this interaction is enhanced by SAHA treatment (figure 3.8), but further investigation is required to elucidate whether E1A is a target of HDAC2, or E1A recruits HDAC2 to promoter-specific histones, or interaction between the

proteins interferes with their respective functions. In summary, greater understanding of the role of HDACs in HAdV infection is necessary, which may enable the design of specific HDAC inhibitors that are more effective antivirals.

5.3 Small molecule screen for the identification of novel HAdV inhibitors

To enable drug discovery and repurposing for anti-HAdV compounds, we designed an efficient fixed-cell screening platform using the Ad-late/RFP (see chapter 4). This approach involved quantifying RFP intensity to identify any compounds that change RFP levels through impacts on virus entry, gene expression, DNA replication, protein translation and protein stability. We subsequently screened at least ~1350 small molecules, and showed that compounds with both robust and modest antiviral activities are successfully detected in the screen. This screening method also identified a few enhancers of RFP fluorescence (data not shown), which may be useful for increasing transgene expression from HAdV-based gene therapy vectors.

We first screened the Prestwick Chemical Library of ~1200 FDA-approved, chemically and pharmacologically diverse compounds. Of the eleven confirmed positive hits, six were validated in cell-based assays (figures 4.3 - 4.5 and table 4.1). The most effective antivirals were cardiotonic steroids (digoxin, digitoxigenin and lanatoside C), which reduced late gene expression from virulent HAdV serotypes 4 and 7, impaired multiple stages of the HAdV lifecycle, and led to 100- to 1000-fold reduction in virus yield (figure 4.4). The antiviral properties of this class of compounds were previously observed by Grosso *et al.*, and their results implicated mislocalization of cellular SR proteins and alteration of E1A mRNA splicing as the underlying mechanism (160). Although the RNA splicing machinery can potentially serve as effective

pharmacological targets due to their necessity in viral RNA splicing, our results suggest that cardiotonic steroids may also affect protein synthesis and/or stability (figure 4.4). Indeed, others have reported that CMV and HSV inhibition by these compounds was a result of impaired viral gene expression, DNA replication, protein synthesis and progeny release (171, 186, 187). Similar to the observations in the HSV study (186), HAdV yield was reduced to a higher extent than viral gene expression/DNA replication, suggesting impairment of virus release (figure 4.4). Cohen *et al.* showed that convallotoxin treatment did not affect CMV RNA transcription or translation, but decreased protein synthesis by preventing methionine transport – this could also be the case for HAdV. Compared to the cardiotonic steroids, the other three validated hits (chemotherapeutic agent cytarabine and corticosteroids dexamethasone and flunisolide) led to modest reductions in virus yield and viral gene expression (figure 4.5). The latter was more pronounced at lower, more clinically relevant MOIs (figure 4.5). It is interesting that corticosteroids, which are immunosuppressive/anti-proliferative agents, have antiviral activity against HAdV. However, this has been observed in the case of other viruses (188), and like SAHA, it may be a consequence of their ability to alter signaling pathways and cellular homeostasis. Overall, further investigation is necessary to understand the underlying molecular mechanism of HAdV inhibition for all six compounds. Following evaluation of effectivity in various cell types and against clinically relevant HAdV serotypes, existing clinical data on safety and kinetics of these FDA-approved drugs may ease their transition to *in vivo* infection studies.

To discover other epigenetic regulators of importance in HAdV infection, we also screened the Cayman Epigenetics Screening Library of 150 small molecules that target epigenetic reader, writer and eraser proteins. Two independent screens were conducted at 0.25 and 1 μ M and,

interestingly, the majority of the positive hits were HDAC inhibitors (table 3.1), which supported our previous results and confirmed the reliability of the screen. We also identified modulators of other epigenetic regulators including chaetocin (inhibits methyltransferase SUV39H1) and lestaurtinib (inhibits various tyrosine kinases). Validation of the inhibitory effects of these compounds would provide us with insights on the epigenetic regulation of HAdV, as well as additional cellular targets for therapeutic intervention.

5.4 Concluding remarks

The costs associated with HAdV-induced disease are significant in terms of medical expenses, lost work hours and loss of life in some populations. Thus, development of novel and effective antiviral therapies is necessary to reduce disease pathogenesis and increase survival rates in severe infections. With the goal of identifying novel anti-HAdV compounds, we investigated the therapeutic potential of HDAC inhibitors. Using wildtype-like RFP-expressing and FLAG-tagged viral constructs, we evaluated the impact of HDAC inhibitors on HAdV-5 lifecycle. Our work is the first to provide evidence on the role of class I HDACs in HAdV infection and the ability of HDAC inhibitors to reduce gene expression from clinically relevant HAdV serotypes. To facilitate antiviral discovery against HAdV, we also designed a fluorescence-based screening method that can efficiently detect anti-HAdV compounds from small molecule libraries in a high-throughput manner. From the pilot screens we conducted, several cardiotonic steroids and modulators of epigenetic regulators were identified as potent HAdV inhibitors. The screening method was sufficiently sensitive to identify compounds with even modest antiviral activity. Overall, our results provide insights on the anti-HAdV properties of several classes of existing drugs, enhance the current knowledge for virus-cell interactions, and lay the groundwork for

discovering additional pharmacological targets and developing more effective (and potentially less toxic) antivirals against HAdV.

References

1. Hoke CH, Jr., Snyder CE, Jr. 2013. History of the restoration of adenovirus type 4 and type 7 vaccine, live oral (Adenovirus Vaccine) in the context of the Department of Defense acquisition system. *Vaccine* 31:1623-32.
2. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* 84:570-3.
3. Hilleman MR, Werner JH. 1954. Recovery of new agent from patients with acute respiratory illness. *Proc Soc Exp Biol Med* 85:183-8.
4. Dhingra A, Hage E, Ganzenmueller T, Bottcher S, Hofmann J, Hamprecht K, Obermeier P, Rath B, Hausmann F, Dobner T, Heim A. 2019. Molecular Evolution of Human Adenovirus (HAdV) Species C. *Sci Rep* 9:1039.
5. Whyte P, Williamson NM, Harlow E. 1989. Cellular targets for transformation by the adenovirus E1A proteins. *Cell* 56:67-75.
6. Eckner R, Ewen ME, Newsome D, Gerdes M, DeCaprio JA, Lawrence JB, Livingston DM. 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev* 8:869-84.
7. Fuchs M, Gerber J, Drapkin R, Sif S, Ikura T, Ogryzko V, Lane WS, Nakatani Y, Livingston DM. 2001. The p400 complex is an essential E1A transformation target. *Cell* 106:297-307.
8. Chow LT, Gelinas RE, Broker TR, Roberts RJ. 1977. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell* 12:1-8.

9. Flint J. 1999. p 17-30, Adenoviruses: Basic Biology to Gene Therapy. Landes Company
10. Berk AJ. 2007. Adenoviridae: The Viruses and Their Replication. , p 2355–2394. *In* Knipe DM, Howley PM (ed), Fields Virology 5ed. Lippincott Williams & Wilkins, Philadelphia, PA, USA.
11. Ben-Israel H, Kleinberger T. 2002. Adenovirus and cell cycle control. *Front Biosci* 7:d1369-95.
12. Thomas GP, Mathews MB. 1980. DNA replication and the early to late transition in adenovirus infection. *Cell* 22:523-33.
13. Lee CS, Bishop ES, Zhang R, Yu X, Farina EM, Yan S, Zhao C, Zeng Z, Shu Y, Wu X, Lei J, Li Y, Zhang W, Yang C, Wu K, Wu Y, Ho S, Athiviraham A, Lee MJ, Wolf JM, Reid RR, He T-C. 2017. Adenovirus-mediated gene delivery: Potential applications for gene and cell-based therapies in the new era of personalized medicine. *Genes & Diseases* 4:43-63.
14. Ahi YS, Mittal SK. 2016. Components of Adenovirus Genome Packaging. *Frontiers in microbiology* 7:1503.
15. Horwitz MS. 2004. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. *J Gene Med* 6 Suppl 1:S172-83.
16. Weitzman MD. 2005. Functions of the adenovirus E4 proteins and their impact on viral vectors. *Front Biosci* 10:1106-17.
17. King CR, Zhang A, Tessier TM, Gameiro SF, Mymryk JS. 2018. Hacking the Cell: Network Intrusion and Exploitation by Adenovirus E1A. *mBio* 9.
18. Berk AJ. 1986. Adenovirus promoters and E1A transactivation. *Annu Rev Genet* 20:45-79.

19. Stephens C, Harlow E. 1987. Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. *Embo j* 6:2027-35.
20. Pelka P, Ablack JN, Torchia J, Turnell AS, Grand RJ, Mymryk JS. 2009. Transcriptional control by adenovirus E1A conserved region 3 via p300/CBP. *Nucleic Acids Res* 37:1095-106.
21. Swaminathan S, Thimmapaya B. 1996. Transactivation of adenovirus E2-early promoter by E1A and E4 6/7 in the context of viral chromosome. *J Mol Biol* 258:736-46.
22. Chattopadhyay D, Ghosh MK, Mal A, Harter ML. 2001. Inactivation of p21 by E1A leads to the induction of apoptosis in DNA-damaged cells. *J Virol* 75:9844-56.
23. Liu F, Green MR. 1994. Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. *Nature* 368:520-5.
24. Parks CL, Shenk T. 1997. Activation of the adenovirus major late promoter by transcription factors MAZ and Sp1. *J Virol* 71:9600-7.
25. Frisch SM, Mymryk JS. 2002. Adenovirus-5 E1A: paradox and paradigm. *Nat Rev Mol Cell Biol* 3:441-52.
26. Gallimore PH, Turnell AS. 2001. Adenovirus E1A: remodelling the host cell, a life or death experience. *Oncogene* 20:7824-35.
27. Badr KR, Parente-Rocha JA, Baeza LC, Ficcadori FS, Souza M, Soares CM, Guissoni ACP, Almeida TN, Cardoso DD. 2019. Quantitative proteomic analysis of A549 cells infected with human adenovirus type 2. *J Med Virol* doi:10.1002/jmv.25439.
28. Blackford AN, Grand RJA. 2009. Adenovirus E1B 55-Kilodalton Protein: Multiple Roles in Viral Infection and Cell Transformation. *J Virol* 83:4000-12.

29. Gonzalez R, Huang W, Finnen R, Bragg C, Flint SJ. 2006. Adenovirus E1B 55-Kilodalton Protein Is Required for both Regulation of mRNA Export and Efficient Entry into the Late Phase of Infection in Normal Human Fibroblasts. *J Virol* 80:964-74.
30. Caravokyri C, Leppard KN. 1996. Human adenovirus type 5 variants with sequence alterations flanking the E2A gene: effects on E2 expression and DNA replication. *Virus Genes* 12:65-75.
31. Hoeben RC, Uil TG. 2013. Adenovirus DNA replication. *Cold Spring Harb Perspect Biol* 5:a013003.
32. Parker EJ, Botting CH, Webster A, Hay RT. 1998. Adenovirus DNA polymerase: domain organisation and interaction with preterminal protein. *Nucleic Acids Res* 26:1240-7.
33. Dekker J, Kanellopoulos PN, Loonstra AK, van Oosterhout JA, Leonard K, Tucker PA, van der Vliet PC. 1997. Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis. *The EMBO journal* 16:1455-1463.
34. de Jong RN, Meijer LAT, van der Vliet PC. 2003. DNA binding properties of the adenovirus DNA replication priming protein pTP. *Nucleic Acids Res* 31:3274-86.
35. Nagata K, Guggenheimer RA, Hurwitz J. 1983. Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. *Proc Natl Acad Sci U S A* 80:6177-81.
36. Chen M, Mermod N, Horwitz MS. 1990. Protein-protein interactions between adenovirus DNA polymerase and nuclear factor I mediate formation of the DNA replication preinitiation complex. *J Biol Chem* 265:18634-42.

37. Mangel WF, Toledo DL, Ding J, Sweet RM, McGrath WJ. 1997. Temporal and spatial control of the adenovirus proteinase by both a peptide and the viral DNA. *Trends Biochem Sci* 22:393-8.
38. Arnberg N. 2013. Adenovirus E3 protein modulates leukocyte functions. *Proceedings of the National Academy of Sciences* 110:19976.
39. Martinez-Martin N, Ramani SR, Hackney JA, Tom I, Wranik BJ, Chan M, Wu J, Paluch MT, Takeda K, Hass PE, Clark H, Gonzalez LC. 2016. The extracellular interactome of the human adenovirus family reveals diverse strategies for immunomodulation. *Nature Communications* 7:11473.
40. Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS. 1996. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* 70:2296-306.
41. Bioinformatics SSIO. DNA Strand Displacement Replication - ViralZone. <https://viralzone.expasy.org/1940>. Accessed April 24.
42. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. 2013. Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med* 15:65-77.
43. Lundstrom K. 2018. Viral Vectors in Gene Therapy. *Diseases* 6.
44. Yang Y, Nunes FA, Berencsi K, Furth EE, Gönczöl E, Wilson JM. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A* 91:4407-11.
45. Amalfitano A, Parks RJ. 2002. Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. *Curr Gene Ther* 2:111-33.

46. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93:13565-70.
47. Ahi YS, Mittal SK. 2016. Components of Adenovirus Genome Packaging. *Front Microbiol* 7.
48. Farley DC, Brown JL, Leppard KN. 2004. Activation of the Early-Late Switch in Adenovirus Type 5 Major Late Transcription Unit Expression by L4 Gene Products. *Journal of Virology* 78:1782.
49. Nevins JR, Wilson MC. 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. *Nature* 290:113-8.
50. Young CS. 2003. The structure and function of the adenovirus major late promoter. *Curr Top Microbiol Immunol* 272:213-49.
51. Prescott JC, Falck-Pedersen E. 1992. Varied poly(A) site efficiency in the adenovirus major late transcription unit. *J Biol Chem* 267:8175-81.
52. Morris SJ, Scott GE, Leppard KN. 2010. Adenovirus late-phase infection is controlled by a novel L4 promoter. *J Virol* 84:7096-104.
53. Saha B, Wong CM, Parks RJ. 2014. The adenovirus genome contributes to the structural stability of the virion. *Viruses* 6:3563-83.
54. Parks RJ. 2005. Adenovirus protein IX: a new look at an old protein. *Mol Ther* 11:19-25.
55. O'Malley RP, Mariano TM, Siekierka J, Mathews MB. 1986. A mechanism for the control of protein synthesis by adenovirus VA RNAI. *Cell* 44:391-400.

56. Aparicio O, Razquin N, Zaratiegui M, Narvaiza I, Fortes P. 2006. Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *J Virol* 80:1376-84.
57. Vachon VK, Conn GL. 2016. Adenovirus VA RNA: An essential pro-viral non-coding RNA. *Virus Res* 212:39-52.
58. Tollefson AE, Ying B, Doronin K, Sidor PD, Wold WS. 2007. Identification of a new human adenovirus protein encoded by a novel late 1-strand transcription unit. *J Virol* 81:12918-26.
59. Ying B, Tollefson AE, Wold WSM. 2010. Identification of a Previously Unrecognized Promoter That Drives Expression of the UXP Transcription Unit in the Human Adenovirus Type 5 Genome. *J Virol* 84:11470-8.
60. Liu H, Jin L, Koh SB, Atanasov I, Schein S, Wu L, Zhou ZH. 2010. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 329:1038-43.
61. Reddy VS, Natchiar SK, Stewart PL, Nemerow GR. 2010. Crystal structure of human adenovirus at 3.5 Å resolution. *Science* 329:1071-5.
62. Nemerow GR, Stewart PL, Reddy VS. 2012. Structure of human adenovirus. *Curr Opin Virol* 2:115-21.
63. San Martín C. 2012. Latest insights on adenovirus structure and assembly. *Viruses* 4:847-77.
64. Chatterjee PK, Vayda ME, Flint SJ. 1985. Interactions among the three adenovirus core proteins. *J Virol* 55:379-86.

65. Korn R, Horwitz MS. 1986. Adenovirus DNA synthesis in vitro is inhibited by the virus-coded major core protein. *Virology* 150:342-51.
66. Anderson CW, Young ME, Flint SJ. 1989. Characterization of the adenovirus 2 virion protein, mu. *Virology* 172:506-12.
67. Ostapchuk P, Suomalainen M, Zheng Y, Boucke K, Greber UF, Hearing P. 2017. The adenovirus major core protein VII is dispensable for virion assembly but is essential for lytic infection. *PLoS Pathog* 13:e1006455.
68. Avgousti DC, Della Fera AN, Otter CJ, Herrmann C, Pancholi NJ, Weitzman MD. 2017. Adenovirus Core Protein VII Downregulates the DNA Damage Response on the Host Genome. *J Virol* 91.
69. Avgousti DC, Herrmann C, Kulej K, Pancholi NJ, Sekulic N, Petrescu J, Molden RC, Blumenthal D, Paris AJ, Reyes ED, Ostapchuk P, Hearing P, Seeholzer SH, Worthen GS, Black BE, Garcia BA, Weitzman MD. 2016. A core viral protein binds host nucleosomes to sequester immune danger signals. *Nature* 535:173-7.
70. Xue Y, Johnson JS, Ornelles DA, Lieberman J, Engel DA. 2005. Adenovirus protein VII functions throughout early phase and interacts with cellular proteins SET and pp32. *J Virol* 79:2474-83.
71. Pérez-Vargas J, Vaughan RC, Houser C, Hastie KM, Kao CC, Nemerow GR. 2014. Isolation and characterization of the DNA and protein binding activities of adenovirus core protein V. *J Virol* 88:9287-96.
72. Matthews DA, Russell WC. 1998. Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *Journal of General Virology* 79:1671-1675.

73. Everitt E, Lutter L, Philipson L. 1975. Structural proteins of adenoviruses: XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology* 67:197-208.
74. Christensen JB, Byrd SA, Walker AK, Strahler JR, Andrews PC, Imperiale MJ. 2008. Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. *J Virol* 82:9086-93.
75. Vellinga J, Van der Heijdt S, Hoeben RC. 2005. The adenovirus capsid: major progress in minor proteins. *J Gen Virol* 86:1581-8.
76. Zhang Y, Bergelson JM. 2005. Adenovirus Receptors. *J Virol* 79:12125-31.
77. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275:1320-3.
78. Zhang Y, Huang W, Ornelles DA, Gooding LR. 2010. Modeling adenovirus latency in human lymphocyte cell lines. *J Virol* 84:8799-810.
79. Lyle C, McCormick F. 2010. Integrin $\alpha\beta 5$ is a primary receptor for adenovirus in CAR-negative cells. *Virol J* 7:148.
80. Wickham TJF, E. J.; Cheresh, D. A.; Nemerow G. R. 1994. Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol* 127:257-64.
81. Wiethoff CM, Wodrich H, Gerace L, Nemerow GR. 2005. Adenovirus Protein VI Mediates Membrane Disruption following Capsid Disassembly. *J Virol* 79:1992-2000.

82. Wickham TJ, Mathias P, Cheresch DA, Nemerow GR. 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* 73:309-19.
83. Leopold PL, Ferris B, Grinberg I, Worgall S, Hackett NR, Crystal RG. 1998. Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. *Hum Gene Ther* 9:367-78.
84. Flatt JW, Butcher SJ. 2019. Adenovirus flow in host cell networks. *Open Biol* 9:190012.
85. Strunze S, Engelke MF, Wang IH, Puntener D, Boucke K, Schleich S, Way M, Schoenenberger P, Burckhardt CJ, Greber UF. 2011. Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. *Cell Host Microbe* 10:210-23.
86. Greber UF, Suomalainen M, Stidwill RP, Boucke K, Ebersold MW, Helenius A. 1997. The role of the nuclear pore complex in adenovirus DNA entry. *Embo j* 16:5998-6007.
87. Greber UF, Willetts M, Webster P, Helenius A. 1993. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 75:477-86.
88. Komatsu T, Haruki H, Nagata K. 2011. Cellular and viral chromatin proteins are positive factors in the regulation of adenovirus gene expression. *Nucleic Acids Res* 39:889-901.
89. Komatsu T, Nagata K. 2012. Replication-uncoupled histone deposition during adenovirus DNA replication. *J Virol* 86:6701-11.
90. Giberson A, Saha B, Campbell K, Christou C, Poulin KL, Parks RJ. 2018. Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication. *Biochemistry and Cell Biology* doi:10.1139/bcb-2018-0117.

91. Tate VE, Philipson L. 1979. Parental adenovirus DNA accumulates in nucleosome-like structures in infected cells. *Nucleic Acids Res* 6:2769-85.
92. Ross PJ, Kennedy MA, Christou C, Risco Quiroz M, Poulin KL, Parks RJ. 2011. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol* 85:3950-8.
93. Anonymous. 2017. Chapter 2 - Virus Replication, p 17-45. *In* MacLachlan NJ, Dubovi EJ (ed), *Fenner's Veterinary Virology (Fifth Edition)* doi:<https://doi.org/10.1016/B978-0-12-800946-8.00002-7>. Academic Press, Boston.
94. Daniell E, Groff DE, Fedor MJ. 1981. Adenovirus chromatin structure at different stages of infection. *Mol Cell Biol* 1:1094-105.
95. Beyer AL, Bouton AH, Hodge LD, Miller OL, Jr. 1981. Visualization of the major late R strand transcription unit of adenovirus serotype 2. *J Mol Biol* 147:269-95.
96. Stuiver MH, Bergsma WG, Arnberg AC, van Amerongen H, van Grondelle R, van der Vliet PC. 1992. Structural alterations of double-stranded DNA in complex with the adenovirus DNA-binding protein. Implications for its function in DNA replication. *J Mol Biol* 225:999-1011.
97. Moreau P, Cournac A, Palumbo GA, Marbouty M, Mortaza S, Thierry A, Cairo S, Lavigne M, Koszul R, Neuveut C. 2018. Tridimensional infiltration of DNA viruses into the host genome shows preferential contact with active chromatin. *Nat Commun* 9:4268.
98. Wodrich H, Guan T, Cingolani G, Von Seggern D, Nemerow G, Gerace L. 2003. Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *Embo j* 22:6245-55.

99. Mangel WF, San Martin C. 2014. Structure, function and dynamics in adenovirus maturation. *Viruses* 6:4536-70.
100. Luger K. 2006. Dynamic nucleosomes. *Chromosome Res* 14:5-16.
101. Kouzarides T. 2007. Chromatin modifications and their function. *Cell* 128:693-705.
102. Jenuwein T, Allis CD. 2001. Translating the histone code. *Science* 293:1074-80.
103. Sterner DE, Berger SL. 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev* 64:435-59.
104. Seto E, Yoshida M. 2014. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb Perspect Biol* 6:a018713.
105. Goldberg AD, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ, Stadler S, Dewell S, Law M, Guo X, Li X, Wen D, Chapgier A, DeKolver RC, Miller JC, Lee YL, Boydston EA, Holmes MC, Gregory PD, Greally JM, Rafii S, Yang C, Scambler PJ, Garrick D, Gibbons RJ, Higgs DR, Cristea IM, Urnov FD, Zheng D, Allis CD. 2010. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* 140:678-91.
106. Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD. 2010. Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc Natl Acad Sci U S A* 107:14075-80.
107. Pradhan SK, Su T, Yen L, Jacquet K, Huang C, Cote J, Kurdistani SK, Carey MF. 2016. EP400 Deposits H3.3 into Promoters and Enhancers during Gene Activation. *Mol Cell* 61:27-38.
108. Schreiner S, Bürck C, Glass M, Groitl P, Wimmer P, Kinkley S, Mund A, Everett RD, Dobner T. 2013. Control of human adenovirus type 5 gene expression by cellular Daxx/ATRX chromatin-associated complexes. *Nucleic Acids Res* 41:3532-50.

109. Placek BJ, Huang J, Kent JR, Dorsey J, Rice L, Fraser NW, Berger SL. 2009. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. *J Virol* 83:1416-21.
110. Giberson AN, Davidson AR, Parks RJ. 2012. Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res* 40:2369-76.
111. Soriano AM, Crisostomo L, Mendez M, Graves D, Frost JR, Olanubi O, Whyte PF, Hearing P, Pelka P. 2019. Adenovirus 5 E1A interacts with E4orf3 to regulate viral chromatin organization. *J Virol* doi:10.1128/jvi.00157-19.
112. Gupta A, Jha S, Engel DA, Ornelles DA, Dutta A. 2012. Tip60 degradation by adenovirus relieves transcriptional repression of viral transcriptional activator E1A. *Oncogene* 32:5017.
113. Hsu E, Pennella MA, Zemke NR, Eng C, Berk AJ. 2018. Adenovirus E1A Activation Domain Regulates H3 Acetylation Affecting Varied Steps in Transcription at Different Viral Promoters. *J Virol* doi:10.1128/jvi.00805-18.
114. Hoti N, Chowdhury W, Hsieh JT, Sachs MD, Lupold SE, Rodriguez R. 2006. Valproic acid, a histone deacetylase inhibitor, is an antagonist for oncolytic adenoviral gene therapy. *Mol Ther* 14:768-78.
115. Horwitz GA, Zhang K, McBrien MA, Grunstein M, Kurdistani SK, Berk AJ. 2008. Adenovirus small e1a alters global patterns of histone modification. *Science* 321:1084-5.
116. Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ, Kurdistani SK. 2008. Epigenetic reprogramming by adenovirus e1a. *Science* 321:1086-8.
117. Chaurushiya MS, Weitzman MD. 2009. Viral manipulation of DNA repair and cell cycle checkpoints. *DNA Repair (Amst)* 8:1166-76.

118. Zhao H, Granberg F, Elfineh L, Pettersson U, Svensson C. 2003. Strategic Attack on Host Cell Gene Expression during Adenovirus Infection. *J Virol* 77:11006-15.
119. Prusinkiewicz MA, Mymryk JS. 2019. Metabolic Reprogramming of the Host Cell by Human Adenovirus Infection. *Viruses* 11.
120. Huang JT, Schneider RJ. 1990. Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine. *Proc Natl Acad Sci U S A* 87:7115-9.
121. Flak MB, Connell CM, Chelala C, Archibald K, Salako MA, Pirlo KJ, Lockley M, Wheatley SP, Balkwill FR, McNeish IA. 2010. p21 Promotes oncolytic adenoviral activity in ovarian cancer and is a potential biomarker. *Mol Cancer* 9:175.
122. Harbour JW, Dean DC. 2000. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev* 14:2393-409.
123. Abbas T, Dutta A. 2009. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* 9:400-14.
124. Fattaey AR, Harlow E, Helin K. 1993. Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. *Mol Cell Biol* 13:7267-77.
125. O'Connor RJ, Hearing P. 2000. The E4-6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. *J Virol* 74:5819-24.
126. Chatton B, Bocco JL, Gaire M, Hauss C, Reimund B, Goetz J, Keding C. 1993. Transcriptional activation by the adenovirus larger E1a product is mediated by members of the cellular transcription factor ATF family which can directly associate with E1a. *Mol Cell Biol* 13:561-70.

127. Vink EI, Zheng Y, Yeasmin R, Stamminger T, Krug LT, Hearing P. 2015. Impact of Adenovirus E4-ORF3 Oligomerization and Protein Localization on Cellular Gene Expression. *Viruses* 7:2428-49.
128. Stracker TH, Carson CT, Weitzman MD. 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature* 418:348-52.
129. Zhao H, Chen M, Valdes A, Lind SB, Pettersson U. 2019. Transcriptomic and proteomic analyses reveal new insights into the regulation of immune pathways during adenovirus type 2 infection. *BMC Microbiol* 19:15.
130. Khanal S, Ghimire P, Dhamoon AS. 2018. The Repertoire of Adenovirus in Human Disease: The Innocuous to the Deadly. *Biomedicines* 6.
131. Kajon AE, Lamson DM, Bair CR, Lu X, Landry ML, Menegus M, Erdman DD, St George K. 2018. Adenovirus Type 4 Respiratory Infections among Civilian Adults, Northeastern United States, 2011-2015(1). *Emerg Infect Dis* 24:201-209.
132. Binder AM, Biggs HM, Haynes AK, Chommanard C, Lu X, Erdman DD, Watson JT, Gerber SI. 2017. Human Adenovirus Surveillance - United States, 2003-2016. *MMWR Morb Mortal Wkly Rep* 66:1039-1042.
133. Musher DM. 2003. How contagious are common respiratory tract infections? *N Engl J Med* 348:1256-66.
134. Joffe M, Wagner SD, Tang JW. 2018. Case report: a fatal case of disseminated adenovirus infection in a non-transplant adult haematology patient. *BMC Infect Dis* 18:58.
135. Kojaoghlanian T, Flomenberg P, Horwitz MS. 2003. The impact of adenovirus infection on the immunocompromised host. *Rev Med Virol* 13:155-71.

136. Ying B, Tollefson AE, Spencer JF, Balakrishnan L, Dewhurst S, Capella C, Buller RM, Toth K, Wold WS. 2014. Ganciclovir inhibits human adenovirus replication and pathogenicity in permissive immunosuppressed Syrian hamsters. *Antimicrob Agents Chemother* 58:7171-81.
137. Keyes A, Mathias M, Boulad F, Lee YJ, Marchetti MA, Scaradavou A, Spitzer B, Papanicolaou GA, Wieczorek I, Busam KJ. 2016. Cutaneous involvement of disseminated adenovirus infection in an allogeneic stem cell transplant recipient. *Br J Dermatol* 174:885-888.
138. Lenaerts L, De Clercq E, Naesens L. 2008. Clinical features and treatment of adenovirus infections. *Rev Med Virol* 18:357-74.
139. Ronchi A, Doern C, Brock E, Pugni L, Sanchez PJ. 2014. Neonatal adenoviral infection: a seventeen year experience and review of the literature. *J Pediatr* 164:529-35.e1-4.
140. Takahashi T, Pereda MA, Bala N, Nagarajan S. 2019. In-hospital mortality of hematopoietic stem cell transplantation among children with nonmalignancies: A nationwide study in the United States from 2000 to 2012. *Pediatr Blood Cancer* 66:e27626.
141. Lee B, Park E, Ha J, Ha IS, Il Cheong H, Kang HG. 2018. Disseminated adenovirus infection in a 10-year-old renal allograft recipient. *Kidney Res Clin Pract* 37:414-417.
142. Schwartz KL, Richardson SE, MacGregor D, Mahant S, Raghuram K, Bitnun A. 2019. Adenovirus-Associated Central Nervous System Disease in Children. *J Pediatr* 205:130-137.
143. Bhatti Z, Dhamoon A. 2017. Fatal adenovirus infection in an immunocompetent host. *Am J Emerg Med* 35:1034.e1-1034.e2.

144. Kajon AE, Ison MG. 2016. Severe Infections with Human Adenovirus 7d in 2 Adults in Family, Illinois, USA, 2014. *Emerging Infectious Disease journal* 22:730.
145. Scott MK, Chommanard C, Lu X, Appelgate D, Grenz L, Schneider E, Gerber SI, Erdman DD, Thomas A. 2016. Human Adenovirus Associated with Severe Respiratory Infection, Oregon, USA, 2013-2014. *Emerg Infect Dis* 22:1044-51.
146. Garcia-Zalisnak D, Rapuano C, Sheppard JD, Davis AR. 2018. Adenovirus Ocular Infections: Prevalence, Pathology, Pitfalls, and Practical Pointers. *Eye Contact Lens* 44 Suppl 1:S1-s7.
147. Chen S, Tian X. 2018. Vaccine development for human mastadenovirus. *J Thorac Dis* 10:S2280-s2294.
148. Jobran S, Kattan R, Shamaa J, Marzouqa H, Hindiye M. 2018. Adenovirus respiratory tract infections in infants: a retrospective chart-review study. *Lancet* 391 Suppl 2:S43.
149. Lion T. 2014. Adenovirus Infections in Immunocompetent and Immunocompromised Patients. *Clin Microbiol Rev* 27:441-62.
150. Lopez SMC, Michaels MG, Green M. 2018. Adenovirus infection in pediatric transplant recipients: are effective antiviral agents coming our way? *Curr Opin Organ Transplant* 23:395-399.
151. Cesaro S, Berger M, Tridello G, Mikulska M, Ward KN, Ljungman P, Van Der Werf S, Averbuch D, Styczynski J. 2018. A survey on incidence and management of adenovirus infection after allogeneic HSCT. *Bone Marrow Transplant* doi:10.1038/s41409-018-0421-0.
152. Caruso Brown AE, Cohen MN, Tong S, Braverman RS, Rooney JF, Giller R, Levin MJ. 2015. Pharmacokinetics and safety of intravenous cidofovir for life-threatening viral

- infections in pediatric hematopoietic stem cell transplant recipients. *Antimicrob Agents Chemother* 59:3718-25.
153. Zecca M, Wynn R, Dalle JH, Feuchtinger T, Vainorius E, Brundage TM, Chandak A, Mozaffari E, Nichols G, Locatelli F. 2019. Association between adenovirus viral load and mortality in pediatric allo-HCT recipients: the multinational AdVance study. *Bone Marrow Transplant* doi:10.1038/s41409-019-0483-7.
 154. Detweiler CJ, Mueller SB, Sung AD, Saullo JL, Prasad VK, Cardona DM. 2018. Brincidofovir (CMX001) Toxicity Associated With Epithelial Apoptosis and Crypt Drop Out in a Hematopoietic Cell Transplant Patient: Challenges in Distinguishing Drug Toxicity From GVHD. *J Pediatr Hematol Oncol* doi:10.1097/mpb.0000000000001227.
 155. Ramsay ID, Attwood C, Irish D, Griffiths PD, Kyriakou C, Lowe DM. 2017. Disseminated adenovirus infection after allogeneic stem cell transplant and the potential role of brincidofovir - Case series and 10 year experience of management in an adult transplant cohort. *J Clin Virol* 96:73-79.
 156. Florescu DF, Schaenman JM. 2019. Adenovirus in solid organ transplant recipients: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. *Clin Transplant* doi:10.1111/ctr.13527:e13527.
 157. Liang Y, Quenelle D, Vogel JL, Mascaro C, Ortega A, Kristie TM. 2013. A novel selective LSD1/KDM1A inhibitor epigenetically blocks herpes simplex virus lytic replication and reactivation from latency. *MBio* 4:e00558-12.
 158. Liang Y, Vogel JL, Arbuckle JH, Rai G, Jadhav A, Simeonov A, Maloney DJ, Kristie TM. 2013. Targeting the JMJD2 histone demethylases to epigenetically control herpesvirus infection and reactivation from latency. *Sci Transl Med* 5:167ra5.

159. Liang Y, Vogel JL, Narayanan A, Peng H, Kristie TM. 2009. Inhibition of the histone demethylase LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency. *Nat Med* 15:1312-7.
160. Grosso F, Stoilov P, Lingwood C, Brown M, Cochrane A. 2017. Suppression of Adenovirus Replication by Cardiotonic Steroids. *J Virol* 91.
161. Marrugal-Lorenzo JA, Serna-Gallego A, Gonzalez-Gonzalez L, Bunuales M, Poutou J, Pachon J, Gonzalez-Aparicio M, Hernandez-Alcoceba R, Sanchez-Cespedes J. 2018. Inhibition of adenovirus infection by mifepristone. *Antiviral Res* 159:77-83.
162. Romanutti C, Bruttomesso AC, Castilla V, Galagovsky LR, Wachsman MB. 2010. Anti-adenovirus activity of epiandrosterone and dehydroepiandrosterone derivatives. *Chemotherapy* 56:158-65.
163. Mentel R, Wegner U. 2000. Evaluation of the efficacy of 2',3'-dideoxycytidine against adenovirus infection in a mouse pneumonia model. *Antiviral Research* 47:79-87.
164. Naesens L, Lenaerts L, Andrei G, Snoeck R, Van Beers D, Holy A, Balzarini J, De Clercq E. 2005. Antiadenovirus activities of several classes of nucleoside and nucleotide analogues. *Antimicrob Agents Chemother* 49:1010-6.
165. Hartline CB, Gustin KM, Wan WB, Ciesla SL, Beadle JR, Hostetler KY, Kern ER. 2005. Ether lipid-ester prodrugs of acyclic nucleoside phosphonates: activity against adenovirus replication in vitro. *J Infect Dis* 191:396-9.
166. Mentel R, Kinder M, Wegner U, von Janta-Lipinski M, Matthes E. 1997. Inhibitory activity of 3'-fluoro-2' deoxythymidine and related nucleoside analogues against adenoviruses in vitro. This paper is dedicated to Prof. Döhner on the occasion of his 65th birthday. *Antiviral Research* 34:113-119.

167. Sanchez-Cespedes J, Martinez-Aguado P, Vega-Holm M, Serna-Gallego A, Candela JJ, Marrugal-Lorenzo JA, Pachon J, Iglesias-Guerra F, Vega-Perez JM. 2016. New 4-Acyl-1-phenylaminocarbonyl-2-phenylpiperazine Derivatives as Potential Inhibitors of Adenovirus Infection. Synthesis, Biological Evaluation, and Structure-activity Relationships. *J Med Chem* 59:5432-48.
168. Sanchez-Cespedes J, Moyer CL, Whitby LR, Boger DL, Nemerow GR. 2014. Inhibition of adenovirus replication by a trisubstituted piperazin-2-one derivative. *Antiviral Res* 108:65-73.
169. Wang X, Zhang Q, Zhou Z, Liu M, Chen Y, Li J, Xu L, Guo J, Li Q, Yang J, Wang S. 2018. Retinoic acid receptor beta, a potential therapeutic target in the inhibition of adenovirus replication. *Antiviral Res* 152:84-93.
170. Lenaerts L, Naesens L. 2006. Antiviral therapy for adenovirus infections. *Antiviral Research* 71:172-180.
171. Waye MMY, Sing CW. 2010. Anti-Viral Drugs for Human Adenoviruses. *Pharmaceuticals* 3:3343-3354.
172. Stray SJ, Johnson JM, Kopek BG, Zlotnick A. 2006. An in vitro fluorescence screen to identify antivirals that disrupt hepatitis B virus capsid assembly. *Nat Biotechnol* 24:358-62.
173. Nun TK, Kroll DJ, Oberlies NH, Soejarto DD, Case RJ, Piskaut P, Matainaho T, Hilscher C, Wang L, Dittmer DP, Gao S-J, Damania B. 2007. Development of a fluorescence-based assay to screen antiviral drugs against Kaposi's sarcoma associated herpesvirus. *Molecular cancer therapeutics* 6:2360-2370.

174. Luthra P, Liang J, Pietzsch CA, Khadka S, Edwards MR, Wei S, De S, Posner B, Bukreyev A, Ready JM, Basler CF. 2018. A high throughput screen identifies benzoquinoline compounds as inhibitors of Ebola virus replication. *Antiviral Research* 150:193-201.
175. Johansen LM, Brannan JM, Delos SE, Shoemaker CJ, Stossel A, Lear C, Hoffstrom BG, Dewald LE, Schornberg KL, Scully C, Lehár J, Hensley LE, White JM, Olinger GG. 2013. FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. *Sci Transl Med* 5:190ra79.
176. Hartline CB, Keith KA, Eagar J, Harden EA, Bowlin TL, Prichard MN. 2018. A standardized approach to the evaluation of antivirals against DNA viruses: Orthopox-, adeno-, and herpesviruses. *Antiviral Res* 159:104-112.
177. Duffy MR, Parker AL, Kalkman ER, White K, Kovalskyy D, Kelly SM, Baker AH. 2013. Identification of novel small molecule inhibitors of adenovirus gene transfer using a high throughput screening approach. *J Control Release* 170:132-40.
178. Andersson EK, Strand M, Edlund K, Lindman K, Enquist P-A, Spjut S, Allard A, Elofsson M, Mei Y-F, Wadell G. 2010. Small-molecule screening using a whole-cell viral replication reporter gene assay identifies 2-{[2-(benzoylamino)benzoyl]amino}-benzoic acid as a novel antiadenoviral compound. *Antimicrobial agents and chemotherapy* 54:3871-3877.
179. Bradley RR, Lynch DM, Iampietro MJ, Borducchi EN, Barouch DH. 2012. Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. *J Virol* 86:625-9.

180. Liu T, Zhou Z, Tian X, Liu W, Xu D, Fan Y, Liao J, Gu S, Li X, Zhou R. 2018. A recombinant trivalent vaccine candidate against human adenovirus types 3, 7, and 55. *Vaccine* 36:2199-2206.
181. Eckstein A, Grossl T, Geisler A, Wang X, Pinkert S, Pozzuto T, Schwer C, Kurreck J, Weger S, Vetter R, Poller W, Fechner H. 2010. Inhibition of adenovirus infections by siRNA-mediated silencing of early and late adenoviral gene functions. *Antiviral Res* 88:86-94.
182. Madison DL, Yaciuk P, Kwok RP, Lundblad JR. 2002. Acetylation of the adenovirus-transforming protein E1A determines nuclear localization by disrupting association with importin-alpha. *J Biol Chem* 277:38755-63.
183. Feng Q, Su Z, Song S, Xu H, Zhang B, Yi L, Tian M, Wang H. 2016. Histone deacetylase inhibitors suppress RSV infection and alleviate virus-induced airway inflammation. *Int J Mol Med* 38:812-22.
184. Tollefson AE, Ying B, Spencer JF, Sagartz JE, Wold WSM, Toth K. 2017. Pathology in Permissive Syrian Hamsters after Infection with Species C Human Adenovirus (HAdV-C) Is the Result of Virus Replication: HAdV-C6 Replicates More and Causes More Pathology than HAdV-C5. *Journal of Virology* 91:e00284-17.
185. Somanath P, Herndon Klein R, Knoepfler PS. 2017. CRISPR-mediated HDAC2 disruption identifies two distinct classes of target genes in human cells. *PLOS ONE* 12:e0185627.
186. Su CT, Hsu JT, Hsieh HP, Lin PH, Chen TC, Kao CL, Lee CN, Chang SY. 2008. Anti-HSV activity of digitoxin and its possible mechanisms. *Antiviral Res* 79:62-70.

187. Cohen T, Williams JD, Opperman TJ, Sanchez R, Lurain NS, Tortorella D. 2016. Convallatoxin-Induced Reduction of Methionine Import Effectively Inhibits Human Cytomegalovirus Infection and Replication. *J Virol* 90:10715-10727.
188. Lancz G, Whitaker-Dowling P, Marsh YV, Bradley G, Eppstein DA, Hackney JF, Hulick-Swardstrom B. 1990. Inhibition of vesicular stomatitis virus replication in dexamethasone-treated L929 cells. *Proc Soc Exp Biol Med* 193:190-6.

Appendix 1: Authorizations

Figure 1.1 Schematic representation of the complex HAdV-5 genome (36 kb)

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Figure 1.2 Illustration of HAdV DNA replication

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Figure 1.3 Structure of the HAdV Virion and Appendix 3.1

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- Citation: Saha B, Wong CM, Parks RJ. 2014. The adenovirus genome contributes to the structural stability of the virion. Viruses 6:3563-83.
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Figure 1.4 Simplified representation of the HAdV lifecycle

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- Citation: Anonymous. 2017. Chapter 2 - Virus Replication, p 17-45. In MacLachlan NJ, Dubovi EJ (ed), Fenner's Veterinary Virology (Fifth Edition) doi:<https://doi.org/10.1016/B978-0-12-800946-8.00002-7>. Academic Press, Boston.

Chapter 2: Human Adenovirus Type 5 Vectors Deleted of Early Region 1 (E1) Undergo Limited Expression of Early Replicative E2 Proteins and DNA Replication in Non-permissive Cells

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

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Chapter 3: Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid Suppresses Human Adenovirus Gene Expression and Replication

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- Citation: Saha B and Parks RJ. (2019) Histone deacetylase inhibitor suberoylanilide hydroxamic acid suppresses human adenovirus gene expression and replication. *J Virol.* 93(12): e00088-19.
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Chapter 4: Development of a novel screening platform for the identification of small molecule inhibitors of human adenovirus

- This article, on which I am the primary author on this article, which was published in Virology, an Elsevier journal. According to the copyright information provided by the publisher, authors have the right to include the article in a dissertation.
- Citation: Saha B, Varette O, Stanford WL, Diallo JS and Parks RJ. (2019) Development of a novel screening platform for the identification of small molecule inhibitors of human adenovirus. *Virology.* 538: 24-34.
- Additional information: <https://www.elsevier.com/about/policies/copyright#personaluse>

Appendix 3.2 Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication

- I am a contributing author on this article, which was published in Biochemistry and Cell Biology, an NRC Research Press journal. The publisher allows authors to reuse the manuscript for non-commercial purposes, as long as the publication is acknowledged appropriately.
- Citation: Giberson A, Saha B, Campbell K, Christou C, Poulin KL, Parks RJ. 2018. Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication. *Biochemistry and Cell Biology* doi:10.1139/bcb-2018-0117.
- Additional information: <https://www.nrcresearchpress.com/page/authors/information/rights>

Appendix 2.1: Description of Adenovirus Constructs

- **Ad-CMV/E1A:** replication-competent ($E1^+$) virus with the E1 region under the control of CMV promoter (AdRP3169)
- **Ad-late/RFP:** replication-competent ($E1^+$) virus with RFP in the E3 region, under the control of MLP (AdRP3089)
- **Ad($E1^-$)-CMV/RFP:** replication-deficient ($E1^-$) virus with RFP in the E1 region, under the control of CMV promoter (AdRP2619)
- **Ad($E1^-$)-late/RFP:** replication-deficient ($E1^-$) virus with RFP in the E3 region, under the control of MLP (AdRP3090)
- **AdE1⁻DBP-F:** replication-deficient ($E1^-$) virus with FLAG-tagged DNA binding protein (AdRP3121)
- **AdE1⁺DBP-F:** replication-competent ($E1^+$) virus with FLAG-tagged DNA binding protein (AdRP3122)
- **AdE1⁺Pol-F:** replication-competent ($E1^+$) virus with FLAG-tagged DNA polymerase (AdRP3000)
- **AdE1⁺TP-F:** replication-competent ($E1^+$) virus with FLAG-tagged terminal protein (AdRP3000)
- **HAdV-4, -5, -7:** wildtype human adenovirus serotypes 4, 5, 7

Appendix 2.2: Supplementary Data

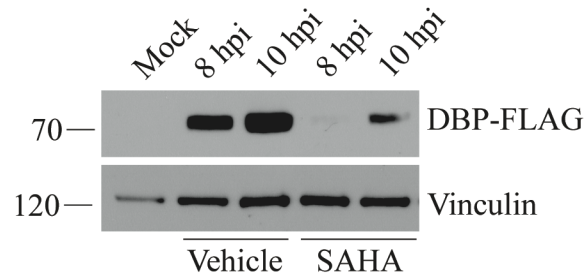


Figure S1. SAHA reduces HAdV DNA binding protein expression during early infection.

A549 cells were infected with the replication-competent AdE1⁺DBP-F at an MOI of 10, and treated with vehicle or 10 μ M SAHA. Expression of the FLAG-tagged DBP in cell lysates was analyzed by immunoblot using an antibody against FLAG.

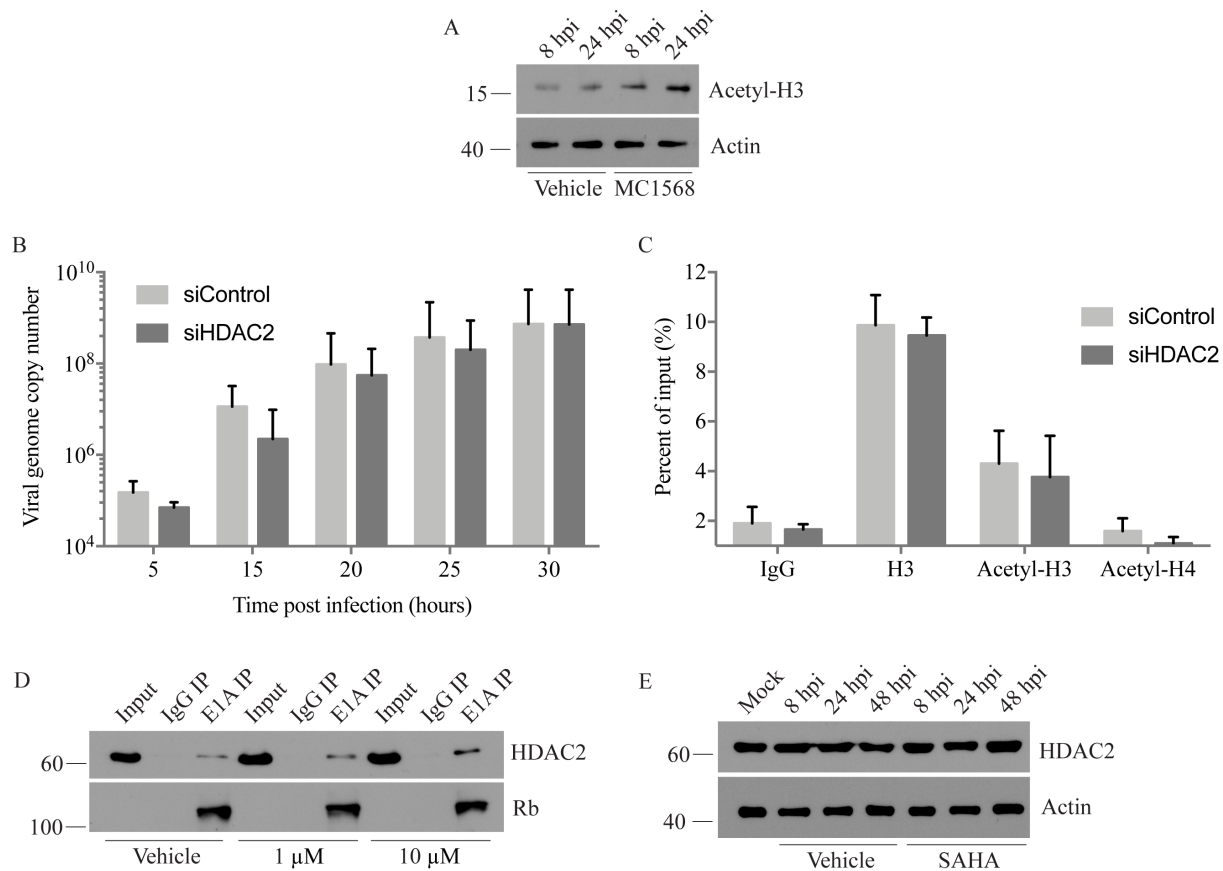


Figure S2. Investigation of HDAC2's role in HAdV infection. A549 cells were infected with Ad-late/RFP (MOI 10) in all experiments. (A) Infected cells were treated with vehicle or 10 μ M MC1568. Cell lysates were collected for immunoblot analysis at the indicated times post infection. (B, C) A549 cells were transfected with 100 nM control or HDAC2 siRNA for 48 hours, and then infected. (B) Total cellular DNA was extracted and viral DNA replication was analyzed at the indicated time points by qPCR with primers to the hexon coding region. (C) Cells were subjected to ChIP at 6 hpi, followed by qPCR with primers specific to HAdV E1A region. (D) co-IP was performed with IgG or E1A (554155, BD Biosciences) antibody on infected cells treated with 0-10 μ M SAHA for 24 hours. Input was 10% of the IP volume. (E) Infected cells were treated with 10 μ M SAHA. HDAC2 levels were analyzed in cell lysates by immunoblot at the indicated times post infection. All error bars represent SD (n=2).

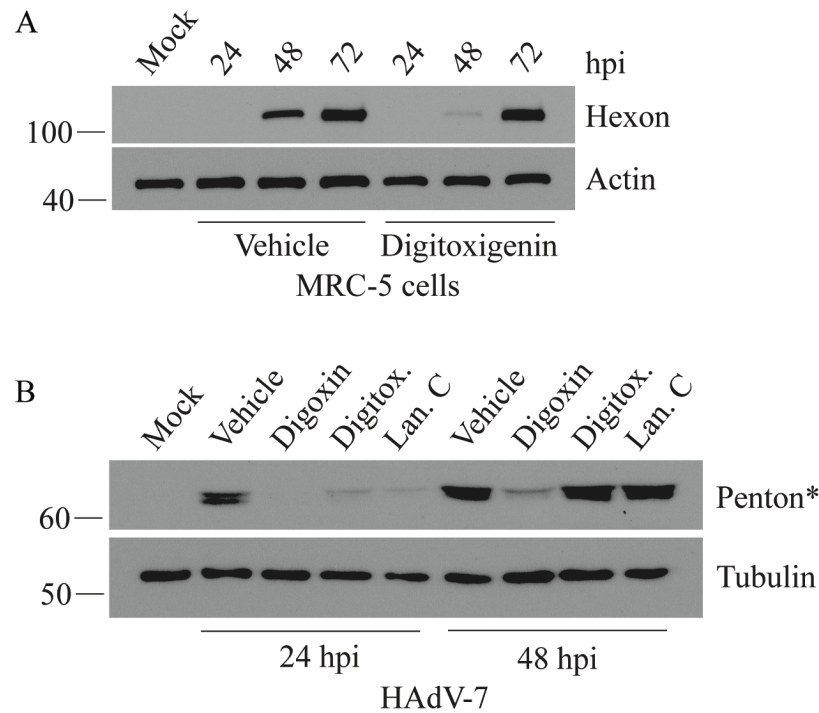


Figure S3. Efficacy of digitoxigenin in primary cells and against HAdV-7. (A) MRC-5 cells were pretreated with digitoxigenin for 2 hours and then infected with Ad-late/RFP (MOI 10). Cell lysates were harvested over 24-72 hours to detect changes in hexon expression by immunoblot. (B) A549 cells were pretreated as in panel A and infected with HAdV-7 (MOI 10). *The protein (detected using an anti-HAdV-5 antibody that cross-reacts with some of the HAdV-4 and HAdV-7 capsid proteins) is most likely penton based on its size of ~60 kDa.

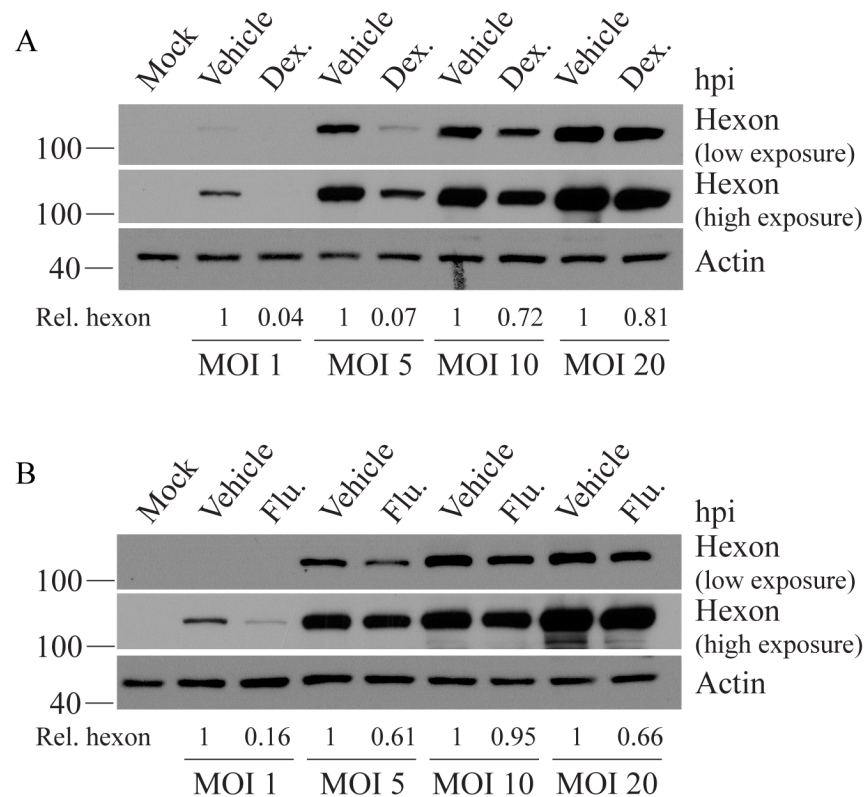


Figure S4. Dexamethasone and flunisolide are more effective in reducing hexon at lower MOIs. (A, B) A549 cells were pretreated with (A) dexamethasone or (B) flunisolide for 2 hours, and infected with Ad-late/RFP at the indicated MOIs. Cell lysates were collected at 24 hpi to analyze hexon expression by immunoblot. Relative hexon levels were determined using the Image J software. Hexon bands were first normalized to actin, followed by calculation of drug-treated band intensities relative to vehicle-treated bands at that MOI.

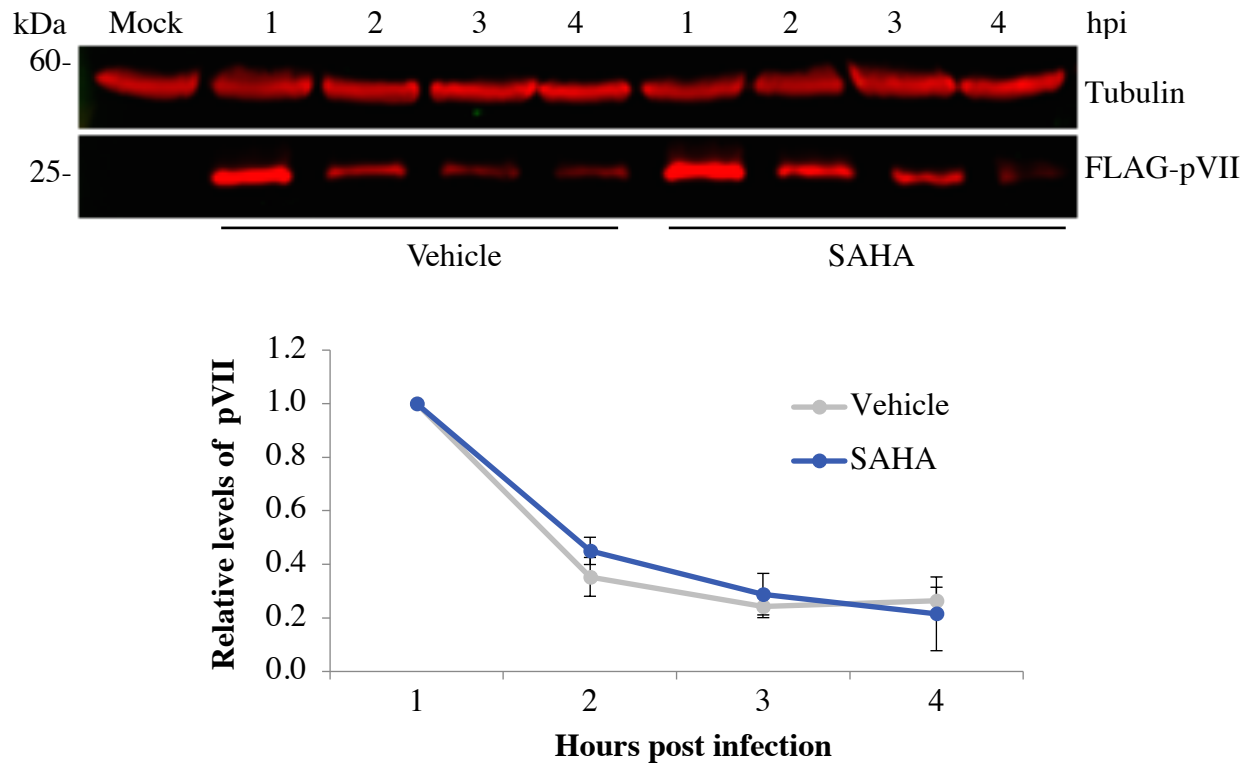


Figure S5. SAHA does not affect pVII loss/degradation in infected cells. A549 cells were infected with MOI 1000 of an E1⁺, replication-competent virus containing FLAG-tagged pVII. A synchronized or cold infection was conducted on ice to allow the majority of virions attached to the cell receptors to internalize simultaneously upon addition of warm media containing vehicle or SAHA. Infected cells were harvested for immunoblot analysis at 1-4 hpi. The intensities of the pVII bands in the immunoblot were quantified, normalized to tubulin and plotted as a fraction of the intensity at 1 hpi in vehicle- or SAHA-treated cells. Error bars represent SD (n=3).

Appendix 2.3: Data from the Prestwick Library Screen

Compound Name	Location in Library ^a	% RFP ^b
Azaguanine-8	01A02	101.4
Allantoin	01A03	113.3
Acetazolamide	01A04	105.2
Metformin hydrochloride	01A05	113.1
Atracurium besylate	01A06	109.6
Isoflupredone acetate	01A07	57.7
Amiloride hydrochloride dihydrate	01A08	103.6
Amprolium hydrochloride	01A09	124.9
Hydrochlorothiazide	01A10	118.5
Sulfaguanidine	01A11	110.3
Meticrane	01B02	96.3
Benzonate	01B03	94.1
Hydroflumethiazide	01B04	133.5
Sulfacetamide sodic hydrate	01B05	91.7
Heptaminol hydrochloride	01B06	106.7
Sulfathiazole	01B07	87.9
Levodopa	01B08	106.3
Idoxuridine	01B09	92.4
Captopril	01B10	91.2
Minoxidil	01B11	92.4
Sulfaphenazole	01C02	75.6
Panthenol (D)	01C03	95.9
Sulfadiazine	01C04	112.4
Norethynodrel	01C05	96.0
Thiamphenicol	01C06	98.6
Cimetidine	01C07	96.2
Doxylamine succinate	01C08	106.2
Ethambutol dihydrochloride	01C09	103.3
Antipyrine	01C10	94.3
Antipyrine, 4-hydroxy	01C11	93.5
Chloramphenicol	01D02	102.7
Epirizole	01D03	106.9
Diprophylline	01D04	93.2
Triamterene	01D05	110.2
Dapsone	01D06	103.0
Troleandomycin	01D07	101.3
Pyrimethamine	01D08	87.9

Hexamethonium dibromide dihydrate	01D09	100.2
Diflunisal	01D10	105.1
Niclosamide	01D11	91.6
Procaine hydrochloride	01E02	107.4
Moxisylyte hydrochloride	01E03	107.7
Betazole hydrochloride	01E04	106.1
Isoxicam	01E05	117.9
Naproxen	01E06	98.8
Naphazoline hydrochloride	01E07	102.7
Ticlopidine hydrochloride	01E08	96.4
Dicyclomine hydrochloride	01E09	83.6
Amyleine hydrochloride	01E10	107.1
Lidocaïne hydrochloride	01E11	90.0
Trichlorfon	01F02	102.0
Carbamazepine	01F03	107.9
Triflupromazine hydrochloride	01F04	99.9
Mefenamic acid	01F05	123.6
Acetohexamide	01F06	105.9
Sulpiride	01F07	111.8
Benoxinate hydrochloride	01F08	93.0
Oxethazaine	01F09	99.6
Pheniramine maleate	01F10	108.9
Tolazoline hydrochloride	01F11	82.6
Morantel tartrate	01G02	100.2
Homatropine hydrobromide (R,S)	01G03	108.4
Nifedipine	01G04	109.7
Chlorpromazine hydrochloride	01G05	102.8
Diphenhydramine hydrochloride	01G06	112.3
Minaprine dihydrochloride	01G07	109.7
Miconazole	01G08	112.4
Isoxsuprine hydrochloride	01G09	130.7
Acebutolol hydrochloride	01G10	114.5
Tolnaftate	01G11	106.7
Todralazine hydrochloride	01H02	94.2
Imipramine hydrochloride	01H03	80.3
Sulindac	01H04	87.5
Amitryptiline hydrochloride	01H05	103.0
Adiphenine hydrochloride	01H06	93.5
Dibucaïne	01H07	95.9
Prednisone	01H08	116.6

Thioridazine hydrochloride	01H09	98.6
Diphepanil methylsulfate	01H10	100.8
Trimethobenzamide hydrochloride	01H11	106.4
Metronidazole	02A02	89.2
Fulvestrant	02A03	94.4
Edrophonium chloride	02A04	87.8
Moroxidine hydrochloride	02A05	77.9
Baclofen (R,S)	02A06	86.3
Acyclovir	02A07	94.9
Diazoxide	02A08	87.3
Amidopyrine	02A09	90.8
Busulfan	02A10	100.1
Pindolol	02A11	76.9
Khellin	02B02	95.2
Zimelidine dihydrochloride monohydrate	02B03	66.8
Azacyclonol	02B04	96.5
Azathioprine	02B05	81.9
Lynestrenol	02B06	83.9
Guanabenz acetate	02B07	72.3
Disulfiram	02B08	90.7
Acetylsalicylsalicylic acid	02B09	88.5
Mianserine hydrochloride	02B10	84.4
Nocodazole	02B11	96.0
R(-) Apomorphine hydrochloride hemihydrate	02C02	99.2
Amoxapine	02C03	88.2
Cyproheptadine hydrochloride	02C04	89.9
Famotidine	02C05	73.1
Danazol	02C06	97.7
Nicorandil	02C07	90.4
Pioglitazone	02C08	80.4
Nomifensine maleate	02C09	85.6
Dizocilpine maleate	02C10	88.4
Oxandrolone	02C11	77.8
Naloxone hydrochloride	02D02	115.3
Metolazone	02D03	102.0
Ciprofloxacin hydrochloride	02D04	93.5
Ampicillin trihydrate	02D05	87.2
Haloperidol	02D06	96.3
Naltrexone hydrochloride dihydrate	02D07	84.3
Chlorpheniramine maleate	02D08	97.0

Nalbuphine hydrochloride	02D09	109.9
Picotamide monohydrate	02D10	105.0
Triamcinolone	02D11	55.7
Bromocryptine mesylate	02E02	97.0
Amfepramone hydrochloride	02E03	77.3
Dehydrocholic acid	02E04	91.3
Tioconazole	02E05	91.7
Perphenazine	02E06	107.1
Mefloquine hydrochloride	02E07	104.7
Isoconazole	02E08	89.5
Spironolactone	02E09	83.1
Pirenzepine dihydrochloride	02E10	95.0
Dexamethasone acetate	02E11	39.4
Glipizide	02F02	103.9
Loxapine succinate	02F03	82.9
Hydroxyzine dihydrochloride	02F04	96.3
Diltiazem hydrochloride	02F05	105.2
Methotrexate	02F06	33.0
Astemizole	02F07	112.1
Clindamycin hydrochloride	02F08	100.3
Terfenadine	02F09	91.6
Cefotaxime sodium salt	02F10	85.7
Tetracycline hydrochloride	02F11	79.5
Verapamil hydrochloride	02G02	118.2
Dipyridamole	02G03	94.3
Chlorhexidine	02G04	80.2
Loperamide hydrochloride	02G05	120.5
Chlortetracycline hydrochloride	02G06	98.7
Tamoxifen citrate	02G07	92.8
Nicergoline	02G08	94.9
Canrenoic acid potassium salt	02G09	98.3
Thiopropazine dimesylate	02G10	78.3
Dihydroergotamine tartrate	02G11	99.9
Erythromycin	02H02	92.3
Chloroxine	02H03	82.9
Didanosine	02H04	83.1
Josamycin	02H05	105.0
Paclitaxel	02H06	94.8
Ivermectin	02H07	84.5
Gallamine triethiodide	02H08	97.0

Neomycin sulfate	02H09	96.7
Dihydrostreptomycin sulfate	02H10	98.7
Gentamicine sulfate	02H11	96.1
Isoniazid	03A02	99.0
Pentylentetrazole	03A03	106.6
Chlorzoxazone	03A04	88.7
Ornidazole	03A05	97.0
Ethosuximide	03A06	96.3
Mafenide hydrochloride	03A07	95.9
Riluzole hydrochloride	03A08	109.5
Nitrofurantoin	03A09	99.8
Hydralazine hydrochloride	03A10	105.0
Phenelzine sulfate	03A11	112.5
Tranexamic acid	03B02	98.6
Etofylline	03B03	99.1
Tranlycypromine hydrochloride	03B04	100.1
Alverine citrate salt	03B05	103.9
Aceclofenac	03B06	109.0
Iproniazide phosphate	03B07	84.6
Sulfamethoxazole	03B08	96.8
Mephenesin	03B09	85.7
Phenformin hydrochloride	03B10	93.1
Flutamide	03B11	84.4
Ampyrone	03C02	99.1
Levamisole hydrochloride	03C03	112.8
Pargyline hydrochloride	03C04	88.1
Methocarbamol	03C05	95.6
Aztreonam	03C06	100.8
Cloxacillin sodium salt	03C07	92.4
Catharanthine	03C08	103.4
Pentolinium bitartrate	03C09	109.0
Aminopurine, 6-benzyl	03C10	97.0
Tolbutamide	03C11	114.5
Midodrine hydrochloride	03D02	112.9
Thalidomide	03D03	90.2
Oxolinic acid	03D04	114.5
Nimesulide	03D05	88.4
Hydrastinine hydrochloride	03D06	105.2
Pentoxifylline	03D07	92.1
Metaraminol bitartrate	03D08	90.9

Salbutamol	03D09	113.4
Prilocaine hydrochloride	03D10	89.7
Camptothecine (S,+)	03D11	77.9
Ranitidine hydrochloride	03E02	94.7
Tiratricol, 3,3',5-triiodothyroacetic acid	03E03	85.7
Flufenamic acid	03E04	88.9
Flumequine	03E05	97.7
Tolfenamic acid	03E06	95.3
Meclofenamic acid sodium salt monohydrate	03E07	95.8
Tibolone	03E08	102.7
Trimethoprim	03E09	98.7
Metoclopramide monohydrochloride	03E10	104.7
Fenbendazole	03E11	100.8
Piroxicam	03F02	119.8
Pyrantel tartrate	03F03	94.9
Fenspiride hydrochloride	03F04	92.0
Gemfibrozil	03F05	116.2
Mefexamide hydrochloride	03F06	109.5
Tiapride hydrochloride	03F07	97.6
Mebendazole	03F08	95.3
Fenbufen	03F09	53.4
Ketoprofen	03F10	106.1
Indapamide	03F11	112.2
Norfloxacin	03G02	89.3
Antimycin A	03G03	76.6
Xylometazoline hydrochloride	03G04	101.9
Oxymetazoline hydrochloride	03G05	94.3
Nifenazone	03G06	94.0
Griseofulvin	03G07	101.2
Clemizole hydrochloride	03G08	103.2
Tropicamide	03G09	87.1
Nefopam hydrochloride	03G10	112.2
Phentolamine hydrochloride	03G11	101.3
Etodolac	03H02	110.1
Scopolamin-N-oxide hydrobromide	03H03	83.1
Hyoscyamine (L)	03H04	119.1
Chlorphensin carbamate	03H05	103.6
Metampicillin sodium salt	03H06	55.7
Dilazep dihydrochloride	03H07	92.4
Ofloxacin	03H08	96.6

Lomefloxacin hydrochloride	03H09	104.6
Orphenadrine hydrochloride	03H10	96.0
Proglumide	03H11	102.6
Mexiletine hydrochloride	04A02	113.8
Flavoxate hydrochloride	04A03	100.4
Bufexamac	04A04	94.5
Glutethimide, para-amino	04A05	84.4
Dropropizine (R,S)	04A06	91.1
Pinacidil	04A07	58.6
Albendazole	04A08	96.5
Clonidine hydrochloride	04A09	105.9
Bupropion hydrochloride	04A10	102.2
Alprenolol hydrochloride	04A11	98.8
Chlorothiazide	04B02	102.3
Diphenidol hydrochloride	04B03	111.9
Norethindrone	04B04	83.6
Nortriptyline hydrochloride	04B05	104.7
Niflumic acid	04B06	44.5
Isotretinoin	04B07	112.1
Retinoic acid	04B08	116.4
Antazoline hydrochloride	04B09	105.1
Ethacrynic acid	04B10	91.3
Praziquantel	04B11	103.9
Ethisterone	04C02	74.2
Tripolidine hydrochloride	04C03	106.4
Doxepin hydrochloride	04C04	98.2
Dyclonine hydrochloride	04C05	96.6
Dimenhydrinate	04C06	114.5
Disopyramide	04C07	110.8
Clotrimazole	04C08	80.0
Vinpocetine	04C09	87.9
Clomipramine hydrochloride	04C10	87.7
Fendiline hydrochloride	04C11	113.5
Vincamine	04D02	92.4
Indomethacin	04D03	95.0
Cortisone	04D04	105.2
Prednisolone	04D05	90.3
Fenofibrate	04D06	101.5
Bumetanide	04D07	141.7
Labetalol hydrochloride	04D08	99.9

Cinnarizine	04D09	119.2
Methylprednisolone, 6-alpha	04D10	108.3
Quinidine hydrochloride monohydrate	04D11	93.7
Fludrocortisone acetate	04E02	100.6
Fenoterol hydrobromide	04E03	95.3
Homochlorcyclizine dihydrochloride	04E04	86.5
Diethylcarbamazine citrate	04E05	103.6
Chenodiol	04E06	96.4
Perhexiline maleate	04E07	100.1
Oxybutynin chloride	04E08	103.7
Spiperone	04E09	90.0
Pyrilamine maleate	04E10	104.4
Sulfinpyrazone	04E11	101.0
Dantrolene sodium salt	04F02	82.2
Trazodone hydrochloride	04F03	85.2
Glafenine hydrochloride	04F04	95.9
Pimethixene maleate	04F05	96.3
Pergolide mesylate	04F06	89.2
Acemetacin	04F07	89.5
Benzydamine hydrochloride	04F08	100.6
Fipexide hydrochloride	04F09	86.7
Mifepristone	04F10	116.7
Diperodon hydrochloride	04F11	120.1
Lisinopril	04G02	94.0
Lincomycin hydrochloride	04G03	82.5
Telenzepine dihydrochloride	04G04	319.5
Econazole nitrate	04G05	77.6
Bupivacaine hydrochloride	04G06	51.2
Clemastine fumarate	04G07	107.4
Oxytetracycline dihydrate	04G08	86.2
Pimozide	04G09	101.3
Amodiaquin dihydrochloride dihydrate	04G10	107.1
Mebeverine hydrochloride	04G11	90.4
Ifenprodil tartrate	04H02	87.1
Flunarizine dihydrochloride	04H03	103.8
Trifluoperazine dihydrochloride	04H04	91.3
Enalapril maleate	04H05	81.5
Minocycline hydrochloride	04H06	115.2
Glibenclamide	04H07	105.0
Guanethidine sulfate	04H08	103.6

Quinacrine dihydrochloride dihydrate	04H09	103.6
Clofilium tosylate	04H10	97.0
Fluphenazine dihydrochloride	04H11	106.4
Streptomycin sulfate	05A02	116.5
Alfuzosin hydrochloride	05A03	91.8
Chlorpropamide	05A04	120.3
Phenylpropanolamine hydrochloride	05A05	74.8
Ascorbic acid	05A06	95.6
Methyldopa (L,-)	05A07	74.1
Cefoperazone dihydrate	05A08	107.4
Zoxazolamine	05A09	90.5
Tacrine hydrochloride hydrate	05A10	59.2
Bisoprolol fumarate	05A11	97.7
Tremorine dihydrochloride	05B02	110.3
Practolol	05B03	119.3
Zidovudine, AZT	05B04	98.0
Sulfisoxazole	05B05	78.0
Zaprinast	05B06	93.7
Chlormezanone	05B07	33.5
Procainamide hydrochloride	05B08	120.1
N6-methyladenosine	05B09	95.1
Guanfacine hydrochloride	05B10	105.1
Domperidone	05B11	100.6
Furosemide	05C02	126.8
Methapyrilene hydrochloride	05C03	78.2
Desipramine hydrochloride	05C04	115.7
Clorgyline hydrochloride	05C05	88.8
Clenbuterol hydrochloride	05C06	84.3
Maprotiline hydrochloride	05C07	169.0
Thioguanosine	05C08	98.9
Chlorprothixene hydrochloride	05C09	86.4
Ritodrine hydrochloride	05C10	87.5
Clozapine	05C11	88.8
Chlorthalidone	05D02	88.3
Dobutamine hydrochloride	05D03	69.2
Moclobemide	05D04	80.8
Clopamide	05D05	80.5
Hycanthone	05D06	90.7
Adenosine 5'-monophosphate monohydrate	05D07	98.8
Amoxicillin	05D08	95.9

Cephalexin monohydrate	05D09	102.0
Dextromethorphan hydrobromide monohydrate	05D10	80.1
Droperidol	05D11	103.4
Bambuterol hydrochloride	05E02	95.3
Betamethasone	05E03	72.8
Colchicine	05E04	83.1
Metergoline	05E05	96.6
Brinzolamide	05E06	98.1
Ambroxol hydrochloride	05E07	69.6
Benfluorex hydrochloride	05E08	38.9
Bepridil hydrochloride	05E09	91.0
Meloxicam	05E10	88.6
Benzbromarone	05E11	93.9
Ketotifen fumarate	05F02	110.0
Debrisoquin sulfate	05F03	92.6
Amethopterin (R,S)	05F04	88.9
Methylergometrine maleate	05F05	135.0
Methiothepin maleate	05F06	89.9
Clofazimine	05F07	104.4
Nafronyl oxalate	05F08	90.9
Bezafibrate	05F09	84.5
Nefazodone HCl	05F10	93.9
Clebopride maleate	05F11	104.0
Lidoflazine	05G02	89.8
Betaxolol hydrochloride	05G03	88.2
Nicardipine hydrochloride	05G04	94.6
ProbucoI	05G05	74.5
Mitoxantrone dihydrochloride	05G06	101.2
GBR 12909 dihydrochloride	05G07	104.1
Carbetapentane citrate	05G08	92.2
Dequalinium dichloride	05G09	86.1
Ketoconazole	05G10	79.7
Fusidic acid sodium salt	05G11	90.2
Terbutaline hemisulfate	05H02	116.0
Ketanserine tartrate hydrate	05H03	90.6
Hemicholinium bromide	05H04	83.2
Kanamycin A sulfate	05H05	76.4
Amikacin hydrate	05H06	79.0
Etoposide	05H07	93.8
Clomiphene citrate (Z,E)	05H08	94.6

Oxantel pamoate	05H09	106.4
Prochlorperazine dimaleate	05H10	78.0
Hesperidin	05H11	91.0
Testosterone propionate	06A02	83.8
Arecoline hydrobromide	06A03	91.9
Thyroxine (L)	06A04	86.9
Idebenone	06A05	98.2
Pepstatin A	06A06	84.8
SR-95639A	06A07	95.5
Adamantamine fumarate	06A08	91.4
Butoconazole nitrate	06A09	88.8
Amiodarone hydrochloride	06A10	89.6
Amphotericin B	06A11	96.4
Androsterone	06B02	110.6
Amifostine	06B03	98.8
Carbarsone	06B04	98.1
Amlodipine	06B05	77.8
Modafinil	06B06	88.1
Bacampicillin hydrochloride	06B07	78.9
Lamivudine	06B08	86.3
Biotin	06B09	71.1
Bisacodyl	06B10	89.0
Erlotinib	06B11	96.0
Suloctidil	06C02	97.5
Zotepine	06C03	114.7
Carisoprodol	06C04	93.1
Cephalosporanic acid, 7-amino	06C05	95.9
Chicago sky blue 6B	06C06	82.4
Buflomedil hydrochloride	06C07	105.7
Dibenzepine hydrochloride	06C08	113.5
Roxatidine Acetate HCl	06C09	97.6
Cholecalciferol	06C10	93.9
Cisapride	06C11	111.3
Pefloxacin	06D02	108.2
Corticosterone	06D03	73.9
Cyanocobalamin	06D04	100.0
Cefadroxil	06D05	114.5
Cyclosporin A	06D06	105.9
Digitoxigenin	06D07	22.9
Digoxin	06D08	21.0

Doxorubicin hydrochloride	06D09	90.7
Carbimazole	06D10	90.4
Epiandrosterone	06D11	112.9
Estradiol-17 beta	06E02	106.6
Clobutinol hydrochloride	06E03	84.4
Gabazine	06E04	95.1
Oxcarbazepine	06E05	97.7
Cyclobenzaprine hydrochloride	06E06	100.7
Carteolol hydrochloride	06E07	88.5
Hydrocortisone base	06E08	58.2
Hydroxytacrine maleate (R,S)	06E09	99.8
Pilocarpine nitrate	06E10	89.8
Dicloxacillin sodium salt	06E11	101.9
Alizapride HCl	06F02	112.6
Stanozolol	06F03	113.7
Calcipotriene	06F04	117.6
Linezolid	06F05	88.0
Mebhydroline 1,5-naphtalenedisulfonate	06F06	119.2
Meclocycline sulfosalicylate	06F07	99.3
Meclozine dihydrochloride	06F08	98.1
Melatonin	06F09	115.5
Butalbital	06F10	87.9
Dinoprost trometamol	06F11	110.1
Tropisetron HCl	06G02	104.7
Cefixime	06G03	96.6
Metrizamide	06G04	84.5
Quetiapine	06G05	90.8
Tosufloxacin hydrochloride	06G06	90.8
Efavirenz	06G07	97.6
Rifapentine	06G08	88.2
Neostigmine bromide	06G09	112.3
Niridazole	06G10	106.3
Ceforanide	06G11	103.4
Vatalanib	06H02	93.8
Itopride	06H03	107.5
Cefotetan	06H04	106.3
Fentiazac	06H05	91.0
Brompheniramine maleate	06H06	107.6
Primaquine diphosphate	06H07	101.0
Progesterone	06H08	113.3

Felodipine	06H09	117.3
Raclopride	06H10	111.9
Closantel	06H11	107.8
Serotonin hydrochloride	07A02	93.3
Cefotiam hydrochloride	07A03	92.7
Rofecoxib	07A04	93.3
Benperidol	07A05	105.2
Cefaclor	07A06	97.7
Colistin sulfate	07A07	89.1
Daunorubicin hydrochloride	07A08	86.6
Dosulepin hydrochloride	07A09	99.0
Ceftazidime pentahydrate	07A10	100.7
Iobenguane sulfate	07A11	97.5
Metixene hydrochloride	07B02	106.0
Nitrofurantoin	07B03	107.1
Omeprazole	07B04	97.4
Propylthiouracil	07B05	97.7
Terconazole	07B06	96.2
Tiaprofenic acid	07B07	82.6
Vancomycin hydrochloride	07B08	100.2
Artemisinin	07B09	130.2
Propafenone hydrochloride	07B10	118.7
Ethamivan	07B11	122.4
Vigabatrin	07C02	100.7
Biperiden hydrochloride	07C03	112.1
Cetirizine dihydrochloride	07C04	100.0
Etifenin	07C05	85.3
Metaproterenol sulfate, orciprenaline sulfate	07C06	100.4
Sisomicin sulfate	07C07	106.0
Sibutramine HCl	07C08	97.0
Resveratrol	07C09	114.7
Bromperidol	07C10	115.1
Cyclizine hydrochloride	07C11	113.0
Fluoxetine hydrochloride	07D02	111.6
Iohexol	07D03	105.2
Norcyclobenzaprine	07D04	107.3
Pyrazinamide	07D05	96.9
Trimethadione	07D06	118.2
Lovastatin	07D07	103.6
Nystatine	07D08	91.4

Budesonide	07D09	62.0
Imipenem	07D10	109.7
Sulfasalazine	07D11	93.3
Lofexidine	07E02	112.1
Thiostrepton	07E03	92.0
Miglitol	07E04	74.0
Tiabendazole	07E05	105.7
Rifampicin	07E06	96.0
Ethionamide	07E07	97.6
Tenoxicam	07E08	97.7
Triflusal	07E09	110.3
Mesoridazine besylate	07E10	93.5
Trolox	07E11	94.5
Pirenperone	07F02	118.8
Isoquinoline, 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydro, hydrochloride	07F03	101.7
Phenacetin	07F04	94.6
Atovaquone	07F05	110.8
Methoxamine hydrochloride	07F06	97.0
(S)-(-)-Atenolol	07F07	104.5
Piracetam	07F08	99.9
Phenindione	07F09	99.2
Thiocolchicoside	07F10	91.1
Clorsulon	07F11	77.2
Ciclopirox ethanolamine	07G02	79.2
Probenecid	07G03	94.1
Betahistine mesylate	07G04	81.5
Tobramycin	07G05	83.6
Tetramisole hydrochloride	07G06	64.4
Pregnenolone	07G07	86.3
Molsidomine	07G08	80.7
Chloroquine diphosphate	07G09	85.5
Trimetazidine dihydrochloride	07G10	95.1
Parthenolide	07G11	99.9
Hexetidine	07H02	109.1
Selegiline hydrochloride	07H03	84.1
Pentamidine isethionate	07H04	104.5
Tolazamide	07H05	89.3
Nifuroxazide	07H06	89.2
Mirtazapine	07H07	70.9

Dirithromycin	07H08	73.0
Gliclazide	07H09	87.5
DO 897/99	07H10	92.6
Prenylamine lactate	07H11	87.7
Ziprasidone Hydrochloride	08A02	83.4
Mevastatin	08A03	100.9
Pyridostigmine iodid	08A04	88.8
Pentobarbital	08A05	86.8
Atropine sulfate monohydrate	08A06	83.0
Eserine sulfate, physostigmine sulfate	08A07	104.6
Itraconazole	08A08	81.7
Acarbose	08A09	95.4
Entacapone	08A10	103.1
Nicotinamide	08A11	94.5
Tetracaine hydrochloride	08B02	103.8
Mometasone furoate	08B03	51.0
Troglitazone	08B04	93.6
Dacarbazine	08B05	103.8
Tenatoprazole	08B06	72.4
Acetpromazine maleate salt	08B07	98.1
Escitalopram	08B08	83.7
Ropinirole HCl	08B09	105.3
Lacidipine	08B10	99.2
Argatroban	08B11	96.9
Reboxetine mesylate	08C02	95.8
Lobelanidine hydrochloride	08C03	98.2
Papaverine hydrochloride	08C04	95.2
Yohimbine hydrochloride	08C05	91.3
Lobeline alpha (-) hydrochloride	08C06	89.8
Alfacalcidol	08C07	115.0
Cilostazol	08C08	108.9
Gаланthamine hydrobromide	08C09	109.5
Azelastine HCl	08C10	107.1
Etretinate	08C11	105.7
Emedastine	08D02	98.7
Etofenamate	08D03	78.5
Zaleplon	08D04	89.0
Diclofenac sodium	08D05	100.0
Exemestane	08D06	79.1
Convolamine hydrochloride	08D07	97.5

Temozolomide	08D08	104.2
Xylazine	08D09	110.5
Celiprolol HCl	08D10	107.1
Zopiclone	08D11	106.6
Tranilast	08E02	106.4
Tizanidine HCl	08E03	97.9
Zafirlukast	08E04	97.6
Butenafine	08E05	95.0
Carbadox	08E06	84.4
Rimantadine	08E07	101.4
Eburnamonine (-)	08E08	89.2
Oxibendazol	08E09	93.4
Ipsapirone	08E10	105.5
Harmaline hydrochloride dihydrate	08E11	100.3
Harmalol hydrochloride dihydrate	08F02	85.0
Harmol hydrochloride monohydrate	08F03	102.6
Harmine hydrochloride	08F04	86.5
Carbidopa	08F05	92.3
Chrysene-1,4-quinone	08F06	93.2
Demecarium bromide	08F07	96.4
Quipazine dimaleate salt	08F08	106.2
Acipimox	08F09	95.3
Diflorasone Diacetate	08F10	48.7
Harmene hydrochloride	08F11	92.6
Methoxy-6-harmalan	08G02	101.4
Amisulpride	08G03	105.8
Pyridoxine hydrochloride	08G04	93.1
Mercaptopurine	08G05	73.3
Cytarabine	08G06	30.2
Racecadotril	08G07	100.8
Folic acid	08G08	98.4
Benazepril HCl	08G09	85.2
Aniracetam	08G10	76.4
Dimethisoquin hydrochloride	08G11	63.2
Alendronate sodium	08H02	93.5
Dipivefrin hydrochloride	08H03	94.4
Thiorphan	08H04	94.5
Tomoxetine hydrochloride	08H05	96.0
Lapatinib ditosylate	08H06	87.3
Penciclovir	08H07	87.1

Levetiracetam	08H08	98.5
Dexfenfluramine hydrochloride	08H09	85.2
Etoricoxib	08H10	90.4
Sertindole	08H11	84.7
Sulmazole	09A02	91.9
Gefitinib	09A03	78.5
Flunisolide	09A04	49.1
N-Acetyl-DL-homocysteine Thiolactone	09A05	89.9
Flurandrenolide	09A06	47.1
Oxiconazole Nitrate	09A07	94.5
Rebamipide	09A08	103.0
Nilvadipine	09A09	88.8
Etanidazole	09A10	98.9
Butirosin disulfate salt	09A11	94.9
Glimepiride	09B02	109.2
Picrotoxinin	09B03	108.4
Mepenzolate bromide	09B04	97.8
Benfotiamine	09B05	83.4
Halcinonide	09B06	63.7
Lanatoside C	09B07	19.5
Benzamil hydrochloride	09B08	92.3
Suxibuzone	09B09	97.1
6-Furfurylaminopurine	09B10	99.6
Avermectin B1a	09B11	86.5
Pranlukast	09C02	109.6
Penicillamine	09C03	125.9
Zileuton	09C04	119.9
Loratadine	09C05	106.1
Clindamycin Phosphate	09C06	92.2
Nisoldipine	09C07	103.5
Foliosidine	09C08	97.2
Acitretin	09C09	109.9
Zonisamide	09C10	102.4
Irsogladine Maleate	09C11	109.0
Dydrogesterone	09D02	102.3
Sumatriptan succinate	09D03	98.5
Opipramol dihydrochloride	09D04	92.1
Nalidixic acid sodium salt	09D05	98.9
Oxacillin Na	09D06	112.9
Beta-Escin	09D07	103.0

Tiludronate disodium	09D08	115.5
Tazobactam	09D09	99.3
Ibandronate	09D10	118.8
Warfarin	09D11	91.7
Pranoprofen	09E02	102.5
Secnidazole	09E03	110.3
Pempidine tartrate	09E04	115.0
Clodronate	09E05	109.1
Nitrarine dihydrochloride	09E06	116.8
Thimerosal	09E07	119.9
Tramadol hydrochloride	09E08	121.7
Estropipate	09E09	132.0
Butylscopolammonium (n-) bromide	09E10	110.7
Irinotecan Hydrochloride	09E11	89.6
Tylosin	09F02	107.2
Citalopram Hydrobromide	09F03	99.4
Promazine hydrochloride	09F04	103.1
Sulfamerazine	09F05	99.5
Venlafaxine	09F06	115.8
Ethotoin	09F07	103.8
3-alpha-Hydroxy-5-beta-androstan-17-one	09F08	112.1
Tetrahydrozoline hydrochloride	09F09	90.5
Hexestrol	09F10	106.0
Cefmetazole sodium salt	09F11	116.3
Trihexyphenidyl-D,L Hydrochloride	09G02	102.2
Succinylsulfathiazole	09G03	102.5
Famprofazone	09G04	101.1
Bromopride	09G05	100.7
Methyl benzethonium chloride	09G06	95.5
Chlorcyclizine hydrochloride	09G07	103.9
Diphenylpyraline hydrochloride	09G08	85.7
Benzethonium chloride	09G09	108.8
Trioxsalen	09G10	90.8
Doxofylline	09G11	86.1
Sulfabenzamide	09H02	97.0
Benzocaine	09H03	88.7
Dipyrone	09H04	98.9
Isosorbide dinitrate	09H05	114.8
Sulfachloropyridazine	09H06	106.0
Pramoxine hydrochloride	09H07	98.9

Finasteride	09H08	120.2
Fluorometholone	09H09	57.8
Cephalothin sodium salt	09H10	123.3
Cefuroxime sodium salt	09H11	100.7
Althiazide	10A02	104.4
Isopyrin hydrochloride	10A03	98.3
Phenethicillin potassium salt	10A04	119.5
Sulfamethoxypyridazine	10A05	104.0
Deferoxamine mesylate	10A06	88.9
Mephentermine hemisulfate	10A07	90.4
Liranaftate	10A08	110.1
Sulfadimethoxine	10A09	99.4
Sulfanilamide	10A10	103.1
Balsalazide Sodium	10A11	102.6
Sulfaquinoxaline sodium salt	10B02	108.2
Streptozotocin	10B03	103.7
Metoprolol-(+,-) (+)-tartrate salt	10B04	100.5
Flumethasone	10B05	45.7
Flecainide acetate	10B06	111.6
Cefazolin sodium salt	10B07	109.6
Atractyloside potassium salt	10B08	120.9
Folinic acid calcium salt	10B09	105.8
Levonordefrin	10B10	109.1
Ebselen	10B11	108.8
Nadide	10C02	105.5
Sulfamethizole	10C03	113.1
Medrysone	10C04	88.8
Flunixin meglumine	10C05	106.1
Spiramycin	10C06	116.1
Glycopyrrolate	10C07	112.4
Cefamandole sodium salt	10C08	101.7
Monensin sodium salt	10C09	133.4
Isoetharine mesylate salt	10C10	114.7
Mevalonic-D, L acid lactone	10C11	112.6
Terazosin hydrochloride	10D02	95.1
Phenazopyridine hydrochloride	10D03	111.3
Demeclocycline hydrochloride	10D04	120.0
Fenoprofen calcium salt dihydrate	10D05	91.2
Piperacillin sodium salt	10D06	114.3
Diethylstilbestrol	10D07	109.6

Chlorotrianisene	10D08	102.1
Ribostamycin sulfate salt	10D09	121.9
Methacholine chloride	10D10	100.4
Pipenzolate bromide	10D11	113.2
Butamben	10E02	93.6
Sulfapyridine	10E03	111.4
Meclofenoxate hydrochloride	10E04	110.4
Furaltadone hydrochloride	10E05	110.6
Ethoxyquin	10E06	139.3
Tinidazole	10E07	127.5
Guanadrel sulfate	10E08	113.7
Vidarabine	10E09	109.7
Sulfameter	10E10	115.2
Isopropamide iodide	10E11	113.5
Alclometasone dipropionate	10F02	70.2
Leflunomide	10F03	103.7
Norgestrel-(-)-D	10F04	107.9
Fluocinonide	10F05	65.6
Sulfamethazine sodium salt	10F06	103.1
Guaifenesin	10F07	122.6
Alexidine dihydrochloride	10F08	108.9
Proadifen hydrochloride	10F09	110.7
Zomepirac sodium salt	10F10	114.7
Cinoxacin	10F11	89.2
Clobetasol propionate	10G02	57.0
Podophyllotoxin	10G03	45.4
Clofibric acid	10G04	100.8
Bendroflumethiazide	10G05	101.8
Dicumarol	10G06	107.6
Methimazole	10G07	93.7
Merbromin	10G08	90.5
Hexylcaine hydrochloride	10G09	82.9
Drofenine hydrochloride	10G10	89.4
Cycloheximide	10G11	55.3
® -Naproxen sodium salt	10H02	107.4
Propidium iodide	10H03	106.5
Cloperastine hydrochloride	10H04	102.6
Eucatropine hydrochloride	10H05	106.1
Isocarboxazid	10H06	109.7
Lithocholic acid	10H07	107.6

Methotrimeprazine maleate salt	10H08	104.5
Dienestrol	10H09	107.3
Pridinol methanesulfonate salt	10H10	96.6
Amrinone	10H11	89.5
Carbinoxamine maleate salt	11A02	90.6
Methazolamide	11A03	92.1
Pyrithyldione	11A04	91.3
Spectinomycin dihydrochloride	11A05	98.9
Piromidic acid	11A06	99.8
Trimipramine maleate salt	11A07	106.8
Chloropyramine hydrochloride	11A08	105.3
Furazolidone	11A09	114.5
Dichlorphenamide	11A10	98.6
Sulconazole nitrate	11A11	113.1
Auranofin	11B02	112.6
Cromolyn disodium salt	11B03	91.3
Bucladesine sodium salt	11B04	116.1
Cefsulodin sodium salt	11B05	103.8
Fosfosal	11B06	106.3
Suprofen	11B07	108.5
Catechin-(+,-) hydrate	11B08	100.9
Nadolol	11B09	93.9
Moxalactam disodium salt	11B10	94.6
Aminophylline	11B11	110.6
Azlocillin sodium salt	11C02	100.1
Clidinium bromide	11C03	114.7
Sulfamonomethoxine	11C04	121.8
Benzthiazide	11C05	106.1
Trichlormethiazide	11C06	93.5
Oxalamine citrate salt	11C07	111.0
Propantheline bromide	11C08	120.5
Viloxazine hydrochloride	11C09	112.6
Dimethadione	11C10	112.4
Ethaverine hydrochloride	11C11	101.3
Butacaine	11D02	102.9
Cefoxitin sodium salt	11D03	125.0
Ifosfamide	11D04	106.8
Novobiocin sodium salt	11D05	99.8
Tetrahydroxy-1,4-quinone monohydrate	11D06	108.8
Indoprofen	11D07	128.9

Carbenoxolone disodium salt	11D08	103.8
Iocetamic acid	11D09	103.6
Ganciclovir	11D10	113.9
Ethopropazine hydrochloride	11D11	114.8
Olanzapine	11E02	129.3
Trimeprazine tartrate	11E03	118.0
Nafcillin sodium salt monohydrate	11E04	99.7
Procyclidine hydrochloride	11E05	111.1
Amiprilose hydrochloride	11E06	96.8
Ethynylestradiol 3-methyl ether	11E07	105.7
(-) -Levobunolol hydrochloride	11E08	122.9
Iodixanol	11E09	106.1
Rolitetraacycline	11E10	116.8
Equilin	11E11	140.8
Paroxetine Hydrochloride	11F02	110.1
Nylidrin	11F03	102.8
Liothyronine	11F04	107.5
Roxithromycin	11F05	100.9
Beclomethasone dipropionate	11F06	69.2
Tolmetin sodium salt dihydrate	11F07	131.4
(+) -Levobunolol hydrochloride	11F08	119.6
Doxazosin mesylate	11F09	119.0
Fluvastatin sodium salt	11F10	114.1
Methylhydantoin-5-(L)	11F11	109.1
Gabapentin	11G02	107.7
Raloxifene hydrochloride	11G03	99.5
Etidronic acid, disodium salt	11G04	113.0
Methylhydantoin-5-(D)	11G05	115.9
Simvastatin	11G06	112.8
Azacytidine-5	11G07	125.9
Paromomycin sulfate	11G08	116.6
Acetaminophen	11G09	97.1
Phthalylsulfathiazole	11G10	119.5
Luteolin	11G11	116.9
Iopamidol	11H02	99.2
Iopromide	11H03	117.0
Theophylline monohydrate	11H04	120.8
Theobromine	11H05	97.8
Reserpine	11H06	115.2
Bicalutamide	11H07	109.4

Scopolamine hydrochloride	11H08	107.8
Ioversol	11H09	121.6
Rabeprazole	11H10	114.6
Carbachol	11H11	121.5
Niacin	12A02	116.9
Bemegride	12A03	108.9
Digoxigenin	12A04	103.1
Meglumine	12A05	99.9
Cantharidin	12A06	115.8
Clioquinol	12A07	109.4
Oxybenzone	12A08	117.6
Promethazine hydrochloride	12A09	119.6
Diacerein	12A10	111.4
Esmolol hydrochloride	12A11	108.9
Cortisol acetate	12B02	96.2
Flubendazol	12B03	105.9
Felbinac	12B04	108.1
Butylparaben	12B05	96.2
Aminohippuric acid	12B06	95.5
N-Acetyl-L-leucine	12B07	114.9
Pipemidic acid	12B08	100.8
Dioxybenzone	12B09	106.8
Adrenosterone	12B10	99.3
Methylatropine nitrate	12B11	125.6
Hymecromone	12C02	96.3
Caffeic acid	12C03	430.6
Diloxanide furoate	12C04	97.6
Metyrapone	12C05	105.5
Urapidil hydrochloride	12C06	129.1
Fluspirilen	12C07	105.1
S-(+)-ibuprofen	12C08	116.6
Ethinodiol diacetate	12C09	108.8
Nabumetone	12C10	95.1
Nisoxetine hydrochloride	12C11	97.8
(+)-Isoproterenol (+)-bitartrate salt	12D02	102.4
Monobenzone	12D03	105.7
2-Aminobenzenesulfonamide	12D04	118.6
Estrone	12D05	108.0
Lorglumide sodium salt	12D06	120.3
Nitrendipine	12D07	117.1

Flurbiprofen	12D08	111.6
Nimodipine	12D09	105.3
Bacitracin	12D10	107.6
L(-)-vesamicol hydrochloride	12D11	112.4
Nizatidine	12E02	93.8
Thioperamide maleate	12E03	104.3
Xamoterol hemifumarate	12E04	102.3
Rolipram	12E05	112.2
Thonzonium bromide	12E06	97.4
Idazoxan hydrochloride	12E07	106.1
Quinapril HCl	12E08	98.6
Nilutamide	12E09	105.0
Ketorolac tromethamine	12E10	117.3
Protriptyline hydrochloride	12E11	93.7
Propofol	12F02	131.0
S(-)Eticlopride hydrochloride	12F03	99.8
Primidone	12F04	121.0
Flucytosine	12F05	107.2
(-)-MK 801 hydrogen maleate	12F06	119.3
Bephenium hydroxynaphthoate	12F07	110.9
Dehydroisoandosterone 3-acetate	12F08	141.4
Benserazide hydrochloride	12F09	104.1
Iodipamide	12F10	111.4
Allopurinol	12F11	113.8
Pentetic acid	12G02	101.1
Bretylium tosylate	12G03	112.5
Pralidoxime chloride	12G04	116.6
Phenoxybenzamine hydrochloride	12G05	111.6
Salmeterol	12G06	136.0
Altretamine	12G07	212.2
Prazosin hydrochloride	12G08	104.1
Timolol maleate salt	12G09	102.1
(+,-)-Octopamine hydrochloride	12G10	105.0
Stavudine	12G11	100.2
Crotamiton	12H02	105.0
Toremifene	12H03	99.7
®-(+)-Atenolol	12H04	90.2
Tyloxapol	12H05	97.1
Florfenicol	12H06	121.7
Megestrol acetate	12H07	104.9

Deoxycorticosterone	12H08	87.5
Urosiol	12H09	98.4
Proparacaine hydrochloride	12H10	105.4
Aminocaproic acid	12H11	90.4
Denatonium benzoate	13A02	114.7
Canrenone	13A03	108.5
Enilconazole	13A04	111.4
Methacycline hydrochloride	13A05	90.1
Floxuridine	13A06	72.3
Sotalol hydrochloride	13A07	114.1
Gestrinone	13A08	99.7
Decamethonium bromide	13A09	102.4
3-Acetamidocoumarin	13A10	99.8
Roxarsone	13A11	90.1
Remoxipride Hydrochloride	13B02	115.7
THIP Hydrochloride	13B03	100.4
Pirlindole mesylate	13B04	106.3
Pronethalol hydrochloride	13B05	102.8
Naftopidil dihydrochloride	13B06	107.0
Tracazolate hydrochloride	13B07	108.2
Zardaverine	13B08	98.0
Memantine Hydrochloride	13B09	96.9
Ozagrel hydrochloride	13B10	109.9
Piribedil hydrochloride	13B11	110.9
Nitrocaramiphen hydrochloride	13C02	111.5
Nandrolone	13C03	94.2
Dimaprit dihydrochloride	13C04	114.6
Oxfendazol	13C05	100.8
Guaiacol	13C06	98.8
Proscillaridin A	13C07	54.7
Pramipexole	13C08	131.4
Norgestimate	13C09	100.1
Chlormadinone acetate	13C10	99.9
Phenylbutazone	13C11	103.2
Gliquidone	13D02	95.3
Pizotifen malate	13D03	99.1
Ribavirin	13D04	104.9
Cyclopenthiazide	13D05	110.4
Fluvoxamine maleate	13D06	100.8
Prothionamide	13D07	105.8

Fluticasone propionate	13D08	77.2
Zuclopenthixol hydrochloride	13D09	94.5
Proguanil hydrochloride	13D10	110.5
Lymecycline	13D11	100.8
Alfadolone acetate	13E02	98.0
Alfaxalone	13E03	108.5
Azapropazone	13E04	113.8
Meptazinol hydrochloride	13E05	117.8
Apramycin	13E06	120.0
Epitiostanol	13E07	122.4
Fursultiamine Hydrochloride	13E08	99.6
Gabexate mesilate	13E09	116.1
Pivampicillin	13E10	106.5
Talampicillin hydrochloride	13E11	90.1
Flucloxacillin sodium	13F02	113.7
Trapidil	13F03	129.2
Deptropine citrate	13F04	116.6
Sertraline	13F05	108.6
Ethamsylate	13F06	120.3
Moxonidine	13F07	103.6
Etilefrine hydrochloride	13F08	101.7
Alprostadil	13F09	127.6
Tribenoside	13F10	112.7
Rimexolone	13F11	78.4
Isradipine	13G02	100.8
Tiletamine hydrochloride	13G03	117.0
Isometheptene mucate	13G04	110.7
Nifurtimox	13G05	151.3
Letrozole	13G06	122.6
Arbutin	13G07	116.9
Tocainide hydrochloride	13G08	98.9
Benzathine benzylpenicillin	13G09	100.0
Risperidone	13G10	93.1
Torsemide	13G11	103.0
Halofantrine hydrochloride	13H02	91.2
Articaine hydrochloride	13H03	104.0
Nomegestrol acetate	13H04	99.2
Pancuronium bromide	13H05	122.7
Molindone hydrochloride	13H06	93.3
Alcuronium chloride	13H07	100.6

Zalcitabine	13H08	93.4
Methyldopate hydrochloride	13H09	102.0
Levocabastine hydrochloride	13H10	101.0
Pyrvinium pamoate	13H11	93.7
Etomidate	14A02	117.9
Tridihexethyl chloride	14A03	119.4
Penbutolol sulfate	14A04	103.8
Prednicarbate	14A05	80.1
Sertaconazole nitrate	14A06	136.3
Repaglinide	14A07	104.3
Piretanide	14A08	117.1
Piperacetazine	14A09	110.0
Oxyphenbutazone	14A10	113.6
Quinethazone	14A11	118.1
Moricizine hydrochloride	14B02	104.3
Iopanoic acid	14B03	90.8
Pivmecillinam hydrochloride	14B04	98.1
Levopropoxyphene napsylate	14B05	109.2
Piperidolate hydrochloride	14B06	93.3
Trifluridine	14B07	106.2
Oxprenolol hydrochloride	14B08	105.2
Ondansetron Hydrochloride	14B09	128.5
Propoxycaine hydrochloride	14B10	122.0
Oxaprozin	14B11	101.3
Phensuximide	14C02	104.6
Ioxaglic acid	14C03	118.7
Naftifine hydrochloride	14C04	121.5
Meprylcaine hydrochloride	14C05	111.6
Milrinone	14C06	108.7
Methantheline bromide	14C07	114.3
Ticarcillin sodium	14C08	128.6
Thiethylperazine malate	14C09	98.7
Mesalamine	14C10	115.2
Vorinostat	14C11	85.9
Imidurea	14D02	110.3
Lansoprazole	14D03	91.9
Bethanechol chloride	14D04	114.2
Cyproterone acetate	14D05	117.6
®-Propranolol hydrochloride	14D06	102.4
Ciprofibrate	14D07	112.5

Formestane	14D08	97.7
Benzylpenicillin sodium	14D09	111.0
Chlorambucil	14D10	109.1
Methiazole	14D11	98.4
(S)-propranolol hydrochloride	14E02	116.7
(-)-Eseroline fumarate salt	14E03	97.2
Isosorbide mononitrate	14E04	104.1
Leucomisine	14E05	107.9
Topiramate	14E06	103.3
D-cycloserine	14E07	98.9
2-Chloropyrazine	14E08	107.8
(+,-)-Synephrine	14E09	112.8
(S)-(-)-Cycloserine	14E10	100.5
Homosalate	14E11	100.6
Spaglumic acid	14F02	109.6
Ranolazine	14F03	110.9
Misoprostol	14F04	119.6
Sulfadoxine	14F05	112.3
Cyclopentolate hydrochloride	14F06	104.5
Estriol	14F07	122.2
(-)-Isoproterenol hydrochloride	14F08	117.1
Sarafloxacin	14F09	115.9
Nialamide	14F10	107.0
Toltrazuril	14F11	113.1
Perindopril	14G02	110.2
Fexofenadine HCl	14G03	126.1
4-aminosalicylic acid	14G04	119.8
Clonixin Lysinate	14G05	108.8
Verteporfin	14G06	103.4
Meropenem	14G07	132.1
Ramipril	14G08	124.4
Mephénytoin	14G09	112.4
Rifabutin	14G10	100.8
Parbendazole	14G11	111.3
Mecamylamine hydrochloride	14H02	105.8
Procarbazine hydrochloride	14H03	90.2
Viomycin sulfate	14H04	89.0
Saquinavir mesylate	14H05	96.3
Ronidazole	14H06	93.5
Dorzolamide hydrochloride	14H07	97.5

Azaperone	14H08	101.6
Cefepime hydrochloride	14H09	92.2
Clocortolone pivalate	14H10	102.0
Nadifloxacin	14H11	99.2
Buspirone hydrochloride	15A02	104.8
Anastrozole	15A03	107.7
Doxycycline hydrochloride	15A04	121.1
Sulbactam	15A05	102.3
Fleroxacin	15A06	113.9
Potassium clavulanate	15A07	112.0
Valproic acid	15A08	126.5
Mepivacaine hydrochloride	15A09	107.8
Rifaximin	15A10	117.2
Estradiol Valerate	15A11	114.3
Acetylcysteine	15B02	103.4
Melengestrol acetate	15B03	81.6
Bromhexine hydrochloride	15B04	119.2
Anethole-trithione	15B05	111.7
Amcinonide	15B06	73.4
Caffeine	15B07	104.4
Carvedilol	15B08	105.0
Methenamine	15B09	104.9
Phentermine hydrochloride	15B10	113.8
Diclazuril	15B11	129.4
Famciclovir	15C02	115.2
Dopamine hydrochloride	15C03	118.8
Cefdinir	15C04	113.4
Carprofen	15C05	133.3
Celecoxib	15C06	105.9
Candesartan	15C07	110.9
Fludarabine	15C08	110.9
Cladribine	15C09	72.1
Vardenafil	15C10	109.3
Fluconazole	15C11	114.3
5-fluorouracil	15D02	116.9
Mesna	15D03	119.0
Mitotane	15D04	121.0
Ambrisentan	15D05	105.0
Triclosan	15D06	132.1
Enoxacin	15D07	116.7

Olopatadine hydrochloride	15D08	104.7
Granisetron	15D09	104.4
Anthralin	15D10	121.4
Lamotrigine	15D11	104.0
Clofibrate	15E02	135.2
Cyclophosphamide	15E03	101.9
Aripiprazole	15E04	111.0
Ethinylestradiol	15E05	122.8
Fluocinolone acetonide	15E06	98.7
Sparfloxacin	15E07	117.4
Desloratadine	15E08	112.9
Clarithromycin	15E09	96.6
Tripeleennamine hydrochloride	15E10	131.0
Tulobuterol	15E11	125.1
Topotecan	15F02	93.8
Atorvastatin	15F03	120.0
Azithromycin	15F04	115.9
Ibudilast	15F05	105.7
Losartan	15F06	135.6
Benztropine mesylate	15F07	105.0
Vecuronium bromide	15F08	113.6
Telmisartan	15F09	128.9
Nalmefene hydrochloride	15F10	115.0
Bifonazole	15F11	106.0
Gatifloxacin	15G02	104.2
Bosentan	15G03	101.0
Gemcitabine	15G04	75.6
Olmesartan	15G05	93.6
Racepinephrine HCl	15G06	110.3
Montelukast	15G07	99.5
Docetaxel	15G08	88.7
Cilnidipine	15G09	81.3
Imiquimod	15G10	114.8
Fosinopril	15G11	99.9
Imatinib	15H02	91.9
Moxifloxacin	15H03	109.4
Formoterol fumarate	15H04	109.5
Rufloxacin	15H05	123.2
Pravastatin	15H06	94.9
Rosiglitazone	15H07	94.9

Rivastigmine	15H08	102.6
Sildenafil	15H09	93.1
Acetylsalicylic acid	15H10	86.1
Hexachlorophene	15H11	117.2

^aThe location of the compound in the library is provided in the following format: XX-Y-ZZ, where XX is the plate number, Y is the row and ZZ is the column (e.g. 15H11 is row H and column 11 on library plate number 15).

^bRFP percentage values were calculated by dividing average (n=3) of RFP intensities in drug-treated cells by the average intensity in vehicle-treated cells.

Appendix 3.1:
**The Adenovirus Genome Contributes to the Structural Stability of
the Virion (Review)**

Review

The Adenovirus Genome Contributes to the Structural Stability of the Virion

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Abstract: Adenovirus (Ad) vectors are currently the most commonly used platform for therapeutic gene delivery in human gene therapy clinical trials. Although these vectors are effective, many researchers seek to further improve the safety and efficacy of Ad-based vectors through detailed characterization of basic Ad biology relevant to its function as a vector system. Most Ad vectors are deleted of key, or all, viral protein coding sequences, which functions to not only prevent virus replication but also increase the cloning capacity of the vector for foreign DNA. However, radical modifications to the genome size significantly decreases virion stability, suggesting that the virus genome plays a role in maintaining the physical stability of the Ad virion. Indeed, a similar relationship between genome size and virion stability has been noted for many viruses. This review discusses the impact of the genome size on Ad virion stability and emphasizes the need to consider this aspect of virus biology in Ad-based vector design.

Keywords: Adenovirus; virion stability; viral vectors; helper-dependent Ad

1. Introduction

Human adenovirus (Ad) is a common pathogen that generally causes minor, self-limiting illnesses in most patients [1]. The basic biology of Ad has been extensively studied for over 60 years, and many aspects of its lifecycle have been elucidated in great detail [2]. This knowledge has allowed for the development of replication-defective Ad vectors that are very efficient at delivering genes to mammalian cells for high-level transgene expression. Indeed, Ad has become the most commonly used vector systems for delivering therapeutic genes in human gene therapy clinical trials [3]. Most Ad vectors are deleted of the essential early region 1 (E1), which renders such vectors incapable of replication in most cell lines [4]. Numerous studies have shown that E1-deleted Ad vectors are ideally suited for studies requiring short-term transgene expression *in vitro* and *in vivo* [4]. Unfortunately, low level viral gene expression from the viral sequences retained in E1-deleted Ad contributes to the immune-mediated loss of the vector and transduced cells *in vivo* [5–7], compromising the ability of these vectors to provide long-term transgene expression. Second generation Ad vectors include deletions in the essential E2 or E4 regions, and, in some studies, prolonged transgene expression has been achieved [4]. If a higher cloning capacity is required, fully-deleted or helper-dependent Ad vectors (hdAd) can be generated by removing all viral protein coding sequences [8].

For both E1-deleted Ad and hdAd, removal of some or all viral protein coding sequences is accompanied by an overall reduction in the size of the genome of the virus from 36 kb for the wildtype (AdWT) virus to as small as 30 kb for E1/E3-deleted Ad and, theoretically, ~500 bp for hdAd. These dramatic reductions in the Ad genome length have uncovered a previously unappreciated relationship between vector genome size and virion stability: viruses with genome size below ~90% of the wildtype size have significantly reduced heat stability. Thus, not all Ad vectors are created equal—E1-deleted Ad vectors containing large transgenes, resulting in an overall genome size of greater than ~33 kb, are more stable than vectors containing smaller transgenes. This relationship appears to be a central tenet for several other viruses as well (e.g., bacteriophage lambda, parvovirus). In this review, we discuss our current understanding of the relationship between genome size and virion stability for Ad and other viruses.

2. Adenovirus Biology

Studies of human Adenovirus (Ad) began with its isolation from adenoid tissue and its identification as the cause of some respiratory infections in the early 1950s [9,10]. Human Ad has a remarkable capacity to spread with patients contracting the disease from as few as five virus particles [11]. Ad-induced acute respiratory disease is common in confined populations, such as day care centers, hospitals, retirement homes, and military training venues [12]. Ad accounts for ~8% of all childhood respiratory tract infections, and can lead to bronchitis, bronchiolitis, or pneumonia, requiring hospitalization in ~25% of diagnosed cases [13]. Ads also cause other severe localized diseases, such as colitis, hemorrhagic cystitis, hepatitis, nephritis, encephalitis, myocarditis, and disseminated disease with multiorgan failure [1,14]. Such diseases can be more serious in pediatric and geriatric populations, and in individuals with suppressed immune systems, such as transplant recipients or patients with AIDS.

The discovery that some human Ads are tumorigenic in rodents [15,16] stimulated intensive research into the physiology, genetics, and molecular biology of Ads. These studies, while giving us a great deal of information about DNA replication, control of gene expression, and tumorigenesis [2], also laid the foundations for the later development of Ads as gene transfer vectors. While over 100 serotypes of Ad have been isolated from mammals, birds, reptiles, and amphibians [2], the subgroup C serotypes 2 (Ad2) and 5 (Ad5) have been the most extensively studied. As for many viruses, Ads express viral proteins that alter the cellular environment to promote viral replication. Studies examining Ad infection and lifecycle have uncovered new information regarding host cell DNA replication, mRNA splicing, tumourigenesis, and control of cell cycle progression [17]. For example, alternative splicing, a ubiquitous process in mammalian cells, was first identified in Ad [18,19]. Similarly, association of Ad proteins, such as E1A, with cellular components has led to the discovery or elucidation of the function of these host proteins. One prominent example is the discovery of a 300 kDa protein that binds to E1A: EP300 (E1A-binding protein, 300-kDa), also known as p300 [20].

2.1. The Ad Genome

The genome of Ad2 was the first to be fully sequenced, and it, as well as the Ad5 genome, is approximately 36 kb and encodes over 40 proteins [17]. Adenoviral coding regions are designated early or late depending on when they are expressed (*i.e.*, before or after DNA replication) (Figure 1) [21]. The early regions E1A, E1B, E2, E3, and E4 are the first regions transcribed and encode proteins involved in activating transcription of other viral regions and altering the cellular environment to promote viral production. The E1A proteins induce mitogenic activity in the host cell and stimulate expression of other viral genes [17]. The E2 proteins mediate viral DNA replication, while E3 and E4 proteins alter host immune responses and cell signaling, respectively [22,23]. Activation of the major late promoter (MLP) following the start of virus DNA synthesis allows expression of the late genes encoding primarily virion structural proteins. The late regions (L1–L5) are transcribed from an alternatively spliced transcript. Recently, it was shown that the regions encoding the L4-22K and L4-33K proteins are initially expressed at low levels from a novel promoter located within the L4 region [24], and these proteins act to fully activate the MLP [25]. There are also four small products produced at intermediate/late times of infection, including the structural protein IX (pIX), and the IVa2 protein that helps package viral DNA into immature virions [26]. The late products, VA RNA I and II, inhibit activation of the interferon response, impede cellular micro-RNA processing, and may influence expression of host genes [27,28]. Located on both ends of the genome are the 100 bp inverted terminal repeats (ITRs), which act as the origin of replication, with the ~200 bp viral packaging sequence positioned next to the left ITR. This genome arrangement is common among Ad species [21].

Even though Ad has been studied in great detail for more than half a century, our knowledge of genes encoded by the virus is still expanding. In 2007, Tollefson *et al.* [29] identified a new open reading frame (ORF) located between the fiber ORF and E3, and termed it U exon. The U exon protein (UXP) is expressed from a unique promoter during late stages of infection, and may play a role in virus DNA replication or RNA transcription [25,29]. Similarly, a recent study using deep cDNA sequencing identified many new alternatively spliced transcripts originating from the Ad genome [30], suggesting

that there may be numerous other new or altered polypeptides produced by Ad in the infected cell. Thus, the Ad genome may still have many secrets that remain to be uncovered.

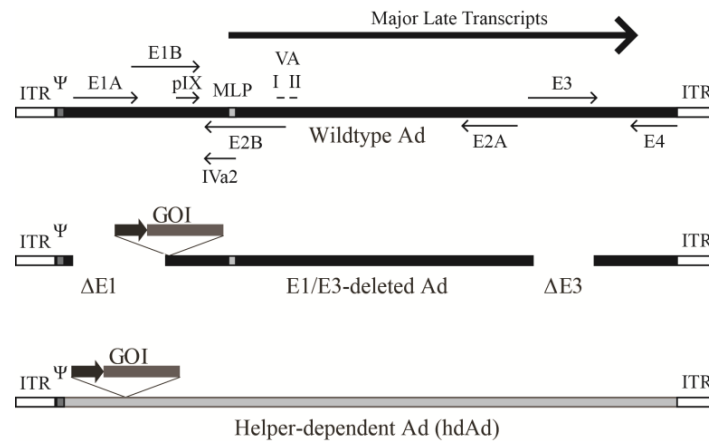


Figure 1. Schematic representation of the adenovirus genome and various adenoviral vectors. (**Top panel**) The Ad5 genome is depicted (not to scale). The early regions are noted as E1–E4 along with the full-length major late transcript from which the L1–L5 transcripts are produced through alternative splicing. Transcripts encoding pIX, IVa2, and VA RNA I and II are also shown. ITR represents the inverted terminal repeats that serve as origins of replication and Ψ is the packaging sequence. The location of the major late promoter is denoted as MLP. Middle panel: A typical early region (E1) deleted Ad vector is shown. The gene of interest (GOI) normally replaces the E1 deletion and its expression is driven by a heterologous promoter (dark arrow). Most of these vectors also have the E3 region removed as it is not essential for viral replication and allows for insertion of a ~8 kb foreign expression cassette. (**Bottom panel**) Helper-dependent Ad schematic. All the DNA encoding viral proteins are removed. Only the ITRs and packaging sequence are required. These vectors usually have non-coding stuffer DNA (grey) to ensure the genome is stable. Adapted from [31].

2.2. The Ad Virion

Ad virions can come in four forms, differing in the quantity of DNA contained in the capsid and whether specific capsid proteins have been processed by the Ad-encoded protease [32,33]. However, only one of these forms, mature virions, are fully infectious. Empty capsids contain no DNA and have the lightest density on a cesium chloride gradient. Light intermediate virions contain only the left end of the Ad genome, whereas heavy intermediates contain the entire genome, but have unprocessed capsid proteins. Mature virions contain the entire genome and have proteolytically processed capsid proteins (IIIa, pVI, pVII, pVIII, pX, pTP, and L1 52/55K). The Ad DNA genome appears to be an important co-factor for conversion of heavy intermediates to mature virions, as treatment of disrupted virions with DNase abolishes protease activity, but this activity can be reactivated through addition of exogenous DNA [34]. This cofactor requirement appears to be simply for a high density negative charge, rather than a specific need for Ad DNA, since any DNA, or even ssDNA or poly-glutamic acid, can function as a cofactor. Thus, the Ad DNA plays an active role in maturation of the Ad virion.

The general structure of the mature Ad virion is conserved among different Ad species. The main “body” of the virion consists of a non-enveloped icosahedral capsid with a diameter of ~70–100 nm surrounding the DNA-protein core (Figure 2). The fiber protein that extends from each vertex of the icosahedron can increase the overall diameter of the virus by more than two-fold. The Ad5 capsid is composed of three major proteins (II, III, and IV) and five minor proteins (Figure 2) [26,35–38]. Trimers of polypeptide II, called hexons, form the triangular facets of the capsid and contribute ~80% of the capsid mass [39]. Pentons are pentamers of polypeptide III that cap the vertices and serve as the base from which fiber (trimers of protein IV) extend outwards. The five minor polypeptides (IIIa, IVa2, VI, VIII, and IX) act to stabilize the capsid or connect the outer capsid to the nucleoprotein core [39]. Within the virion, the viral DNA is bound to the basic proteins VII, V, and Mu [40–42]. Protamine-like protein VII condenses and compacts the DNA, forming a central dense core and facilitates DNA packaging within the physical constraints of the capsid [43]. Parts of the protein-wrapped DNA form spherical structures, termed adenosomes, that are pushed into the vertex regions of the inner capsid [44]. Protein V is believed to form a shell surrounding the protein VII-DNA complex and serves as a key linkage protein between the DNA core and inner capsid [44–47]. Protein V is reported to interact directly with penton protein [48,49]. Protein V also interacts indirectly with penton and peripentonal hexon through protein IIIa, and again indirectly with the remainder of hexon through protein VI [36,37,48,50–52]. A very recent refinement of the position of some the minor Ad capsid proteins has suggested that protein V interacts with both protein VI and VIII on the interior of the capsid (underneath the vertex regions), and that protein IIIa is located largely on the exterior of the capsid [38]. Interestingly, protein V is only found in the genus Mastadenoviruses (which includes human, simian, porcine and canine adenoviruses, amongst others), but not in Aviadenovirus, Atadenovirus and Siadenovirus [21]. Pre-mu is a 79 amino acid protein that may also be involved in compacting the viral DNA [53]. Cleavage of pre-mu by the Ad protease is speculated to relax the viral DNA in preparation for delivery to the nucleus [53,54]. Overall, the nucleoprotein core does not have an ordered structure that is visible in cryo-EM images [55], unlike bacteriophage T4 and lambda or Herpes virus [55–57], which has prevented elucidation of its true structure and conclusive identification of all points of contact between the core and inner capsid.

2.3. Packaging of Ad DNA into the Capsid

The process and proteins involved in mediating Ad DNA packaging into the capsid are beginning to be elucidated. As mentioned, the packaging sequence contained in the Ad DNA is approximately 200 bp and is located immediately adjacent to the left inverted terminal repeat (ITR) and before the E1 coding sequence [58]. Its placement at the left end of the genome leads to Ad DNA being encapsidated in a polar fashion, and virions that have packaged only subgenomic DNA fragments only contain left-end sequences [59]. However, the packaging sequence can function equally well when transposed to the right end of the Ad genome [58], and, for such a virus, the polar packaging is reversed. Ad vectors have been created that contain packaging sequences at both ends of the genome [60–62], which arguably may enhance the efficiency of vector DNA packaging, as it can initiate at either end. The packaging sequence becomes non-functional when moved more than ~300 bp internal from the end of the genome [58].

The packaging sequence contains seven functionally redundant elements referred to as A repeats (TTTGN8CG) [63,64]. The virus-encoded IVa2 protein binds the CG motif of the A repeat either as a homodimer, or in complex with the L4 22K protein, which itself binds the TTTG motif in the A repeat [65,66]. L4 22K must form a complex with IVa2 in order to successfully bind the packaging sequence [67,68]. The L4 33K protein, which shares amino-terminal homology with the L4 22 K protein, has also been found bound to the DNA packaging sequence, but again only in the presence of IVa2 [69]. Other proteins involved in the DNA packaging process include L1-52/55K [70–72] and IIIa [73]. Loss or mutation of any of these proteins results in an inability to form mature virions. However, such viruses are still capable of forming empty capsids which lack any viral DNA, suggesting that capsids are preformed and do not “nucleate” on the packaging sequence [70,71]. Indeed several groups have shown that the packaging sequence can be entirely deleted from the Ad DNA and significant amounts of empty capsid are still formed [74–76]. Thus, it is believed that the Ad capsid forms first within the nucleus and the Ad DNA is subsequently spooled into the preformed capsids [77]. IVa2 may be part of a portal structure, and act as the ATPase-dependent motor that facilitates spooling of the Ad DNA into the preformed capsids [26,78]. Consistent with this idea, the IVa2 protein is found at only one vertex within the mature Ad virion [26].

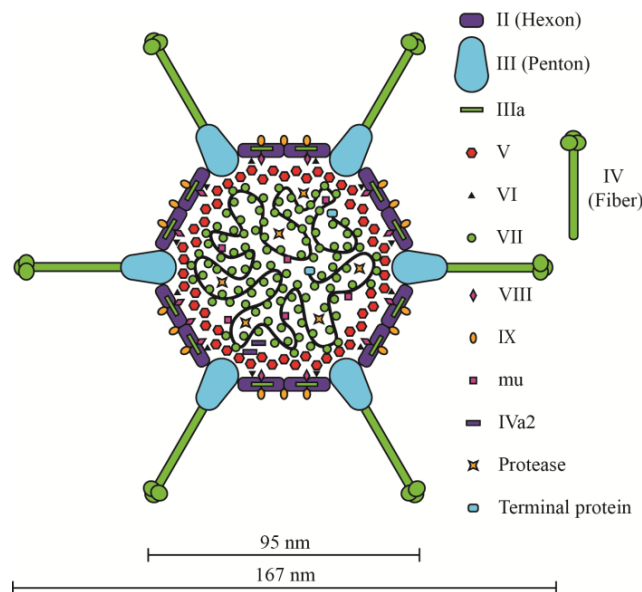


Figure 2. Model of the Ad5 virion, based on data provided in [26,35–38]. Adapted from [79].

2.4. Adenovirus Infection

Ad5 entry into host cells is initiated by the binding of fiber protein to the coxsackie and adenovirus receptor (CAR), which is expressed on the surface of many cell types [80,81]. Ad5 can also use heparin sulfate glycosaminoglycans (HSGs) on the cell surface for infection [82]. This process can be achieved through either direct binding of fiber to HSG or through a bridging interaction mediated by binding of Ad hexon to various blood factors or the complement component C4-binding protein [83–85]. An arginine-glycine-aspartic acid (RGD) motif in penton protein then interacts with membrane $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins to induce Ad internalization via receptor-mediated endocytosis into clathrin-coated

vesicles, which eventually form endosomes [86]. The virus evades degradation by lysing the endosomal membrane and escaping from the early endosome [87,88]. The virion then migrates to the nucleus by travelling along microtubules [87], and the capsid proteins are dismantled in a step-wise manner during transport [89]. The capsid proteins are degraded in the cytoplasm while the protein VII-viral DNA complex makes its way into the nucleus through the nuclear pore [42,89,90]. Entry into the nucleus is followed by early gene expression, viral DNA replication, late gene expression, and assembly of progeny virions [2]. During at least the early stage of viral gene expression, AdWT (and Ad vector) DNA uses cellular histones to form DNA structures similar to cellular chromatin, which appears necessary for efficient viral gene expression [31,79,91–93].

3. The Ad Genome Size and Virion Stability

3.1. Early Region 1-Deleted Ad Vectors

Ad-based vectors are one of the most commonly used vehicles for delivery of transgenes into mammalian cells in research laboratories worldwide, and in human gene therapy clinical trials [3]. As such, several studies have explored their cloning capacity for foreign DNA and how this impacts upon the function and stability of the vector. Although most of the following information is gleaned from studies of human Ad serotype C, given the overall similarity between human and other Ads, it is likely (but not certain) that these principles hold true to all Ads in general. Studies have shown that the Ad capsid can accommodate up to ~108% of the wildtype genome length [94,95]. However, such vectors tend to be unstable and undergo spontaneous genome rearrangement, usually resulting in a loss of the encoded transgene and a reduction in the overall genome size to closer to wildtype length [94]. Vectors with an overall genome size of ~105% of the wildtype genome length tend to be stable, resulting in a functional cloning capacity of ~8 kb in E1/E3-deleted Ad vectors [94] (Figure 2). On the other end of the spectrum, E1/E3-deleted Ads encoding expression cassettes for a fluorescent protein or short-hairpin RNA may have a genome size much smaller than wildtype Ad, in the range of 30–32 kb, and these genomes are relatively stable (*i.e.*, no evident selection for rearrangement) [96,97]. Thus, E1/E3-deleted Ad vectors can vary greatly in their overall genome size from as small as 30 kb to over 38 kb. These E1-deleted vectors are grown in E1-complementing cell lines, such as the extensively used 293 cell line [98].

3.2. Helper-Dependent Ad Vectors

The latest generation of Ad vectors, hdAds, do not encode any viral proteins and only need the packaging signal for DNA packaging into the capsid and the ITRs to allow for genome replication [99] (Figure 2). Thus, hdAd vectors allow for almost 38 kb in cloning capacity. Since hdAd are devoid of most if not all viral genes, they lack the ability to replicate autonomously and typically rely on an accompanying “helper” virus to provide replicative and packaging functions *in trans*. Early systems used to generate hdAd vectors relied on either wildtype or E1-deleted helper viruses to provide these necessary functions [100–105]. As cesium chloride density gradient-based purification separates virions based on the genome length (which directly affects the density of the virion [61]), the hdAd could be partially purified away from the helper virus by centrifugation. Interestingly, hdAd with very

small genome sizes tended to undergo spontaneous rearrangement to increase the length of the genome to closer to wildtype size [101,102], an early suggestion that there was an optimal genome size for Ad DNA packaging.

An alternative system for generating Ad vectors deleted of most viral protein coding sequences involved the use of an E1-deleted Ad containing one loxP site, a target for the bacteriophage P1 Cre recombinase, located immediately down-stream from the transgene cassette in the E1 region and a second site inserted within the E3 region [106]. In the presence of Cre, the loxP sites were recombined, excising the Ad coding sequences located between the two loxP sites, and leaving a genome of approximately 9 kb. The E4 coding region was retained in these vectors. In this system, the unrecombined vector itself acts as the complementing helper. Interestingly, the protein content of the virions containing the 9 kb genome was quite aberrant, with many extra protein bands which were postulated to be nonspecific degradation products.

The current system for generating hdAd involves the use of an E1/E3-deleted helper virus that contains a packaging sequence flanked by Cre or Flp recombinase recognition sites and a 293-based cell line that expresses the corresponding recombinase [99,107–110]. This combination results in a helper virus that can replicate and provide all the trans-acting functions necessary for replication and packaging of the hdAd, but the helper virus is deficient in its ability to package its own DNA due to Cre- or Flp-mediated removal of the viral packaging sequence. Additional improvements to the original Cre/loxP system for hdAd preparation have involved modifying the packaging sequence to reduce the chances of recombination between the helper virus and the hdAd genomes, which could result in loss of one of the loxP sites and outgrowth of the modified helper virus [111,112].

Early during the development and characterization of hdAd, we, and others, showed that hdAd vectors with genomes below ~27 kb in size spontaneously rearrange their DNA to increase the genome size [61,101,102]. Indeed, this property can be used to design hdAd vectors containing two (or more) copies of a desired transgene, as a vector constructed to be 15 kb in size will be packaged as a mix of direct and inverted dimers, effectively giving a vector genome size of 30 kb [61,62,113]. However, most hdAd are constructed of a size greater than at least 27 kb to promote stable genome structure, frequently through the inclusion of non-coding “stuffer” DNA [114,115]. For example, a 5 kb expression cassette would require ~22 kb of stuffer DNA to reach the 27 kb genome size minimal limit. Importantly, the nature of the stuffer DNA can significantly affect vector function. An hdAd containing 22 kb of eukaryotic stuffer DNA expressed its transgene ~10-fold higher *in vitro* and *in vivo* compared to a vector with an identical expression cassette but containing stuffer DNA derived from prokaryotic DNA [114]. Similar results have been observed by others [116]. Subsequent studies showed that the prokaryotic stuffer DNA was subjected to epigenetic regulation which down-regulated expression of the associated transgene [115]. Nevertheless, when constructed optimally, hdAd are associated with a high transduction efficiency and low toxicity, and can provide long-term and high levels of transgene expression in a variety of species [4,8,117,118].

3.3. Virion Instability of Ad Vectors

In an attempt to devise a new method for hdAd production that results in vector of greater purity, we designed a novel approach which takes advantage of the DNA packaging restrictions imposed on

Ad capsids deficient in protein IX (pIX) [119]. pIX is a minor capsid protein that assists in holding the facets of the virion together. Capsids deficient in pIX are believed to accommodate a maximum of 35 kb of DNA, and thus cannot package a full-sized AdWT genome [120,121]. pIX-deficient virions are also heat labile [122]. We hypothesized that a helper virus with a genome greater than 35 kb could be grown in 293 cells expressing pIX while in normal 293 cells, which encode but do not naturally express pIX [123,124], the helper virus would be unable to produce viable virions due to the inability to package its own large genome into pIX-deficient capsids. As a result, hdAd with a genome smaller than 35 kb (e.g., 29.6 kb [119]) would be selectively packaged during hdAd propagation. This new system functioned largely as expected [119]. Although the hdAd generated with this system were heat labile, as expected since the virions were deficient in pIX, this effect appeared to be independent of pIX, as hdAd virions with an identical DNA genome and containing pIX in the virion showed similar heat sensitivity [125]. Interestingly, heat stability could be rescued simply by increasing the size of the hdAd genome from 29.6 kb to ~34 kb. Similar results were obtained when E1/E3-deleted Ad vectors were used. E1/E3-deleted Ad with a genome larger than ~33 kb were just as stable as AdWT while those smaller than ~32 kb showed increased instability as genomes decreased in size (Figure 3) [125]. While capsid morphology and protein content did not differ between the various Ad vectors, the capsids of Ads that had smaller genomes quickly fell apart when heated [125]. Specifically, heating resulted in a release of the vertex proteins (fiber, penton, and, likely, the peripentonal hexon), followed by complete disintegration of the capsid structure. This suggests that the biophysical connections, which normally hold these proteins in place, are compromised when the genome is reduced in size. Thus, the Ad genome size contributes to the physical stability of Ad.

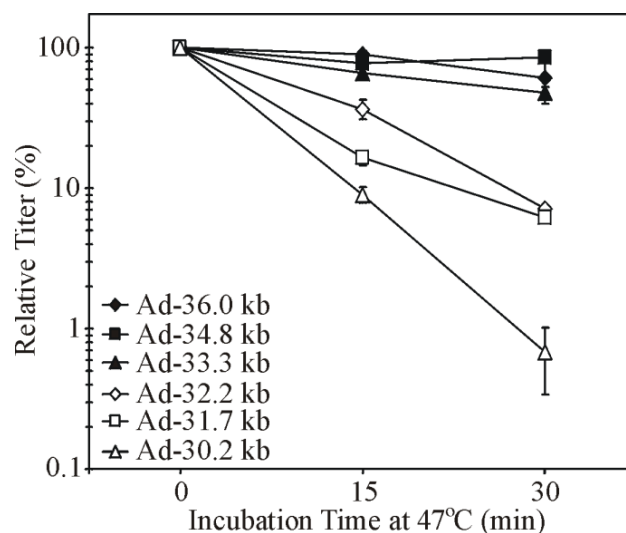


Figure 3. Ad genome size affects Ad heat stability. E1/E3-deleted Ad vectors with genome size ranging from 30.2 to 34.8 kb and wildtype Ad (AdWT) were heated at 47 °C over a timecourse of 30 min. The viral titer was determined at each timepoint to assess virion stability. Viruses with genomes over ~33.3 kb were as stable as the 36 kb AdWT. Viruses with smaller genomes exhibited increased instability as genome sizes decreased. Adapted from [125,126].

The mechanism by which Ad DNA contributes to virion stability is not yet fully elucidated. In the virion, the nucleoprotein core extends into the vertex regions of the inner capsid, and makes direct contact with penton and peripentonal hexon. This region is destabilized during virus endocytosis at the early stages of infection. Binding of Ad penton to membrane integrins may cause conformational changes in the peripentonal capsid region [89], resulting in liberation of protein VI which functions to lyse the endosomal membrane [88]. Since timely removal of the vertex proteins is crucial for efficient infection, this process may be highly regulated and require proper positioning of all viral proteins involved. Packaging of the tightly condensed full-length genome into the capsid may position the DNA correctly within the vertex regions to allow the proper protein chain of connections to stabilize the vertex proteins. Reducing the size of the genome may affect correct positioning and prevent the interaction of these proteins, ultimately destabilizing the capsid.

The mechanism of virion instability may also vary depending on the size of the DNA. Virions with very small genomes (~9 kb) show aberrant protein content [106,127], suggesting that the small genome may affect proper maturation of the virion. As the Ad DNA acts as a cofactor for activation of the Ad protease [34], it is possible that the small genome is not capable of efficiently activating the protease, or loose packaging of the viral DNA may affect the ability of an active protease to interact with its substrate. For vectors with genomes of ~29 kb, there was no obvious defect in protein content [125], suggesting the protease was capable of efficiently mediating virion maturation. A recent study by Benevento *et al.* [128] used quantitative proteomics to characterize the protein composition of Ad virus, and also the effect of thermal denaturation on virion disassembly. It is likely that a similar approach could be used to delve into the mechanism by which the Ad genome affects virion stability.

4. Studies of Genome Size and Virion Stability in Other Viruses

The relationship between genome size and capsid stability has been studied in other viruses as well. For bacteriophage lambda, another icosahedral virus, genomes smaller than 78% of the wildtype length show reduced infectivity while genomes larger than 106% are not packaged [129]. The tight packaging of bacteriophage DNA into the capsid causes significant electrostatic repulsion, which results in an increase in internal pressure [129–131]. This internal pressure is not only used to inject the phage DNA into bacteria during the infection process, but also directly influences capsid strength and stability. A previous study by Ivanovska *et al.* [129] observed that phages with smaller genomes were half as strong as those with full length genomes, as measured by atomic force microscopy. Thus, the pressure exerted by the packaged DNA makes the capsid physically stronger. In terms of Ad, increased capsid pressure due to electrostatic interactions are unlikely to play a role in capsid stability since the basic proteins VII, V, and μ likely function to neutralize these charges in the packaged DNA. However, atomic force microscopy measurements of the “strength” of Ad virions containing varying lengths of DNA could be very informative [132,133].

Studies with other viruses, such as the single-stranded DNA (ssDNA) mouse parvovirus minute virus (MVM), also show that the genome is important in capsid stability. When the capsid strength of empty and wildtype MVM capsids were compared, those containing DNA were stronger by 140% [134]. MVM DNA binds to 60 concavities of the inner capsid and interacts with the VP2 capsid protein to increase capsid thickness, strength and stability [134]. Mutation of key amino acid residues that

mediate the DNA-VP2 interaction reduced virus infectivity, heat stability, and virion rigidity [135,136]. Similar observations and mechanism have been proposed for genome-mediated capsid stabilization of the cowpea chlorotic mottle virus (CCMV) and bean pod mottle virus (BPMV) [137–139].

An inverse relationship between genome size and capsid stability was recently observed in adeno-associated viruses (AAVs). AAVs are small, non-enveloped, ssDNA viruses that also belong to the *Parvoviridae* family. Horowitz *et al.* studied the effects of increasing heat on AAV capsids containing genomes varying from 72% to 123% of the wild-type length [140]. AAVs with smaller genomes required higher temperatures to induce capsid dissociation compared to wildtype AAV. These researchers suggested that packaging of sub-genomic DNA resulted in reduced internal pressure, which lessened the outward-directed pressures on the capsid, essentially increasing the stability. Several researchers have examined the ability of AAV capsids to accommodate large genomes, after an initial report suggested that very large ssDNA genomes could be found in virions (~180% of the wildtype genome length [141]). These three follow-up studies clearly showed that AAV has an upper limit of ssDNA packaging of ~5.2 kb, and that attempts to package larger genomes simply resulted in the packaging of sub-genomic fragments, which was associated with a dramatic decrease in vector recovery [142–144]. Apparent packaging of larger genomes was likely the result of recombination between these subgenomic fragments in transduced cells, resulting in restoration of the intact large genome. The observation that attempts to package very large genomes is accompanied by a significant drop in virus recovery once again illustrates the importance of optimal genome size in vector design.

Thus, many icosahedral viruses have a very strong preference for genome size, with an associated disruption in virus stability and viability when this preferred size is dramatically altered. The mechanism by which the nucleic acid contributes to capsid stability likely depends on the virus and the nature of genetic material (*i.e.*, dsDNA, ssDNA, or RNA).

5. Implications for Vector Design

The relationship between the genome size and virion stability is important to consider when designing and cloning novel vectors. As shown in Figure 3 [125], a ~32 kb Ad vector is less stable than a ~35 kb vector. However, how this innate instability impacts vector function is not completely clear. Ad vectors deficient in pIX have a similar virion instability as vectors with small genomes, and pIX-deficient vectors reportedly have an enhanced ability to activate peripheral blood mononuclear cells [145]. Thus, unstable Ad vectors may have a greater tendency to elicit anti-vector immune responses. If capsids of Ad vectors with small genomes undergo significant disassembly in the early endosome, due to this inherent instability, the vector DNA may be exposed to pathogen recognition receptors, such as toll-like receptor 9 (TLR9), which can recognize the high CpG content of Ad DNA and initiate innate immune response signaling cascades [146,147]. Moreover, if vectors with small genomes have a slight defect in their ability to escape the early endosome, due to inappropriate release of the endosomal disrupting protein VI [88], enhanced innate immune activation can also occur due to transit of the vector into the late endosome [148,149]. A similar phenomenon could occur for AAV vectors with small genomes, as their enhanced stability could alter their intracellular trafficking and engagement of cellular immune pattern recognition receptors. Indeed, it has been suggested that

non-coding “stuffer” DNA could be incorporated into the AAV vector DNA to increase the size of the genome closer to wildtype size to preserve natural vector stability and function [140]. As such, the overall genome size is important to consider in the design of vectors derived from a number of different viruses.

6. Conclusions

Viruses have been under selective pressure for millions of years, and have optimized the design of their genome to promote rapid and efficient expression of viral genes within the infected host cell. It is clear that these same evolutionary pressures have optimized the relationship between the genome size and the capsid that is responsible for delivering the viral genetic material to the host. For adenovirus, we, and others, have shown that genomes below 75% or above 105% of the wildtype genome length tend to rearrange to achieve a size closer to wildtype length. However, although genome stability can be achieved at 75% of the wildtype genome length [61], virion stability is only achieved with a genome size of ~90% of the wildtype genome length. Many other viruses show a similar dependence between genome size and capsid stability, suggesting this is a general property for many viruses.

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Author Contributions

B.S., C.M.W. and R.J.P. wrote the article.

Conflicts of Interest

The authors declare no conflict of interest.

References and Notes

1. Wold, W.S.; Horwitz, M.S. Adenoviruses. In *Fields Virology*, 5th ed.; Knipe, D.M., Howley, P.M., Eds.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2007; pp. 2396–2436.
2. Berk, A.J. Adenoviridae: The Viruses and Their Replication. In *Fields Virology*, 5th ed.; Knipe, D.M., Howley, P.M., Eds.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2007; pp. 2355–2394.
3. Ginn, S.L.; Alexander, I.E.; Edelstein, M.L.; Abedi, M.R.; Wixon, J. Gene therapy clinical trials worldwide to 2012—An update. *J. Gene Med.* **2013**, *15*, 65–77.
4. Amalfitano, A.; Parks, R.J. Separating fact from fiction: Assessing the potential of modified adenovirus vectors for use in human gene therapy. *Curr. Gene Ther.* **2002**, *2*, 111–133.

5. Yang, Y.; Jooss, K.U.; Su, Q.; Ertl, H.C.; Wilson, J.M. Immune responses to viral antigens *versus* transgene product in the elimination of recombinant adenovirus-infected hepatocytes *in vivo*. *Gene Ther.* **1996**, *3*, 137–144.
6. Yang, Y.; Li, Q.; Ertl, H.C.; Wilson, J.M. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **1995**, *69*, 2004–2015.
7. Yang, Y.; Nunes, F.A.; Berencsi, K.; Furth, E.E.; Gonczol, E.; Wilson, J.M. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 4407–4411.
8. Palmer, D.J.; Ng, P. Helper-dependent adenoviral vectors for gene therapy. *Hum. Gene Ther.* **2005**, *16*, 1–16.
9. Rowe, W.P.; Huebner, R.J.; Gilmore, L.K.; Parrott, R.H.; Ward, T.G. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med.* **1953**, *84*, 570–573.
10. Hilleman, M.R.; Werner, J.H. Recovery of new agents from patients with acute respiratory illness. *Proc. Soc. Exp. Biol. Med.* **1954**, *85*, 183–188.
11. Musher, D.M. How contagious are common respiratory tract infections? *N. Engl. J. Med.* **2003**, *348*, 1256–1266.
12. Hoke, C.H., Jr.; Snyder, C.E., Jr. History of the restoration of adenovirus type 4 and type 7 vaccine, live oral (Adenovirus Vaccine) in the context of the Department of Defense acquisition system. *Vaccine* **2013**, *31*, 1623–1632.
13. Rocholl, C.; Gerber, K.; Daly, J.; Pavia, A.T.; Byington, C.L. Adenoviral infections in children: The impact of rapid diagnosis. *Pediatrics* **2004**, *113*, e51–e56.
14. Horwitz, M.A. Adenoviruses. In *Fields Virology*; Fields, B.N., Knipe, D.M., Howley, P.M., Eds.; Lippincott-Raven Publishers: Philadelphia, PA, USA, 1996; pp. 2149–2171.
15. Trentin, J.J.; Yabe, Y.; Taylor, G. The quest for human cancer viruses. *Science* **1962**, *137*, 835–841.
16. Yabe, Y.; Trentin, J.J.; Taylor, G. Cancer induction in hamsters by human type 12 adenovirus. Effect of age and of virus dose. *Proc. Soc. Exp. Biol. Med.* **1962**, *111*, 343–344.
17. Flint, J. Organization of the adenoviral genome. In *Adenoviruses: Basic Biology to Gene Therapy*; Seth, P., Ed.; R.G. Landes Company: Austin, TX, USA, 1999; pp. 17–30.
18. Berget, S.M.; Moore, C.; Sharp, P.A. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 3171–3175.
19. Chow, L.T.; Gelinas, R.E.; Broker, T.R.; Roberts, R.J. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell* **1977**, *12*, 1–8.
20. Harlow, E.; Whyte, P.; Franza, B.R., Jr.; Schley, C. Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* **1986**, *6*, 1579–1589.
21. Davison, A.J.; Benko, M.; Harrach, B. Genetic content and evolution of adenoviruses. *J. Gen. Virol.* **2003**, *84*, 2895–2908.
22. Horwitz, M.S. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. *J. Gene Med.* **2004**, *6* (Suppl. S1), S172–S183.

23. Weitzman, M.D. Functions of the adenovirus E4 proteins and their impact on viral vectors. *Front. Biosci.* **2005**, *10*, 1106–1117.
24. Morris, S.J.; Scott, G.E.; Leppard, K.N. Adenovirus late-phase infection is controlled by a novel L4 promoter. *J. Virol.* **2010**, *84*, 7096–7104.
25. Ying, B.; Tollefson, A.E.; Wold, W.S. Identification of a previously unrecognized promoter that drives expression of the UXP transcription unit in the human adenovirus type 5 genome. *J. Virol.* **2010**, *84*, 11470–11478.
26. Christensen, J.B.; Byrd, S.A.; Walker, A.K.; Strahler, J.R.; Andrews, P.C.; Imperiale, M.J. Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. *J. Virol.* **2008**, *82*, 9086–9093.
27. O'Malley, R.P.; Mariano, T.M.; Siekierka, J.; Mathews, M.B. A mechanism for the control of protein synthesis by adenovirus VA RNAI. *Cell* **1986**, *44*, 391–400.
28. Aparicio, O.; Razquin, N.; Zaratiegui, M.; Narvaiza, I.; Fortes, P. Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *J. Virol.* **2006**, *80*, 1376–1384.
29. Tollefson, A.E.; Ying, B.; Doronin, K.; Sidor, P.D.; Wold, W.S. Identification of a new human adenovirus protein encoded by a novel late l-strand transcription unit. *J. Virol.* **2007**, *81*, 12918–12926.
30. Zhao, H.; Chen, M.; Pettersson, U. A new look at adenovirus splicing. *Virology* **2014**, *456–457*, 329–341.
31. Wong, C.M.; McFall, E.R.; Burns, J.K.; Parks, R.J. The role of chromatin in adenoviral vector function. *Viruses* **2013**, *5*, 1500–1515.
32. Ostapchuk, P.; Hearing, P. Control of adenovirus packaging. *J. Cell. Biochem.* **2005**, *96*, 25–35.
33. Guimet, D.; Hearing, P. The adenovirus L4–22K protein has distinct functions in the posttranscriptional regulation of gene expression and encapsidation of the viral genome. *J. Virol.* **2013**, *87*, 7688–7699.
34. Mangel, W.F.; McGrath, W.J.; Toledo, D.L.; Anderson, C.W. Viral DNA and a viral peptide can act as cofactors of adenovirus virion proteinase activity. *Nature* **1993**, *361*, 274–275.
35. Russell, W.C. Adenoviruses: Update on structure and function. *J. Gen. Virol.* **2009**, *90*, 1–20.
36. Liu, H.; Jin, L.; Koh, S.B.; Atanasov, I.; Schein, S.; Wu, L.; Zhou, Z.H. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* **2010**, *329*, 1038–1043.
37. Reddy, V.S.; Natchiar, S.K.; Stewart, P.L.; Nemerow, G.R. Crystal structure of human adenovirus at 3.5 Å resolution. *Science* **2010**, *329*, 1071–1075.
38. Reddy, V.S.; Nemerow, G.R. Structures and organization of adenovirus cement proteins provide insights into the role of capsid maturation in virus entry and infection. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 11715–11720.
39. Rux, J.J.; Burnett, R.M. Adenovirus structure. *Hum. Gene Ther.* **2004**, *15*, 1167–1176.
40. Maizel, J.V., Jr.; White, D.O.; Scharff, M.D. The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. *Virology* **1968**, *36*, 126–136.

41. Russell, W.C.; Laver, W.G.; Sanderson, P.J. Internal components of adenovirus. *Nature* **1968**, *219*, 1127–1130.
42. Chatterjee, P.K.; Vayda, M.E.; Flint, S.J. Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32P-labelled *in vivo*. *J. Mol. Biol.* **1986**, *188*, 23–37.
43. Mirza, M.A.; Weber, J. Structure of adenovirus chromatin. *Biochim. Biophys. Acta* **1982**, *696*, 76–86.
44. Newcomb, W.W.; Boring, J.W.; Brown, J.C. Ion etching of human adenovirus 2: Structure of the core. *J. Virol.* **1984**, *51*, 52–56.
45. Everitt, E.; Sundquist, B.; Pettersson, U.; Philipson, L. Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. *Virology* **1973**, *52*, 130–147.
46. Brown, D.T.; Westphal, M.; Burlingham, B.T.; Winterhoff, U.; Doerfler, W. Structure and composition of the adenovirus type 2 core. *J. Virol.* **1975**, *16*, 366–387.
47. Perez-Vargas, J.; Vaughan, R.C.; Houser, C.; Hastie, K.M.; Kao, C.C.; Nemerow, G.R. Isolation and characterization of the DNA and protein binding activities of adenovirus core protein V. *J. Virol.* **2014**, *88*, 9287–9296.
48. Everitt, E.; Lutter, L.; Philipson, L. Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology* **1975**, *67*, 197–208.
49. Fabry, C.M.; Rosa-Calatrava, M.; Moriscot, C.; Ruigrok, R.W.; Boulanger, P.; Schoehn, G. The C-terminal domains of adenovirus serotype 5 protein IX assemble into an antiparallel structure on the facets of the capsid. *J. Virol.* **2009**, *83*, 1135–1139.
50. Chatterjee, P.K.; Vayda, M.E.; Flint, S.J. Interactions among the three adenovirus core proteins. *J. Virol.* **1985**, *55*, 379–386.
51. Stewart, P.L.; Fuller, S.D.; Burnett, R.M. Difference imaging of adenovirus: Bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.* **1993**, *12*, 2589–2599.
52. Saban, S.D.; Silvestry, M.; Nemerow, G.R.; Stewart, P.L. Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. *J. Virol.* **2006**, *80*, 12049–12059.
53. Anderson, C.W.; Young, M.E.; Flint, S.J. Characterization of the adenovirus 2 virion protein, mu. *Virology* **1989**, *172*, 506–512.
54. Perez-Berna, A.J.; Marabini, R.; Scheres, S.H.; Menendez-Conejero, R.; Dmitriev, I.P.; Curiel, D.T.; Mangel, W.F.; Flint, S.J.; San Martin, C. Structure and uncoating of immature adenovirus. *J. Mol. Biol.* **2009**, *392*, 547–557.
55. Fabry, C.M.; Rosa-Calatrava, M.; Conway, J.F.; Zubieta, C.; Cusack, S.; Ruigrok, R.W.; Schoehn, G. A quasi-atomic model of human adenovirus type 5 capsid. *EMBO J.* **2005**, *24*, 1645–1654.
56. Booy, F.P.; Newcomb, W.W.; Trus, B.L.; Brown, J.C.; Baker, T.S.; Steven, A.C. Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. *Cell* **1991**, *64*, 1007–1015.

57. Lepault, J.; Dubochet, J.; Baschong, W.; Kellenberger, E. Organization of double-stranded DNA in bacteriophages: A study by cryo-electron microscopy of vitrified samples. *EMBO J.* **1987**, *6*, 1507–1512.
58. Hearing, P.; Samulski, R.J.; Wishart, W.L.; Shenk, T. Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* **1987**, *61*, 2555–2558.
59. Daniell, E. Genome structure of incomplete particles of adenovirus. *J. Virol.* **1976**, *19*, 685–708.
60. Morsy, M.A.; Gu, M.C.; Zhao, J.Z.; Holder, D.J.; Rogers, I.T.; Pouch, W.J.; Motzel, S.L.; Klein, H.J.; Gupta, S.K.; Liang, X.; *et al.* Leptin gene therapy and daily protein administration: A comparative study in the ob/ob mouse. *Gene Ther.* **1998**, *5*, 8–18.
61. Parks, R.J.; Graham, F.L. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J. Virol.* **1997**, *71*, 3293–3298.
62. Gilbert, R.; Dudley, R.W.; Liu, A.B.; Petrof, B.J.; Nalbantoglu, J.; Karpatti, G. Prolonged dystrophin expression and functional correction of mdx mouse muscle following gene transfer with a helper-dependent (guttled) adenovirus-encoding murine dystrophin. *Hum. Mol. Genet.* **2003**, *12*, 1287–1299.
63. Ostapchuk, P.; Hearing, P. Minimal cis-acting elements required for adenovirus genome packaging. *J. Virol.* **2003**, *77*, 5127–5135.
64. Grable, M.; Hearing, P. Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J. Virol.* **1990**, *64*, 2047–2056.
65. Zhang, W.; Imperiale, M.J. Interaction of the adenovirus IVa2 protein with viral packaging sequences. *J. Virol.* **2000**, *74*, 2687–2693.
66. Ostapchuk, P.; Anderson, M.E.; Chandrasekhar, S.; Hearing, P. The L4 22-kilodalton protein plays a role in packaging of the adenovirus genome. *J. Virol.* **2006**, *80*, 6973–6981.
67. Ewing, S.G.; Byrd, S.A.; Christensen, J.B.; Tyler, R.E.; Imperiale, M.J. Ternary complex formation on the adenovirus packaging sequence by the IVa2 and L4 22-kilodalton proteins. *J. Virol.* **2007**, *81*, 12450–12457.
68. Wu, K.; Orozco, D.; Hearing, P. The adenovirus L4–22K protein is multifunctional and is an integral component of crucial aspects of infection. *J. Virol.* **2012**, *86*, 10474–10483.
69. Ali, H.; LeRoy, G.; Bridge, G.; Flint, S.J. The adenovirus L4 33-kilodalton protein binds to intragenic sequences of the major late promoter required for late phase-specific stimulation of transcription. *J. Virol.* **2007**, *81*, 1327–1338.
70. Hasson, T.B.; Soloway, P.D.; Ornelles, D.A.; Doerfler, W.; Shenk, T. Adenovirus L1 52- and 55-kilodalton proteins are required for assembly of virions. *J. Virol.* **1989**, *63*, 3612–3621.
71. Gustin, K.E.; Imperiale, M.J. Encapsidation of viral DNA requires the adenovirus L1 52/55-kilodalton protein. *J. Virol.* **1998**, *72*, 7860–7870.
72. Perez-Romero, P.; Tyler, R.E.; Abend, J.R.; Dus, M.; Imperiale, M.J. Analysis of the interaction of the adenovirus L1 52/55-kilodalton and IVa2 proteins with the packaging sequence *in vivo* and *in vitro*. *J. Virol.* **2005**, *79*, 2366–2374.
73. Ma, H.C.; Hearing, P. Adenovirus structural protein IIIa is involved in the serotype specificity of viral DNA packaging. *J. Virol.* **2011**, *85*, 7849–7855.

74. Stilwell, J.L.; McCarty, D.M.; Negishi, A.; Superfine, R.; Samulski, R.J. Development and characterization of novel empty adenovirus capsids and their impact on cellular gene expression. *J. Virol.* **2003**, *77*, 12881–12885.
75. Muruve, D.A.; Petrilli, V.; Zaiss, A.K.; White, L.R.; Clark, S.A.; Ross, P.J.; Parks, R.J.; Tschopp, J. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* **2008**, *452*, 103–107.
76. Wu, K.; Guimet, D.; Hearing, P. The adenovirus L4–33K protein regulates both late gene expression patterns and viral DNA packaging. *J. Virol.* **2013**, *87*, 6739–6747.
77. Ostapchuk, P.; Hearing, P. Regulation of adenovirus packaging. *Curr. Top. Microbiol. Immunol.* **2003**, *272*, 165–185.
78. Ostapchuk, P.; Hearing, P. Adenovirus IVa2 protein binds ATP. *J. Virol.* **2008**, *82*, 10290–10294.
79. Giberson, A.N.; Davidson, A.R.; Parks, R.J. Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res.* **2012**, *40*, 2369–2376.
80. Bergelson, J.M.; Cunningham, J.A.; Droguett, G.; Kurt-Jones, E.A.; Krithivas, A.; Hong, J.S.; Horwitz, M.S.; Crowell, R.L.; Finberg, R.W. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* **1997**, *275*, 1320–1323.
81. Tomko, R.P.; Xu, R.; Philipson, L. HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 3352–3356.
82. Smith, T.A.; Idamakanti, N.; Rollence, M.L.; Marshall-Neff, J.; Kim, J.; Mulgrew, K.; Nemerow, G.R.; Kaleko, M.; Stevenson, S.C. Adenovirus serotype 5 fiber shaft influences *in vivo* gene transfer in mice. *Hum. Gene Ther.* **2003**, *14*, 777–787.
83. Shayakhmetov, D.M.; Gaggar, A.; Ni, S.; Li, Z.Y.; Lieber, A. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J. Virol.* **2005**, *79*, 7478–7491.
84. Waddington, S.N.; McVey, J.H.; Bhella, D.; Parker, A.L.; Barker, K.; Atoda, H.; Pink, R.; Buckley, S.M.K.; Greig, J.A.; Denby, L.; *et al.* Adenovirus Serotype 5 Hexon Mediates Liver Gene Transfer. *Cell* **2008**, *132*, 397–409.
85. Kalyuzhniy, O.; Di Paolo, N.C.; Silvestry, M.; Hofherr, S.E.; Barry, M.A.; Stewart, P.L.; Shayakhmetov, D.M. Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes *in vivo*. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5483–5488.
86. Wickham, T.J.; Mathias, P.; Cheresch, D.A.; Nemerow, G.R. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **1993**, *73*, 309–319.
87. Leopold, P.L.; Ferris, B.; Grinberg, I.; Worgall, S.; Hackett, N.R.; Crystal, R.G. Fluorescent virions: Dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. *Hum. Gene Ther.* **1998**, *9*, 367–378.
88. Wiethoff, C.M.; Wodrich, H.; Gerace, L.; Nemerow, G.R. Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J. Virol.* **2005**, *79*, 1992–2000.
89. Greber, U.F.; Willetts, M.; Webster, P.; Helenius, A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* **1993**, *75*, 477–486.

90. Strunze, S.; Engelke, M.F.; Wang, I.H.; Puntener, D.; Boucke, K.; Schleich, S.; Way, M.; Schoenenberger, P.; Burckhardt, C.J.; Greber, U.F. Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. *Cell Host Microbe* **2011**, *10*, 210–223.
91. Komatsu, T.; Haruki, H.; Nagata, K. Cellular and viral chromatin proteins are positive factors in the regulation of adenovirus gene expression. *Nucleic Acids Res.* **2011**, *39*, 889–901.
92. Komatsu, T.; Nagata, K. Replication-uncoupled histone deposition during adenovirus DNA replication. *J. Virol.* **2012**, *86*, 6701–6711.
93. Ross, P.J.; Kennedy, M.A.; Christou, C.; Risco Quiroz, M.; Poulin, K.L.; Parks, R.J. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J. Virol.* **2011**, *85*, 3950–3958.
94. Bett, A.J.; Prevec, L.; Graham, F.L. Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* **1993**, *67*, 5911–5921.
95. Wellington, C.L.; Brunham, L.R.; Zhou, S.; Singaraja, R.R.; Visscher, H.; Gelfer, A.; Ross, C.; James, E.; Liu, G.; Huber, M.T.; *et al.* Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1. *J. Lipid Res.* **2003**, *44*, 1470–1480.
96. Hubberstey, A.V.; Pavliv, M.; Parks, R.J. Cancer therapy utilizing an adenoviral vector expressing only E1A. *Cancer Gene Ther.* **2002**, *9*, 321–329.
97. Mizuguchi, H.; Funakoshi, N.; Hosono, T.; Sakurai, F.; Kawabata, K.; Yamaguchi, T.; Hayakawa, T. Rapid construction of small interfering RNA-expressing adenoviral vectors on the basis of direct cloning of short hairpin RNA-coding DNAs. *Hum. Gene Ther.* **2007**, *18*, 74–80.
98. Graham, F.L.; Smiley, J.; Russell, W.C.; Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **1977**, *36*, 59–74.
99. Parks, R.J.; Chen, L.; Anton, M.; Sankar, U.; Rudnicki, M.A.; Graham, F.L. A helper-dependent adenovirus vector system: Removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 13565–13570.
100. Mitani, K.; Graham, F.L.; Caskey, C.T.; Kochanek, S. Rescue, propagation, and partial purification of a helper virus- dependent adenovirus vector. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 3854–3858.
101. Fisher, K.J.; Choi, H.; Burda, J.; Chen, S.J.; Wilson, J.M. Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* **1996**, *217*, 11–22.
102. Haecker, S.E.; Stedman, H.H.; Balice-Gordon, R.J.; Smith, D.B.; Greelish, J.P.; Mitchell, M.A.; Wells, A.; Sweeney, H.L.; Wilson, J.M. *In vivo* expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. *Hum. Gene Ther.* **1996**, *7*, 1907–1914.
103. Kumar-Singh, R.; Chamberlain, J.S. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. *Hum. Mol. Genet.* **1996**, *5*, 913–921.
104. Kumar-Singh, R.; Farber, D.B. Encapsidated adenovirus mini-chromosome-mediated delivery of genes to the retina: Application to the rescue of photoreceptor degeneration. *Hum. Mol. Genet.* **1998**, *7*, 1893–1900.

105. Kochanek, S.; Clemens, P.R.; Mitani, K.; Chen, H.H.; Chan, S.; Caskey, C.T. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5731–5736.
106. Lieber, A.; He, C.Y.; Kirillova, I.; Kay, M.A. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors *in vitro* and *in vivo*. *J. Virol.* **1996**, *70*, 8944–8960.
107. Hardy, S.; Kitamura, M.; Harris-Stansil, T.; Dai, Y.; Phipps, M.L. Construction of adenovirus vectors through Cre-lox recombination. *J. Virol.* **1997**, *71*, 1842–1849.
108. Ng, P.; Beauchamp, C.; Eveleigh, C.; Parks, R.; Graham, F.L. Development of a FLP/rt system for generating helper-dependent adenoviral vectors. *Mol. Ther.* **2001**, *3*, 809–815.
109. Umana, P.; Gerdes, C.A.; Stone, D.; Davis, J.R.; Ward, D.; Castro, M.G.; Lowenstein, P.R. Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat. Biotechnol.* **2001**, *19*, 582–585.
110. Parks, R.J.; Eveleigh, C.M.; Graham, F.L. Use of helper-dependent adenoviral vectors of alternative serotypes permits repeat vector administration. *Gene Ther.* **1999**, *6*, 1565–1573.
111. Palmer, D.; Ng, P. Improved system for helper-dependent adenoviral vector production. *Mol. Ther.* **2003**, *8*, 846–852.
112. Sandig, V.; Youil, R.; Bett, A.J.; Franlin, L.L.; Oshima, M.; Maione, D.; Wang, F.; Metzker, M.L.; Savino, R.; Caskey, C.T. Optimization of the helper-dependent adenovirus system for production and potency *in vivo*. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1002–1007.
113. Morsy, M.A.; Gu, M.; Motzel, S.; Zhao, J.; Lin, J.; Su, Q.; Allen, H.; Franlin, L.; Parks, R.J.; Graham, F.L.; *et al.* An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7866–7871.
114. Parks, R.J.; Bramson, J.L.; Wan, Y.; Addison, C.L.; Graham, F.L. Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. *J. Virol.* **1999**, *73*, 8027–8034.
115. Ross, P.J.; Kennedy, M.A.; Parks, R.J. Host cell detection of noncoding stuffer DNA contained in helper-dependent adenovirus vectors leads to epigenetic repression of transgene expression. *J. Virol.* **2009**, *83*, 8409–8417.
116. Schiedner, G.; Hertel, S.; Johnston, M.; Biermann, V.; Dries, V.; Kochanek, S. Variables affecting *in vivo* performance of high-capacity adenovirus vectors. *J. Virol.* **2002**, *76*, 1600–1609.
117. Parks, R.J. Improvements in adenoviral vector technology: Overcoming barriers for gene therapy. *Clin. Genet.* **2000**, *58*, 1–11.
118. Brunetti-Pierri, N.; Ng, T.; Iannitti, D.; Cioffi, W.; Stapleton, G.; Law, M.; Breinholt, J.; Palmer, D.; Grove, N.; Rice, K.; *et al.* Transgene expression up to 7 years in nonhuman primates following hepatic transduction with helper-dependent adenoviral vectors. *Hum. Gene Ther.* **2013**, *24*, 761–765.
119. Sargent, K.; Ng, P.; Eveleigh, C.; Graham, F.L.; Parks, R.J. Development of a size restricted pIX deleted helper virus for amplification of helper dependent adenovirus vectors. *Gene Ther.* **2004**, *11*, 504–511.

120. GhoshChoudhury, G.; Haj-Ahmad, Y.; Graham, F.L. Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *EMBO J.* **1987**, *6*, 1733–1739.
121. Parks, R.J. Adenovirus protein IX: A new look at an old protein. *Mol. Ther.* **2005**, *11*, 19–25.
122. Colby, W.W.; Shenk, T. Adenovirus type 5 virions can be assembled *in vivo* in the absence of detectable polypeptide IX. *J. Virol.* **1981**, *39*, 977–980.
123. Louis, N.; Eveleigh, C.; Graham, F.L. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology* **1997**, *233*, 423–429.
124. Sargent, K.; Meulenbroek, R.A.; Parks, R.J. Activation of adenoviral gene expression by protein IX is not required for efficient virus replication. *J. Virol.* **2004**, *78*, 5032–5037.
125. Smith, A.C.; Poulin, K.L.; Parks, R.J. DNA genome size affects the stability of the adenovirus virion. *J. Virol.* **2009**, *83*, 2025–2028.
126. Kennedy, M.A.; Parks, R.J. Adenovirus virion stability and the viral genome: Size matters. *Mol. Ther.* **2009**, *17*, 1664–1666.
127. Shayakhmetov, D.M.; Li, Z.Y.; Gaggar, A.; Gharwan, H.; Ternovoi, V.; Sandig, V.; Lieber, A. Genome size and structure determine efficiency of postinternalization steps and gene transfer of capsid-modified adenovirus vectors in a cell-type-specific manner. *J. Virol.* **2004**, *78*, 10009–10022.
128. Benevento, M.; Di Palma, S.; Snijder, J.; Moyer, C.L.; Reddy, V.S.; Nemerow, G.R.; Heck, A.J. Adenovirus composition, proteolysis, and disassembly studied by in-depth qualitative and quantitative proteomics. *J. Biol. Chem.* **2014**, *289*, 11421–11430.
129. Ivanovska, I.; Wuite, G.; Jonsson, B.; Evilevitch, A. Internal DNA pressure modifies stability of WT phage. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 9603–9608.
130. Tzlil, S.; Kindt, J.T.; Gelbart, W.M.; Ben-Shaul, A. Forces and pressures in DNA packaging and release from viral capsids. *Biophys. J.* **2003**, *84*, 1616–1627.
131. Evilevitch, A.; Roos, W.H.; Ivanovska, I.L.; Jeembaeva, M.; Jonsson, B.; Wuite, G.J. Effects of salts on internal DNA pressure and mechanical properties of phage capsids. *J. Mol. Biol.* **2011**, *405*, 18–23.
132. Ortega-Esteban, A.; Perez-Berna, A.J.; Menendez-Conejero, R.; Flint, S.J.; San Martin, C.; de Pablo, P.J. Monitoring dynamics of human adenovirus disassembly induced by mechanical fatigue. *Sci. Rep.* **2013**, *3*, 1434.
133. Snijder, J.; Reddy, V.S.; May, E.R.; Roos, W.H.; Nemerow, G.R.; Wuite, G.J. Integrin and defensin modulate the mechanical properties of adenovirus. *J. Virol.* **2013**, *87*, 2756–2766.
134. Carrasco, C.; Carreira, A.; Schaap, I.A.; Serena, P.A.; Gomez-Herrero, J.; Mateu, M.G.; de Pablo, P.J. DNA-mediated anisotropic mechanical reinforcement of a virus. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13706–13711.
135. Reguera, J.; Grueso, E.; Carreira, A.; Sanchez-Martinez, C.; Almendral, J.M.; Mateu, M.G. Functional relevance of amino acid residues involved in interactions with ordered nucleic acid in a spherical virus. *J. Biol. Chem.* **2005**, *280*, 17969–17977.
136. Carrasco, C.; Castellanos, M.; de Pablo, P.J.; Mateu, M.G. Manipulation of the mechanical properties of a virus by protein engineering. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 4150–4155.

137. Li, T.; Chen, Z.; Johnson, J.E.; Thomas, G.J., Jr. Conformations, interactions, and thermostabilities of RNA and proteins in bean pod mottle virus: Investigation of solution and crystal structures by laser Raman spectroscopy. *Biochemistry* **1992**, *31*, 6673–6682.
138. Da Poian, A.T.; Johnson, J.E.; Silva, J.L. Protein-RNA interactions and virus stability as probed by the dynamics of tryptophan side chains. *J. Biol. Chem.* **2002**, *277*, 47596–47602.
139. Michel, J.P.; Ivanovska, I.L.; Gibbons, M.M.; Klug, W.S.; Knobler, C.M.; Wuite, G.J.; Schmidt, C.F. Nanoindentation studies of full and empty viral capsids and the effects of capsid protein mutations on elasticity and strength. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 6184–6189.
140. Horowitz, E.D.; Rahman, K.S.; Bower, B.D.; Dismuke, D.J.; Falvo, M.R.; Griffith, J.D.; Harvey, S.C.; Asokan, A. Biophysical and ultrastructural characterization of adeno-associated virus capsid uncoating and genome release. *J. Virol.* **2013**, *87*, 2994–3002.
141. Allocca, M.; Doria, M.; Petrillo, M.; Colella, P.; Garcia-Hoyos, M.; Gibbs, D.; Kim, S.R.; Maguire, A.; Rex, T.S.; Di Vicino, U.; *et al.* Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J. Clin. Invest.* **2008**, *118*, 1955–1964.
142. Dong, B.; Nakai, H.; Xiao, W. Characterization of genome integrity for oversized recombinant AAV vector. *Mol. Ther.* **2010**, *18*, 87–92.
143. Lai, Y.; Yue, Y.; Duan, D. Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome ≥ 8.2 kb. *Mol. Ther.* **2010**, *18*, 75–79.
144. Wu, Z.; Yang, H.; Colosi, P. Effect of genome size on AAV vector packaging. *Mol. Ther.* **2010**, *18*, 80–86.
145. de Vrij, J.; van den Hengel, S.K.; Uil, T.G.; Koppers-Lalic, D.; Dautzenberg, I.J.; Stassen, O.M.; Barcena, M.; Yamamoto, M.; de Ridder, C.M.; Kraaij, R.; *et al.* Enhanced transduction of CAR-negative cells by protein IX-gene deleted adenovirus 5 vectors. *Virology* **2011**, *410*, 192–200.
146. Yamaguchi, T.; Kawabata, K.; Koizumi, N.; Sakurai, F.; Nakashima, K.; Sakurai, H.; Sasaki, T.; Okada, N.; Yamanishi, K.; Mizuguchi, H. Role of MyD88 and TLR9 in the innate immune response elicited by serotype 5 adenoviral vectors. *Hum. Gene Ther.* **2007**, *18*, 753–762.
147. Appledorn, D.M.; Patial, S.; McBride, A.; Godbehere, S.; Van Rooijen, N.; Parameswaran, N.; Amalfitano, A. Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 *in vivo*. *J. Immunol.* **2008**, *181*, 2134–2144.
148. Fejer, G.; Drechsel, L.; Liese, J.; Schleicher, U.; Ruzsics, Z.; Imelli, N.; Greber, U.F.; Keck, S.; Hildenbrand, B.; Krug, A.; *et al.* Key role of splenic myeloid DCs in the IFN- α response to adenoviruses *in vivo*. *PLoS Pathog.* **2008**, *4*, e1000208.
149. Teigler, J.E.; Kagan, J.C.; Barouch, D.H. Late endosomal trafficking of alternative serotype adenovirus vaccine vectors augments antiviral innate immunity. *J. Virol.* **2014**, *88*, 10354–10363.

Appendix 3.2:

Human Adenoviral DNA Association with Nucleosomes Containing Histone Variant H3.3 During the Early Phase of Infection is Not Dependent on Viral Transcription or Replication

Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication

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Abstract: Adenovirus (Ad) DNA undergoes dynamic changes in protein association as the virus progresses through its replicative cycle. Within the virion, the Ad DNA associates primarily with the virus-encoded, protamine-like protein VII. During the early phase of infection (~6 h), the viral DNA showed declining association with VII, suggesting that VII was removed from at least some regions of the viral DNA. Within 6 h, the viral DNA was wrapped into a repeating nucleosome-like array containing the histone variant H3.3. Transcription elongation was not required to strip VII from the viral DNA or for deposition of H3.3. H3.1 did not associate with the viral DNA at any point during infection. During the late phase of infection (i.e., active DNA replication ~12–24 h), association with H3 was dramatically reduced and the repeating nucleosome-like pattern was no longer evident. Thus, we have uncovered some of the changes in nucleoprotein structure that occur during lytic Ad infection.

Key words: adenovirus, chromatin, histones.

Résumé : L'ADN d'un adénovirus (Ad) subit des changements dynamiques en matière d'association avec les protéines au fur et à mesure de la progression du virus à travers son cycle de réplication. À l'intérieur d'un virion, l'ADN de l'Ad s'associe principalement avec la protéine codée par le virus, la protéine VII de type protamine. Durant la phase précoce d'infection (~6 h), l'on assiste au déclin de l'association de l'ADN viral avec la protéine VII suggérant que la protéine VII était enlevée de certaines régions de l'ADN viral. En moins de 6 h, l'ADN était enroulé dans un réseau répétitif de type nucléosome comportant le variant d'histone H3.3. L'élongation de la transcription n'était pas requise pour enlever la protéine VII de l'ADN viral ou déposer H3.3. H3.1 ne s'associait à l'ADN viral à aucun moment durant l'infection. Durant la phase tardive de l'infection (c.-à-d. la réplication active de l'ADN à ~12–24 h), l'association avec H3 était considérablement réduite et le patron répétitif de type nucléosome n'était plus apparent. Ainsi, les auteurs ont mis au jour certains des changements de la structure des nucléoprotéines qui surviennent durant l'infection à un Ad lytique. [Traduit par la Rédaction]

Mots-clés : adénovirus, chromatine, histones.

Introduction

In eukaryotic cells, genomic DNA is packaged into chromatin, a highly organized complex of DNA and proteins that encodes epigenetic information governing gene expression and cell identity (Luger 2006). Numerous studies have shown the active role that histones play in gene regulation, through post-translational modification of their N-terminal ends and their ability to serve as docking/recognition sites for other proteins (Jenuwein and Allis 2001; Kouzarides 2007). It is within this cellular context that human adenovirus (Ad) DNA must function to efficiently express the viral genes necessary for reconfiguring the cellular environment to one more conducive to virus replication and to generate the viral structural proteins necessary for progeny virion formation. Yet, the dynamic changes in nucleoprotein structure that the virus undergoes during its lifecycle, and the cellular proteins involved in mediating these events, are poorly understood (Giberson et al. 2012; Wong et al. 2013).

Within the icosahedral capsid, Ad DNA associates with 3 highly basic proteins, VII, V, and μ (mu) (Maizel et al. 1968; Russell et al. 1968; Chatterjee et al. 1986; Fabry et al. 2005; Saha et al. 2014). Protein VII is the main protein responsible for wrapping and condensing the viral DNA (Mirza and Weber 1982). The protein VII – DNA nucleoprotein complex in the virion is organized into a central dense core, from which extend 12 large spherical units that are directed towards each vertex (Brown et al. 1975; Newcomb et al. 1984). Somewhat surprisingly, a recent study found that virions can be formed in the absence of protein VII, suggesting that the DNA-condensing function of protein VII is not absolutely required for packaging of the viral DNA into capsids (Ostapchuk et al. 2017). However, virions deficient in protein VII show a reduced ability to escape from the endosome and show compromised gene expression and replication, suggesting VII is important for a subsequent stage of the lifecycle. Protein V is believed to form a shell around the protein VII – DNA complex (Everitt et al. 1973; Brown et al.

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1975) and tethers the protein-VII-wrapped DNA to the inner capsid (Everitt et al. 1975; Chatterjee et al. 1985; Stewart et al. 1993; Matthews and Russell 1998). Viruses deficient in protein V can be recovered, but these viruses formed small plaques and had reduced thermostability and infectivity (Ugai et al. 2007). Pre-mu may aid in wrapping and condensing the viral DNA (Anderson et al. 1989), and cleavage of pre-mu by the Ad-encoded protease may relax the viral nucleoprotein structure before it enters the nucleus (Pérez-Berná et al. 2009). Immediately after infection, it is the protein-VII-wrapped DNA that enters the nucleus (Chatterjee et al. 1986). There are conflicting data regarding the degree to which VII remains associated with the viral DNA and for how long (Chatterjee et al. 1986; Xue et al. 2005; Haruki et al. 2006; Chen et al. 2007; Spector 2007; Walkiewicz et al. 2009; Karen and Hearing 2011; Komatsu et al. 2011; Ross et al. 2011; Komatsu and Nagata 2012; Wang et al. 2013). However, *in vitro* studies have shown that the protein-VII-bound DNA structure does not allow for efficient transcription or replication (Matsumoto et al. 1993; Okuwaki and Nagata 1998), suggesting that the complex must be remodeled in some way to permit efficient gene expression.

Interestingly, recent studies have shown that VII from the incoming virus can form a complex with nucleosomes present on cellular chromatin, which functions to sequester the high mobility group B1 (HMGB1) protein and prevent its release (Avgousti et al. 2016). Since release of HMGB1 protein from chromatin acts as a danger signal to activate innate immunity (Kang et al. 2014), VII is able to actively repress antiviral immune signaling. Similarly, protein VII association with cellular chromatin has been shown to reduce the DNA damage response within the cell (Avgousti et al. 2017). Thus, protein VII may have important functions in regulating cellular gene expression in addition to viral genes.

Dissociation of protein VII and remodeling of the Ad genome are likely key steps in transitioning the viral DNA to a transcriptionally active template and modifying the cellular environment to be more favorable for virus replication. Thus, targeting Ad nucleoprotein remodeling may represent a point of intervention to prevent Ad replication and spread. In this study, we examined some of the dynamic and complex changes in nucleoprotein structure that occur on Ad DNA during lytic infection.

Materials and methods

Cell lines and culture conditions

A549 (CCL 185; ATCC) and 293 cells [obtained from Frank Graham, McMaster University, Hamilton, Ontario (Graham et al. 1977)] were cultured as described previously (Ross and Parks 2009). The pCMV-H3.3-YFP plasmid, which expresses yellow fluorescent protein (YFP)-tagged H3.3, was obtained from Addgene (Watertown, Massachusetts, USA), and the construction of pCMV-H3.1-YFP has been described previously (Ross et al. 2011). These plasmids were transfected into A549 cells and stable colonies were selected with 200 µg/mL of hygromycin (Invitrogen), then maintained in culture medium (minimal essential medium, 10% fetal bovine serum, 2 mmol/L glutamax, 100 U penicillin, and 1x antibiotic-antimycotic (Invitrogen)) supplemented with 50 µg/mL of hygromycin, creating A549 H3.1-YFP and A549 H3.3-YFP cells. A549shCTRL and A549shHIRA cells were created by infecting A549 cells with lentivirus (produced using the Thermo Scientific Trans-Lentiviral packaging kit and pTripZ plasmids (Open Biosystems)) and selected with 1.5 µg/mL of puromycin (Sigma). Individual clones were isolated and tested for their ability to knockdown expression of HIRA by immunoblot analysis. The shRNA/RFP expression cassette in these cells was induced with 1 µg/mL of doxycycline (Sigma) and monitored by the expression of RFP. In experiments involving the transcription elongation inhibitor DRB (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; Sigma), cells were pretreated for 1 h with 100 µmol/L DRB in culture media and maintained in DRB throughout the duration of the experiment.

Virus culture and infections

All viruses are based on human Ad serotype 5. AdVII-FLAG (E1+, replication-competent) contains a FLAG epitope tag on the C-terminus of protein VII, as previously described (Smith et al. 2009; Ross et al. 2011). The presence of the FLAG tag on protein VII has no discernible deleterious effect on virus viability. Viruses were propagated on 293N3S cells (Graham 1987), purified by cesium chloride buoyant density centrifugation using standard procedures, and titered on 293 cells as described previously (Ross and Parks 2009). For most experiments, cells were infected with Ad diluted in phosphate-buffered saline (PBS). After 1 h at 37 °C, medium was added to the cells and they were incubated in a humidified CO₂ incubator at 37 °C until the desired time point. For synchronized infections, the cells were incubated on ice in the presence of virus for 30 min, allowing the virus to attach but not enter the cells. Infection started when warm media was added and the cells were placed in a 37 °C incubator.

Immunoblotting

Immunoblotting was performed using standard techniques (BioRad). To obtain cell lysates, the medium was removed and 2x protein sample buffer (62.5 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% sodium dodecyl sulphate (SDS), 0.01% bromophenol blue, and 5% β-mercaptoethanol) was added. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A semi-dry protein transfer protocol was used to transfer the proteins to polyvinylidene fluoride (PVDF) membrane (Millipore). PVDF membranes were probed with the following primary antibodies: anti-FLAG M2 (1:10 000 dilution; Rockland), anti-α-tubulin (1:10 000; Calbiochem), anti-green fluorescent protein (GFP) that cross-reacts with YFP (1:2000; Invitrogen), anti-Ad fiber (clone 4D2, 1:10 000; Neo-Markers), and anti-Ad 5 E1A (M58, 1:10 000; Neo-Markers). Anti-H3 (catalog No. 2650; 1:10 000; Cell Signaling) was used in the majority of experiments (unless otherwise indicated), and recognizes the C-terminus of the native H3 protein, but does not recognize the YFP-fusion proteins. Anti-H3 specific to the N-terminus of the protein (PA1-46377, ThermoScientific) was used to determine the relative amounts of H3-YFP to endogenous H3. Both H3 antibodies recognize H3.1 and H3.3. Secondary antibodies contained horse radish peroxidase coupled to either goat anti-rabbit IgG or anti-mouse IgG (catalog No. 170-6515 and 170-6516, respectively; BioRad). Binding of the secondary antibody was detected using the Pierce ECL Western Blotting Substrate and visualized using X-ray film (ThermoScientific).

Reverse-transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed using standard techniques (Invitrogen). RNA was isolated using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. First strand synthesis was conducted using random hexamers and M-MLV reverse transcriptase (Invitrogen), and then treated with RNase H. PCR was conducted using E1A primers (Table 1) and Taq DNA polymerase (Invitrogen) at an annealing temperature of 60 °C for 30 cycles.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (Ross et al. 2009, 2011). Confluent A549, A549 H3.1-YFP, or A549 H3.3-YFP cells in 5 dishes 15 cm in diameter (~1.25 × 10⁸ cells) were infected with virus AdVII-FLAG at a multiplicity of infection (MOI) of 10. At the indicated time points, cells were fixed by adding formaldehyde to a final concentration of 1% for 10 min. Fixation was quenched with 0.125 mol/L glycine (final concentration) and after 5 min of incubation, the monolayers were rinsed twice with cold PBS and scraped into PBS supplemented with protease inhibitors (Complete mini EDTA-free; Roche). All subsequent buffers contained protease inhibitors. The cells were pelleted (1500g, 10 min, 20 °C) and stored at

Table 1. Primer sequences.

Primer	Sequence (5'–3')
E1A, forward	GTC CGG TTT CTA TGC CAA ACC TTG
E1A, reverse	CAA ACT CCT CAC CCT CTT CAT CCT
Hexon, forward	CTT ACC CCC AAC GAG TTT GA
Hexon, reverse	GGA GTA CAT GCG GTC CTT GT
Human β -actin, forward	CTG AAC CCT AAG GCC AAC CGT
Human β -actin, reverse	CCG TCA GGC AGC TCA TAG CT
Human GAPDH, forward	GGC CGG TGC TGA GTA TGT CG
Human GAPDH, reverse	TTC AAG TGG GCC CCG GCC TT
Human RPL30, exon 3	No. 7014 from Cell Signaling

–80 °C. To isolate nuclei, the cells were re-suspended in 2 mL of cell swelling buffer (25 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.1% NP-40, and 1 mmol/L DTT) and incubated on ice for 15 min followed by Dounce homogenization (10–15 strokes using pestle B). The nuclei were pelleted by centrifugation (2000g, 5 min, 4 °C) and lysed in 1 mL of sonication buffer with 1% SDS (50 mmol/L HEPES (pH 7.0), 140 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, and 0.1% sodium-deoxycholate). Chromatin was sheared to an average size of 300 bp by sonication in a Diagenode Bioruptor for 40 cycles (30 s on, 1 min off) at 4 °C. Sheared chromatin was diluted 8-fold with ChIP dilution buffer (sonication buffer without SDS). Diluted chromatin was precleared with 20 μ L of a 50% slurry of protein G magnetic beads (Invitrogen) (which had been equilibrated with ChIP buffer with 1 μ g sheared herring sperm DNA and 1 μ g of BSA) for at least 2 h at 4 °C with constant rotation. Antibody (2 μ g) was incubated with 40 μ L of a 50% slurry of protein G magnetic beads for each sample for at least 2 h. Precleared chromatin (1 mL) was incubated overnight with antibody–bead complex at 4 °C with constant rotation. Antibodies used for ChIP analysis were rabbit IgG anti-GFP (A-11122; Invitrogen), anti-FLAG M2 (Rockland), anti-H3 (catalog No. 2650; Cell Signaling), or control IgG. After immunoprecipitation, beads were rinsed twice each with low-salt immune complex wash buffer (ICWB; 50 mmol/L HEPES (pH 7.0), 140 mmol/L NaCl, 0.1% SDS, 1 mmol/L EDTA, 1% Triton X-100, and 0.1% sodium-deoxycholate), high-salt ICWB (50 mmol/L HEPES (pH 7.0), 500 mmol/L NaCl, 0.1% SDS, 1 mmol/L EDTA, 1% Triton X-100, and 0.1% sodium-deoxycholate), lithium chloride ICWB (20 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, 250 mmol/L LiCl, 0.5% NP40, and 0.5% sodium-deoxycholate), and Tris–EDTA (TE). The beads were then incubated twice for 10 min with 0.2 mL elution buffer (50 mmol/L Tris–HCl (pH 8.0), 1 mmol/L EDTA, and 1% SDS). Eluates were combined, NaCl and RNase A were added to 200 mmol/L and 10 μ g/mL, respectively, and the samples were incubated for 4 to 6 h at 65 °C. After crosslink reversal, 4 μ L of 0.5 mmol/L EDTA and 10 μ g of proteinase K were added to the samples and incubated for 2 h at 42 °C. DNA was purified by phenol–chloroform extraction and precipitated with twice the volume of 100% ethanol, 0.1 mol/L sodium acetate, and 100 μ g of glycogen. ChIP DNA was suspended in 100 μ L H_2O , and input DNA sample represents 0.02% of the sample in the IP. Quantitative PCR (qPCR) was performed using SYBR Green JumpStart Taq ReadyMix (Sigma), in accordance with the manufacturer's instructions, with the exception that the reaction was performed in a total volume of 20 μ L. The following primer sets for qPCR were used: E1A, Hexon, and Human RPL30 Exon 3 (see Table 1 for sequences). The PCR was performed on a MX3000P (Stratagene/Agilent, Mississauga, Ont.), with 40 amplification cycles and a 60 °C annealing temperature. Data was analyzed and presented as a percentage of input.

Micrococcal nuclease accessibility assays

A549 cells ($\sim 2.0 \times 10^7$ cells) in 15 cm dishes were infected with AdVII-FLAG at an MOI of 100 (for isolation of DNA at 6 hours post infection (hpi)) or an MOI of 10 (for isolation of DNA at 18 hpi). We used a higher MOI of Ad for our early time point to ensure that

there was sufficient viral DNA to detect in the Southern blot, which was not a concern for the later time point that benefited from amplification of the viral DNA during normal virus replication. At the indicated time points after infection, nuclei were isolated as described previously (Challberg and Kelly 1979; Ross et al. 2011). Isolated nuclei in aliquots of 0.2 mL ($\sim 4.0 \times 10^6$ nuclei) were treated with 200 U of micrococcal nuclease (MNase; NEB) and incubated for 0, 2, 3, 4, and 5 min at 37 °C. Digests were terminated by addition of 50 μ L 5 $\times$ stop solution (20 mmol/L Tris–HCl (pH 8.0), 50 mmol/L EDTA, and 2.5% SDS) and digested with 4 μ g of proteinase K and 1 μ g of RNase overnight at 42 °C. DNA was purified by phenol–chloroform extraction, ethanol precipitated, and resuspended in TE. Purified DNA (6 μ g) was separated on 1.25% agarose gels, transferred to a nylon membrane, and hybridized with a digoxigenin-labeled fragment from pCC100 plasmid (an Ad genomic plasmid corresponding to the AdVII-FLAG virus) or a bacterial artificial chromosome containing the cellular human SMN locus (RP164H30). DNA labeling and probe detection were performed using the digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche), in accordance with the manufacturer's instructions, and visualized with autoradiography. The cellular and viral probes were applied to separate, duplicate membranes.

Results

Protein VII exhibits declining association with the Ad DNA during the early phase of infection

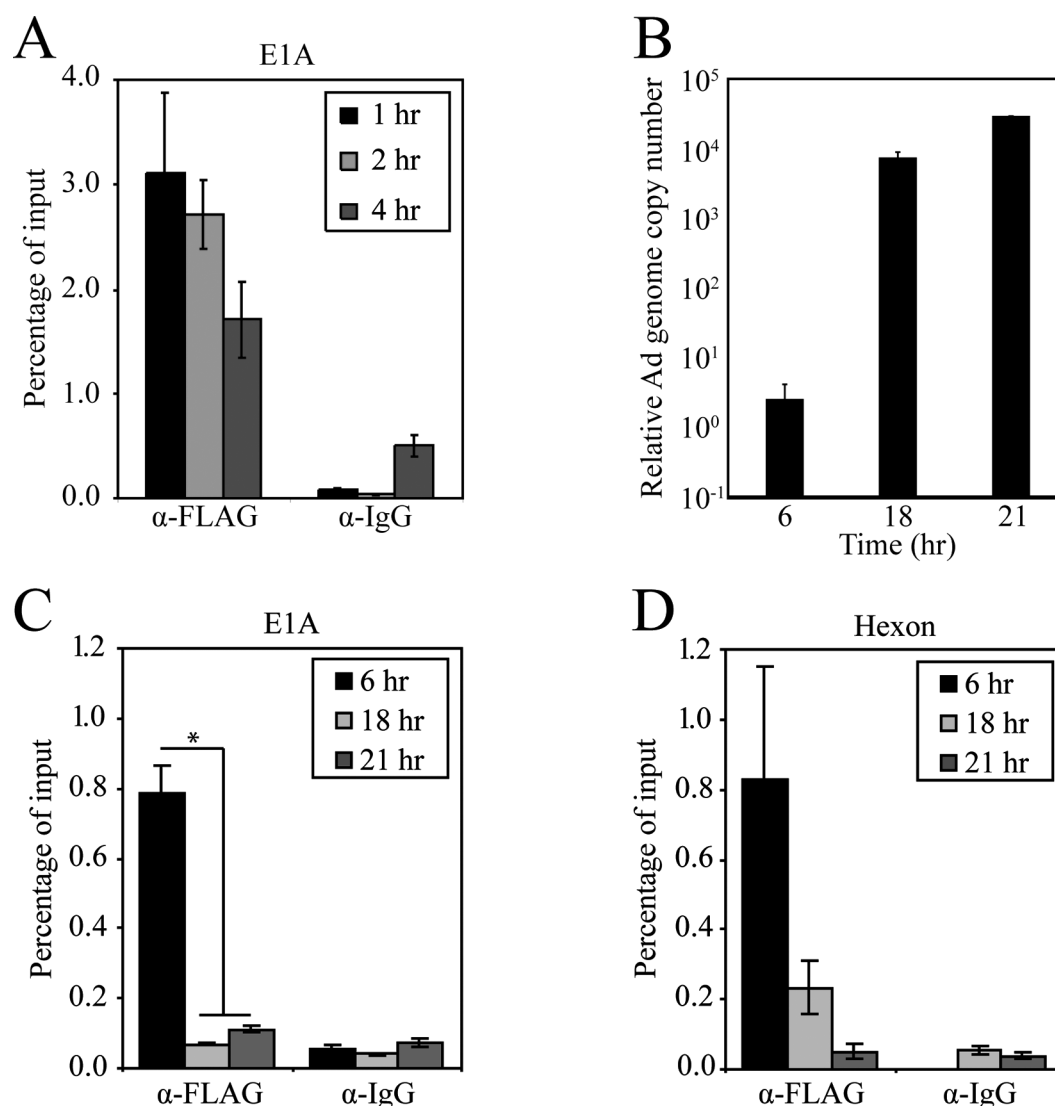
The existing literature on protein VII association with the Ad genome report conflicting data. Some studies suggest that VII association with the Ad DNA remains relatively constant during the early phase of infection (1–12 hpi), whereas others have shown a gradual or more rapid dissociation of VII soon after the viral DNA reaches the nucleus (reviewed in (Giberson et al. 2012; Wong et al. 2013)). To address whether there is a decrease in VII association with the Ad DNA during the early phase of infection, we performed ChIP using our FLAG-tagged VII Ad at an MOI of 10. In this virus, the FLAG is located on the C-terminus of the protein and does not affect virus growth. As shown in Figure 1A, we observed a trend towards a decrease in VII association with Ad DNA over the first 4 h of infection, but this did not reach statistical significance.

We next examined VII association with viral DNA at later times of infection, focusing on time points before and after viral DNA replication (which occurs at around 15 hpi for E1+ viruses in A549 cells). Between 6 and 18 hpi, we observed approximately a 3-log increase in the quantity of viral DNA within the cells (Fig. 1B). At 6 hpi, VII association was reduced to about 25% of that observed at 1 hpi (Fig. 1A versus 1C), and the extent of association with the late hexon region was similar to that of the early E1 region at this time point (Figs. 1C and 1D). Association of VII with the viral genome remained very low during the remainder of infection, suggesting that only a fraction of the newly replicated viral DNA at late times of infection is wrapped in pre-VII for production of progeny virions or that the VII-wrapped DNA is masked from ChIP due to already being incorporated into capsids.

Transcription elongation on the Ad genome is not required for removal of protein VII

There is some debate regarding the requirement for active transcription for the removal of protein VII from the Ad DNA. Although some studies have shown that VII appeared to remain associated with the viral DNA for longer periods of time when transcription elongation was inhibited (Chen et al. 2007; Karen and Hearing 2011), other studies did not see a difference (Komatsu et al. 2011; Ross et al. 2011). To address the effect of transcription on association of the Ad genome with protein VII, we used the well-characterized inhibitor of transcription elongation DRB

Fig. 1. Protein VII association with adenovirus (Ad) DNA during infection. A549 cells were infected with AdVII-FLAG at a multiplicity of infection of 10 for all experiments. (A) Infected cells were processed for ChIP at 1, 2, and 4 hours post infection (hpi) with the indicated antibodies. The resulting DNA samples were analyzed by qPCR with primers specific for the E1A region. (B) DNA was isolated from AdVII-FLAG-infected cells at 6, 18, and 21 hpi and analyzed for genome copy number by qPCR. (C and D) Cells were processed for ChIP at 6, 18, and 21 hpi as in (A). qPCR analysis was conducted for 2 viral regions, E1A (C) and hexon (D). The median and standard errors are shown ($n = 2$), analyzed in duplicate.



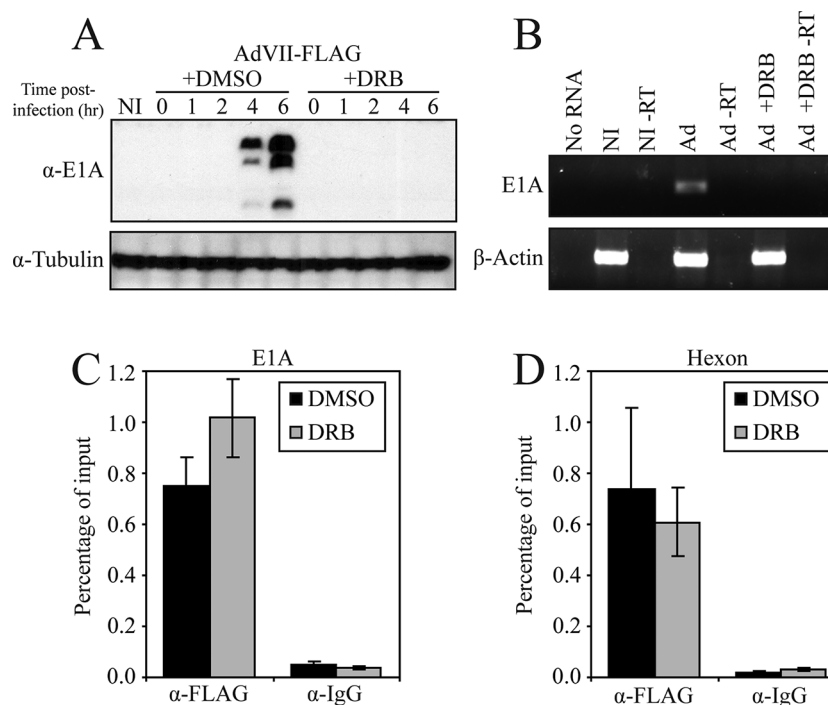
(Yankulov et al. 1995). A549 cells were infected with AdVII-FLAG in the absence or presence of the RNA polymerase inhibitor DRB, crude protein samples were isolated at varying times post-infection, and assayed for E1A or VII-FLAG by immunoblot. E1A protein expression can be detected as early as 4 hpi by immunoblot (Fig. 2A), and no E1A protein expression was detected in cells treated with DRB. Similarly, no mRNA transcript was detected from the E1A region at 6 hpi in cells treated with DRB (Fig. 2B), indicating that this concentration of DRB was effective. We next examined whether treatment of cells with DRB altered VII association with the viral DNA by ChIP. A549 cells were treated with DRB, or left untreated, and 1 h later infected with AdVII-FLAG at an MOI of 10. At 6 hpi, the cellular proteins and DNA within the infected cells were crosslinked and processed for ChIP. As shown in Fig. 2C, treatment with DRB had no significant effect on the level of VII association with the Ad E1A region. Similarly, DRB had no effect on VII association with the region encoding hexon (Fig. 2D), part of the late transcription unit that is only expressed after DNA replication. Taken together, these data indicate that

there is a slow loss of VII from the viral DNA over the first 6 h of infection, and transcription elongation on the viral genome is not required for this loss.

Ad DNA assembles into a repeating nucleosome-like structure during the early phase of infection, but not during the late phase

We next investigated whether Ad DNA was wrapped in a repeating nucleosome-like structure at early and late times of infection using a micrococcal nuclease (MNase) digestion assay. A549 cells were infected with AdVII-FLAG at an MOI of 100 (for the early 6 h time point) or 10 (for the late 18 h time point). Isolated nuclei were treated with MNase for varying times, the DNA was purified, subjected to Southern blot, and probed for viral or cellular sequences. At both early and late times of infection, the cellular DNA was found to produce a clear laddering pattern (Figs. 3A and 3B). At the 6 h time point (Fig. 3A), the viral DNA was visualized as a repeating pattern of similar size to the cellular fragments, indicating that at least some of the viral DNA had assembled into a repeating

Fig. 2. Protein VII association with adenovirus (Ad) DNA is not dependent on transcription. (A) A549 cells were treated with a transcription elongation inhibitor (5,6-dichlorobenzimidazole 1- β -D-ribofuranoside; DRB) or vehicle (dimethyl sulfoxide; DMSO) and synchronously infected at a multiplicity of infection (MOI) of 1000. At varying times points after infection, infected cell lysates were probed for Ad E1A and tubulin (loading control). (B) A549 cells were treated as described in (A). RNA was harvested at 6 hours post infection (hpi) and then subjected to RT-PCR for viral (E1A) and control (β -actin) sequences. NI, not infected; and NI-RT, not infected and no reverse transcriptase added. (C and D) A549 cells were treated with DRB or vehicle (DMSO) and infected with AdVII-FLAG at an MOI of 10. Cells were processed for ChIP at 6 hpi with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for 2 viral regions, (C) E1A and (D) hexon. The median and standard error are shown ($n = 2$), analyzed in duplicate.



nucleosome-like structure. We also observed a significant amount of high molecular weight, MNase-resistant viral DNA, suggesting that some of the incoming viral DNA is either compacted into a high molecular weight, MNase-resistant nucleoprotein structure, perhaps still tightly bound in VII, or may still be protected in the viral capsid. At the 6 h time point, although the viral DNA did display a repeating pattern, it appeared less distinct than that of the cellular DNA. At the 18 h time point, we again observed a large, MNase-resistant DNA species (Fig. 3B), which may represent newly encapsidated DNA that is fully protected from MNase digestion. However, we did not observe a laddering pattern, and the protected viral DNA appeared at a size consistent with mononucleosomes. The less distinct repeating pattern observed at early times of infection and the lack of a repeating nucleosome structure at late times of infection may reflect either the presence of widely spaced nucleosomes, as has been suggested for Ad previously (Beyer et al. 1981), or unstable nucleosomes, as has been shown for other viruses such as herpes simplex virus (HSV) (Lacasse and Schang 2010).

Ad DNA associates with histones during the early phase of infection

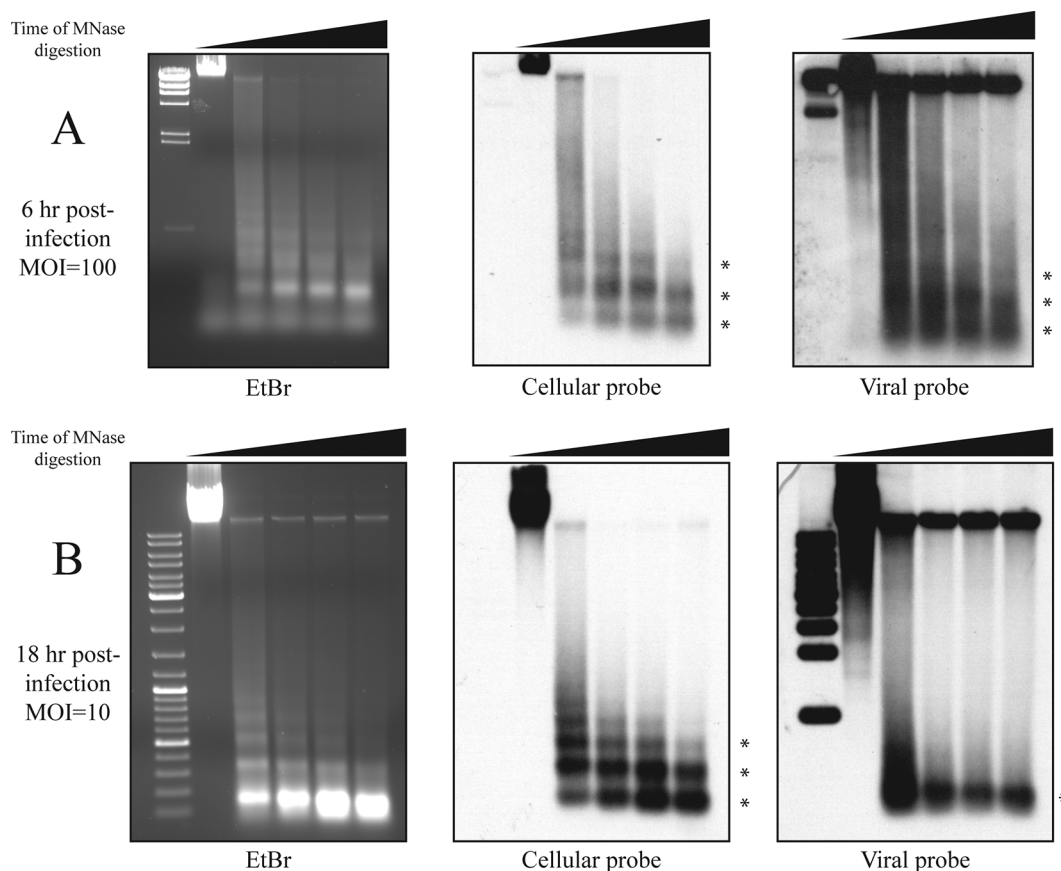
The fragmentation pattern observed in the MNase assay during the early phase of infection suggests that the viral DNA does indeed associate with histones and assembles into nucleosome-like structures. To confirm this, we performed ChIP analysis with an α -H3 antibody and examined the resulting DNA for H3 association with representative early (E1A) and late (hexon) regions of the Ad genome. We chose H3 for our analysis because, in combination with H4, it is the first histone deposited on DNA during nucleosome formation and, as such, H3-H4 deposition acts as a rate limiting step in chromatin formation (Li et al. 2012). Furthermore,

deciphering which H3 species (H3.1 or H3.3) is deposited on the DNA can provide insight into the mechanism of histone deposition (Tagami et al. 2004). As shown in Fig. 4A, we observed robust association of H3 with the E1A region at 6 hpi, but only very low association at later time points. This observation is consistent with the reduced wrapping in nucleosomes seen in the MNase digestion assay at late times of infection (Fig. 3B). We observed only low levels of association of H3 with hexon at both early and late times of infection (Fig. 4B). As expected, the cellular RPL30 control locus showed high level association with H3 at all time points (Fig. 4C). We next asked whether transcription elongation was required for deposition of histones on the Ad DNA. As in our previous experiment, A549 cells were pre-treated with DRB, infected with Ad, and assayed for association with H3 at 6 hpi by ChIP. We did not observe a difference in association with H3 for either Ad E1A or hexon regions, or the cellular RPL30 locus in the presence of DRB (Figs. 4D–4F). Thus, transcription elongation does not appear to be required for deposition of histones on the Ad DNA.

Ad DNA preferentially associates with H3 variant H3.3 throughout infection

There are 2 main variants of H3 typically found within nucleosomes, H3.3 and H3.1. H3.1 is deposited in a replication-coupled manner on newly synthesized DNA following S phase, whereas H3.3 is deposited on actively transcribed regions independent of DNA replication (Tagami et al. 2004). To address the specific species of H3 with which Ad DNA preferentially associates, we established stable A549 cell lines that expressed YFP-tagged versions of either H3.1 or H3.3. We performed a series of preliminary characterization experiments to ensure that forced expression of these histones did not adversely affect cell or virus growth. We exam-

Fig. 3. Adenovirus (Ad) DNA assembles into a repeating nucleosome-like structure early during infection. A549 cells were infected with AdVII-FLAG at a multiplicity of infection (MOI) of (A) 100 or (B) 10. The samples were processed for a micrococcal nuclease (MNase) digestion assay at (A) 6 or (B) 18 hours post infection (hpi). The ethidium bromide (EtBr)-stained agarose gel (showing bulk cellular chromatin) and Southern blots using cellular and viral probes are displayed. The gradient bars above each image denote increasing incubation times with MNase. Bands representing DNA that was protected from MNase cleavage are indicated by asterisks. Representative of $n = 2$ blots. For (A), the DNA ladder is λ HindIII. For (B), the DNA ladder is GeneRuler DNA Ladder Mix.



ined the level of expression of the tagged histones and showed that both cell lines expressed these variants at a similar level (Fig. 5A). We next examined the level of expression of H3.1-YFP or H3.3-YFP relative to endogenous H3, using an antibody recognizing the unmodified N-terminus of the proteins. Using a dilution series of the native and fusion protein extracts from cells, we determined that both H3.1-YFP and H3.3-YFP were expressed at a level of about 1/10th the endogenous H3 protein (Figs. 5B and 5C). We also examined growth of the H3.1-YFP and H3.3-YFP expressing cells compared with the parental A549 cell line and found that the growth kinetics were unaltered (data not shown). Finally, we examined Ad gene expression and recovery on the 3 cell lines and showed that a similar level of viral late gene expression was achieved with each cell line (Fig. 5D) and similar titers of virus were recovered from all 3 cell lines (Fig. 5E). Taken together, these results suggest that forced expression of H3.1-YFP or H3.3-YFP at a level of 1/10th the endogenous H3 does not adversely affect cell or virus growth.

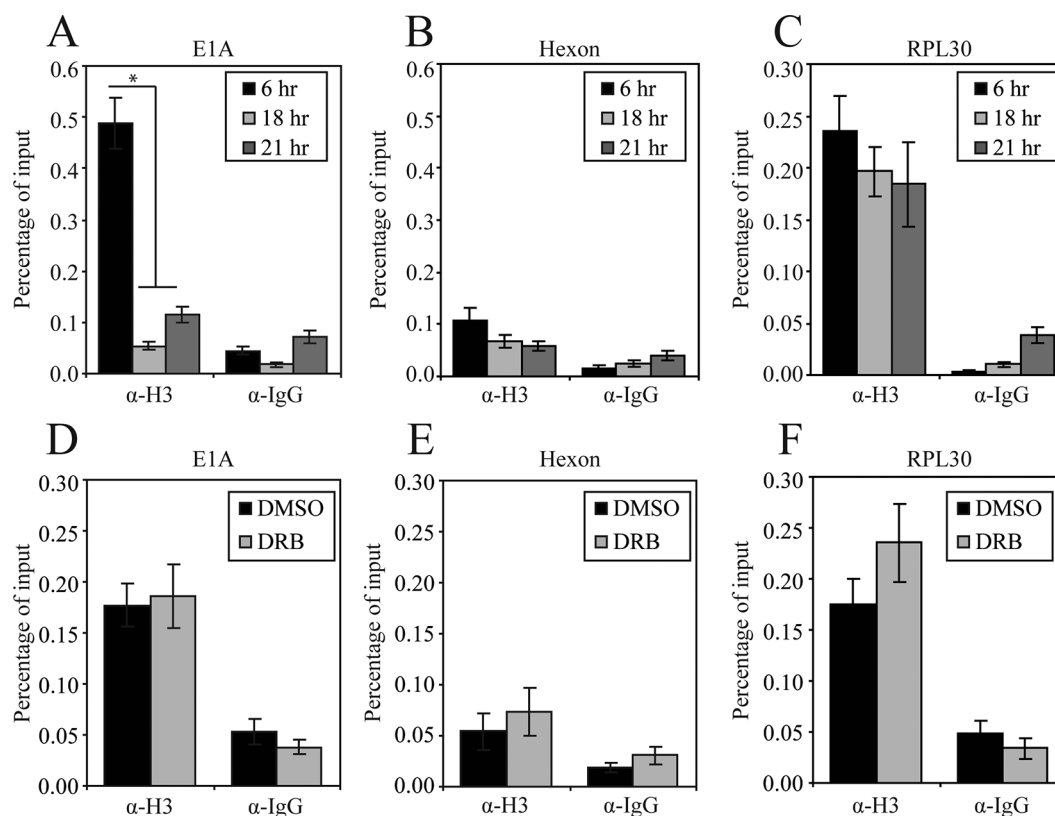
To determine the identity of the H3 variant that associates with Ad DNA, we infected parental A549 cells or cells expressing H3.1-YFP or H3.3-YFP with Ad at an MOI of 10 and processed the cells for ChIP at 6 or 18 hpi using antibody to GFP (cross-reacts with the YFP tag on the histones) or IgG. During the early phase of infection, we observed that the E1A region mainly associated with H3.3, and not with H3.1 (Fig. 5F). We also detected reduced association of the E1A region with H3.3 at late times of infection (Fig. 5F). The late hexon region showed low-level association with H3.3 at the early time

point, but slightly higher association at later times of infection (Fig. 5G). Again, H3.1-YFP was not found to be associated with this region of the viral DNA at either time point during infection. Taken together, these results indicate that Ad DNA preferentially associates with nucleosomes containing the histone variant H3.3.

Knockdown of histone variant-specific chaperone HIRA does not impact H3 deposition on Ad DNA

Our previous work showed that the histone variant H3.3-specific chaperone HIRA plays a role in the deposition of H3 on Ad vector DNA (Ross et al. 2011). Indeed, knockdown of HIRA reduced helper-dependent Ad (hdAd) DNA association with H3 and reduced expression of a vector-encoded transgene, suggesting that assembly of the hdAd into a nucleosome-like structure was important for expression of genes encoded in the vector. Thus, we asked whether HIRA was involved in deposition of H3 on Ad. We created an A549-derived cell line that contained an inducible shRNA construct targeted to HIRA (designated A549shHIRA), as well as a control cell line encoding a nontargeted shRNA (A549shCTRL). With the addition of 1 μ g/mL of doxycycline for 3 days, expression of the shRNA was induced and a knockdown of HIRA of 85%–90% was achieved (Fig. 6A). In the normal A549 or the A549shCTRL cell line, addition of doxycycline had no effect on the levels of HIRA. As an initial test of the effect of HIRA knockdown on viral growth and gene expression, we infected the cells with Ad at an MOI of 10 and examined early (E1A) and late (fiber) protein expression in the cells. As shown in Fig. 6A, knockdown of HIRA had no effect on the

Fig. 4. Adenovirus (Ad) DNA associates with cellular histones, which is not dependent of viral transcription. (A, B, and C) A549 cells were infected with AdVII-FLAG at a multiplicity of infection (MOI) of 10. Cells were processed for ChIP at 6, 18, and 21 hours post infection (hpi) with the indicated antibodies. (D, E, and F) A549 cells were treated with 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB) or vehicle (dimethyl sulfoxide; DMSO) for 1 h, infected as described above and processed for ChIP at 6 hpi. DNA obtained from the ChIP assays were subjected to qPCR analysis for 2 viral regions, E1A (A and D) and hexon (B and E) as well as a cellular locus RPL30 (C and F). For all panels, the mean and standard error are shown ($n = 3$ assays), analyzed in duplicate.



level of E1A protein levels measured at 8 h post-infection, as all cell lines exhibited near identical levels of E1A in the presence or absence of doxycycline. Furthermore, the cellular levels of fiber protein at 18 h post-infection were similar in control cells and those in which HIRA had been significantly depleted (Fig. 6B). Knockdown of HIRA also did not affect the amount of virus recovered over a 48 h time course of infection (data not shown). We next performed ChIP analysis to examine H3 association with the viral DNA and a representative cellular locus. As shown in Fig. 6C, as expected induction of shCTRL did not affect H3 association with the viral E1A region but, surprisingly, knockdown of HIRA also did not affect association of the viral DNA with H3. A similar relationship was shown for the cellular RPL30 locus: induction of neither shCTRL nor shHIRA in the A549 cells affected association with H3 (Fig. 6D). Taken together, these results suggest that, unlike our previous studies using hdAd, HIRA is not involved in deposition of H3 on incoming wildtype Ad DNA or that there are other proteins within the cell that can functionally compensate for loss of HIRA to deposit H3 on the Ad DNA.

Discussion

In the virion, the Ad DNA is condensed through the action of protein VII, a protamine-like protein, and it is the VII-wrapped DNA that enters the nucleus of infected cells. Cell-free studies have suggested that the protein-VII-wrapped DNA structure is refractory to virus replication and transcription and must be remodeled to efficiently perform these processes (Matsumoto et al. 1993; Okuwaki and Nagata 1998). Whether remodeling of the Ad genome

involves the removal of VII or if it is simply shifted along the genome to expose enhancer and promoter regions during the initial phase of virus gene expression is not clear. Immunofluorescence-based studies have suggested that there is stable association of VII during the early phase of infection (Xue et al. 2005; Chen et al. 2007; Walkiewicz et al. 2009; Karen and Hearing 2011). A confounding factor in these studies is the observation that VII from infecting virions can apparently form discrete foci in the absence of the viral genome (Wang et al. 2013), indicating that there is not a one-to-one relationship between Ad genome and VII foci in the nucleus. Furthermore, mature protein VII can mask direct detection of the incoming viral DNA in some fluorescence-based analyses (Krzywkowski et al. 2017). We observed a trend towards reduced association of VII with the DNA over the first 4 h of infection; however, this did not reach significance (Fig. 1A). By 6 hpi, association of the viral DNA with VII was reduced to approximately 25% of levels observed 1 hpi, and both our representative early (E1) and late (hexon) regions showed a similar level of association with VII at the 6 h time point (Figs. 1C and 1D), suggesting that remodeling of VII does not correlate with transcriptional activity of the region. Indeed, further studies confirmed that inhibition of transcription elongation has no effect on removal of VII (Fig. 2).

Using an MNase digestion assay, we observed a pattern for Ad DNA that is consistent with a repeating nucleosome-like array at 6 hpi (Fig. 3). Previous studies have also observed Ad DNA assembling into nucleosome-like structures (Sergeant et al. 1979; Beyer et al. 1981; Daniell et al. 1981; Déry et al. 1985), although not all

Fig. 5. Adenovirus (Ad) DNA preferentially associates with histone variant H3.3. (A) Immunoblot analysis of whole cell lysates showing exogenous H3-YFP tagged variants (α -GFP) and endogenous H3 (α -H3) in uninfected cells. (B and C) Immunoblot analysis of serial dilutions of A549 H3.1-YFP (B) or A549 H3.3-YFP (C) lysates probed with an H3 antibody (α -H3(N)) that recognizes the N-terminus of both endogenous and YFP-tagged forms of H3 to estimate the relative levels of expression, indicated by the boxed bands. (D and E) Cells (A549, A549 H3.1-YFP, or A549 H3.3-YFP) were infected with AdVII-FLAG at a multiplicity of infection (MOI) of 10 and processed for immunoblot analysis for Ad fiber protein at varying times post-infection (D) or virus yield quantification at 24 hours post infection (hpi) via plaque assay (E). (F and G) Cells (A549, A549 H3.1-YFP, or A549 H3.3-YFP) were infected with AdVII-FLAG at an MOI of 10 and processed for ChIP at 6 and 18 hpi with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for E1A (F) and hexon (G). Of note, the GFP antibody cross-reacts with the YFP tag on H3.1 and H3.3. The mean and standard error are shown ($n = 2$), analyzed in duplicate. NI, not infected.

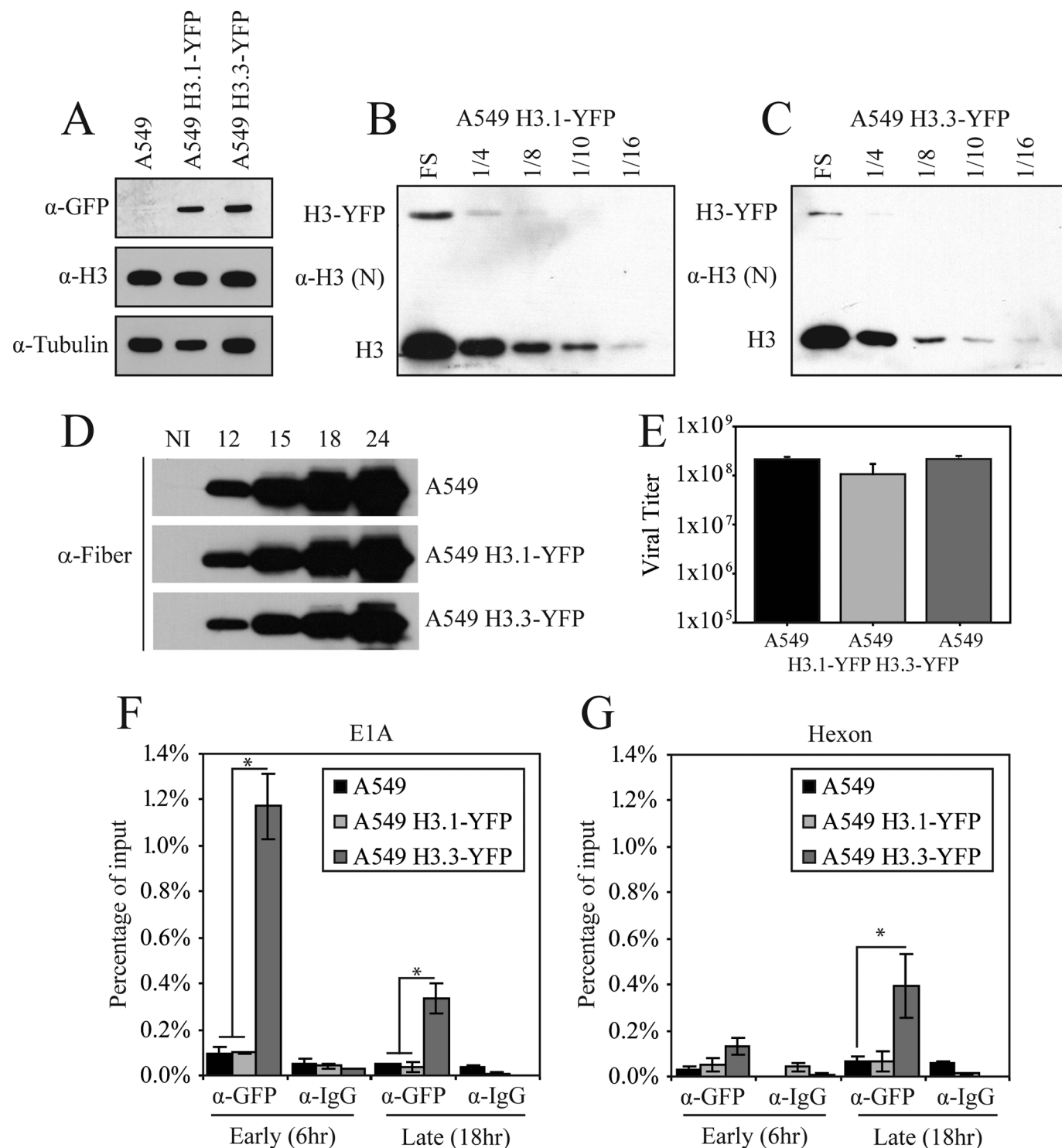
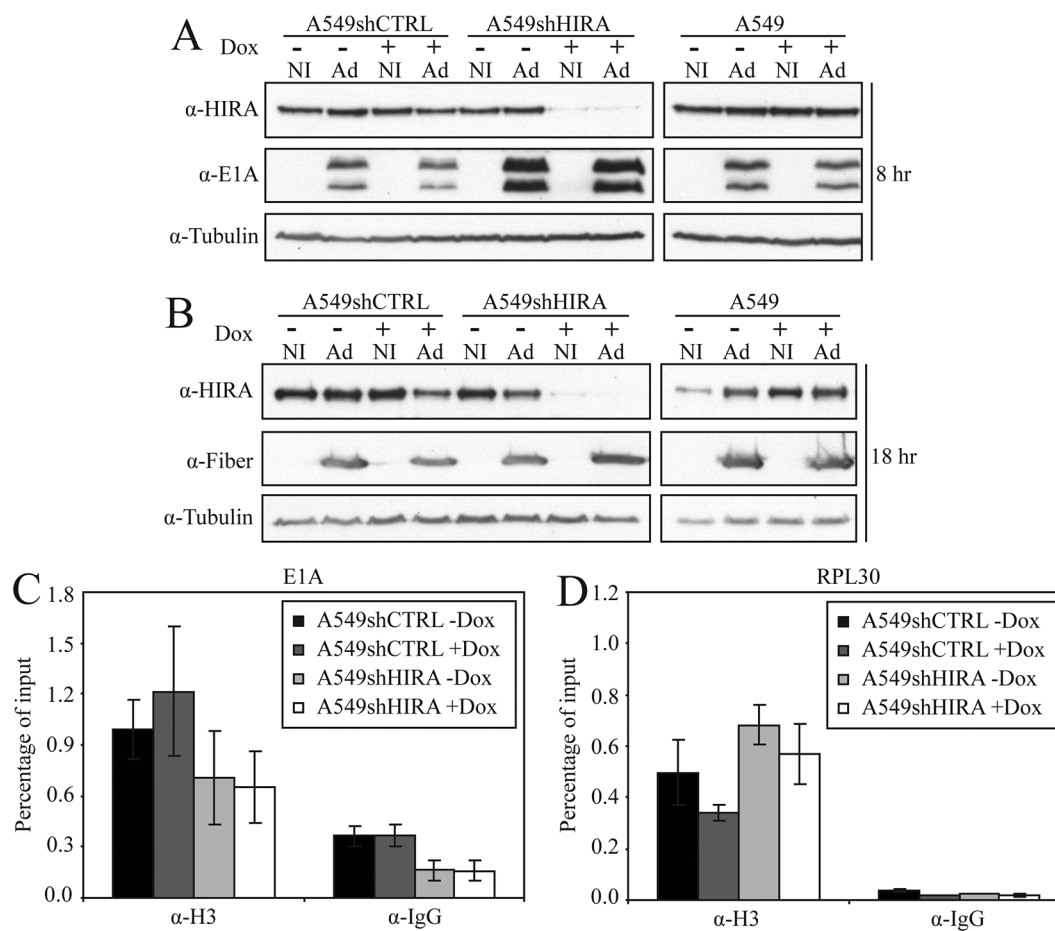


Fig. 6. Knockdown of the H3.3-specific histone chaperone HIRA does not affect adenovirus (Ad) gene expression or Ad DNA histone association. A549, A549shCTRL, and A549shHIRA cells were treated with vehicle or doxycycline to induce expression of the shRNA construct. Seventy-two hours later, the cells were infected with AdVII-FLAG at a multiplicity of infection of 10. (A and B) Whole cell lysates were harvested at 8 (A) or 18 (B) hours post infection (hpi) and subjected to immunoblot analysis for E1A (early gene) or fiber (late gene), respectively. (C and D) Cells were also processed for ChIP at 6 hpi with the indicated antibodies and the DNA samples obtained were analyzed by qPCR for the viral E1A region (C) and the cellular RPL30 locus (D). The mean and standard error are shown ($n = 2$), analyzed in duplicate. NI, not infected.



(Wong and Hsu 1988). The observation that Ad associates with all core histones (H2A-H2B and H3-H4) (Komatsu et al. 2011; Komatsu and Nagata 2012) suggests that Ad associates with true nucleosomes similar to cellular DNA. It has been estimated that up to 40% of infecting Ad DNA is contained in nucleosomes at 3 hpi, and all regions of the genome are represented in MNase-sensitive fractions (Sergeant et al. 1979). This repeating nucleosome-like pattern was not retained at late time of infection (Fig. 3B), where the viral DNA migrated primarily at a size of ~100 bp, which is more consistent with mononucleosomes. This observation is consistent with previous studies showing that the density of nucleosome-like structures associated with the Ad was approximately one-tenth that of cellular chromatin at late times of infection, as judged by electron microscope, suggesting that the Ad-DNA-associated nucleosomes were either widely spaced (Beyer et al. 1981) or unstable (i.e., exhibiting a rapid on-off rate) (Lacasse and Schang 2010). During late infection, we also observed a decline in H3 association with the E1 region to approximately 20% of that observed early in infection (Fig. 4A). Interestingly, we observed only very low-level association of the hexon region with H3 both early and late in infection (Fig. 4B). Although the percent association of H3 with the Ad genome at late times of infection is low, the number of genome copies present in the cell increases dramatically (Fig. 1B), so the absolute number of genomes associated with histones is likely still significant. As with our studies of VII re-

moval, we did not observe a difference in H3 association with the E1 or hexon regions in the presence or absence of DRB (Figs. 4D and 4E). Our data further suggest that nucleosomes may not be deposited uniformly across the Ad genome (compare Figs. 4A and 4B). Specific DNA elements that may signal or initiate histone deposition on the Ad genome have yet to be elucidated.

Through ChIP-qPCR analysis, we have shown that the Ad DNA preferentially associates with H3 variant H3.3 (Fig. 5). H3.3 is considered a replacement histone for cellular DNA, which is deposited onto promoters and enhancers by EP400 (Pradhan et al. 2016), within actively transcribed genes by HIRA (Goldberg et al. 2010), and on telomeres and pericentric DNA repeats by DAXX (Drané et al. 2010; Goldberg et al. 2010; Lewis et al. 2010). H3.3 is also preferentially deposited on incoming pronuclear DNA in *Drosophila* and on HSV DNA early in infection, both through the action of HIRA (Loppin et al. 2005; Placek et al. 2009). Recent studies have suggested that the rapid deposition of H3.3 mediated by HIRA may be a mechanism to protect naked DNA from damage (Schneiderman et al. 2012). Thus, initial removal of VII from sections of the Ad DNA may provide a signal to recruit cellular chaperones to wrap the viral DNA in nucleosomes containing H3.3. Our previous work showed that hdAd vectors associate with histone variant H3.3, and that this is mediated by the chaperone HIRA (Ross et al. 2011), but this does not appear to be the case for wildtype Ad. Alternatively, there may be other proteins within the

cell that can compensate for loss of HIRA to deposit H3 on the Ad DNA. We did not determine whether the H3 deposited on the Ad DNA in HIRA-knockdown cells is still H3.3, but we and others have never observed H3.1 association with Ad DNA, suggesting that it likely is. [Schreiner et al. \(2013\)](#) have shown that DAXX contributes to establishing the chromatin state of Ad DNA in the infected cell nucleus. Knockdown of DAXX resulted in enhanced viral gene expression and virus recovery, which was attributed to reduced chromatin density on the viral DNA. DAXX may function to place histones and nucleosomes on incoming viral DNA in an attempt to regulate or reduce spurious expression of the viral genes, which likely explains why Ad targets DAXX for degradation early in infection ([Schreiner et al. 2010](#)). DAXX-mediated deposition of H3.3 on human cytomegalovirus DNA is also important for gene silencing during establishment of latent infection ([Saffert and Kalejta 2007](#)). In contrast, HIRA may be involved in depositing H3.3 in a manner that promotes gene expression, as has been observed for HSV-1 ([Placek et al. 2009](#)) and our previous work using Ad-based vectors containing a heterologous CMV promoter ([Ross et al. 2011](#)). Thus, for many nuclear viruses, it is clear that the cell rapidly detects the naked or viral protein-wrapped DNA and engages pathways designed to incorporate the DNA into nucleosomes, thus exerting some level of regulatory/epigenetic control on the DNA ([Knipe et al. 2013](#)). The signals that guide whether repressive or activating chromatin is placed on the DNA have yet to be elucidated for Ad, but may represent potential therapeutic targets to limit virus-induced disease.

We showed that neither protein VII (Figs. 1D and 1E) nor histones (Figs. 4A and 4B) appear to be the major proteins that bind the Ad DNA during late infection. Naked DNA is not well tolerated in the nucleus of a cell ([Ray-Gallet et al. 2011](#)), and it is unlikely that newly replicated DNA is devoid of protein. Ad DNA replication employs a mechanism in which one of the two strands of the dsDNA genome is replicated in a continuous manner, while the second displaced strand of ssDNA is at least temporarily wrapped in the virus-encoded DNA binding protein (DBP) to protect it ([Berk 2007](#)). DBP also binds dsDNA, but with lower affinity ([Stuiver et al. 1992](#)). Given the very high abundance of DBP during late infection ([Saha and Parks 2017](#)), it is possible that Ad dsDNA coated in DBP may be the major nucleoprotein species within the cell. Although binding of DBP can enhance transcription from the major late promoter in a cell-free system ([Zijdeveld et al. 1994](#)), newly replicated DNA does not appear to be the template for late transcription ([Brison et al. 1979](#)). Thus, it is likely that the infected cell nucleus contains a mix of genomes associated with different proteins for specific functions. A small population may remain associated with histones to facilitate late gene expression, while a second major population may undergo DNA replication and remain primarily associated with DBP. The relatively low affinity of DBP for dsDNA may aid in switching to a pre-VII wrapped structure immediately before packaging of the viral DNA into the newly formed capsids. Recent studies have suggested that the Ad replicative centers may have subcompartments with specific regions dedicated to gene expression, replication, or viral packaging ([Komatsu et al. 2016](#)). Further studies are required to elucidate the complex events that dictate the nucleoprotein structure of the Ad DNA late in infection.

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References

- Anderson, C.W., Young, M.E., and Flint, S.J. 1989. Characterization of the adenovirus 2 virion protein, mu. *Virology*, **172**(2): 506–512. doi:10.1016/0042-6822(89)90193-1. PMID:2800334.
- Avgousti, D.C., Herrmann, C., Kulej, K., Pancholi, N.J., Sekulic, N., Petrescu, J., et al. 2016. A core viral protein binds host nucleosomes to sequester immune danger signals. *Nature*, **535**(7610): 173–177. doi:10.1038/nature18317. PMID:27362237.
- Avgousti, D.C., Della Fera, A.N., Otter, C.J., Herrmann, C., Pancholi, N.J., and Weitzman, M.D. 2017. Adenovirus core protein VII down-regulates the DNA damage response on the host genome. *J. Virol.* **91**(20): e01089–17. doi:10.1128/JVI.01089-17.
- Berk, A.J. 2007. Adenoviridae: the viruses and their replication. In *Fields virology*. Edited by D.M. Knipe and P.M. Howley. Lippincott Williams & Wilkins, Philadelphia. pp. 2355–2394.
- Beyer, A.L., Bouton, A.H., Hodge, L.D., and Miller, O.L., Jr. 1981. Visualization of the major late R strand transcription unit of adenovirus serotype 2. *J. Mol. Biol.* **147**(2): 269–295. doi:10.1016/0022-2836(81)90441-1. PMID:7288880.
- Brison, O., Kédinger, C., and Chambon, P. 1979. Adenovirus DNA template for late transcription is not a replicative intermediate. *J. Virol.* **32**(1): 91–97. PMID:232191.
- Brown, D.T., Westphal, M., Burlingham, B.T., Winterhoff, U., and Doerfler, W. 1975. Structure and composition of the adenovirus type 2 core. *J. Virol.* **16**(2): 366–387. PMID:1152144.
- Challberg, M.D., and Kelly, T.J., Jr. 1979. Adenovirus DNA replication in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **76**(2): 655–659. doi:10.1073/pnas.76.2.655. PMID:284391.
- Chatterjee, P.K., Vayda, M.E., and Flint, S.J. 1985. Interactions among the three adenovirus core proteins. *J. Virol.* **55**(2): 379–386. PMID:4020954.
- Chatterjee, P.K., Vayda, M.E., and Flint, S.J. 1986. Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32P-labelled in vivo. *J. Mol. Biol.* **188**(1): 23–37. doi:10.1016/0022-2836(86)90477-8. PMID:3712442.
- Chen, J., Morral, N., and Engel, D.A. 2007. Transcription releases protein VII from adenovirus chromatin. *Virology*, **369**(2): 411–422. doi:10.1016/j.virol.2007.08.012. PMID:17888479.
- Daniell, E., Groff, D.E., and Fedor, M.J. 1981. Adenovirus chromatin structure at different stages of infection. *Mol. Cell. Biol.* **1**(12): 1094–1105. doi:10.1128/MCB.1.12.1094. PMID:7346714.
- Déry, C.V., Toth, M., Brown, M., Horvath, J., Allaire, S., and Weber, J.M. 1985. The structure of adenovirus chromatin in infected cells. *J. Gen. Virol.* **66**(12): 2671–2684. doi:10.1099/0022-1317-66-12-2671. PMID:4067582.
- Drané, P., Ouarrhni, K., Depaux, A., Shuaib, M., and Hamiche, A. 2010. The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. *Genes Dev.* **24**(12): 1253–1265. doi:10.1101/gad.566910. PMID:20504901.
- Everitt, E., Sundquist, B., Pettersson, U., and Philipson, L. 1973. Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. *Virology*, **52**(1): 130–147. PMID:4139804.
- Everitt, E., Lutter, L., and Philipson, L. 1975. Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology*, **67**(1): 197–208. PMID:808902.
- Fabry, C.M., Rosa-Calatrava, M., Conway, J.F., Zubietta, C., Cusack, S., Ruigrok, R.W., and Schoehn, G. 2005. A quasi-atomic model of human adenovirus type 5 capsid. *EMBO J.* **24**(9): 1645–1654. doi:10.1038/sj.emboj.7600653. PMID:15861131.
- Giberson, A.N., Davidson, A.R., and Parks, R.J. 2012. Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res.* **40**(6): 2369–2376. doi:10.1093/nar/gkr1076. PMID:22116065.
- Goldberg, A.D., Banaszynski, L.A., Noh, K.M., Lewis, P.W., Elsaesser, S.J., Stadler, S., et al. 2010. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell*, **140**(5): 678–691. doi:10.1016/j.cell.2010.01.003. PMID:20211137.
- Graham, F.L. 1987. Growth of 293 cells in suspension culture. *J. Gen. Virol.* **68**(3): 937–940. doi:10.1099/0022-1317-68-3-937. PMID:3819703.
- Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**(1): 59–72. doi:10.1099/0022-1317-36-1-59. PMID:886304.
- Haruki, H., Okuwaki, M., Miyagishi, M., Taira, K., and Nagata, K. 2006. Involvement of template-activating factor I/SET in transcription of adenovirus early genes as a positive-acting factor. *J. Virol.* **80**(2): 794–801. doi:10.1128/JVI.80.2.794-801.2006. PMID:16378981.
- Jenuwein, T., and Allis, C.D. 2001. Translating the histone code. *Science*, **293**(5532): 1074–1080. doi:10.1126/science.1063127. PMID:11498575.
- Kang, R., Chen, R., Zhang, Q., Hou, W., Wu, S., Cao, L., et al. 2014. HMGB1 in health and disease. *Mol. Aspects Med.* **40**: 1–116. doi:10.1016/j.mam.2014.05.001. PMID:25010388.
- Karen, K.A., and Hearing, P. 2011. Adenovirus core protein VII protects the viral genome from a DNA damage response at early times after infection. *J. Virol.* **85**(9): 4135–4142. doi:10.1128/JVI.02540-10. PMID:21345950.

- Knipe, D.M., Lieberman, P.M., Jung, J.U., McBride, A.A., Morris, K.V., Ott, M., et al. 2013. Snapshots: chromatin control of viral infection. *Virology*, **435**: 141–156. doi:10.1016/j.virol.2012.09.023. PMID:23217624.
- Komatsu, T., and Nagata, K. 2012. Replication-uncoupled histone deposition during adenovirus DNA replication. *J. Virol.* **86**(12): 6701–6711. doi:10.1128/JVI.00380-12. PMID:22496236.
- Komatsu, T., Haruki, H., and Nagata, K. 2011. Cellular and viral chromatin proteins are positive factors in the regulation of adenovirus gene expression. *Nucleic Acids Res.* **39**(3): 889–901. doi:10.1093/nar/gkq783. PMID:20926393.
- Komatsu, T., Robinson, D.R., Hisaoka, M., Ueshima, S., Okuwaki, M., Nagata, K., and Wodrich, H. 2016. Tracking adenovirus genomes identifies morphologically distinct late DNA replication compartments. *Traffic*, **17**(11): 1168–1180. doi:10.1111/tra.12429. PMID:27492875.
- Kouzarides, T. 2007. Chromatin modifications and their function. *Cell*, **128**(4): 693–705. doi:10.1016/j.cell.2007.02.005. PMID:17320507.
- Krzywkowski, T., Ciftci, S., Assadian, F., Nilsson, M., and Punga, T. 2017. Simultaneous single-cell in situ analysis of human adenovirus type 5 DNA and mRNA expression patterns in lytic and persistent infection. *J. Virol.* **91**(11): e00166-17. doi:10.1128/JVI.00166-17. PMID:28298601.
- Lacasse, J.J., and Schang, L.M. 2010. During lytic infections, herpes simplex virus type 1 DNA is in complexes with the properties of unstable nucleosomes. *J. Virol.* **84**(4): 1920–1933. doi:10.1128/JVI.01934-09. PMID:20007274.
- Lewis, P.W., Elsaesser, S.J., Noh, K.M., Stadler, S.C., and Allis, C.D. 2010. Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc. Natl. Acad. Sci. U.S.A.* **107**(32): 14075–14080. doi:10.1073/pnas.1008850107. PMID:20651253.
- Li, Q., Burgess, R., and Zhang, Z. 2012. All roads lead to chromatin: multiple pathways for histone deposition. *Biochim. Biophys. Acta, Gene Regul. Mech.* **1819**(3–4): 238–246. doi:10.1016/j.bbaggm.2011.06.013. PMID:21763476.
- Loppin, B., Bonnefoy, E., Anselme, C., Laurençon, A., Karr, T.L., and Couble, P. 2005. The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. *Nature*, **437**(7063): 1386–1390. doi:10.1038/nature04059. PMID:16251970.
- Luger, K. 2006. Dynamic nucleosomes. *Chromosome Res.* **14**(1): 5–16. doi:10.1007/s10577-005-1026-1. PMID:16506092.
- Maizel, J.V., Jr., White, D.O., and Scharff, M.D. 1968. The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. *Virology*, **36**(1): 126–136. doi:10.1016/0042-6822(68)90122-0. PMID:5669983.
- Matsumoto, K., Nagata, K., Ui, M., and Hanaoka, F. 1993. Template activating factor I, a novel host factor required to stimulate the adenovirus core DNA replication. *J. Biol. Chem.* **268**(14): 10582–10587. PMID:8486711.
- Matthews, D.A., and Russell, W.C. 1998. Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *J. Gen. Virol.* **79**(7): 1671–1675. doi:10.1099/0022-1317-79-7-1671. PMID:9680130.
- Mirza, M.A., and Weber, J. 1982. Structure of adenovirus chromatin. *Biochim. Biophys. Acta, Gene Struct. Expression*, **696**(1): 76–86. doi:10.1016/0167-4781(82)90012-4. PMID:7082670.
- Newcomb, W.W., Boring, J.W., and Brown, J.C. 1984. Ion etching of human adenovirus 2: structure of the core. *J. Virol.* **51**(1): 52–56. PMID:6726895.
- Okuwaki, M., and Nagata, K. 1998. Template activating factor-I remodels the chromatin structure and stimulates transcription from the chromatin template. *J. Biol. Chem.* **273**(51): 34511–34518. doi:10.1074/jbc.273.51.34511. PMID:9852120.
- Ostapchuk, P., Suomalainen, M., Zheng, Y., Boucke, K., Greber, U.F., and Hearing, P. 2017. The adenovirus major core protein VII is dispensable for virion assembly but is essential for lytic infection. *PLoS Pathog.* **13**(6): e1006455. doi:10.1371/journal.ppat.1006455.
- Pérez-Berná, A.J., Marabini, R., Scheres, S.H., Menéndez-Conejero, R., Dmitriev, I.P., Curiel, D.T., et al. 2009. Structure and uncoating of immature adenovirus. *J. Mol. Biol.* **392**(2): 547–557. doi:10.1016/j.jmb.2009.06.057. PMID:19563809.
- Placek, B.J., Huang, J., Kent, J.R., Dorsey, J., Rice, L., Fraser, N.W., and Berger, S.L. 2009. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. *J. Virol.* **83**(3): 1416–1421. doi:10.1128/JVI.01276-08. PMID:19004946.
- Pradhan, S.K., Su, T., Yen, L., Jacquet, K., Huang, C., Côté, J., et al. 2016. EP400 deposits H3.3 into promoters and enhancers during gene activation. *Mol. Cell*, **61**(1): 27–38. doi:10.1016/j.molcel.2015.10.039. PMID:26669263.
- Ray-Gallet, D., Woolfe, A., Vassias, I., Pellentz, C., Lacoste, N., Puri, A., et al. 2011. Dynamics of histone H3 deposition in vivo reveal a nucleosome gap-filling mechanism for H3.3 to maintain chromatin integrity. *Mol. Cell*, **44**(6): 928–941. doi:10.1016/j.molcel.2011.12.006. PMID:22195966.
- Ross, P.J., and Parks, R.J. 2009. Construction and characterization of adenovirus vectors. *Cold Spring Harbor Protoc.* doi:10.1101/pdb.prot5011.
- Ross, P.J., Kennedy, M.A., and Parks, R.J. 2009. Host cell detection of noncoding stuffer DNA contained in helper-dependent adenovirus vectors leads to epigenetic repression of transgene expression. *J. Virol.* **83**(17): 8409–8417. doi:10.1128/JVI.00796-09. PMID:19515759.
- Ross, P.J., Kennedy, M.A., Christou, C., Risco Quiroz, M., Poulin, K.L., and Parks, R.J. 2011. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J. Virol.* **85**(8): 3950–3958. doi:10.1128/JVI.01787-10. PMID:21307207.
- Russell, W.C., Laver, W.G., and Sanderson, P.J. 1968. Internal components of adenovirus. *Nature*, **219**(5159): 1127–1130. doi:10.1038/2191127a0. PMID:5675626.
- Saffert, R.T., and Kalejta, R.F. 2007. Human cytomegalovirus gene expression is silenced by Daxx-mediated intrinsic immune defense in model latent infections established in vitro. *J. Virol.* **81**(17): 9109–9120. doi:10.1128/JVI.00827-07. PMID:17596307.
- Saha, B., and Parks, R.J. 2017. Human adenovirus type 5 vectors deleted of early region 1 (E1) undergo limited expression of early replicative E2 proteins and DNA replication in non-permissive cells. *PLoS One*, **12**(7): e0181012. doi:10.1371/journal.pone.0181012. PMID:28700677.
- Saha, B., Wong, C.M., and Parks, R.J. 2014. The adenovirus genome contributes to the structural stability of the virion. *Viruses*, **6**(9): 3563–3583. doi:10.3390/v6093563. PMID:25254384.
- Schneiderman, J.L., Orsi, G.A., Hughes, K.T., Loppin, B., and Ahmad, K. 2012. Nucleosome-depleted chromatin gaps recruit assembly factors for the H3.3 histone variant. *Proc. Natl. Acad. Sci. U.S.A.* **109**(48): 19721–19726. doi:10.1073/pnas.1206629109. PMID:23150573.
- Schreiner, S., Wimmer, P., Sirma, H., Everett, R.D., Blanchette, P., Groitl, P., and Dobner, T. 2010. Proteasome-dependent degradation of Daxx by the viral E1B-55K protein in human adenovirus-infected cells. *J. Virol.* **84**(14): 7029–7038. doi:10.1128/JVI.00074-10. PMID:20484509.
- Schreiner, S., Bürck, C., Glass, M., Groitl, P., Wimmer, P., Kinkley, S., et al. 2013. Control of human adenovirus type 5 gene expression by cellular Daxx/ATRAX chromatin-associated complexes. *Nucleic Acids Res.* **41**(6): 3532–3550. doi:10.1093/nar/gkt064. PMID:23396441.
- Sergeant, A., Tigges, M.A., and Raskas, H.J. 1979. Nucleosome-like structural subunits of intranuclear parental adenovirus type 2 DNA. *J. Virol.* **29**(3): 888–898. PMID:448800.
- Smith, A.C., Poulin, K.L., and Parks, R.J. 2009. DNA genome size affects the stability of the adenovirus virion. *J. Virol.* **83**(4): 2025–2028. doi:10.1128/JVI.01644-08. PMID:19036812.
- Spector, D.J. 2007. Default assembly of early adenovirus chromatin. *Virology*, **359**(1): 116–125. doi:10.1016/j.virol.2006.09.005. PMID:17034827.
- Stewart, P.L., Fuller, S.D., and Burnett, R.M. 1993. Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.* **12**(7): 2589–2599. PMID:8334984.
- Stuiver, M.H., Bergsma, W.G., Arnberg, A.C., van Amerongen, H., van Grondelle, R., and van der Vliet, P.C. 1992. Structural alterations of double-stranded DNA in complex with the adenovirus DNA-binding protein. Implications for its function in DNA replication. *J. Mol. Biol.* **225**(4): 999–1011. PMID:1319498.
- Tagami, H., Ray-Gallet, D., Almouzni, G., and Nakatani, Y. 2004. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell*, **116**(1): 51–61. doi:10.1016/S0092-8674(03)01064-X. PMID:14718166.
- Ugai, H., Borovjagin, A.V., Le, L.P., Wang, M., and Curiel, D.T. 2007. Thermostability/infectivity defect caused by deletion of the core protein V gene in human adenovirus type 5 is rescued by thermo-selectable mutations in the core protein X precursor. *J. Mol. Biol.* **366**(4): 1142–1160. doi:10.1016/j.jmb.2006.11.090. PMID:17208253.
- Walkiewicz, M.P., Morral, N., and Engel, D.A. 2009. Accurate single-day titration of adenovirus vectors based on equivalence of protein VII nuclear dots and infectious particles. *J. Virol. Methods*, **159**(2): 251–258. doi:10.1016/j.jviromet.2009.04.010. PMID:19406166.
- Wang, I.H., Suomalainen, M., Andriasyan, V., Kilcher, S., Mercer, J., Neef, A., et al. 2013. Tracking viral genomes in host cells at single-molecule resolution. *Cell Host Microbe*, **14**(4): 468–480. doi:10.1016/j.chom.2013.09.004. PMID:24139403.
- Wong, C.M., McFall, E.R., Burns, J.K., and Parks, R.J. 2013. The role of chromatin in adenoviral vector function. *Viruses*, **5**(6): 1500–1515. doi:10.3390/v5061500. PMID:23771241.
- Wong, M.L., and Hsu, M.T. 1988. Psoralen-cross-linking study of the organization of intracellular adenovirus nucleoprotein complexes. *J. Virol.* **62**(4): 1227–1234. PMID:2831383.
- Xue, Y., Johnson, J.S., Ornelles, D.A., Lieberman, J., and Engel, D.A. 2005. Adenovirus protein VII functions throughout early phase and interacts with cellular proteins SET and pp32. *J. Virol.* **79**(4): 2474–2483. doi:10.1128/JVI.79.4.2474-2483.2005. PMID:15681448.
- Yankulov, K., Yamashita, K., Roy, R., Egly, J.M., and Bentley, D.L. 1995. The transcriptional elongation inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription factor IIH-associated protein kinase. *J. Biol. Chem.* **270**(41): 23922–23925. doi:10.1074/jbc.270.41.23922. PMID:7592583.
- Zijderveld, D.C., d'Adda di Fagagna, F., Giacca, M., Timmers, H.T., and van der Vliet, P.C. 1994. Stimulation of the adenovirus major late promoter in vitro by transcription factor USF is enhanced by the adenovirus DNA binding protein. *J. Virol.* **68**(12): 8288–8295. PMID:7966622.

Appendix 4:
Curriculum Vitae

Briti Saha

Regenerative Medicine Program, Ottawa Hospital Research Institute
501 Smyth Road, Ottawa, ON K1H 8M2

Education

Ph.D. in Biochemistry with Specialization in Human and Molecular Genetics

University of Ottawa 2013 – 2019

B.Sc. Honours with Specialization in Biochemistry (*magna cum laude*)

University of Ottawa 2009 – 2013

Scholarships and Awards

Academic and research awards:

Ontario Graduate Scholarship	2016 – 2018
Excellence Scholarship, uOttawa	2015 – 2018
Queen Elizabeth II Graduate Scholarship (QEII-GSST)	2015 – 2016
Ph.D. Admission Scholarship, uOttawa	2015, 2018
Faculty of Medicine Award of Excellence in Mentorship, uOttawa	2015
Syed Sattar Master's Student Award for research excellence, uOttawa	2015
Master's Admission Scholarship, uOttawa	2013 – 2015
Dean's Honour List, uOttawa	2012 – 2013
Merit Scholarship, uOttawa	2012 – 2013
Undergraduate Research Opportunity Program (UROP) Award, uOttawa	2011 – 2012
Undergraduate Admission Scholarship, uOttawa	2009 – 2010

Presentation and conference travel awards:

3 rd place in poster presentation competition, OHRI Research Day	2017
CEEHRC Network Travel Award	2017
Conference Travel Grant from Graduate Student Association, uOttawa	2016, 2017
Travel Award for Chromatin Control of Viral Infection Meeting	2016
Travel Award for DNA Tumour Virus Meeting	2016
Best Poster Award, Ottawa Hospital Dept. of Medicine Research Day	2016
Faculty of Graduate Studies Conference Travel Grant, uOttawa	2015 – 2017
Department of Biochemistry Travel Award, uOttawa	2015 – 2017
Outstanding Poster Award, ASGCT 18 th Annual Meeting	2015
2 nd place in biochemistry research seminar competition, uOttawa	2015
3 rd place in biochemistry poster presentation competition, uOttawa	2014

Research Experience

Doctoral Researcher

September 2013 – June 2019

Dr. Robin Parks' Lab, Ottawa Hospital Research Institute

- Research project objective was to identify and validate novel antiviral compounds and pharmacological targets for therapeutic intervention in human adenovirus (Ad) infections

- Generated viral vectors and developed a high-content screening platform to determine effect of small molecules on virus gene expression and replication
- Screened small-molecule libraries and identified several compounds that exhibit antiviral activity against clinically relevant Ad serotypes
- Validated positive hits by investigating their impacts on virus lifecycle and cell viability
- Investigated virus-cell interactions via cell-based assays and elucidated a previously unreported role of histone deacetylases in Ad infection
- Acquired diverse technical skills in virology, molecular biology and biochemistry including viral vector production, mammalian cell culture, cell transfection/infection, molecular cloning, small molecule screens, SDS-PAGE, Western blotting, qPCR, RT-PCR, fluorescence microscopy, chromatin/protein immunoprecipitation, etc.

Honours Research Student

August 2012 – April 2013

Dr. Thien-Fah Mah's Lab, Faculty of Medicine, University of Ottawa

- Research objective was to study the expression of the *Pseudomonas aeruginosa* (PA) *ndvB* gene, which confers biofilm-specific antibiotic resistance to the bacteria
- Created a PA plasmid encoding a reporter gene under the control of the *ndvB* promoter for investigating the temporal and spatial promoter activity in biofilms
- Acquired technical skills in bacteria culture, bacterial/yeast cloning and bactericidal concentration assays

Undergraduate Research Student

October 2011 – February 2012

Dr. Martin Pelchat's Lab, Faculty of Medicine, University of Ottawa

- Studied the interaction of hepatitis delta virus (HDV) RNA with a cellular regulatory protein to determine the role of HDV in inducing oncogene expression

Publications

Saha B, Varette O, Stanford WL, Diallo JS and Parks RJ. (2019) Development of a novel screening platform for the identification of small molecule inhibitors of human adenovirus. *Virology*. 538: 24-34.

Saha B and Parks RJ. (2019) Histone deacetylase inhibitor SAHA suppresses human adenovirus gene expression and replication. *J Virol*. 93(12): e00088-19.

Giberson AN, **Saha B**, Campbell K, Christou C, Poulin KL, Parks RJ. (2018) Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication. *Biochem Cell Biol*. 96(6): 797-807.

Hall CW, Hinz AJ, Gagnon LB, Zhang L, Nadeau JP, Copeland S, **Saha B** and Mah TF. (2018) Expression of the *Pseudomonas aeruginosa* biofilm antibiotic resistance gene *ndvB* requires the RpoS stationary phase sigma factor. *Appl Environ Microbiol*. 84(7): e02762-17.

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

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

Saha B and Parks RJ. Towards novel antiviral therapies against human adenovirus: Prospective small molecules for clinical development. (In preparation)

Presentations

Oral presentations:

Saha B, Varette O, Diallo J-S and Parks RJ. (2018) Identification of cellular targets and small molecules inhibiting adenovirus replication. 2nd Symposium of the Canadian Society for Virology. Halifax, Canada.

Saha B, Varette O, Diallo J-S and Parks RJ. (2016) Identification of cellular targets and small molecules inhibiting adenovirus replication. 12th International Adenovirus Meeting. Barsinghausen, Germany.

Saha B, Varette O, Diallo J-S and Parks RJ. (2016) Identification of cellular targets and small molecules inhibiting adenovirus replication. DNA Tumour Virus Meeting. Montreal, Canada.

Poster presentations:

Saha B, Varette O, Diallo J-S and Parks RJ. (2018) Identification of pharmacological targets and small molecules inhibiting adenovirus replication. Biochemistry, Microbiology and Immunology Scientific Symposium. Montebello, Quebec.

Saha B, Varette O, Diallo J-S and Parks RJ. (2017) Investigating epigenetic regulation of the adenovirus genome as a potential therapeutic target in infection. 4th Canadian Conference on Epigenetics. Whistler, Canada.

Saha B, Varette O, Diallo J-S and Parks RJ. (2017) Identifying pharmacological enhancers of adenoviral vector production and transgene expression. 4th Ottawa International Conference on Neuromuscular Biology, Disease and Therapy. Ottawa, Canada.

Saha B, Varette O, Diallo J-S and Parks RJ. (2017) Identification of pharmacological targets and small molecules inhibiting adenovirus replication. Ottawa Hospital Research Institute Research Day and The Ottawa Hospital Department of Medicine Research Day. Ottawa, Canada.

Saha B, Varette O, Diallo J-S and Parks RJ. (2017) Identification of pharmacological targets and small molecules inhibiting adenovirus replication. EMBO Conference on Hijacking Host Signaling and Epigenetic Mimicry during Infections. Paris, France.

Saha B, Varette O, Diallo J-S, Stanford W and Parks RJ. (2017) Epigenetic regulation of the adenovirus genome and identification of cellular factors as potential pharmacological targets in adenovirus infection. 60th Annual Conference of the Canadian Society of Molecular Biosciences. Ottawa, Canada.

Saha B, Varette O, Diallo J-S, Stanford W and Parks RJ. (2016) Identification of cellular targets and small molecules inhibiting adenovirus replication. Ottawa Hospital Research Institute Research Day and The Ottawa Hospital Department of Medicine Research Day. Ottawa, Canada.

Saha B, Diallo J-S and Parks RJ. (2016) Epigenetic regulation of the adenovirus genome and identification of cellular epigenetic modifiers as potential therapeutic targets in adenovirus infection. 3rd Canadian Conference on Epigenetics and Epigenomics in Development and Disease. Esterel, Canada.

Saha B, Diallo J-S and Parks RJ. (2016) Epigenetic regulation of the adenovirus genome and identification of cellular epigenetic modifiers as potential therapeutic targets in adenovirus infection. Chromatin Control of Viral Infections Workshop. Bethesda, U.S.A.

Saha B, Diallo J-S, Stanford W and Parks RJ. (2015) Identifying pharmacological enhancers of adenoviral vector replication and transgene expression. 3rd Ottawa International Conference on Neuromuscular Biology, Disease and Therapy. Ottawa, Canada.

Saha B, Diallo J-S and Parks RJ. (2015) Identifying pharmacological enhancers of oncolytic adenoviral vector replication and transgene expression. American Society of Gene and Cell Therapy 18th Annual Meeting. New Orleans, U.S.A.

Professional Involvements

Reviewer October 2017 – October 2018
University of Ottawa Journal of Medicine

- Reviewed submitted articles; provided constructive criticism and decision on article acceptance for publication

VP Finance May 2017 – April 2018
Biochemistry, Microbiology and Immunology Graduate Student Association (BMIGSA), University of Ottawa

- Managed the annual budget, allocated funds for various events, monitored expenditures and oversaw all other financial affairs of the BMIGSA
- Received custody of BMIGSA funds and acted as chief signing officer

VP External and Departmental Director May 2014 – April 2017
BMIGSA and Graduate Student Association (GSAED), University of Ottawa

- Represent the BMIGSA and the graduate students of the BMI department in GSAED Board of Directors; act as a liaison between the councils
- Assist in the organization and advertisement of academic, social and fundraising events

Graduate Student Representative April 2014 – June 2019
Ottawa Hospital Research Institute (OHRI) Trainee Committee

- Present concerns of OHRI graduate students to the committee (e.g. regarding training environment, equipment) and discuss potential solutions

Fundraising and Events Assistant

January 2012 – May 2014

“Team Diabetes” Chapter, University of Ottawa

- Participated in the organization and advertisement of events to raise funds for the Canadian Diabetes Association to support research in diabetes
- Assisted club executives with raising awareness about diabetes and promoting healthy living within the student community

Co-Chair of Marketing and Communications

September 2012 – May 2013

Spirit of the Capital Youth Awards Executive Committee, Youth Ottawa

- Managed media relations and program promotion through various social media platforms

Work Experience

Data Collector/Analyst, Alumni Trajectory Project

February 2019 – June 2019

Faculty of Medicine, University of Ottawa

- Gathered information on career trajectories of alumni from graduate programs at the Faculty of Medicine with the purpose of implementing courses, workshops and resources to increase success of graduates in the workplace
- Worked within a committee to administer surveys for information collection, analyzed data from respondents and prepared a detailed Alumni Perspective Report

Teaching Assistant for Undergraduate Courses

January 2014 – April 2018

Department of Chemistry and Biomolecular Sciences, University of Ottawa

- Supervised undergraduate students during lab sessions, taught lab contents and evaluated lab reports for introductory biochemistry course
- Proctored and corrected examinations for courses on molecular biology, biochemistry and biopharmaceutical science

Laboratory Coordinator Assistant

May 2013 – August 2013

Undergraduate Biochemistry Laboratory, University of Ottawa

- Created mutants of the T7 RNA polymerase enzyme and assessed the effect of the mutations on enzyme activity and efficiency
- Tested experiments designed for undergraduate biochemistry and molecular biology laboratory courses to optimize results and experimental conditions

Laboratory Technician Assistant

May 2011 – August 2011

Undergraduate Biochemistry Laboratory, University of Ottawa

- Assisted laboratory personnel with preparation of reagents and maintenance of laboratory stations, equipment and chemical inventories

Ambassador for Biochemistry Program

October 2010 – October 2011

Undergraduate Biochemistry Program, University of Ottawa

- Discussed the undergraduate biochemistry program with prospective students
- Demonstrated basic lab techniques

Volunteer Experience

Peer Mentor

January 2017 – June 2019

Department of Biochemistry, Microbiology and Immunology, University of Ottawa

- Assist new students in becoming familiar with their graduate study program in Biochemistry or Microbiology and Immunology
- Provide guidance, support and advice when required

Poster Session Organizer for Brain Health Research Day

January 2017 – June 2017

Brain and Mind Research Institute, University of Ottawa

- Scheduled poster presentations, coordinated judging assignments and finalized awards

Volunteer Educator

September 2013 – Present

Let's Talk Science, University of Ottawa Chapter

- Present various scientific concepts and related hands-on activities in classrooms and community events to engage youth, and inspire them to pursue studies in science
- Judge high school and undergraduate science projects, and evaluate presentations

Patient Ambassador

May 2010 – March 2013

The Canadian Cancer Society and the Ottawa Hospital Cancer Centre

- Guided patients through completing their electronic symptom assessments
- Informed new patients of various support programs offered by the cancer centre
- Escorted patients into examination rooms and delivered their medical charts to oncologist

Media Coverage

[Ottawa Hospital team gets funding boost to cure common cold.](#) Featured on CBC News.

December 2015. (Television)

[Ottawa researchers tackle the common — and costly — cold.](#) The Ottawa Citizen and Vancouver Sun. December 2015. (Print and online)