UNDERSTANDING MECHANISMS OF ADENO-ASSOCIATED VIRUS VECTOR TRANSDUCTION THROUGH PHARMACOLOGICAL AND BIOLOGICAL APPROACHES

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ABSTRACT

Sarah Cathlin Nicolson: Understanding mechanisms of Adeno-associated virus vector transduction through pharmacological and biological approaches
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Gene therapy using Adeno-associated virus vectors (rAAV) has garnered much promise recently due to impressive clinical performance for several indications. Indeed, the lack of pathogenicity, broad tissue tropism among several serotypes, and ability to replace greater than 90 percent of the viral genome with a therapeutic transgene makes rAAV an attractive tool to achieve therapeutic benefit. However, recent gene therapy trials have highlighted a particular challenge that has hindered widespread applicability. While administration to local, immunopriviledged sites results in robust, long term expression of therapeutic transgenes; systemic delivery is seemingly restricted to a certain dose, as administration of vectors above this threshold seems to trigger an immune response resulting in decreased transgene expression over time. Thus, a major goal in the field is to identify ways to enhance transduction efficiency of vectors. In this dissertation, two approaches are explored that may provide insight into the future development of more effective vectors. First, a high throughput, small molecule screen was designed and executed to identify and characterize compounds resulting in enhanced transduction. These compounds were validated *in vitro* and clinically relevant compounds were explored *in vivo*. Secondly, a mechanism for rAAV2 nuclear entry, a step in subcellular trafficking thought to be a barrier to efficient transduction, was characterized. Efforts in capsid design or pharmacological approaches to overcome cellular barriers to transduction could result in enhanced transduction in a mechanism distinct from those already being targeted. These

results provide a foundation for further advancements in pharmacological screens and rational vector design in order to achieve widespread clinical utility of rAAV.

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AAP	Adeno-associated virus assembly activating protein
AAV	Adeno-associated virus
AAVS1	Adeno-associated virus integration site 1
Ad-DBP	Adenovirus DNA binding protein
ALT	alanine aminotransferase
ASPA	aspartoacylase
AST	aspartate aminootransferase
ATM	ataxia telangiectasia mutated
BBB	blood brain barrier
BUN	blood urea nitrate
CLIC/GEEC	clathrin-independent carriers/GPI-enriched early endosomal compartments
CK	creatine kinase
co-IP	co-immunoprecipitation
cNLS	classical nuclear localization signal
CNS	central nervous system
CUPID	calcium upregulation by percutaneous administration of gene therapy in cardiac disease
DNMT3B	DNA methyltransferase 3B
EGFR	epidermal growth factor receptor
EGFR-PTK	epidermal growth factor receptor protein tyrosine kinase
ER	endoplasmic reticulum

fAAV	fragmented Adeno-associated virus
FGFR	fibroblast growth factor receptor
FIX	factor IX
FOXM1	forkhead box protein M1
GAD65	glutamate decarboxylase, 65 kilodaltons
GAD67	glutamate decarboxylase, 67 kilodaltons
HBV	hepatitis B virus
HGFR	hepatocyte growth factor receptor
HSV-1	herpes simplex virus 1
hTERT	human telomerase reverse transcriptase
HU	hydroxyurea
ITR	inverted terminal repeat
LCA	Leber congenital amaurosis
LPLD	lipoprotein lipase deficiency
MRN	Mre11/Rad50/Nbs1
MTOC	microtubule organizing center
MVM	minute virus of mice
NHF1	normal human fibroblast 1
NLS	nuclear localization signal
NPC	nuclear pore complex
PCNA	proliferating cell nuclear antigen
PDGFR	platelet derived growth factor receptor
PEI	polyethyleneimine

qPCR	quantitative polymerase chain reaction
RFC	replication factor C
RPA	replication protein A
RPE65	retinal pigment epithelium-specific protein, 65 kilodaltons
SAHA	suberanilohydroxamic acid
ssAAV	single-stranded Adeno-associated virus
scAAV	self-complementary Adeno-associated virus
WGA	wheat germ agglutinin
VG	vector genome
VG/cell	vector genomes/cell

CHAPTER 1

Introduction

Gene therapy and the emergence of rAAV

Gene therapy has been a long sought after strategy to combat genetic disorders or acquired diseases. Since the 1970s, the idea of introducing a copy of a functional gene to someone with a defective counterpart has been pursued in myriad fields using an array of delivery strategies. More recently, gene therapy has been pursued as a way to introduce a therapeutic protein for acquired disease. Monogenetic disorders, the primary target for congenital gene therapy, affect approximately one percent of births worldwide. Acquired diseases, such as Alzheimer's disease, Parkinson's disease, and cancer, could benefit from gene therapy through the addition of a therapeutic protein or RNAi. The perceived clinical utility is growing, as can be evidenced by the approximately 1800 gene therapy clinical trials initiated to date (wiley.co.uk/genetherapy/clinical). While most are phase I trials, a growing number are reporting phase II and phase III statuses. In fact, the first ever approved gene therapy has emerged: Glybera® is a treatment for lipoprotein lipase deficiency (LPLD) currently approved in the European Union. This endpoint represents a longtime goal held by so many and will hopefully mark the beginning of exciting new developments in the future.

Indeed, the promising pre-clinical and clinical data seen especially in the past decade has resulted from the persistence of a field that suffered many setbacks in the decades before. Stemming from a gene therapy trial utilizing retroviral vectors for hematopoietic stem cells, the

importance of controlling for integration of the transgene into human chromosomes was highlighted when 2 out of 10 children treated for Severe Combined Immunodeficiency Disorder developed leukemia shortly thereafter. The death of Jesse Gelsinger in 1999 due to hyper-immune complications from an Adenoviral-based gene therapy resulted in serious scrutiny from the regulatory community and stalled gene therapy progress at the time. However, these events inspired the field to investigate alternative viral vector approaches; namely, viral vectors that did not invoke a strong immune response and did not function exclusively by integrating their genome into host cell chromosomes.

Concomitant with the aforementioned adverse events came the emergence of promising studies with Adeno-associated virus (AAV), a small parvovirus which had been shown to facilitate long term gene expression in a multitude of tissue types and myriad animal models. AAV vectors (rAAV) are nonpathogenic and delivered transgenes persist in host cells as episomes rather than integrating into host chromosomes. To date, there have been over 10 serotypes and 100 variants identified, which infect a wide range of human tissues with varying levels of efficacy. For these reasons, AAV has become a very promising vector for gene therapy applications.

The first demonstration of rAAV-mediated gene transfer into murine and human cells occurred in 1984 (1). To date, there have been over 100 clinical trials initiated utilizing rAAV (http://www.abedia.com/wiley). The results of completed and ongoing trials have consistently demonstrated that rAAV vector administraton and persistence is safe and well tolerated by humans (2) **(Figure 1.1A)**. The first AAV-mediated gene therapy that clearly demonstrated long-term efficacy was reported in two clinical trials for Leber congenital amaurosis (LCA), an autosomal recessive disease that results in blindness. In these particular trials, patients with a

deficiency in the human retinal pigment epithelium-specific 65 (RPE65) gene were treated with an rAAV2 vector carrying the correct copy. One trial was performed in London, while the other was completed in Philadelphia (3-5). Differences in the trials included the promoter selected for each construct (constitutive in the US trial, endogenous RPE65 promoter in the UK) as well as the selected patient population (adolescents in the UK, pediatrics with less progressive disease as well as adults in the US). While visual function improved in only one of the three patients tested in the UK, all patients the US trial reported objective and subjective improvement in vision, with the greatest improvement seen in the pediatric patients. It remains to be determined if the rAAV2_RPE65-mediated therapy will prevent further degeneration in the UK patients who did not report improvements in vision.

Efficacy using rAAV-mediated gene therapy has also been reported for Parkinson's disease and congestive heart failure. In the Parkinson's disease trial, patients were given a 1:1 infusion of glutamate decarboxylase (GAD) 65 and GAD67 encapsidated by AAV2 delivered to the center of the subthalamic nucleus (6). At 6 months post-infusion, the unified Parkinson's disease rating scale (UPDRS) had decreased by 8.1 points in the AAV2_GAD cohort as compared to a decrease of 4.7 points in the sham operation group. Clinical improvement was also noted in the 6 month follow-up study in the AAV2_GAD-treated cohort. In the Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trial, patients received either placebo or low-dose, medium-dose, or high-dose sarcoplasmic reticulum Ca2+-ATPase (SERCA2a) gene encapsidated by AAV1 (7). Administration was performed via a percutaneous intracoronary infusion and primary endpoints were evaluated 6 months post-administration. Improvements or stabilization in several clinical parameters, including 6-minute walk test, peak maximum oxygen consumption, left ventricular end-systolic

volume, time to clinical events, and cardiovascular events, was observed for the high-dose cohort.

Long-term improvements in clinical outcomes has been demonstrated for a congenital neurological disease, Canavan disease (8). In this trial, patients were treated with rAAV2 carrying the aspartoacylase gene (ASPA) administered via intraparenchymal delivery at six sites in the brain. Safety profiles and clinical outcomes were recorded for at least 5 years post-treatment. In the AAV2_ASPA-treated cohort, clinical outcomes such as decreased N-acetylaspartate (NAA), slowed progression of brain atrophy, improvement in seizure frequency, and general clinical stabilization were reported.

The emergence of such promising results in rAAV-mediated gene therapy has been substantiated by the first ever approved gene therapy therapeutic: Glybera® (alipogene tiparvovec). Glybera® is an rAAV1-based vector carrying a lipoprotein lipase variant (LPLS447X) for the treatment of lipoprotein lipase deficiency (LPLD). It was approved by the European Commission in November 2012. Familial LPLD affects between one in 500,000 to one in 1,000,000 people (9) and is caused by the lack of a functional LPL gene. LPL is produced in and secreted from skeletal muscle and adipose tissue and is transported to the limunal surface of blood vessels. The primary role of LPL is in the metabolism of triglycerides, chylomicrons, and very low-density lipoproteins. Clinical manifestations of LPLD include severe hypertriglyceridemia and hyperchylomicronemia which can lead to several complications, including acute pancreatitis. Prior to the approval of Glybera®, the only treatment available was dietary restriction to a low fat diet, as enzyme replacement therapy was not an option due to the short half life of LPL.

Three clinical trials were performed to assess the safety and efficacy of Glybera® for LPLD. In the first clinical trial, eight patients were administered either a low-dose or high-dose of AAV1_LPLS447X via 40-60 intramuscular injections (10). Decrease in plasma triglyceride levels were noted, but this decrease was transient and thought to be mediated by an immune response. The second clinical trial was conducted in 14 patients who received an immunosuppression regimen in addition to AAV1_LPLS447X (11). Decreases in plasma triglyceride levels were reported, but were still transient, suggesting that an immune response was not responsible for the fleeting measurements. Despite the disappointing measured outcome, signs of clinical improvement were notable out to two years post-administration. These included increased patient tolerance for certain foods, changes in overall blood lipid profile, and decreased incidences of pancreatitis, in addition to persistent expression of the LPLS447X transgene. Since a discrepancy in clinical outcome and plasma triglyceride levels was found, a third clinical trial was initiated with predetermined outcomes including incidences of abdominal pain, pancreatitis, and chylomicron plasma clearance. Five patients were treated with rAAV1_LPLS447X and reported decreased abdominal pain, decreased incidences of pancreatitis, and improved overall lifestyle in addition to improvements in chylomicron metabolism out to two years post-administration (12). These studies, in combination with a retrospective analysis of 22 of the 27 treated patients overall that showed a decrease in the incidence and severity of pancreatitis (9), provided the evidence that resulted in approval of Glybera® by the European Commission.

Clinical limitations of rAAV-mediated gene therapy

The influx of promising results from clinical trials and the emergence of a clinically approved rAAV-based gene therapy has reinvigorated the field and provided excellent proof-of-concept studies that warrant broader investigations into rAAV-mediated gene therapy. However, systemic application of rAAV-based gene therapy has been met with challenges of low levels of transgene expression or loss of transgene expression over time, thought to be mediated by a cytotoxic T cell response to the AAV capsid. These complications are highlighted by the earlier Hemophilia B trials, where patients were treated with three doses of rAAV2 carrying Factor IX (FIX) and assessed for circulating FIX, bleeding episodes, and general clinical condition. In a phase I/II, dose escalation trial with seven patients, therapeutic levels of FIX were achieved with the highest vector dose (13). However, these levels returned to baseline by eight weeks post-administration and correlated with a transient rise in liver aspartate aminotransferase (AST) and alanine aminotransfease (ALT). It was later determined that the rise in liver enzymes and corresponding decrease in FIX expression was due to capsid-mediated CD8+ T-cell cytotoxiciy (14). No rise in liver enzymes was observed with the patients receiving a lower dose of vector; however, therapeutic levels of FIX were not achieved either.

In the most recent dose-escalation Hemphilia B clinical trial, patients received peripheral vein-administered rAAV8 encoding a self-complementary, codon-optimized FIX gene (15). Generally FIX expression and clinical outcome correlated with vector dose. Four of the six patients were able to discontinue FIX infusions, although 2 of these patients required bolus injections of FIX after severe injury and one patient required FIX prophylaxis prior to surgery. The remaining two patients (one from the low dose cohort and one from the medium dose cohort) that still received infusions were able to increase the interval at which the infusions were

administered, which indicated clinical improvement. While therapeutic FIX levels were achieved in this trial, a similar observation to that of the 2006 trial was made regarding the two participants receiving the high-dose of rAAV8_FIX **(Figure 1.1B)**. Specifically, the higher levels of vector administration corresponded to a rise in liver AST and ALT at seven and nine weeks, respectively. Liver AST and ALT levels decreased upon administration of immunosuppressants, and circulating FIX levels stabilized once again. Again, the rise in liver enzymes corresponded with an increase in a capsid-specific CD8+ T cell response. A significant increase in capsid-specific T cell response was also recorded for the intermediate-dose cohort, but not the low dose cohort. While demonstrating promising therapeutic value, systemic administration of rAAV at levels required to achieve therapeutic efficacy is limited by host immune responses, which were not predicted by animal studies (16).

With the exception of Glybera®, the paradox of sub-therapeutic protein expression but no detectable host immune response on one end, and transient therapeutic protein expression corresponding with a host immune response on the other, has become the inflection point in the field of gene therapy today. In order to improve efficacy, the efficiency of viral transduction must be enhanced. This would allow lower levels of vector while still achieving therapeutic levels of protein. Transduction involves all of the viral steps needed in order to achieve gene expression, which include cell surface binding, subcellular trafficking, nuclear entry, and uncoating, among others. In order to improve transduction efficiency, it has become paramount to understand AAV vector biology, including the physical properties of the viral vector as well as the cellular barriers that limit efficient transduction.

Origins and biology of AAV and rAAV

AAV was first discovered in the 1960s as a contaminant in Adenovirus preparations (17, 18). At the time, AAV was referred to as a "defective virus" since it could not carry out a lytic infectious life cycle on its own. Rather, AAV requires a helper virus, such as Adenovirus, in order to complete a productive life cycle (17, 19). The helper virus functions to increase the efficiency of several steps in the AAV life cycle, including nuclear transport of AAV (20), double stranded DNA formation (21, 22), and the conversion to circular DNA (23, 24). The DNA-binding protein and E1A protein of Adenovirus serve to increase AAV transcription by releasing the p5 promoter from Rep mediated repression (25-27). Furthermore, Adenoviral proteins can increase the maturation and translation of AAV mRNAs (28-30). Finally, the helper virus facilitates AAV genome replication [86-88] and egress from the host cell (31). While most helper functions have been delineated with Adenovirus, other viruses, as well as several agents of cellular stress, have been shown to provide helper function for the AAV life cycle. Such examples include Herpes Simplex Virus (32), Vaccinia virus (33), Human Papillomavirus (34, 35), hydroxyurea (36) and UV light in the presence of SV40 T antigen (37). Because AAV relies on the helper functions of other viruses or certain forms of cellular stress, it has been classified as a *dependovirus*, a genus of the parvovirus family.

The AAV genome consists of a linear 4.7 kb single-stranded DNA molecule that can be either positive or negative sense **(Figure 1.2B)**. Two genes, termed *rep* and *cap*, flanked by two 145 base pair inverted terminal repeats (ITRs) comprise this small genome. The ITRs, found at both ends of the genome, provide many functions during the AAV life cycle. Specific sequences within the ITRs are bound and nicked by the large Rep proteins, which facilitates replication of the genome (38). The ITRs form hairpins on either end of the genome, which serve to prime

leading strand synthesis for the rest of the genome through a strand displacement mechanism **(Figure 1.2C)**. The small Rep proteins also recognize sequences on the ITRs in order to facilitate packaging into the capsid (39). Due to their structure and high G-C content, the ITRs are highly recombinogenic and are paramount to the concatamerization, circularization, and chromosomal integration of the AAV genome in host cell nuclei. In fact, due to this property, ITRs are being explored in gene editing applications (40).

Like many other viruses, the AAV genome makes use of alternative promoters, multiple start codons and alternative splicing in order to transcribe and translate its eight known proteins. The *rep* gene encodes four non-structural proteins—Rep78, Rep68, Rep52, and Rep40—through alternative promoters (p5 and p19) and splicing variants. The two larger proteins, Rep78 and Rep68, have been shown to be important for genome replication and transcriptional control (41). Due to the homology of the Rep binding element on the ITRS and a specific location on human chromosome 19 termed AAVS1, the larger Rep proteins can facilitate site-specific integration of the AAV genome into this location (42, 43). The small Rep proteins, Rep52 and Rep40, have been shown to have ATP-dependent helicase activity and are important for packaging the AAV genome into preformed capsids during viral assembly within the nucleus (39).

The *cap* gene encodes the three structural proteins, termed VP1, VP2, and VP3, that comprise the viral capsid. Additionally, *cap* encodes a non-structural protein, Adeno-associated virus assembly-activating protein (AAP). Similarly to Rep, these proteins are produced through alternative start codons and splice variants. VP1, VP2, and VP3 are driven by a third promoter, p40, and share the same C-terminal region and stop codon, but differ in their start codons. Thus, VP2 and VP1 have different N-terminal regions that provide extra functionality over VP3 alone. The VP1- and VP2- unique regions contain stretches of basic amino acids that are thought to

serve as nuclear localization sequences (NLSs). The VP1-unique region (VP1up) contains a third basic region as well as a phospholipase 2 (PLA2) domain. The capsid proteins combine in an approximately 1:1:10 ratio to form the 60 monomer icosahedral (T=1) viral capsid (44, 45). The outer surface of the capsid is comprised mainly of VP3 and the corresponding shared C-terminus of VP1 and VP2. The VP1- and VP2- unique regions are thought to lie underneath the capsid surface and exist as less rigid structures, as they are not identifiable in any crystal structures to date. The capsid assembly process requires AAP, which serves to target translated capsid proteins to the nucleolus and facilitate proper assembly (46).

One of the many features that makes AAV a compelling gene therapy vector is the fact that virtually the entire viral genome can be replaced with a therapeutic transgene. Due to their importance in Rep recruitment and DNA priming, the ITRs are the only *cis*-elements of the AAV genome required for replication and encapsidation of the genome. In vector production, *rep* and *cap* can be provided in *trans*. Production is carried out through a triple transfection method, whereby *rep* and *cap*, devoid of ITRs, are provided by one plasmid; a transgene, flanked by AAV ITRs, is provided in another plasmid, and helper virus proteins that facilitate efficient replication and encapsidation are provided in a third plasmid. On the molecular level, Rep and helper proteins facilitate the replication of the ITR-flanked transgene and package it into preformed capsids. The resulting product is termed recombinant AAV (rAAV). Since the encapsidated transgenes of rAAV no longer encode Rep, site-specific integration into chromosome 19 does not occur in gene therapy applications, and sustained gene expression is primarily due to circularized rAAV episomes within host nuclei. As the process for synthesizing the opposite strand of ssDNA is rate-limiting in gene therapy applications, research into making this step more efficient has yielded self-complementary rAAV vectors (scAAV).

scAAV transgenes are approximately half the size of single-stranded rAAV transgenes and contain a mutation in the Rep nicking site on one ITR. This results in a transgene that can self-anneal, thus bypassing the need for second-strand synthesis, which allows for faster and more efficient gene expression (47-49). Thus, the triple transfection method is advantageous because it allows for modular construction of AAV vectors; i.e., different transgene cassettes (ssAAV or scAAV) can be incorporated into the capsids of various AAV serotypes.

The initial discovery of AAV, identified as AAV2, followed by its development as a gene therapy vector, has prompted much interest in the discovery of alternative serotypes. Indeed, to date 12 serotypes have been discovered in humans and non-human primates, along with hundreds of variant species sequenced from human tissue samples (50) with capsid homology ranging from 49-99% (51). These serotypes display diverse tissue tropisms, which has been advantageous in the design of gene therapy vectors for tissue-specific diseases (i.e. heart disease, neurological disorders, etc.) **(Figure 1.3)**. For example, AAV8 infects liver cells between 10- and 100-fold more efficiently than AAV2 (52), while in muscle, AAV1 is between 5-12 fold more efficient than AAV2 (53). A more recently discovered serotype, AAV9, has been shown to have more efficient heart and neuronal transduction compared to other serotypes (54). Development of serotypes other than AAV2 as gene therapy vectors provides an immune advantage as well. AAV2 is the most prevalent serotype in the human population, with the first infection occurring in 30-80% of the population in early childhood. Less seroprevalent serotypes include AAV6, which is estimated to exist in approximately 30% of the population, followed by AAV5 (10-20%), and AAV7 and AAV8 (6%) (52, 55-57). While more recently identified serotypes display great promise in mouse models and in the clinic, research into their biological

properties is limited by their poor performance in cell culture compared to AAV2. Because of this, caution must be and has been taken in translating *in vitro* findings to *in vivo* applications.

The differences in tissue tropism and neutralizing antibody response stem from the capsid architecture mainly comprised by capsid protein VP3. Indeed, while the core of the VP3 monomer consists of a fairly conserved β-barrel motif, looped regions that extend beyond the core of the capsid and interface with other monomers are highly varied among the serotypes. To date, capsid structures have been solved for eight serotypes of AAV (45, 58-64). Distinct protrusions at the three-fold axis of symmetry vary in structure between serotypes and have important roles in both receptor binding and recognition by neutralizing antibodies. The prototypical AAV serotype, AAV2, has been shown to require heparan sulfate proteoglycan (HSPG) as a primary receptor for transduction (65) and amino acid residues on the three-fold protrusions required for this interaction have been elucidated (66-68). AAV9, a serotype that is emerging as the lead vector candidate for cardiac and CNS applications, has recently been shown to utilize galactose as its primary receptor (69, 70). Shortly thereafter, a binding pocket, located on within the three-fold axis of symmetry and consisting of five amino acids, was determined to be the capsid region important for galactose interactions (71, 72). While AAV1 and AAV6 have been shown to utilize N-linked sialic acid as primary receptors (73), the structural determinants for receptor binding have yet to be elucidated.

AAV subcellular trafficking and cellular barriers to transduction

While serotype-specific tissue tropism has emerged as an effective strategy to boost transduction efficiency in certain cell types, mounting evidence suggests that viral trafficking once inside the cell serves as a major barrier to efficient transduction. It has thus become

paramount to understand the parameters within host cells that govern permissible viral transit to the nucleus, where subsequent uncoating and gene expression occur. The majority of the interactions between AAV and host cells has been elucidated with AAV2 and common tissue-culture cells such as HeLa cells. In assessing these studies, it appears that wild type AAV2 infection and rAAV transduction share very similar subcellular trafficking patterns, and differ in genome persistence (integration and episome formation vs. episomal alone) and possibly capsid conformational changes and uncoating that might be more efficient depending on the content of the genome within the capsid. A schematic of the general trafficking pattern of AAV is displayed in **Figure 1.4**. AAV first binds to its primary receptor and then forms an interaction with a co-receptor. In the case of AAV2, several co-receptors have been identified, including αvβ5 integrin (74), fibroblast growth factor receptor type 1 (FGFR1) (75), hepatocyte growth factor receptor (HGFR) (76), α5β1 integrin (77), and the laminin receptor (78). Some of the co-receptors for other serotypes have been identified, including the platelet derived growth factor receptor (PDGFR) for AAV5 (79) epidermal growth factor receptor (EGFR) for AAV6 (80), and the laminin receptor for AAV8 and AAV9 (78). Tissue tropism is thought to be due to, in part, the combination of primary and co-receptor preferences among different serotypes. In fact, a "click-to-fit" model has been proposed that describes the requirement of viral co-interaction between select primary and co-receptors that facilitates internalization (77).

Internalization is thought to occur through several pathways, including receptor-mediated endocytosis (81, 82), entry through the clathrin-independent carriers and GPI-enriched endocytic compartment (CLIC-GEEK) pathway (83), and possibly macropinocytosis (84). Internalization might be dependent on the pathway that is prototypical to the co-receptor/native ligand interaction and not necessarily induced by virus binding or activation of signaling cascades.

Depending on the route of internalization, this process may be clathrin- and dynamin-dependent (81, 82, 85), or independent (83) but ultimately results in vesicular trafficking, facilitated by the microtubule network (86). In order to proceed to the nucleus, AAV must escape from the endosome, either entering the cytosol or perhaps by traversing the ER-Golgi network through retrograde transport (85). AAV then enters the nucleus as an intact particle (87) and is targeted to the nucleolus. It is thought that in situations that favor viral reproduction (or gene expression in the case of rAAV), AAV mobilizes from the nucleolus and uncoats. Finally, the single-stranded genome is converted to the double stranded form by host cell factors or anneals with a complementary genome, whereby gene expression ensues (21, 88, 89). While the general pathway of AAV trafficking might be conserved for some serotypes, there is evidence that supports that some serotypes might traffic to the nucleus using either different pathways or can more efficiently navigate the prototypical endo-lysosomal pathway. For example, recent studies have shown that rAAV1 and rAAV5 converge on and enter the nucleus at a much more rapid rate than AAV2 (90). This study suggests that cellular tropism is determined by intracellular fates of different serotypes in addition to receptor binding capabilities.

While the general pathway of subcellular trafficking is known, specific details are still being resolved. A more thorough understanding of how AAV navigates to the nucleus, releases its genome, and achieves persistent gene expression is critical in order to design more efficient gene therapy vectors. Currently, there are several known major barriers to transduction, including cell surface binding and internalization, endosomal escape, nuclear entry, and second-strand genome synthesis/persistent gene expression. AAV is limited in its ability to bind cell surfaces based on receptor availability and abundance. Strategies are currently underway to

rationally design capsids that have higher receptor binding affinity or incorporate targeting peptides. These strategies will be discussed in detail later.

Perhaps the largest barrier to efficient transduction is endosomal escape and subsequent transit to the nucleus. It has been shown that AAV enters host cell nuclei as an intact particle; however, both direct and indirect fluorescence microscopy studies have consistently showed that the majority of viral particles remain distal to the nucleus, converging in the region of the Golgi apparatus and the microtubule organizing center (MTOC). As AAV navigates the subcellular space through the endo-lysosomal route, endosomes become acidified as they mature into late endosomes and eventually lysosomes. It is thought that this acidification serves as a trigger, inducing a conformational change in the AAV capsid that allows the release of VP1up from the inside of the capsid (91, 92) **(Figure 1.5)**. In order for this process to occur efficiently, AAV particles must be carrying a genome, as VP1up is significantly less exposed when empty particles converge in the perinuclear region(87). VP1up contains a PLA2 domain that is thought to mediate release from the endosome by hydrolyzing phospholipid substrates that comprise the endosomal membrane or Golgi lipids (93, 94). Indeed, empty particles or viral particles that do not contain VP1up cannot escape endosomal compartments and do not transit to the nucleus (87, 95). VP1up, as well as the unique region of VP2, also contain stretches of basic regions that resemble well-known NLSs (92, 96). When these regions are not present, as in the case of particles made with only VP3 subunits, or cannot be exposed, as in the case of empty particles, nuclear entry of viral particles is significantly limited. Furthermore, mutations to these basic regions, rendering them functionally inert, also ablate nuclear entry (95, 96). It is therefore postulated that efficient endosomal and subsequent nuclear entry require conditions that allow for efficient exposure of VP1up, effective phospholipid cleavage to allow for egress from the

endosome, and likely NLS-mediated targeting to the nucleus. An alternative pathway for nuclear entry has been proposed, whereby viral particles traffic to the nucleus via retrograde transport through the Golgi apparatus and the endoplasmic reticulum (ER). The phospholipase domain on VP1up could then facilitate nuclear entry through lysis of the nuclear lamina. Regardless of the route of entry, the sheer volume of perinuclear, and not intranuclear, particles that are seen during the course of infection strongly suggest that the process of endosomal escape and/or nuclear entry is extremely inefficient. It is therefore possible that enhancing the efficiency of one or both of these processes could enhance transduction.

Second strand synthesis and genome persistence

The preponderance of evidence suggests that AAV translocates to the nucleus as an intact particle; therefore, it must undergo uncoating in order to allow for gene expression. A recent study has suggested that AAV trafficks to the nucleolus prior to uncoating (87). While the exact reasons for this are unknown, it is speculated that the vectors are sequestered in this sub-nuclear space until favorable conditions in the cell allow for vector mobilization and completion of transduction. Once uncoated, the single-stranded genome must convert to a double stranded genome in order for replication (for wt AAV) and gene expression (wtAAV and rAAV) to occur. This is achieved through cellular replication machinery, including DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and replication protein A (RPA), and the Adenovirus DNA binding protein (Ad-DBP) if Adenovirus is present (97, 98). In the wtAAV setting, Rep will cleave the double stranded molecule at the ITR nicking stem, which will eventually allow for completion of the ITR synthesis and eventual strand separation. This double-stranded monomer is the major replicative form of AAV. If DNA replication machinery

begins to synthesize another DNA strand before Rep cleaves the nicking stem, a longer, double-stranded dimer forms, which represents the minor replicative form of AAV DNA. Due to the highly recombinogenic nature of the ITRs, these dimers concatamerize and form stable episomes within the nuclei (24, 99, 100). Second strand synthesis was first discovered to be rate-limiting upon the observation that Adenovirus could significantly enhance rAAV transduction in addition to wtAAV infection (21). These observations were substantiated by *in vivo* experiments revealing that co-infection of mouse hepatocytes with Adenovirus could facilitate transgene expression, whereas transduction with rAAV alone resulted in rAAV DNA-containing hepatocyes, but no gene expression (101). Second-strand synthesis, as well as persistence of gene expression, are thought to be regulated in part by host DNA repair proteins. This is largely due to the fact that the secondary hairpin structure of the AAV ITR resembles a damaged DNA product. Currently, the relationship between AAV DNA and DNA repair machinery appears two-sided: on one hand, double-stranded AAV DNA relies on DNA repair machinery to facilitate concatamerization and circularization; thus ensuring genome stability and persistence. On the other hand, several lines of evidence have demonstrated that this repair machinery inhibits gene expression. For example, Mre11 and NBS1 of the Mre11-Rad50-Nbs1 (MRN) complex, as well as ATM, have been shown to be important for efficient scAAV genome circularization *in vitro* (102). However, the MRN complex and ATM have also been shown to limit rAAV transduction, likely through direct binding in the case of MRN (103, 104). Understanding the speficic roles that these DNA damage proteins play in AAV genome stability is paramount to overcoming this barrier to efficient and sustained gene expression.

Approaches to enhance transduction

Clearly, there are distinct cellular barriers inhibiting the full transduction potential of rAAV vectors. These impediments can be extracellular, as in the case of receptor binding and internalization; and intracellular, as in the case of subcellular trafficking, endosomal escape, nuclear entry, uncoating, second strand synthesis, and longevity of gene expression. From a gene therapy perspective, the overarching issue with inefficient transduction is the onset of the host immune response at what seems to be a certain threshold of introduced vector particles. As a higher dose of vector is introduced to override the shortfalls of inefficient transduction, the immune response counters through the formation of neutralizing antibodies to the capsid or T cell-mediated response against capsid proteins. This immune response is likely mounted more easily if more capsids are available for the proteasomal degradation and antigen presentation that are innate to any antigen presenting cell. These postulations are supported by the Hemophilia B clinical trials previously discussed. The growing number of pre-clinical and clinical studies have converged to a point that has prompted many investigators to discover approaches to enhance transduction efficiency. These strategies include enhancing receptor binding and host cell internalization through rational and library-based approaches to enhance receptor binding and host cell internalization, pharmacological approaches to alter the subcellular space into a more favorable environment for transduction, and rational design of capsids and genomes to overcome well-characterized cellular barriers.

Rational and random mutagenesis to enhance receptor binding

Cell surface receptor binding and subsequent internalization can be enhanced through enhancing the interactions between rAAV vectors and receptors. Two approaches, directed evolution and rational mutagenesis, have been devised that involve mutating key residues on

viral capsids in order to enhance these interactions. Directed evolution is a library-based approach that employs chimeric capsids generated by DNA shuffling, capsids derived from natural serotypes with the exception of an insertional peptide library at a specific location, or a combination of both (105-110). Error-prone PCR has also been utilized to add an additional level of variation to these libraries. Vectors that are efficient at transduction are "panned" out of the host cells or host cell nuclei through Adenovirus-mediated replication or PCR amplification of viral genomes. The amplified clones are then reconstructed into a new batch of vectors for the next round of selection. One example of successful directed evolution initially focused on primary human astrocytes, a target for gene therapy that is limited by inefficient transduction (108). In this study, three libraries were combined, including an AAV2-based library with random capsid mutations, a chimeric library produced from DNA shuffling, and a second AAV2-based library with a random peptide library inserted onto the capsid surface. The resulting clones isolated from the screen demonstrated between three- and five- fold improved astrocyte transduction *in vivo* compared to rAAV2. Next generation directed evolution approaches have involved cycling libraries *in vivo*, which allows for selection of clones that are both targeted to specific tissue types as well as detargeted from undesirable tissues. This approach is highlighted in a study that aimed to isolate chimeric rAAV clones that could cross a seizure-compromised blood brain barrier (BBB) upon systemic administration (107). Two clones were isolated that demonstrated enhanced transduction in the regions of the brain that had been damaged by seizures while also exhibiting decreased transduction of off-target tissues.

Directed evolution studies have demonstrated success in isolating clones that have increased specificity as well as transduction efficiency in target tissue types. These approaches have several limitations, however. First, isolated clones have been shown to vary from

experiment to experiment. In the case of the astrocyte study, an attempt to replicate the isolation of the variants recouped from the first cycling series failed to generate these clones a second time, highlighting the variation in results between different trials. Second, depending on the model used and the selective pressure employed, resulting clones might be specific for select cell types, disease models, or organisms. Third, utilizing PCR to amplify clones that have successfully entered host cells, the only approach currently available for in vivo biopanning, also results in the amplification of variants that may have entered the cell but are unable to traffick to the nucleus and uncoat. Thus, while a powerful method for finding relevant and efficient novel variants, in vivo biopannning requires secondary screening of multiple clones. Finally, attempts to define capsid determininants for cell speficic transduction have proven to be unfruitful. This has been disappointing, given the potential power that could be gleaned from understanding how specific regions of the capsid, or specific amino acid conformations, facilitate efficient transduction. Therefore, while directed evolution can be a powerful approach to producing novel vectors under specialized circumstances, its widespread utility in enhancing rAAV transduction is yet to be realized.

Pharmacological approaches to overcome intracellular barriers

Another approach to enhancing transduction is through the use of small molecules that alter the cellular environment to be more permissive to rAAV transduction. The first demonstration of the utility of small molecules was a study that characterized the effect of DNA damaging agents on transduction (111). In this study, the authors tested the effects of *cis*-platinum, along with UV and gamma irradiation on rAAV transduction efficiency. Treatment with *cis*-platinum resulted in an approximately 20-fold enhancement in transduction 48 hours after the addition of rAAV, while UV irradiation and gamma irradiation enhanced rAAV

transduction between 50- and 80-fold. The effects were especially pronounced in non-dividing cells, suggesting that perhaps these agents promote DNA synthesis or alter the dynamics of repair proteins. In a similar study, the effects of other DNA synthesis inhibitors, as well as Topoisomerase inhibitors, on rAAV transduction was studied (112). Cells were pre-treated with Hydroxyurea (HU), which inhibits ribonucleotide reductase and depletes deoxynucleotide pools, camptothecin, which inhibits topoisomerase I, and etoposide, which inhibits topoisomerase II, prior to infection with rAAV. For all treatments, the frequency of transduction of stationary human fibroblast cultures was increased by over 50-fold (over 100-fold for HU treatment). The positive effects on transduction persisted for over 10 days, suggesting a long term effect. HU and etoposide were also shown to increase the number of stably transduced cells as well, suggesting that application of these small molecules may increase non-specific integration of rAAV genomes into host chromosomal DNA. Taken together, these results also suggest that recruitment of DNA synthesis and/or change in repair factors function serves to enhance rAAV transduction.

The most thoroughly characterized enhancing pharmacologicals to date are small molecules that inhibit the cellular proteasome. The initial study demonstrating the utility of proteasome inhibitors utilized a model that represents human airway epithelia, a target for rAAV-mediated gene therapy for cystic fibrosis (113). Normally, these cells are permissive to rAAV transduction from the basolateral side, but are refractory to transduction from the apical side. Treatment of these cells with DNA synthesis and topoisomerase inhibitors did not enhance apical-side transduction. However, accumulation of single-stranded genomes within these cells was observed, suggesting that rAAV could enter these cells but was likely getting trapped in an unknown cellular compartment. Application of the tri-peptide proteasome inhibitor N-acetyl-L-

leucyl-L-leucyl-L-norleucinal (LLnL) could enhance rAAV transduction by greater than 200 fold. These results were especially encouraging because efficient transduction of airway epithelia has impeded progress for rAAV-mediated therapy of cystic fibrosis. In a second study, the same group utilized the proteasome inhibitor MG132 in addition to doxorubicin (which has both topoisomerase II inhibiting as well as proteasome inhibiting properties) to augment transduction of primary cystic firbosis epithelia with rAAV2 and rAAV5 carrying the CFTR gene (114). Since these studies, proteasome inhibitors have been used to enhance transduction several serotypes in a variety of cell types, including synoviocytes (115), keratinocytes (116), intestinal epithelial cells (117), and liver cells (118). Velcade (bortezomib), the first proteasome inhibitor approved for use by the FDA, was shown to enhance rAAV8-delivered Factor VIII levels by 3-to 6-fold in a hemophilia A mouse model and significantly reduced the number of bleeding episodes in hemophilia A dogs (119).

The mechanism driving enhanced rAAV transduction is thought to involve increased bulk flow of particles into the nucleus, where more single-stranded genomes can be converted to the transcriptionally active double-stranded molecule (113, 120). Indeed, microscopic analysis of the infectious pathway of rAAV2 in HeLa cells in the presence of the proteasome inhibitor MG132 revealed a significant increase in nucleolar accumulation of particles which corresponded to an enhancement in transduction by almost two log orders of magnitude (87). This effect may be dependent on the ability of rAAV to effectively navigate earlier trafficking steps in specific cell types, since proteasome inhibitors are not universally effective. In fact, a study investigating the effects of MG132 on endothelial cells demonstrated that proteasome inhibition could enhance transduction of the endothelial cell line EAhy-926 by approximately 12-fold; however it failed to enhance the transduction of endothelial cells *in vivo* (121).

Similarly, Bortezomib failed to provide any enhancement in transduction of cardiac cells by rAAV9-SERCA2a nor improvement in cardiac function in a rodent model of heart failure (122). Furthermore, the effect of proteasome inhibitors may be multidimensional and include post-trafficking mechanisms, as application of MG132 13, 21, and 28 days post-infection of rAAV2-IL-10 induced gene expression when measured a few days following each administration (115).

Clearly, the impressive effects on transduction seen with various pharmacological agents is promising for gene therapy applications, especially when considering the low permissivity to transduction of some cell types that are critical for specific gene therapy applications. Widespread use of these agents has thus far been limited, however. As topoisomerase inhibitors pose the risk of increasing non-specific integration into the host chromosome, this possibility would need to be fully explored and characterized *in vivo*. While bortezomib seems to be the most promising target for pharmaco-gene therapy, its side effects in humans are grave and include gastrointestinal issues, neuropathy, thrombocytopenia, liver failure and even death (123-125). Therefore, further exploration in new targets for drug enhanced rAAV transduction is necessary in order to move forward with widespread clinical application.

Rational design to overcome intracellular barriers

While capsid mutation and small molecules have driven much of the understanding of AAV and rAAV biology, applying the inverse strategy- i.e. understanding rAAV biology to drive vector modification or the discovery of small molecules- can also be an effective way to improve upon the current limits of transduction. The two best examples of this strategy include the development of self-complementary vectors and surface tyrosine-mutated capsids, which are discussed below.

The pursuit of a more efficient rAAV genome emerged from the initial observation that Adenovirus, specifically the E4Orf6 protein, facilitated increased double-stranded rAAV genomes in cells compared to cells that were transduced with rAAV alone (21). Interestingly, total uncoated rAAV genomes were equivalent between the two conditions, suggesting that Adenovirus enhanced rAAV second-strand synthesis. The increase in second-strand synthesis correlated with significantly enhanced β-galactosidase activity, suggesting that conversion of the single-stranded genome to the transcriptionally active double-stranded form was a rate-limiting step in transduction. This observation was corroborated in an *in vivo* setting, when it was demonstrated that murine hepatocytes harbor sufficient rAAV genomes to support transduction, however transgene expression is minimal but can be augmented through co-infection with Adenovirus (101). It was thus hypothesized that delivery of an already double-stranded rAAV genome might bypass the rate-limiting step of second strand synthesis and lead to enhanced transduction. Indeed, production of rAAV vectors using ITR-flanked transgenes of approximately half the size of the wild type genome resulted in rAAV vectors that could deliver duplexed molecules that demonstrated significantly improved gene expression both *in vitro* and *in vivo* (48). Further development of the self-complementary vector ensued, resulting in a duplex transgene flanked by two ITRs on one end and a mutated ITR on the other (49). The single ITR had a mutation in the D element to eliminate the Rep nicking stem, which allowed for a much higher percentage of self-complementary genomes to be packaged. Analysis of transduction *in vivo* revealed faster gene expression kinetics and increased transduction efficiency. Since their inception, self-complementary vectors have been encapsidated in multiple serotypes and developed for a variety of gene therapy applications, including vectors being investigated in the clinic (15).

The development of rAAV vectors that demonstrated superior subcellular trafficking emerged concomitantly with the discovery of proteasome inhibitors as enhancers of rAAV transduction. An initial study investigating the role of EGFR protein tyrosine kinase (EGFR) signaling on rAAV2 transduction revealed that rAAV2 capsids are phosphorylated by EGFR, which leads to subsequent ubiquitination and proteasomal degradation (126). Particles that could evade proteasomal degradation were hypothesized to be able to traffick more efficiently to the nucleus. Rational design coupled with molecular modeling identified seven surface exposed tyrosine residues that could be targets of EGFR. Mutation of these residues to phenylalanine on the rAAV2 capsid resulted in an approximately 10-fold increase in transduction of Hela cells as well as an almost 30-fold increase in transduction of mouse hepatocytes at a log order lower vector dose *in vivo* (127). The improved transduction efficiency at lower doses was confirmed to be due to increased particle trafficking to the nucleus. To date, several studies have demonstrated significantly enhanced transduction efficiency *in vitro*, including hematopoietic stem cells (128), fibroblasts and mesenchymal stem cells (129), as well as *in vivo* in the mouse retina (130) and for the correction of murine hemophilia B (131). The tyrosine mutation strategy has also been expanded to several serotypes, but has demonstrated variable success. Improvements were seen in rAAV6-mediated gene transfer to murine skeletal muscle (132), rAAV9-mediated delivery to neonatal mice (133), and rAAV2-, rAAV8-, and rAAV9-mediated delivery to the murine retina (134, 135). However, the positive effects of tyrosine mutants on transduction is not universal, as studies investigating rAAV8 and rAAV9 tyrosine mutants for gene transfer to skeletal muscle and cardiac tissue demonstrated no significant improvement compared to the wild type counterparts (136).

Objectives of dissertation

While great strides have been made in the development of rAAV gene therapy in the past two decades, there is a demonstrated need for a new generation of rAAV vectors or delivery strategies that provide significantly enhanced transduction. One has to look no further than the latest hemophilia gene therapy trial to understand the barriers imposed by the immune system when attempting to enhance gene transfer merely through increased viral dose. The objectives for this dissertation were to investigate ways to improve transduction efficiency of rAAV through pharmacological and biological approaches. In Chapter 2, we describe an approach to discover novel compounds that enhance transduction through a small molecule screen. In Chapter 3, we identify cellular factors that are involved in the nuclear entry of rAAV2 and provide a foundation to drive the development of vectors that are more efficient at nuclear entry. Improvements in vector design as well as advancements in pharmaco-gene therapy will ultimately allow for widespread application of rAAV-mediated gene transfer for a variety of diseases.

A.

Vector	Indication
1	Pompe Disease
	Alpha-1 antitrypsin deficiency
	Limb Girdle Muscular Dystrophy
	Becker Muscular Dystrophy
	Lipoprotein Lipase Deficiency
	Chronic Heart Failure
2	Retinal Disease
	Leber's Congenital Amaurosis
	HIV vaccine
	Cystic Fibrosis
	Canavan's Disease
	Hemophilia B
	Batten's Disease
	Parkinson's Disease
	Choroideremia

Vector	Indication
2.5	Duchenne Muscular Dystrophy
8	Hemophilia B
9	Spinal Muscular Atrophy
rh.10	Batten's Disease
	Metachromatic Leukodystrophy
rh.74	Alpha-sarcoglycan Deficiency
Undisclosed	Stage IV Gastric Cancer
	Alzheimer's Disease
	Cystic Fibrosis
	Hepatitis C
	Mucopolysaccharoidosis Type III A
	Arthritis

B.

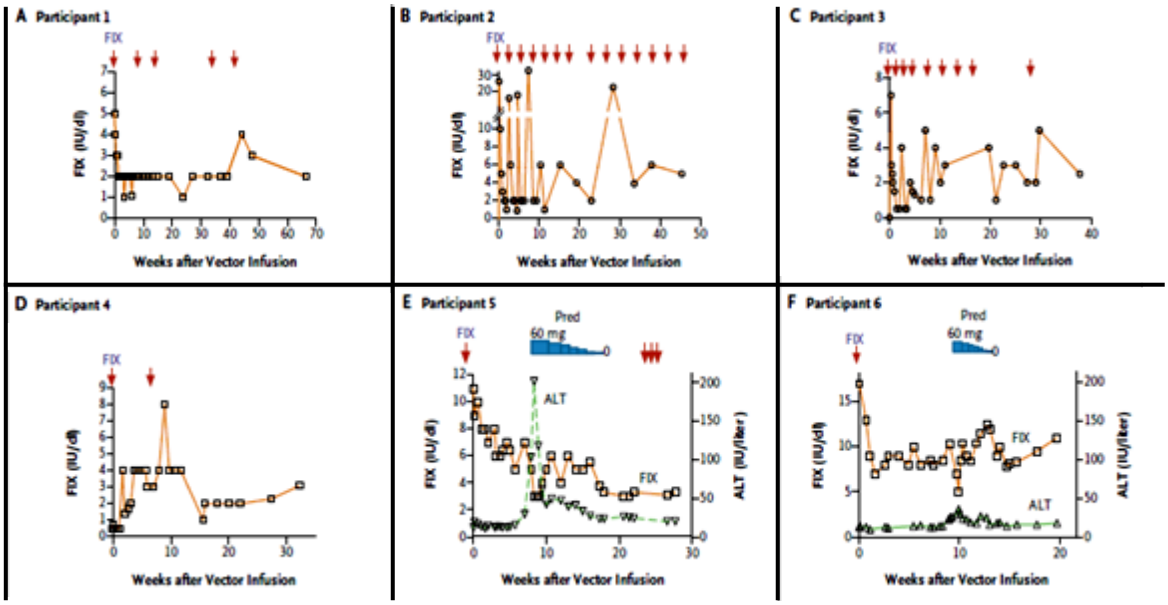
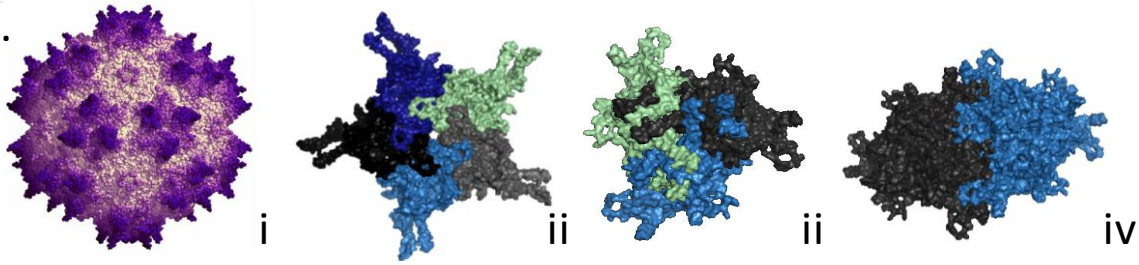
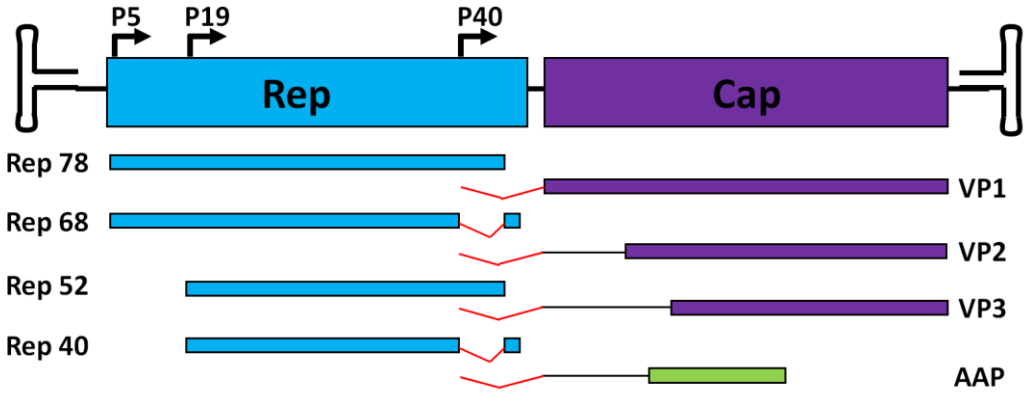


Figure 1.1. Brief overview of selected clinical trials with rAAV. **A**. rAAV clinical trials listed on clinicaltrials.gov as of June 2014, broken down by serotype and indication. **B**. Most recent Hemophilia B trial, highlighting the dose-immune response paradox. Figure adapted from Nathwani et al. "Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B." The New England Journal of Medicine 365: 2357-2365 (2011).

A.


i
ii
ii
iv

B.


P5
P19
P40
Rep
Cap
Rep 78
Rep 68
Rep 52
Rep 40
VP1
VP2
VP3
AAP

C.

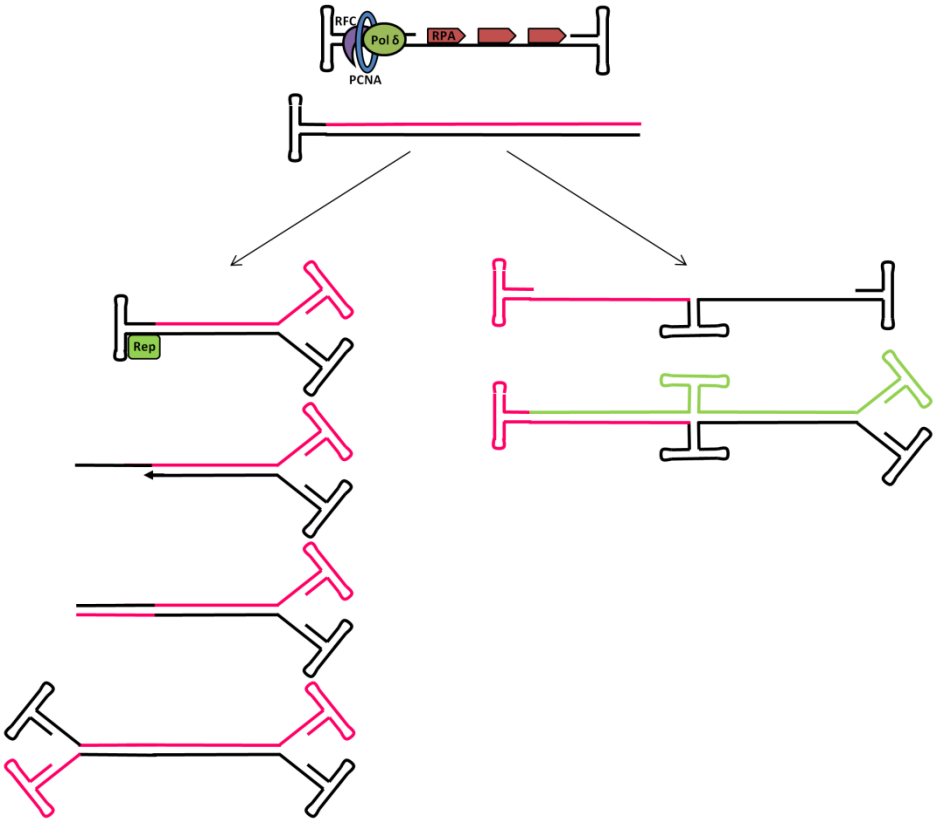
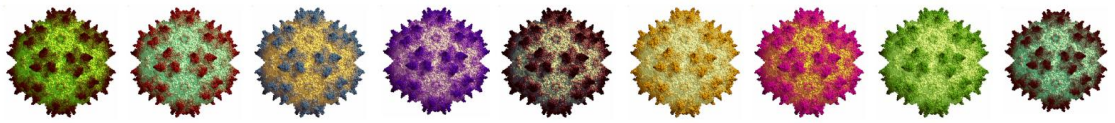

RFC
Pol δ
RPA
PCNA
Rep

Figure 1.2. Biological characteristics of AAV. A. The 25 nm capsid is comprised of 60 protein monomers that form the outer shell. (i) Axes of symmetry can be found at the 5-fold pore (ii), the 3-fold peaks (iii), and the 2-fold interface (iv). B. AAV genome and gene expression. Rep and Cap give rise to structural and non-structural proteins required for replication, capsid assembly, and genome packaging through multiple start codons and alternative splicing. C. Replication of the genome. Self-primed, leading strand synthesis occurs with the help of cellular replication machinery. i. The ITR is then cleaved by Rep at the nicking stem and copied from the other end. Replication repeats through a strand displacement mechanism. ii. The dimeric replicative form occurs for a minor portion of replicated genomes. In this case, synthesis of a new strand occurs before Rep cleaves the nicking stem. This molecule is eventually resolved by Rep nicking.



Serotype	1	2	3	4	5	6	7	8	9
Primary receptor	N-linked sialic acid	HSPG	HSPG, SA	O-linked SA	N-linked SA	N-linked SA, HSPG	Unknown	LamininR,	Galactose
Co receptor		αvβ5, α5β1 integrins, HGFR, FGFR, LamR	LamininR	Unknown	PDGFR	EGFR	Unknown	Unknown	LamininR
Tissue tropism	muscle, lung	liver, neurons, retina?	Unknown	eye, astrocytes	eye, muscle, lung?	muscle, lung	muscle, liver	liver	heart, lung, liver, cns

Figure 1.3. AAV serotypes display a wide range of receptor binding and tissue tropism. Displayed are the AAV serotypes that are currently being explored as platforms for gene therapy vectors. Largely due to capsid topology, different serotypes bind to different primary receptors and co-receptors. It is believed that this combination of receptor binding, among other factors, contributes to the wide variety of tissue tropism seen with these serotypes.

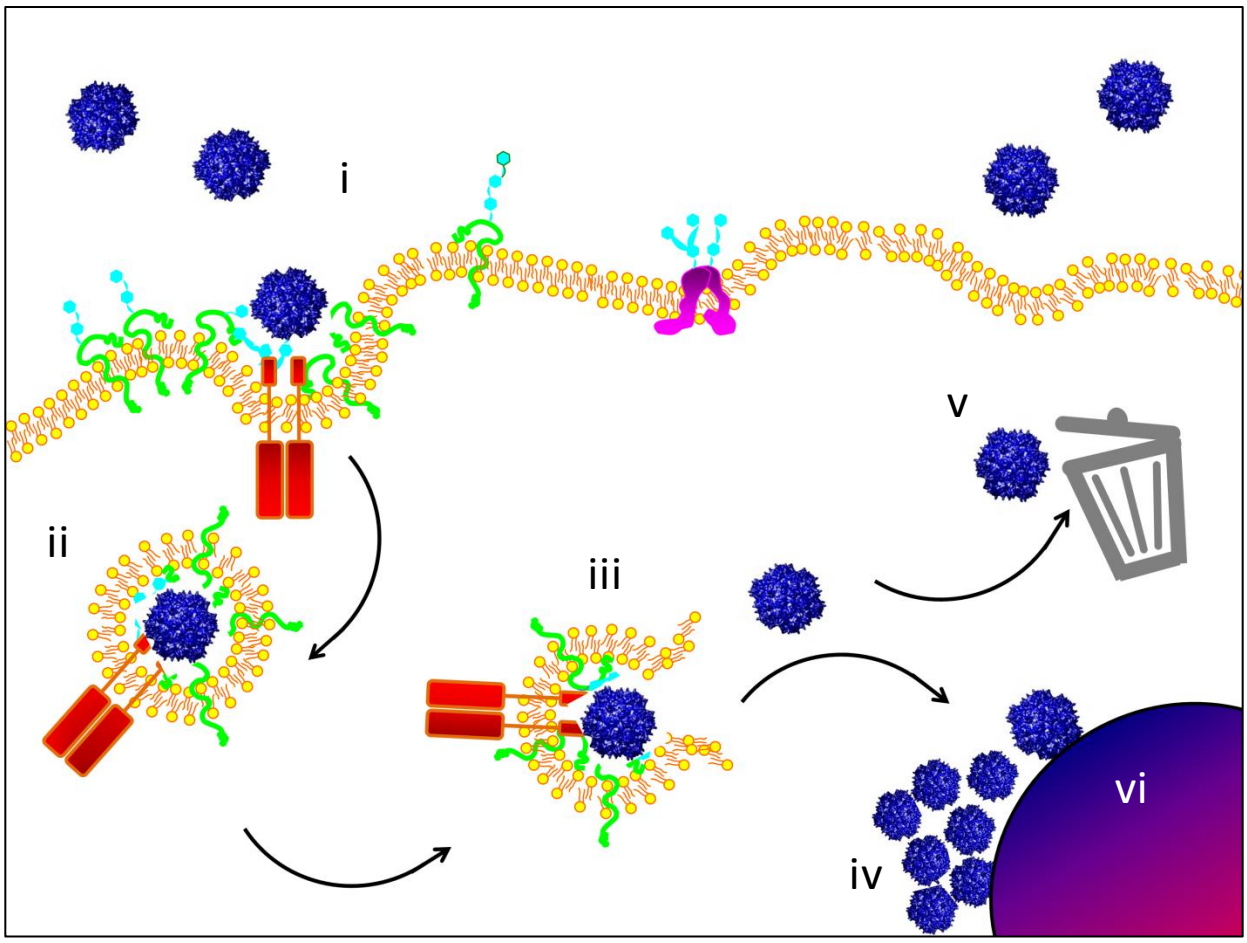


Figure 1.4. Helper-free AAV and rAAV entry and subcellular trafficking (here, both referred to as AAV). i. AAV binds its primary receptor and co-receptor. ii. Internalization occurs through receptor-mediated endocytosis, entry via the CLIC/GEEC pathway, or another mechanism. iii. AAV trafficks along the endolysosomal pathway. As endosomes mature and become more acidic, a conformational change within the capsid exposes VP1up. Endosomal escape ensues. From here, AAV either iv) converges at the perinuclear region or v) is diverted to a less favorable subcellular space, such as the proteasome. In the preferred pathway, AAV enters the nucleus, where subsequent uncoating, second-strand synthesis, and gene expression occur (vi).

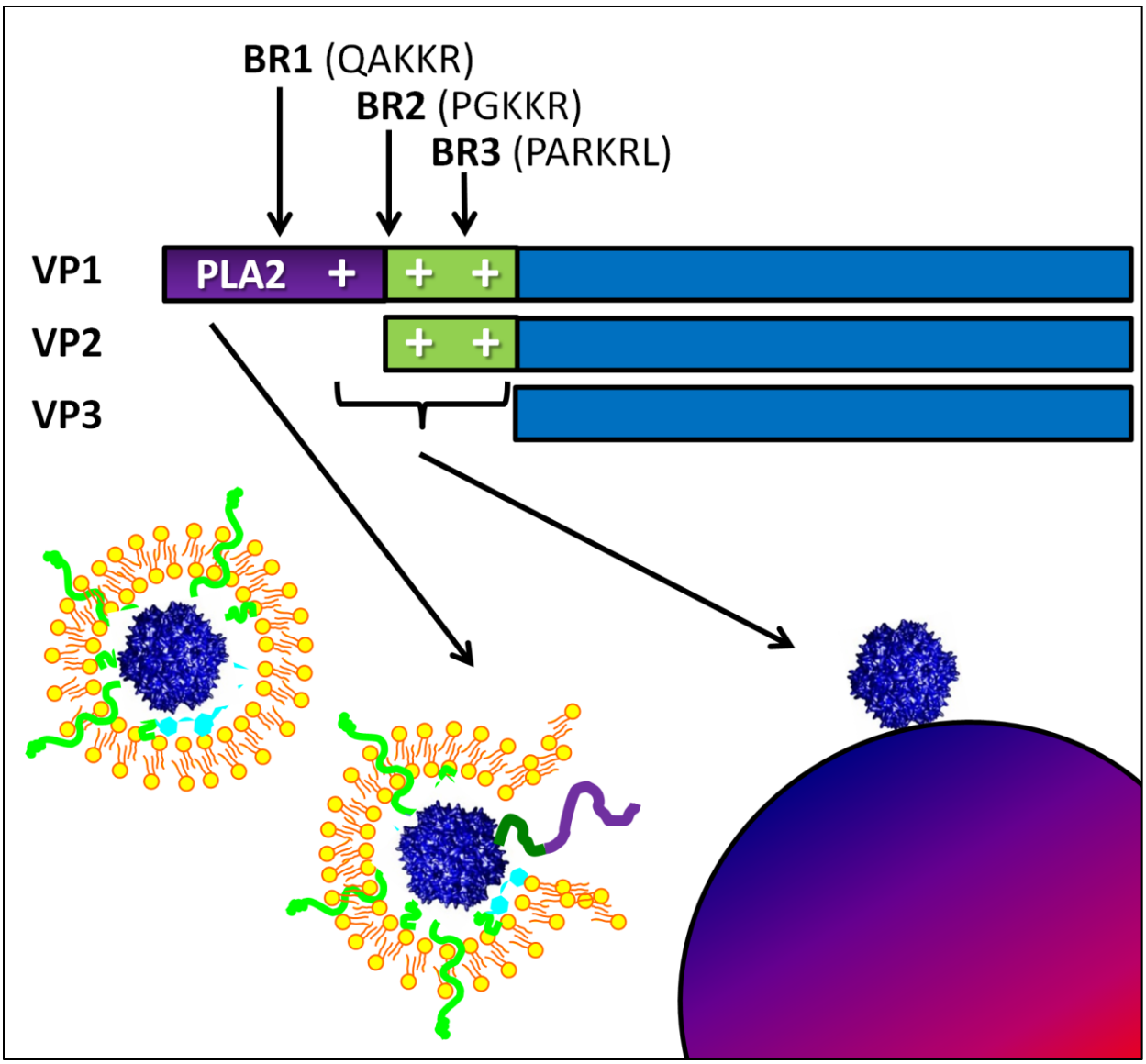


Figure 1.5. Domains of VP1 and VP2 that are essential for infectivity and transduction. VP1up conains a phospholipase A2 domain that is thought to mediate escape from the endosomal compartment. Additionally, three basic regions, especially BR2 and BR3, are necessary for viral entry into the nucleus and transduction. VP2 also contains BR2 and BR3.

CHAPTER 2

Identification and validation of small molecules that enhance recombinant Adeno-associated virus transduction following high throughput screen

Summary

Gene therapy applications using recombinant Adeno-associated virus vectors (rAAV) have recently seen success for indications such as Leber congenital amaurosis, hemophilia B, and lipoprotein lipase deficiency. While the success of these clinical trials is promising, preclinical and clinical data have indicated that there are still challenges facing the widespread applicability of rAAV. Specifically, in systemic applications, the amount of vector that can be administered appears to be limited by a host immune response to the viral capsid. Thus, efforts are underway to enhance the transduction efficiency of the viral vectors in order to achieve therapeutic levels of gene expression at a vector dose that is below the immunological response threshold. Strategies to combat this challenge have included random and rational capsid design, transgene optimization, and co-administration with pharmaceuticals that have been shown to have potentiating effects on transduction. However, these strategies are often limited to specific serotypes or tissue types. In an attempt to identify novel compounds that enhance a wide variety of rAAV vectors, we performed a high-throughput small molecule screen. In doing so, we discovered both novel and previously identified compounds that are capable of enhancing transduction. We compared the identified compounds' abilities to impact transduction in several human cell lines and with single-stranded, self-complementary, and oversized, fragment vectors. Finally, we compared all of the clinically-relevant compounds identified in the screen for their

ability to enhance rAAV transduction *in vivo*. This study provides a foundation upon which more diverse libraries of compounds can be tested in order to find more small molecules that can potentiate rAAV transduction through a variety of pathways.

Introduction

Adeno-associated viral vectors have emerged to be one of the most promising types of vectors for gene therapy. Indeed, recent and ongoing clinical trials have reported improvements in patients with hemophilia B (15) Parkinson's disease (6, 137), Leber congenital amaurosis (3, 4, 138, 139), and Canavan disease (8). Such clinical successes have led to the approval of rAAV-mediated gene therapy in the European Union for the treatment of lipoprotein lipase deficiency (LPLD) (140). Enthusiasm for using rAAV vectors stems from the unique properties of the virus itself. As naturally occurring AAV requires a helper virus such as Adenovirus or Herpes Simplex Virus in order to carry out a productive infection, AAV on its own is not known to cause disease in man. AAV vectors can carry transgenes that are devoid of greater than 90% of viral genetic material, thus limiting viral gene transfer in clinical applications. Furthermore, naturally occurring serotypes and engineered capsids have been shown to display diverse tissue tropism, as well as the ability to infect both dividing and non-dividing cells (for a review, see (141)). From a vector perspective, the use of AAV for gene therapy applications is only limited by the size of the viral capsid, which accommodates a transgene of approximately 4.7 kb (142). However, even the size limitations of rAAV vectors are being challenged with the development of trans-splicing and fragment vector technology; that is, transgene cassettes that rely on cellular recombination pathways to restore a full length, large genome upon delivery by multiple rAAV vectors (143-149).

Despite its remarkable safety profile, gene therapy using AAV vectors has had limited success in applications requiring systemic delivery, namely to non-immunoprivileged sites (as opposed to direct injection of various tissues or to immunoprivileged regions). Results from clinical trials utilizing either rAAV2 or rAAV8 to deliver human Factor IX to hemophilia B patients have suggested that capsid-specific cytotoxic T lymphocytes (CTLs) might have eliminated the majority of transduced cells, thus impeding successful gene expression (13, 15). In these dose-escalation trials, therapeutic protein levels were achieved at the highest vector doses administered; however, these levels were transient and their decline corresponded with a rise in liver transaminases, suggestive of transduced cell death. There was no rise in liver enzymes detected at the lower doses tested, suggesting that gene transfer to achieve protein levels that would be deemed therapeutic is limited by the amount of capsid that can be delivered to the host (14). It has thus been postulated that if transduction efficiency could be improved (including both gene transfer and expression), fewer vectors would be required to achieve a given level of gene expression, and thus a clinically relevant therapeutic protein level could be obtained without eliciting a host immune response (150).

Several strategies have been employed in order to enhance transduction efficiency. Both rational mutagenesis and library-based approaches have been designed to create capsids that have increased transduction efficiency for particular tissue types as well as decreased tropism for non-target organs such as the liver (105, 107, 151-154). Barriers within the intracellular trafficking pathway have also been circumvented based on rationally engineered capsids (127, 155). Improvements to transgene design, including self-complementary rAAV and tissue-specific promoters, ensure faster and more robust onset of gene expression in target tissues (48, 156-158). Finally, enhancing transduction efficiency through the use of pharmacological agents

has been explored. Previous work has shown that topoisomerase inhibitors and anthracyclines can enhance transduction in both *in vitro* and *in vivo* (112, 114, 159-164). Proteasome inhibitors, particularly the FDA-approved Velcade (bortezomib), has been shown to enhance transduction both *in vitro* and in both small and large animal models (114, 119, 162). Collectively, these strategies have been important in reaching the level of transduction required for therapeutic benefit without eliciting a host immune response; however, efficient transduction in a wide variety of clinical applications is still a major goal that is actively being pursued.

In this study, we performed a high-throughput, small molecule screen with the purpose of discovering compounds that enhance rAAV2 transduction. We confirmed the transduction augmentation of the previously identified topoisomerase II inhibitors, anthracyclines, and proteasome inhibitors, in addition to identifying several novel compounds. The ability of these compounds to enhance transduction was confirmed *in vitro* using several human cell lines and several AAV serotypes. These compounds were shown to be effective in enhancing self-complementary (scAAV) as well as oversized, fragment (fAAV) vector transduction in addition to single-stranded vectors. We further examined the activity of compounds identified in our screen that are currently approved for clinical use *in vivo*. Our results demonstrate a simple, effective method of discovering compounds that enhance rAAV2 transduction. We anticipate that this approach can be applied to vectors derived from other serotypes and in other cell lines. Furthermore, our design provides a foundation to investigate the plethora of commercially available compound libraries that span the small molecule and FDA-approved drug milieu.

Materials and Methods

Cell culture. HeLa cells, U87 cells, normal human fibroblasts (NHF1s) and HEK-293 cells were grown in Dulbecco's modified Eagles medium that was supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin (complete DMEM). HepG2 cells were grown in RPMI-1640 medium, supplemented as described above. All cell lines were maintained at 37°C and 5% CO2.

Virus production. Virus was produced in HEK-293 cells as previously described (68). Briefly, using Polyethylenimine (PEI) Max (Polysciences), cells were triple transfected with a Rep and Cap plasmid (pXR2, pXR8, or pXR2.5), an inverted terminal repeat-flanked transgene plamid (single-stranded pTR-CBA-Luciferase, self-complementary pTR-CMV-gaussia-Luciferase, or oversized pTR-CBA-Luciferase) and the pXX6-80 helper plasmid. Between 48-72 h posttransfection, cells were harvested and virus was purified by cesium chloride gradient density centrifugation overnight at 55,000 rpm. Fractions that contained peak virus titers were dialyzed in dialysis buffer (1X phosphate-buffered saline (PBS), 0.5% Sorbitol, calcium chloride (0.5 mM) and magnesium chloride (1 mM). Titers were calculated by quantitative PCR (qPCR) according to established procedures (33) by using a LightCycler 480 instrument with Sybr green PCR master mix. Conditions used for the reaction were as follows: 1 cycle at 95°C for 10 min; 45 cycles at 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s; and 1 cycle at 95°C for 30 s, 65°C for 1 min, and 99°C for acquisition.

Compound screen, 384-well format. HeLa cells were plated at least 18 h prior to compound treatment and infection at a density of 8 X 10^3 cells/well. Compounds were prepared in

complete DMEM so that delivery would yield a final concentration of 1 uM. Compounds were added directly to wells. Two hours post-treatment, rAAV2-CBA-Luciferase was administered at a dose of 1000 vg/cell. Cells were harvested 24 h post transduction by media removal followed by incubation with Passive Lysis Buffer (Promega) for 15 min. Luciferase activity was measured in accordance with the manufacturer's instructions (Promega). Luciferase activity was measured either a Perkin Elmer 1450 MicroBeta TriLux LSC and luminescence counter or a Perkin Elmer 2450 MicroBeta^2 microplate counter. Compounds that enhanced transduction greater than 2 fold over DMSO treatment were considered hits for further study. Cell viability was measured using the CellTiterGlo luminescent cell viability assay (Promega).

Secondary screen, 96-well format. HeLa cells were plated at least 18 h prior to compound treatment and infection at a density of 2 X 10^4 cells/well. Compounds were prepared in complete DMEM at a concentration of 10 uM. Medium was replaced with medium containing each compound. Two hours post-treatment, rAAV2-CBA-Luciferase was administered at a dose of 500 vg/cell. Cells were harvested 24 h post infection via incubation with Passive Lysis Buffer (Promega) for 15 min. Luciferase activity was measured in accordance with the manufacturer's instructions (Promega). Luciferase activity was measured with a Wallac 1420 Victor3 plate reader. Compounds that enhanced transduction greater than 5 fold over DMSO treatment were considered hits for further study.

Transduction assays. Cells were plated at least 18 h prior to infection in 96 well plates at a density of 2 X 10^4 cells/well. Compound treatment was performed 2h prior to infection. Compounds were either provided by the Drug Testing Program or were commercially available

(Teniposide, Sigma; Nanaomycin, Apex Bio; Daunorubicin, Vorinostat, LC Laboratories). Cells were infected with purified rAAV2-CBA_Luc at 500 vector genomes/cell. Cells were harvested 24 h post infection and luciferase activity was determined as described above. For scAAV, cells were treated as described above. At 24h post infection, 20 µL of media was transferred to a black 96-well plate. The luciferase assay was performed using coelenterazine (Nanolight) as the reagent. Briefly, the coelenterazine was resuspended to 10 mg/mL in methanol. To make the working solution, the concentrated stock was dissolved in TE buffer containing NaCl (0.6 M) at a 1:200 dilution. The working solution was added to wells at a 1:1 ratio of media:coelenterazine and luciferase activity was recorded as described.

Transfection Assays. 2 X 10^6 HeLa cells were plated at least 18 h prior to transfection in a 10 cm plate. Cells were transfected with 1 ug pTR-CBA-Luc using PEI Max. 24 h post-transfection, cells were plated in a 96 well plate at a density of 2 X 10^4 cells/well. 48 h post-transfection, cells were treated with the indicated drugs. At 72 h post-transfection, cells were harvested and luciferase activity was measured as described above.

Animal Studies. Housing and handling of BALB/c mice used in the current study were carried out in compliance with National Institutes of Health guidelines and approved by the IACUC at the University of North Carolina Chapel Hill. All drugs and rAAV-Luc were co-administered through the intravenous route (tail vein) in a total volume of 200 µL (normalized with lX PBS). 24 hours post administration, blood was collected and serum was assessed for blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase (CK). Bioluminescence of Luc expression was visualized by using a Xenogen IVIS

Lumina imaging system (PerkinElmer) following intraperitoneal injection of luciferin substrate (120 uL, Nanolight). Image acquisition and analysis were carried out by using Living Image software.

Results

Primary and Secondary Screen

The overall goal of our study was to identify and characterize small molecules that enhanced rAAV transduction *in vitro* and *in vivo* if the hit was currently FDA approved. Two screens were performed to identify such compounds. The first was more stringent screen using a 1 uM final compound concentration to identify hits capable of potentiating rAAV2 transduction at low concentrations. A schematic of the set-up, which utilized a 384-well format, is outlined in Figure 1A, where we utilized a 384-well format. Column 1 (i) contained no virus and no compounds, column 2 (ii) contained rAAV2 but no compounds. Column 24 (iv) contained 1 uM MG132 as a positive control. Column 23 (iii) contained rAAV2 with only DMSO (vehicle control). The compounds were administered in duplicate. Finally, each plate contained two sets of the given controls and compounds, whereby rows 1-8 (top, gray) assayed for transduction activity and rows 9-16 (bottom, white) were assayed for viability using the CellTiterGlo system. As the vast majority of AAV biology has been discovered using AAV2 or rAAV2 in HeLa cells, our primary and secondary screens were performed using these parameters. Owing to its wide range in output and linear relationship to vector dose, the CBA-luciferase transgene, and thus luciferase activity, was chosen as the reporter for transduction efficiency. As a substantial number of hits were not identified from the 384-well screen, a less stringent screen using a 96-well format was employed **(Figure 2.1B)**. This secondary screen utilized a 10 uM final

compound concentration in tandem with employing rAAV2 to HeLa cells at 500 vg/cell. The format of this screen utilized DMSO added to all wells in column 1 (i) as a vehicle control, while two wells in column 12 (ii) contained MG132 as a positive control. Cell viability was not assessed at this stage. A combined list of the compounds that were pursued further is provided in **Table 2.1**.

In the primary screen, an initial "hit" was defined as a compound that enhanced transduction by at least 2-fold **(Supplemental Table S2.1)**. From this selection, we chose to move forward with compounds that had known functions based on existing literature. These compounds included bortezomib, pyrromycin, daunorubicin, nanaomycin, bleomycin, and tetrocarcin A. Bortezomib, an FDA-approved proteasome-inhibiting agent, has been explored for rAAV applications in a variety of cell lines, animal models, and using different serotypes(118, 119, 165-167). Thus, the identification of bortezomib within the screen inadvertently served as an internal control in validating our experimental set-up. Pyrromycin and daunorubucin, both from the anthracycline class, are capable of both proteasome- and topoisomerase-inhibition, and could therefore potentiate rAAV transduction through a combination of these mechanisms. In fact, a related anthracycline, doxorubicin, has also been characterized for its enhancement of rAAV transduction (114, 162). A similarly structured molecule, nanaomycin A, has been shown to generate oxygen free radicals and cause DNA damage (168, 169). Additionally, nanaomycin A has been recently identified as an inhibitor of DNA methyltransferase 3B (DNMT3B) (170), thereby inhibiting epigenetic repression of gene expression. The glycopeptide antibiotic bleomycin, which is known to cause DNA double-strand breaks (171), was also identified to augment transduction. Finally, tetrocarcin A, which has been shown to inhibit the anti-apoptotic function of Bcl-2 (172) and induce ER stress (173), was also

identified. Our screen yielded several compounds that are known to interefere with microtubule dynamics, including colchisine, vincristine, and vinblastine, among others; however, these were excluded from further study as it has already been demonstrated that these microtubule inhibitors inhibit the cell cycle at low concentrations (174), which is likely the mechanism behind their transduction enhancement. In fact, at higher concentrations, these drugs have been shown to inhibit viral trafficking to the nucleus as well as transduction (84, 86).

In the secondary screen, an initial "hit" was defined as a compound that enhanced transduction by at least 4-fold **(Supplemental Table S2.2)**. Identified compounds with already known mechanisms of action were further pursued. However, it is noteworthy to mention that several compounds with no known function were identified as hits, enhancing transduction by between 5-10 fold. These compounds could warrant validation in further studies. Several of the compounds that augmented transduction were in agreement with previously published results. Of the hits, the compounds were divided amongst several categories. Topoisomerase II inhibitors from both the epipodophyllotoxin and anthracycline groups were a large subset of the hits identified. These included teniposide, etoposide, menogaril, and pyrromycin. Etoposide has been previously identified as having transduction-potentiating activity, likely through mechanisms related to DNA damage repair proteins induced by etoposide-mediated inhibition of topoisomerase II (112). Another anthracycline, menogaril, was under developmental testing as a chemotherapeutic, but its development was halted. Vorinostat (suberanilohydroxamic acid, SAHA) belongs to the class of histone deacetylase inhibitors, which function in cells to cause an accumulation of acetylated histones and transcription factors, which in general increases gene expression (175, 176). In addition to bortezomib, two compounds with known proteasome-inhibiting activity were idenfied: physalin B (177) and siomycin (178). In addition, Physalin B

has also been shown to have anti-inflammatory and other immunomodulatory activity (179). The proteasome inhibiting activity of siomycin has been correlated with its role as an inhibitor of the transcription factor forkhead box M1 (FOXM1) (178, 180, 181). Finally, other compounds with DNA-damaging properties were identified, including the sesquiterpene lactone parthenin, which has been shown to induce chromatid breaks (182), as well as the diaziridinylbenzoquinone RH1, a DNA crosslinker (183).

Dose optimization of compounds in HeLa cells

In order to further characterize the hits from our screen, we wanted to determine an optimal concentration that would result in the greatest enhancement of transduction without causing overt toxicity **(Supplemental Figure S2.1)**. Although not identified in our screen, we included the anthracycline analogue doxorubicin into our studies, as it had been previously been identified to enhance transduction in airway epithelia (114, 162), intestinal epithelia (117), and neuronal cells (163) and is approved by the FDA. To avoid potential side effects caused by DMSO, the final concentration of compounds was limited to 100 uM for high-dose applications. It is therefore possible that compounds that were both well-tolerated by cells and that substantially enhanced transduction at higher concentrations (such as etoposide and bleomycin), may be capable of enhancing transduction even more than what is shown. For some compounds, the therapeutic window was quite small in that there was a very sharp decrease in cell viability as the concentration of the compound was increased. A notable example was Menogaril, which at 5 uM showed an over 10-fold enhancement and approximately 100% viability, but at 10 uM resulted in an approximately 50% reduction in viability, which corresponded to a reduction in transduction enhancement as well. The epipodophyllins teniposide and etoposide, as well as the DNA damaging agent bleomycin, were the strongest augmenters in this assay, each increasing

transduction by over 20-fold. The proteasome inhibitors bortezomib and physalin B also showed enhancement by over 10-fold. This approach was used to optimize dosing for several other cell lines (discussed below) but it was found that the effective doses used *in vitro* remained relatively consistent for each compound.

Effects of compounds on vector transduction versus general gene expression

Given the identified compounds' impressive utility across multiple cell lines, it was important to verify that the observed increase in gene expression was due to a mechanism related specifically to rAAV vectors and not simply to a global increase in transcriptional activity. To this end, HeLa cells were transfected with pTR_CBA_Luc, the transgene plasmid used to generate the above studied single-stranded vector constructs, and assayed for enhancement of luciferase activity following compound administration **(Figure 2.2)**. Most compounds elicited a negligible change in luciferase activity. For example, physalin B induced an approximately 2-fold increase in plasmid luciferase expression, compared to a greater than 10-fold increase in transduction, Some of the compounds even induced a decrease in gene expression. The only exception was the HDAC inhibitor Vorinostat, which increased luciferase expression of the transfected plasmid to a similar extent as incoming vectors. While the potential to enhance gene expression through this mechanism could have potential utility in gene therapy applications, Vorinostat was excluded from further analysis within this work to avoid incorrect interpretation of mechanistic action.

Characterization of compound activity in human cell lines.

Given the ability of the identified compounds to enhance the transduction of rAAV2 in HeLa cells **(Figure 2.3A)**, we wanted to explore the utility of these compounds in different cell types. The first such cell line examined was U87, a human glioblastoma cell line that is

amenable to rAAV2 transduction **(Figure 2.3B)**. Overall, the fold enhancement of each of the compounds was very similar to what was observed in HeLa cells, with a few exceptions. While teniposide enhanced transduction by approximately 40 fold in HeLa cells, enhancement was seen at 20-fold in U87 cells. Interestingly, while bortezomib enhanced transduction by approximately 15-fold in HeLa cells, enhancement was only approximately 3-fold in U87 cells. However, the level of enhancement obtained with by another compound with proteasome-inhibiting activity, physalin B, remained the same in both cell lines (approximately 10-fold). We next evaluated these compounds in HepG2 cells, a hepatocellular carcinoma cell line, as the liver is the site of transduction for systemically delivered rAAV2 **(Figure 2.3C)**. For treatment with pyrromycin, nanaomycin A, physalin B, bortezomib, and tetrocarcin A, transduction enhancement was observed at approximately the same magnitude of that seen with HeLa cells. Treatment with the epipodophyllotoxins, anthracyclines, siomycin, and RH-1 resulted in enhancements 4-12 fold greater than that observed in HeLa cells. Notably, Menogaril, which enhanced rAAV2 transduction in HeLa cells by approximately 12-fold, enhanced transduction in HepG2 cells by 138-fold. Finally, our compound collection was applied to NHF1 cells (a kind gift from Dr. William Kaufman), a diploid cell line derived from neonatal foreskin and immortalized by the expression of telomerase reverse transcriptase (hTERT) that displays contact inhibition and stationary growth once confluent (184) **(Figure 2.3D)**. Again, treatment with the epipodophyllotoxins and anthracyclines (especially menogaril) significantly enhanced transduction at magnitudes much greater than what was observed in HeLa cells. Treatment with bleomycin also enhanced transduction to a similar level. These results suggest that general topoisomerase inhibition, either through application of epipodophyllotoxins or anthracyclines, consistently yields the greatest enhancement in transduction, while proteasome inhibition by

bortezomib is more variable and depends on cell type. It is possible that the enhancing ability of these compounds depends on the abundance of their actionable targets within cells. Thus, these results highlight the differences in response to pharmacological modulation in different cell types, which may influence the type of agent chosen to complement rAAV-mediated gene therapy in a given the target tissue.

Characterization of transduction-specific compound activity with vectors comprised of differentially formed transgenes.

Recent and emerging gene therapy applications have utilized transgenes that differ in form from the prototypical single-stranded AAV genome. For example, self-complementary vectors contain a transgene whereby a third inverted terminal repeat (ITR) in the middle of the transgene is mutated to eliminate the Rep nicking stem (48). This yields a double-stranded gene product, thus eliminating the rate-limiting step of second-strand synthesis and providing faster, more robust onset of expression (21, 48). Other emerging vectors designed to deliver large transgenes are derived from the forced packaging of oversized genomes into the viral capsid(145-147, 149, 185, 186). It is thought that the packaged DNA becomes truncated at the 5' end once AAV has reached its packaging limit, but delivery of these transgene "fragments" still results in expression of the intact transgene through a reannealing process modulated by the homologous recombination protein Rad51C (186). However, the transduction efficiency of fAAV is impaired compared to rAAV or scAAV.

We therefore wanted to test the capabilities of the screen-identified compounds in enhancing the transduction of scAAV and fragment rAAV in HeLa cells. For scAAV, a vector carrying a cytomegalovirus promoter-driven gaussia luciferase transgene (CMV-gLuc) was utilized. For fAAV, a previously described vector carrying the same promoter and transgene as

the single-stranded vector (CBA-Luc), but with an additional 2.3-kb "stuffer sequence" inserted into an intronic region upstream of the luciferase (186) was utilized. A schematic of each of these vector transgenes is provided in **Figure 2.4A**. We applied the same optimized concentration of each compound and tested their ability to enhance scAAV and fAAV transduction at the same vector dose. With the exception of daunorubicin and tetrocarcin A, the compounds still enhanced scAAV transduction, but the magnitude of the effect was significantly reduced **(Figure 2.4B)**. This suggests that these compounds may also be acting at the step of second-strand synthesis. Since we still observed enhancement of scAAV, albeit at a reduced capacity compared to ssAAV, it is likely that these compounds augment steps in transduction either before second-strand synthesis (such as subcellular trafficking, nuclear entry, or uncoating) or in establishing the persistence of gene expression following second-strand synthesis. In contrast, application of the compounds in conjunction with fAAV2 enhanced transduction to a greater extent as compared to rAAV2 **(Figure 2.4C)**. Notably, treatment with teniposide, bortezomib, or bleomycin enhanced transduction by over 250-fold, which was an 8-10 fold increase over the enhancement seen in rAAV2. This result is particularly interesting as each of these drugs produces a different reported effect within cells. In untreated cells, fAAV2 transduction was approximately 21-28 fold lower than intact rAAV2, which is in agreement with previously reported results comparing fAAV2 and intact rAAV2 at 5000 vg/cell (186). Treatment with any of the compounds restored transduction of fAAV2 to levels i) above untreated intact rAAV2 levels and ii) within 2-10 fold of drug-treated intact rAAV2 levels, depending on which compound is used. Taken together, these results suggest that the identified compounds can enhance the transduction efficiency of vectors with different forms of transgenes, and disproportionately benefits fAAV2 transduction in HeLa cells.

In vivo analysis of FDA-approved hits

Owing to the appeal of repurposing FDA approved drugs to augment rAAV-mediated gene transfer, we performed an *in vivo* comparison of rAAV2 transduction potentiation capabilities of the hits identified in our screen that are already FDA approved. Because of their impressive performance in several cell lines and with different transgene types, teniposide, etoposide, and bleomycin were utilized in this study. Though doxorubicin has been previously investigated as a transduction-enhancing pharmaceutical, our *in vitro* studies suggested that another anthracycline, daunorubicin, consistently out-performed doxorubicin; therefore, Daunotubicin was included for *in vivo* analysis. Finally, bortezomib- which has been extensively characterized in relation to enhancing transduction of a variety of rAAV serotypes *in vitro* and *in vivo* and was also identified in our screen- was included. The doses selected for *in vivo* study were chosen based on conversions from the FDA-approved dosing in humans to the murine equivalent based on body surface area and established Km factors (187) **(Table 2.2)**. As teniposide and etoposide are recommended for use over a wide range of doses, they were administered in this study at a conservative dose of 20 mg/kg (60 mg/m^2 human equivalent dose). Each pharmaceutical was co-administered with 1x10^{11} vg rAAV2-CBA-Luc through tail vain injection in age-matched female Balb/c mice. Two vehicle cohorts were included in the study. We evaluated toxicity by measuring levels of Blood Urea Nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT), and creatine kinase (CK). These levels appeared within normal ranges and were comparable to the vehicle-treated mice. Average levels were similar between drug-treated and vehicle-treated cohorts. In general, variability among treatment groups was noticed, and could be due to either an immune response against the

luciferase transgene in some mcie, as has been observed before, or perhaps an effect of the vehicle (DMSO) on *in vivo* transduction.

Due to the impressive enhancement we observed *in vitro* at 24 hours post transduction, an early time point of gene expression was measured to determine whether the pharmaceuticals could also provide rapid, high-level gene expression *in vivo*. At 48h post-administration, all of the selected drugs enhanced transduction *in vivo*, but to varying degrees **(Figure 2.6a)**. Consistent with what was seen *in vitro*, the epipodophyllotoxin and anthracycline topoisomerase II inhibitors enhanced transduction to the greatest extent. Notably, co-treatment with teniposide enhanced transduction by an average of approximately 100-fold **(Figure 2.6b)**. While teniposide and etoposide have the same mechanism of action in cells (i.e. inhibition of the relegation of DNA ends through intereference of the DNA-topoisomerase complex), teniposide was a more potent augmenter of transduction at this time point. Upon assessment of transduction 8 days post-administration, enhanced transduction was noted among all of the drug-treated cohorts **(Figure 2.6a)**. Teniposide co-administration yielded the greatest enhancement in transduction at this time point, followed by bortezomib. Daunorubicin co-treatment also yielded impressive transduction enhancement at the 8 day timepoint. Bleomycin and etoposide co-treatment produced only modest enhancement of transduction efficiency. This result could be reflective of the *in vivo* dose limitations in order to reflect clinical levels of each drug.

Because co-administration of rAAV2 with teniposide showed the most robust transduction enhancement, we tested this compound for its ability to enhance transduction of other rAAV vectors (**Figure 2.7)**. We chose to evaluate rAAV9 and rAAV8 due to current interest in using these vectors for clinical applications. Because of the robust enhancement observed for fAAV2, we chose to test the ability of teniposide to enhance fAAV8 *in vivo* as well.

Due to the difficulty in producing high- titer fAAV8, mice were administered only 5 X 10^{10} vg. Interestingly, while enhancement of rAAV2 transduction was the most effective with teniposide, this level of enhancement was not observed with rAAV9 or rAAV8, suggesting either serotype-dependent effects or saturation of luciferase activity with these vectors. At 48 h post-transduction, we observed modest enhancements in both rAAV9 and rAAV8 with co-administration of teniposide. At 8 days post-transduction, transduction levels were similar between teniposide-treated and vehicle-treated rAAV9 and rAAV8 cohors, with only a small enhancement in transduction observed. Further work exploring lower doses of these vectors in vivo may reveal if teniposide co-administration can show higher levels of enhancement. The greatest enhancement observed with teniposide co-administration was in mice treated with fAAV8, thus reflecting observations *in vitro* with fAAV2. At 48 h post-transduction, the teniposide-treated cohort exhibited 34-fold higher levels of luciferase activity than the vehicle-treated cohort. This enhancement was even greater at 8 days post transduction, with the teniposide-treated cohort exhibiting 86-fold higher luciferase activity than the vehicle-treated cohort.

Taken together, these results confirmed our observations *in vitro* and corroborated that the greatest transduction augmentation arises from co-treatment with topoisomerase or proteasome inhibitors. Furthermore, teniposide was shown to have the greatest effect on fAAV8 transduction.

Discussion

Recent gene therapy applications using rAAV have been met with both success and challenges that depend on indication, route of administration, serotype, and vector dose utilized.

Indeed, clinical efficacy for one indication, lipoprotein lipase deficiency, has been achieved to the extent that the first ever gene therapy product, an rAAV1 vector carrying a lipoprotein lipase transgene, is now approved for use in the European Union (140). Results from clinical trials have exposed the current limitations of rAAV-mediated gene therapy, one of which is the ability to achieve robust, long-term therapeutic gene expression without eliciting an immune response induced by high vector doses. Several strategies have been employed to combat this challenge, including capsid and transgene modification as well as pharmaceutical intervention; however they have been met with varied success and may be limited to a particular serotype or tissue target. For example, the elimination of certain tyrosines on the capsids of AAV2 and AAV6 has allowed for enhanced transduction efficiency and lower rates of proteasomal degradation (127, 132, 155); however, it has been shown that this strategy cannot be applied to rAAV9 and attempts with rAAV8 have produced mixed results (134, 136). Similarly, the proteasome inhibitor bortezomib has been shown to enhance transduction of both rAAV2 and rAAV8 vectors, resulting in increased transgene expression in both small and large animal models (118, 119, 167); however, a recent report has shown that this strategy cannot be applied for rAAV9-mediated therapy for cardiac failure (122). Thus, the need for a robust, widely applicable strategy to enhance transduction would be highly beneficial in the translation of rAAV-mediated gene therapy to a broad range of applications.

In this study, we employed a high-throughput small molecule screen to identify compounds capable of enhancing rAAV2 transduction. Several hits were identified, including some of novel origin and some that have been previously defined. These compounds were shown to have varying activity on vectors comprised of different forms of transgenes, as well as some differential effects in a variety of cell lines. Finally, selected hits that are currently being

used in clinical applications were validated *in vivo*. Overall, the greatest enhancers of transduction were drugs that inhibit topoisomerase II, in particular the epipodophyllotoxin class and the anthracyclines. Generalizing from the entire collection of our identified hits, it seems that rAAV can be enhanced by two mechanisms: i) inhibition of the proteasome, and ii) induction of DNA damage, which corroborates previously published results (104, 111, 112, 162).

The epipodophyllotoxin and anthracycline topoisomerase II inhibitors form complexes with DNA and topoisomerase II, an enzyme required for the prevention of DNA supercoiling in eukaryotes. The topoisomerase II cycle includes i) binding and cleavage of duplexed DNA, ii) passage of a second strand of DNA through the complex, and iii) re-ligation of the broken DNA ends (188). Both classes of topoisomerase II inhibitors prevent the re-ligation step of this cycle, thus causing both single-stranded and double-stranded DNA damage. While the direct role of topoisomerase II in the mechanism of rAAV transduction enhancement cannot be ruled out, previous research suggests that the DNA damage response, an indirect result of topoisomerase II inhibition, is likely the main contributor to the increase in transduction observed following use of these compounds (103, 112, 189). Indeed, this screen identified several topoisomerase II-independent DNA-damaging agents, such as bleomycin, parthenin, and RH1, as potentiators of rAAV2 transduction. In fact, Bleomycin enhanced transduction to similar levels as Teniposide and Etoposide treatment in U87 cells (Figure 2A). Several groups have shown that proteins involved in the DNA damage response, including both homologous recombination and nonhomologous end-joining, interact with incoming viral and vector genomes and have important roles in genome processing. These processes are thought to include the conversion of the single-stranded genome into double-stranded DNA (21, 190), opening of ITR hairpins (191), concatamerization and circularization (102, 104, 192, 193), and transgene expression (194).

Notably, the Mre11/Rad50/Nbs1 (MRN) complex, important for double-strand break repair, recombination, and telomere maintenance, has been shown to bind to AAV ITRs, negatively affecting rAAV transduction, wtAAV replication, and double-stranded rAAV DNA accumulation (103, 189). It is currently thought that cellular DNA damage, induced by radiation, small molecules, or other means, may serve as a "decoy," recruiting the MRN complex to the sites of damage and away from single-stranded AAV DNA, thus allowing for double strand conversion. Interestingly, Choi et al observed that ATM, Mre11, and NBS1 are required for scAAV DNA circularization *in vitro* and ATM and DNA-PK(CS) are required for scAAV DNA circularization *in vivo* (102). Taken together, these results suggest that DNA damage response proteins may have a dual function in rAAV DNA processing and the positive or negative effects may depend on the state of the DNA within the cell.

Proteasome inhibition is thought to be beneficial to rAAV transduction through a mechanism that differs from a DNA damage response. It is believed that inhibition of the proteasome facilitates bulk flow of particles away from degradation pathways, thereby redirecting them to routes that favor transduction (i.e. nuclear translocation) (87, 113, 167). Bortezomib, the only currently FDA-approved proteasome inhibitor, has been used in conjunction with rAAV to enhance transduction in hemophilia A and hemophilia B models (119). Its effects seem to be thus far limited to these applications, as a recent study using rAAV9 to deliver SERCA2a to preserve cardiac function in a rat model demonstrated no additional benefit when bortezomib was co-administered (122). Indeed, our results suggest that the effects of proteasome inhibiton by bortezomib are cell-type dependent, as bortezomib-mediated enhancement was not observed in U87 and NHF cell lines, and not as robust in HepG2 cells as compared to HeLa cells. Interestingly, two compounds were identified which have been

previously implicated in proteasome inhibition- physalin B and siomycin- which out-performed bortezomib in U87, HepG2, and NHF cell lines. These compounds may either inhibit proteasome function through a mechanism that is different than that of bortezomib or may perform additional functions in cells that are beneficial to rAAV2 transduction.

The level of involvement of DNA damage response proteins and proteasome inhibition seems to differ between ssAAV2, scAAV2, and fAAV2. While the ssAAV2 and fAAV2 used in this study share identical promoters and luciferase genes, the scAAV2 cassette differed in promoter (CMV vs. CBA) as well as transgene (gaussia vs. firefly luciferase). Therefore, a direct comparison can be made between ssAAV2 vectors and fAAV2 vectors, but considerations must be made when evaluating the performance of the scAAV2 vector. Initial conclusions seem to indicate that, for all of the compounds identified in our screen, transduction enhancement seemed to be less pronounced for scAAV2. This finding is in agreement with the current theory of how DNA damage proteins may be inhibitory for ssAAV DNA, since scAAV DNA does not require second-strand synthesis and would therefore be unaffected by any proteins limiting this type of processing. Since it has been shown that Mre11 and ATM are required for scAAV genome circularization, it will be interesting to evaluate the long-term effects of these compounds on scAAV gene expression in the future. Interestingly, some enhancement of scAAV2 transduction was observed with the co-treatment with the epipodophyllotoxins and anthracyclines, suggesting that these compounds might enhance transduction by mechanisms in addition to facilitating second-strand synthesis.

Perhaps the most striking observation was the dramatic increase in transduction seen for fAAV2 treated with the compounds identified in this screen. Notably, each compound boosted fAAV2 transduction to levels greater than ssAAV2 treated with vehicle alone. Previous studies

have shown that fAAV2 transduction relies on the annealing of sections of vector DNA that comprise the entire oversized transgene cassette (186). This process is heavily dependent upon Rad51C, a single-stranded DNA binding protein involved in supporting Rad51-mediated homologous recombination during DNA double strand break repair. Therefore, it is possible that in the case of fAAV2, cellular DNA damage serves two purposes: i) de-repression of the single-stranded cassette by inhibitory proteins such as the MRN complex, and ii) recruitment of homologous recombination proteins such as Rad51C to facilitate the annealing of fragment DNA. Interestingly, treatment with bortezomib also resulted in high levels of fAAV2 enhancement. Treatment with proteasome inhibitors has been shown to increase the sheer volume of vector particles that reach the nucleus. It is possible that the deficit observed in unassisted fAAV2 transduction is simply a numbers game; i.e. as more particles, and therefore assuming more uncoated genomes, transit to the nucleus, there is an increased chance for fragment strand reannealing. Alternatively, inhibition of the proteasome may also inhibit the degradation of proteins that might be essential for fAAV2 reannealing. Indeed, work by Bennett and Knight has shown that proteasome-mediated degradation of Rad51 occurs during DNA repair, and this process is regulated in part by Rad51C (195). Additionally, the proteasome has been shown to be associated with double strand breaks and has been suggested to play a role in degrading proteins upon completion of DNA repair in yeast (196). Regardless of mechanism, our results suggest that the deficit in fAAV2 transduction can be restored to ssAAV2 levels through pharmacological intervention; thus paving the way for future studies in pharmaco-gene therapy for large gene applications.

Overall, this work validates a method of screening compounds to identify novel enhancers of rAAV transduction. Through this process, several compounds were identified that

have been previously characterized for such purposes, and several novel compounds were discovered as well. Identified hits were validated in several cell lines and with several rAAV transgene cassette configurations. It was further demonstrated that the currently approved therapeutics identified in our screen could also enhance rAAV2 transduction *in vivo*. With the exception of tetrocarcin A (a weak enhancer), all of the identified hits have known mechanisms of action relating either to DNA damage or proteasome inhibition, two cellular functions well known for their implications in rAAV transduction. As the library of compounds studied here originates from a repository dedicated to the discovery of novel cancer chemotherapeutics, it is not surprising that the majority of identified hits relate to two pathways that are common targets for cancer treatment. Other compound libraries not targeted for cancer therapeutics may contain compounds that affect other critical stages of rAAV transduction, including endocytosis, endosomal trafficking and escape, or nuclear entry, and thus may warrant further investigation using the methodology developed herein. Therefore, future studies should include expanding this approach to identify compounds that benefit rAAV trafficking steps, with a potential ultimate goal of using a combination of drugs that act synergistically to enhance transduction to clinically beneficial levels.

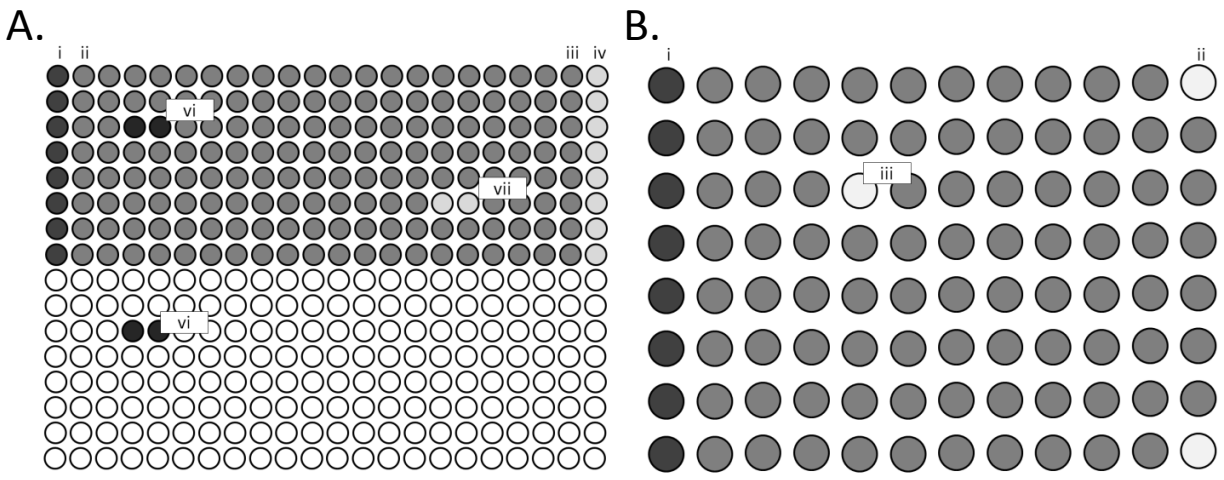
While the compounds that induced DNA damage seemed to enhance rAAV transduction to the greatest extent, caution is necessary before potentially moving forward with any of these drugs. Though the primary mechanism of DNA persistence is through concatamerization and circularization, random integration has been demonstrated for rAAV (197-202). Indeed, a previous study has shown that integration of rAAV DNA *in vitro* increased upon treatment with etoposide (112, 203). While the epipodophyllotoxins have been shown to produce breaks in DNA "hot spots," (204-206) a thorough assessment of *in vivo* integration events would need to

be carried out in order to gauge the risks of this kind of pharmaco-gene therapy. Alternatively, the discovery or development of molecules that could exert the same function for enhanced transduction as the topoisomerase inhibitors, without causing genotoxicity, would be expected to be highly beneficial. Finally, effects of these agents on long term transduction will need to be evaluated. It is tempting to envision a scenario where inducing DNA damage through one of these agents could reactivate persistent-but-silenced rAAV episomes, thus eliminating the need for additional administration of vector if transgene expression falls below therapeutic levels.

With the success of recent clinical trials, the widespread utility of rAAV-mediated gene therapy is becoming an exciting possibility. One of the remaining hurdles towards this end is achieving a level of transgene expression that provides clinical benefit without inducing an immune response that will ultimately result in baseline levels of gene expression. Developments in pharmaco-gene therapy, in addition to capsid and transgene modification, may provide a foundation toward achieving this goal and thus may broaden the clinical utility of rAAV vectors.

Acknowledgements

Animal Studies were performed within the LCCC Animal Studies Core Facility at the University of North Carolina at Chapel Hill. The LCCC Animal Studies Core is supported in part by an NCI Center Core Support Grant (CA16086) to the UNC Lineberger Comprehensive Cancer Center. Serum markers for toxicity were evaluated by the UNC Clinical Chemistry core facility. All rAAV titers were calculated by Sophia Shih.



384-well format	96-well format
Final drug concentration: 1 µM	Final drug concentration: 10 µM
Vector dose: 1000 vg/cell	Vector dose: 500 vg/cell

Figure 2.1. Diagrammatic set-up of both screens used in this study. A. 384-well screen format. Cells were treated with compound in duplicate, and each plate was configured as a dual set-up: the top half was assessed for transduction activity, and the bottom half was assessed for cell viability. i, no rAAV2, no treatment; ii, rAAV2 only; iii, rAAV2 with DMSO; iv, rAAV2 with MG132; vi, example of a compound that induced toxicity and therefore transduction; vii, example of a "hit." B. 96-well screen format. Cells were treated with compound in single wells, and viability was not assessed at this stage. i. rAAV2 with DMSO; ii, rAAV2 with MG132; iii, example of a "hit."

Compound Name	NSC ID	Clinical Use	Used with AAV?	Group	Known mechanism of action
Teniposide	122819	Chemotherapy		Podophyllotoxin	Topoisomerase II inhibition
Etoposide	141540	Chemotherapy	Russell et al, PNAS, 1995		
Bleomycin	125066	Chemotherapy, plantar warts		Glycopeptide antibiotic	DNA damage
Parthenin	85239			Sesquiterpene lactone	
RH-1	697726	Phase I, solid malignancies		diaziridinylbenzoquinone	
Vorinostat	701852	Chemotherapy		HDAC Inhibitor	HDAC inhibition
Nanaomycin A	267461			DNMT3B inhibitor	DNMT3B inhibition
Menogaril	269148			Anthracycline	DNA intercalation, topoisomerase II inhibition, polymerase inhibition, free radical damage to DNA
Pyrromycin	267229				
Doxorubicin*		Chemotherapy	Yan et al, JVI, 2004		
Danorubicin	82151	Chemotherapy			
Bortezomib	681239	Chemotherapy	Nathwani, Gene Ther, 2009 etc.	dipeptide	Proteasome inhibition
Physalin B	287088			Physalin	
Siomycin	285116			Thiazole Antibiotic	
Tetrocarcin A	333856			Microbial metabolite	BCL-2 inhibitor

Table 2.1. Compounds that were characterized in this study. Compounds that emerged as hits were selected based on known function in cells or clinical utility.

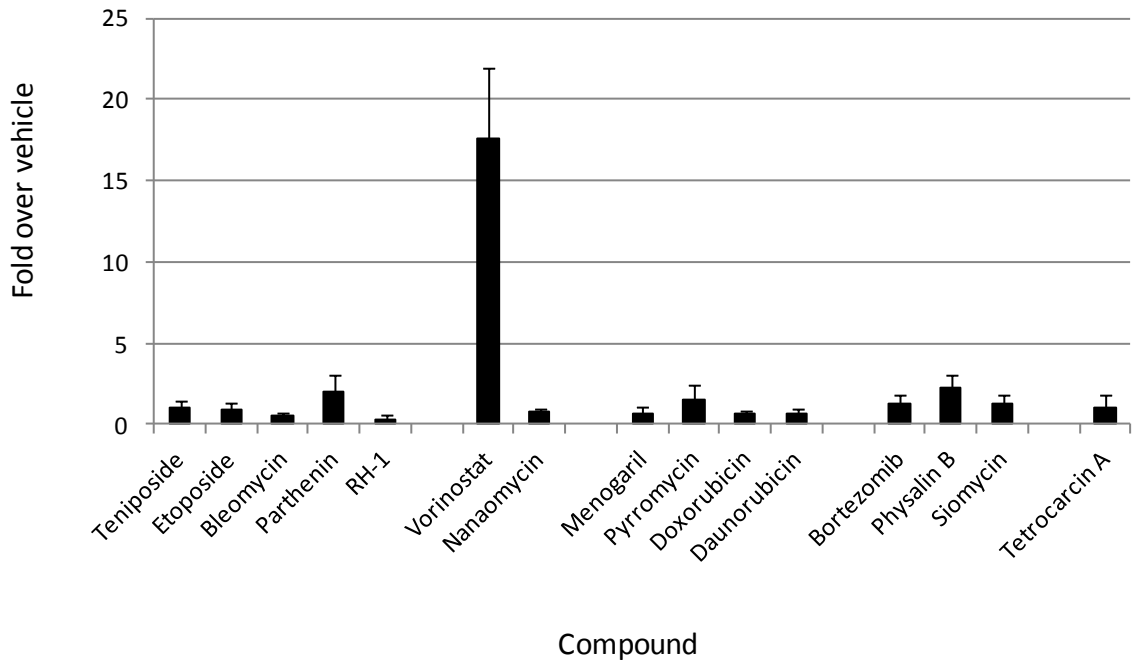


Figure 2.2. Effect of compounds on plasmid gene expression. HeLa cells were transfected with pCBA-Luc and treated with the compounds identified in our screen. Transduction was assessed 24h after compound treatment. Of the hits, Vorinostat appears to be the only compound that enhances plasmid gene expression.

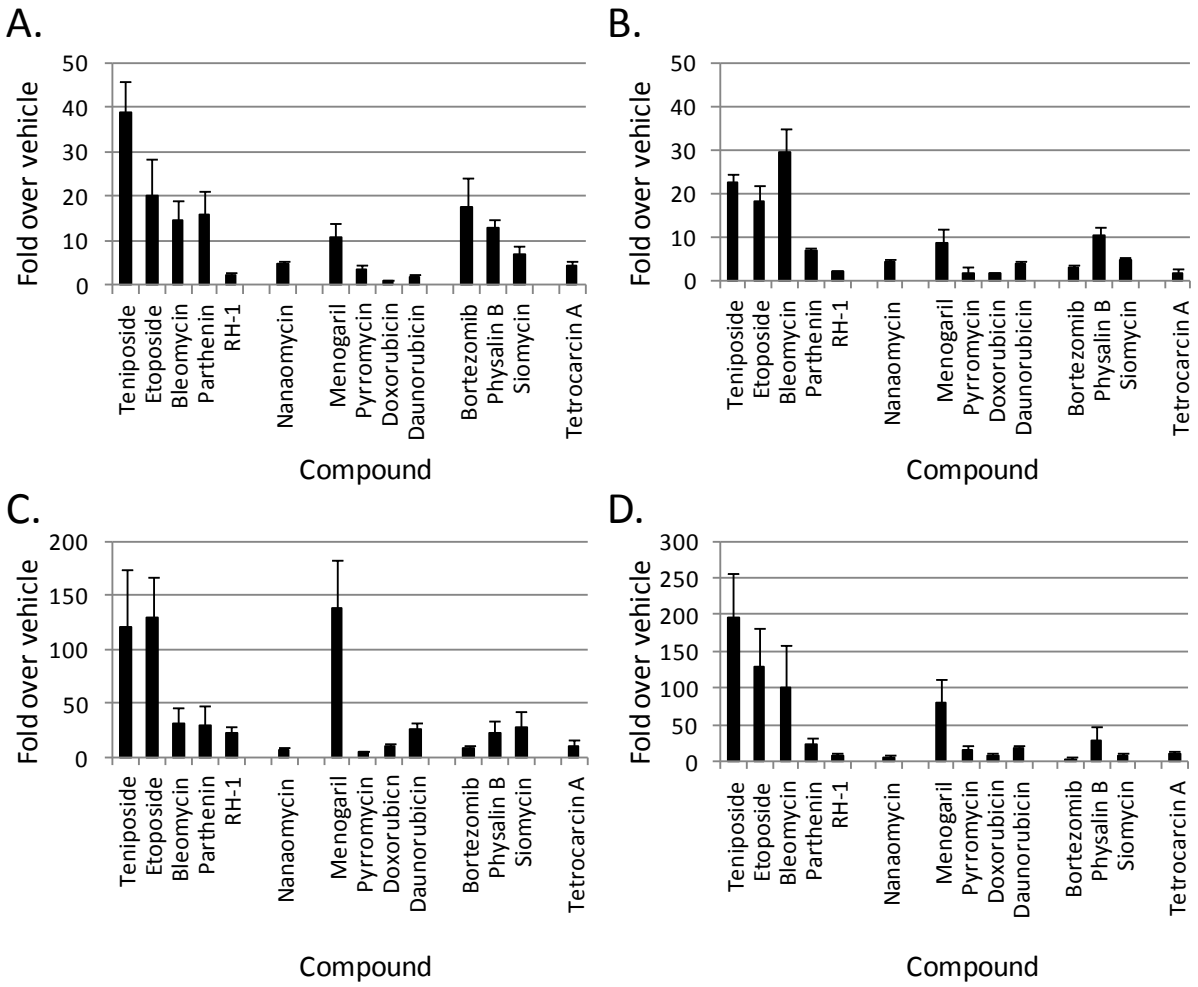


Figure 2.3. Effect of compounds on human cell lines. Cells were treated with the identified compounds or dose-appropriate DMSO and later rAAV2_CBA-Luc. Transduction was assessed 24 hours after drug treatment. A. HeLa cells. B. U87 cells. C. HepG2 cells. D. NHF1 cells.

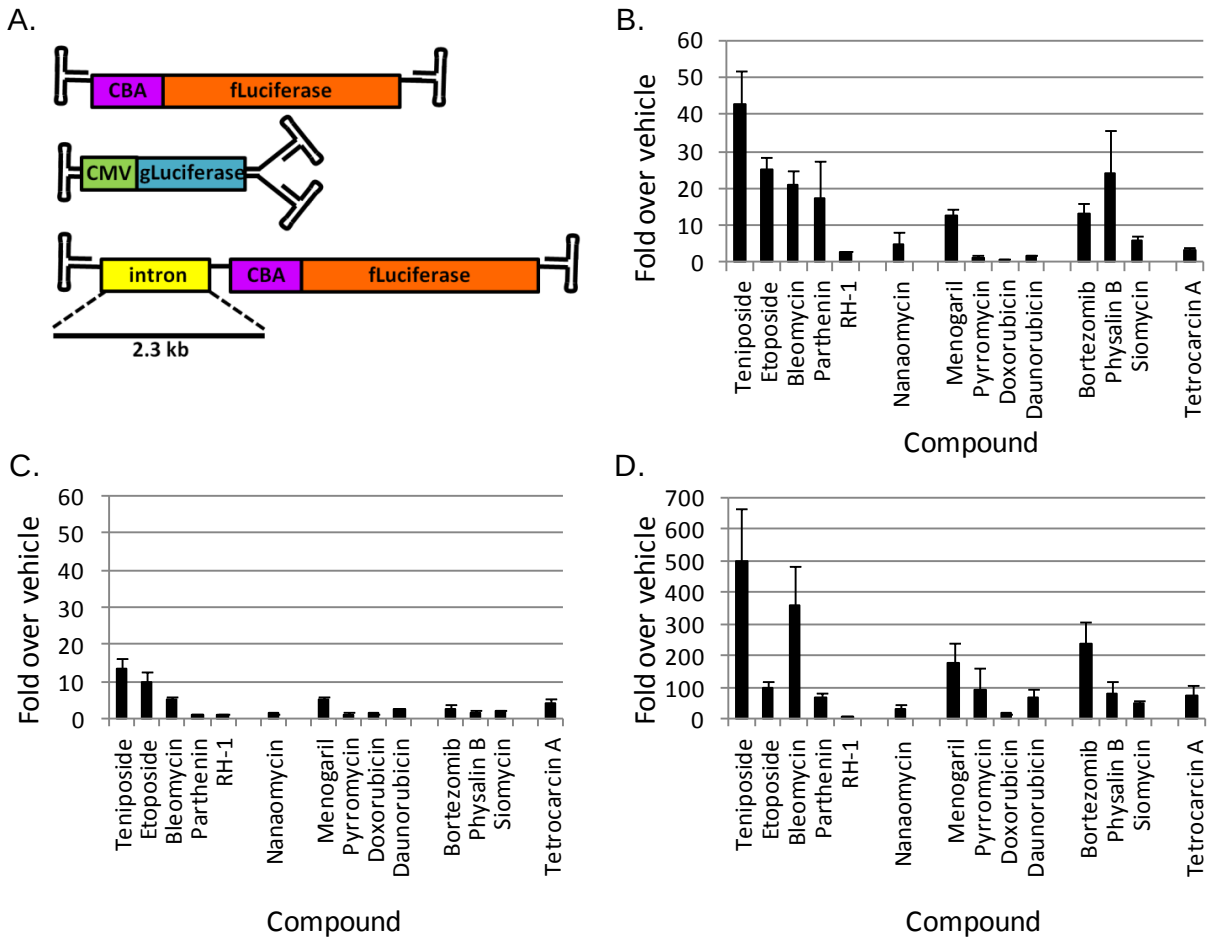


Figure 2.4. Effect of identified compounds on vectors with different forms of transgenes. A. Schematic of each of the trasngene cassettes utilized in this study. Top, single-stranded, intact; middle, self-complementary; bottom, fragment. HeLa cells were treated with each of the identified compounds and single-stranded rAAV2 (B), scAAV2 (C), or fAAV2 (D) or dose-appropriate DMSO. Luciferase activity was measured 24 h post compound treatment.

Drug	Clinical Dose	Dose given in vivo[1]	Whole blood concentration[2]
Teniposide	30-180 mg/m^2	20 mg/kg	381 µM
Etoposide	35-100 mg/m^2	20 mg/kg	430 µM
Daunorubicin	45 mg/m^2	15 mg/kg	332 µM
Bortezomib	1.3 mg/m^2	0.433 mg/kg	14.1 µM
Bleomycin	10-20 units/m^2	6.67 mg/kg	29 µM

Table 2.2. **Parameters and toxicological analysis of in vivo compound administration.** A. Summary of *in vivo* dosage used in study. [1]Murine Equivalent Dose (MED) based on conversion of dose approved in humans using mouse Km value of 3. [2]Whole blood concentration was based on the molecular weight of each compound, the average weight of a mouse (25 g) and the average volume of whole blood (79 mL/kg).

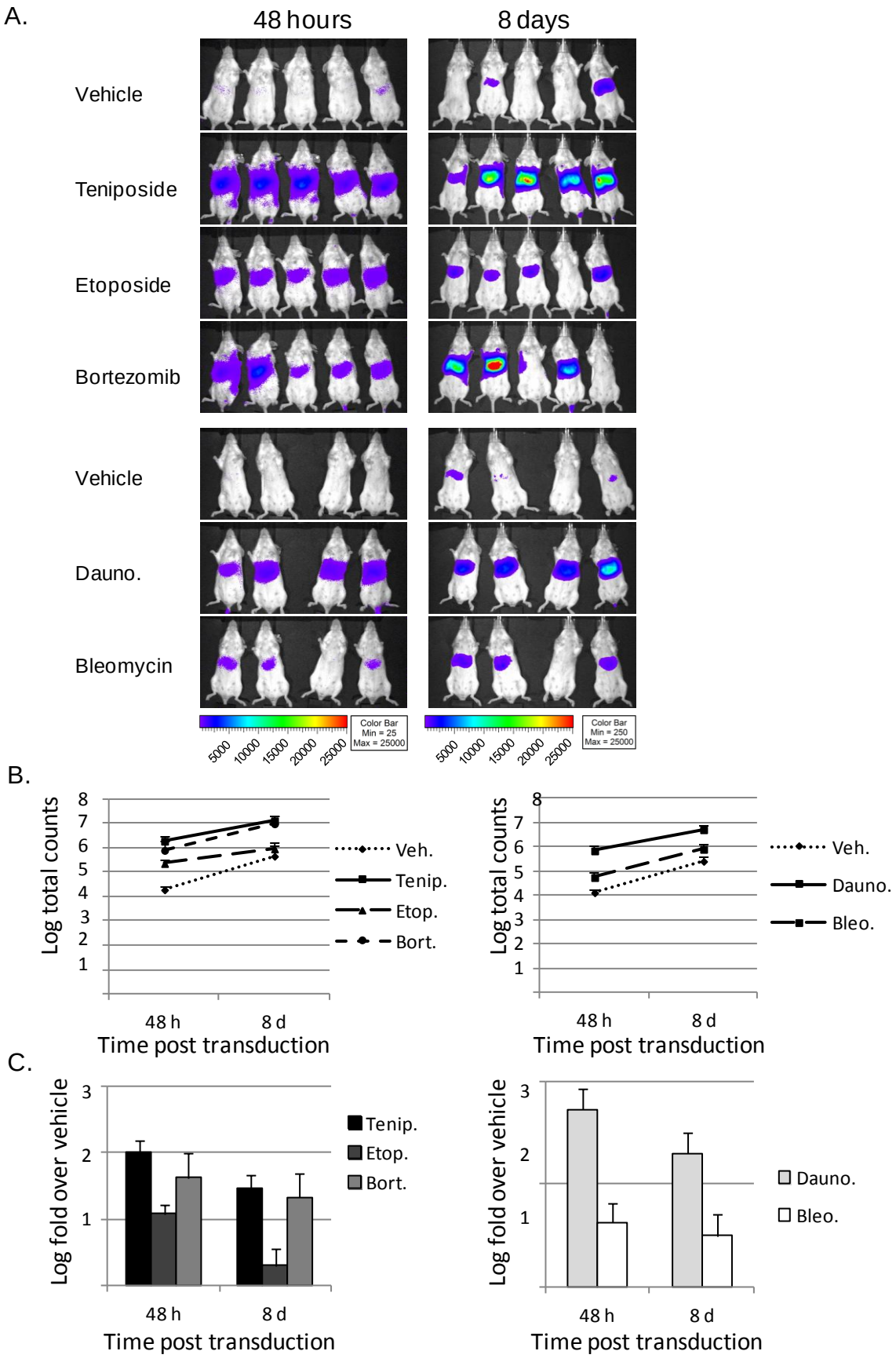

A.
48 hours
8 days
Vehicle
Teniposide
Etoposide
Bortezomib
Vehicle
Dauno.
Bleomycin
Color Bar
Min = 25
Max = 25000
5000
10000
15000
20000
25000
Color Bar
Min = 250
Max = 25000
B.
Log total counts
Veh.
Tenip.
Etop.
Bort.
48 h
8 d
Time post transduction
Log total counts
Veh.
Dauno.
Bleo.
48 h
8 d
Time post transduction
C.
Log fold over vehicle
Tenip.
Etop.
Bort.
48 h
8 d
Time post transduction
Log fold over vehicle
Dauno.
Bleo.
48 h
8 d
Time post transduction

Figure 2.5. **Analysis of transduction enhancement by compounds *in vivo*.** A. Live bioluminescent imaging of mice that were co-treated with rAAV2_CBA-Luc and each of the clinically relevant compounds, imaged 48 h and 8 d post-transduction. B. Quantification of bioluminescent imaging. C. Fold difference in luciferase expression over vehicles.

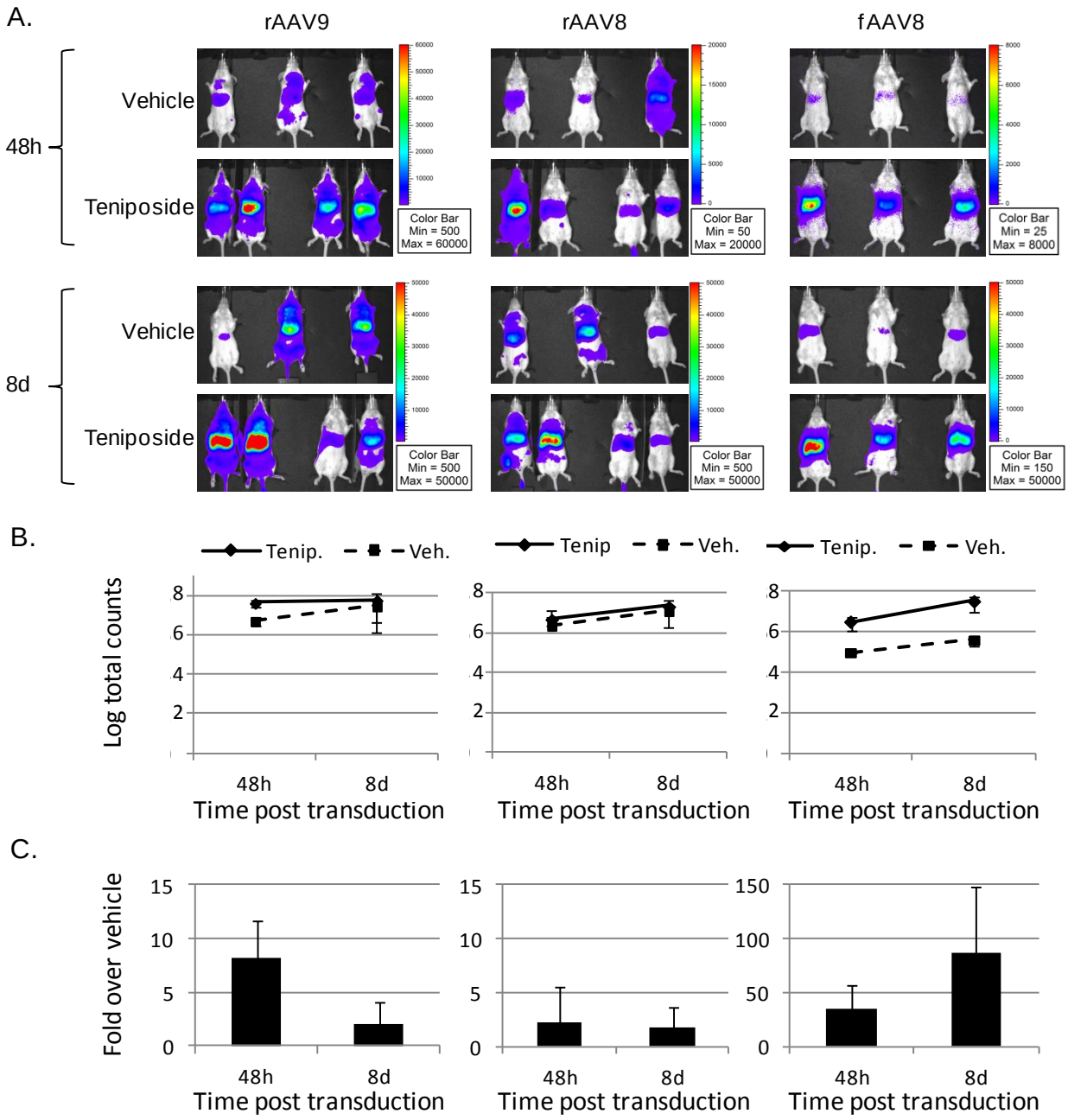


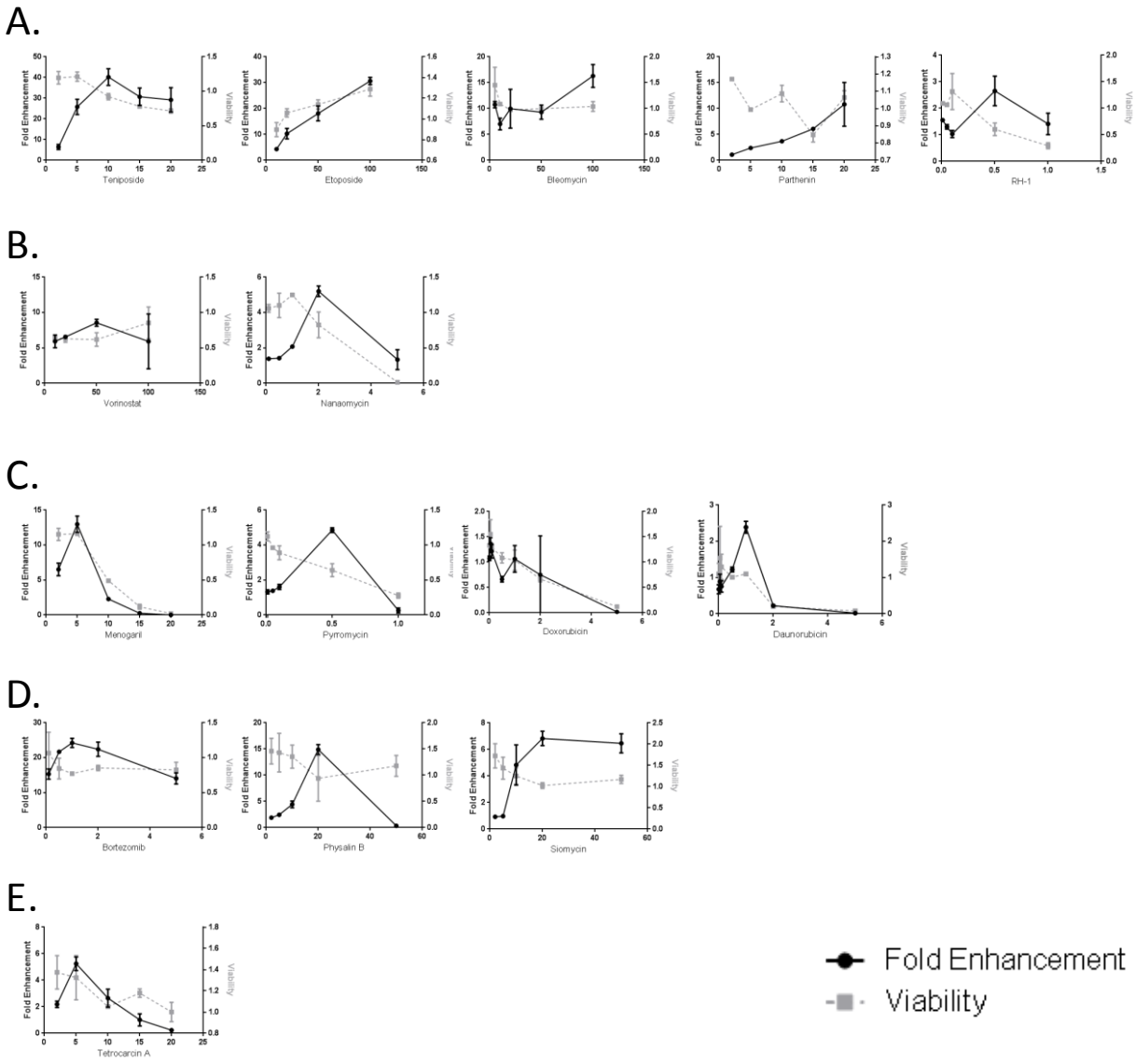
Figure 2.7. Analysis of transduction enhancement by other rAAV vectors with teniposide *in vivo*. A. Live bioluminescent imaging of mice that were co-treated with teniposide and rAAV9 (left), rAAV8 (center), and fAAV8 (right), imaged 48 h and 8 d post-transduction. To avoid pixel saturation, at 8 days post-transduction, image acquisition was 2 min for rAAV9, 1 min for rAAV8, and 5 min for fAAV8. B. Quantification of bioluminescent imaging. C. Fold difference in luciferase expression over vehicle.

NSC id	Name (if known)	Cellular function	Fold enhancement in screen
757	Colchisine	Microtubule dynamics	2.23
82151	Daunorubicin	Topoisomerase II inhibitor	2.01
67574	Vincristine	Microtubule dynamics	2.77
153858	Maytansine	Microtubule dynamics	2.13
267461	Nanaomycin A	DNA methyltransferase IIIB inhibitor	2.18
332598	Rhizoxin	Microtubule dynamics	2.66
333856	Tetrocarcin A, sodium salt	Inhibition of BCL-2	2.85
338250			3.74
325014	Bactobolin	Antibiotic	3.15
400978	Illudin M	DNA damage	2.76
63001			2.19
100120			3.42
93427			2.27
13380			2.06
111194			3.41
102742			2.14
126226			2.21
151252			2.65
159398			3.17
187675			2.29
607097	Quinocarmycin analog	DNA damage, interacts with radixin	2.37
330770			3.39
652287			2.00
659997			2.12
403148	Anthricin	Akt/mTOR signalling inhibitor	2.29
632841			2.02
17946	3-Desmethyl Colchicine	Microtubule dynamics	2.38
126127			2.23
687849			2.91
24818	Podophilox	Unknown, related to epipodophyllotixins	2.19
49842	Vinblastine	Microtubule dynamics	2.25
36354	Trimethylcolchicinic acid	Microtubule dynamics	3.76
208734	Aclarubicin	Topoisomerase inhibitor	2.52
267229	Pyrromycin	Topoisomerase inhbiitor	6.87
125066	Bleomycin	DNA damage	2.10

Supplementary Table S2.1. List of hits and known function (if any) for compounds initially identified in 384-well screen.

NSC id	Name (if known)	Cellular function	Fold enhancement in screen
697923			4.76
5426			7.27
697923			4.77
632839			4.75
376248	Nordracorubin		4.84
642048			4.89
670224			5.30
7525	Scillaren A		6.43
658139			6.06
7530	Strophanthoside-K		5.80
659999			9..6
407286	Ellagic Acid	Proteasome inhibition, anti-inflammatory	7.47
59984			7.4
5426			7.00
98937			6.2
311727			5.00
623051			4.7
93427			4.7
243928			4.4
338250			4.35
673622			4
298197			4

Supplementary Table S2.2. List of hits and known function (if any) for compounds initially identified in 96-well screen.



Supplementary Figure S2.1. Example of dose-optimization for HeLa cells. HeLa cells were treated with increasing concentrations of the A) DNA damaging agents, B)anthracyclines, C) transcriptional activators, D) proteasome inhibitors, or e) tetrocarcin A. Cells were assessed for viability using the CellTiterGlo system (right Y axis) as well as transduction efficiency (left Y axis).

CHAPTER 3

Recombinant Adeno-associated virus can utilize host cell nuclear import machinery to enter the nucleus

Summary

Our previous small molecule screen identified several compounds that enhanced transduction; however, as somewhat of a surprise most of these compounds' known mechanismns of action in cells included DNA damage induction or proteasome inhibition. These are two pathways that are well-characterized to be impediments to efficient transduction. To target other possible cellular barriers to efficient transduction, we decided to investigate the mechanism of nuclear entry in order to create a foundation for the design of vectors or identification of pharmacological interventions to enhance this step in subcellular trafficking. Previous studies suggest that the majority of rAAV accumulates in the perinuclear region of cells, presumably unable to traffic into the nucleus. rAAV nuclear translocation remains ill-defined; therefore, we performed microscopy, genetic, and biochemical analyses *in vitro* in order to understand this mechanism. Lectin blockade of the nuclear pore complex (NPC) resulted in inhibition of nuclear rAAV2. Visualization of fluorescently-labeled particles revealed that rAAV2 localized to Importin-β-dense regions of cells in late trafficking steps. Additionally, siRNA knockdown of Importin-β partially inhibited rAAV2 nuclear translocation and inhibited transduction by 50-70%. Furthermore, co-immunopreciptation (IP) analysis revealed that capsid

[1] This chapter is adapted from an article which previously appeared in the Journal of Virology. The original citation is as follows: Nicolson, Sarah C. and R. Jude Samulski. "Recombinant adeno-associated virus utilizes host cell nuclear import machinery to enter the nucleus." *Journal of Virology* 88: 4132-4144 (2014).

proteins from rAAV2 could interact with Importin-β and that this interaction was sensitive to the small GTPase Ran. More importantly, mutations to key basic regions in the rAAV2 capsid severely inhibited interactions with Importin-β. We tested several other serotypes and found that the extent of Importin-β interaction varied, suggesting that different serotypes may utilize alternative import proteins for nuclear translocation. Co-IP and siRNA analyses were used to investigate the role of other karyopherins and suggested that rAAV2 may utilize multiple import proteins for nuclear entry. Taken together, our results suggest that rAAV2 interacts with Importin-β alone or in complex with other karyopherins and enters the nucleus via the NPC. These results may lend insight into the design of novel AAV vectors that have enhanced nuclear entry capability and transduction potential.

Introduction

Adeno-associated virus (AAV) is a dependovirus in the family *Parvoviridae* that cannot replicate on its own (17). For this reason, recombinant adeno-associated viruses (rAAV) have recently garnered much attention in the field of gene therapy (reviewed in (2)). Several serotypes have been discovered that transduce various tissue types with high efficiency (reviewed in (141)). Despite its promise, widespread use of AAV vectors has been hindered by their relatively low transduction efficiency. Thus, much interest in the field has been directed towards the rational and combinatorial design of enhanced AAV vectors that can overcome transduction barriers at the level of receptor binding, subcellular trafficking, and transgene expression.

It has become apparent that subcellular trafficking presents multiple barriers to successful rAAV transduction, which involves movement of the vector from the host cell surface into the

nucleus, where uncoating and subsequent gene expression occur (84, 120, 207, 208). These events are mediated by interactions between host cell proteins and the three capsid proteins, VP1, VP2, and VP3 (209, 210). In the case of rAAV2, the vector binds to primary and co-receptors such as heparan sulfate proteoglycan and αvβ5 integrins (65, 74, 75). The particle is internalized through receptor-mediated endocytosis via clathrin-coated pits or through the CLIC/GEEC pathway (81, 83). rAAV2 then trafficks along the endo-lysosomal route, accumulating near the golgi compartment and the microtubule organizing center (MTOC, (82, 85, 86, 121, 211, 212)). rAAV2 harbors domains buried within the capsid surface that are critical for further subcellular trafficking and nuclear entry (91, 92, 213). At some point prior to escape from the endosome, rAAV2 undergoes a conformational change, leading to the exposure of the unique N-terminal ends of VP1 and VP2, termed VP1up (91, 92). VP1up contains a phospholipase A2 (PLA2) domain that is thought to mediate escape from the endosomal compartment (93) as well as 3 putative nuclear localization sequences (NLSs) (95, 96). Upon endosomal escape, rAAV2 enters the nucleus as an intact particle (87), where subsequent uncoating and gene expression occur. Currently, the mechanism of nuclear entry by rAAV vectors is unknown. Studies into the intracellular trafficking of rAAV2 have revealed a common pattern: while the majority of particles traffic to a perinuclear region associated with the MTOC, most of these particles remain distal to the nucleus and never translocate (86, 120, 207). These particles are eventually degraded by host proteasomes and likely presented as antigens on the cell surface (214). If this barrier to nuclear entry could be overcome, two benefits could be achieved. First, extra virions in the nucleus could potentially bolster transduction efficiency by contributing to greater transgene expression. Secondly, movement of virions from the cytosol into the nucleus could reduce the number of capsids susceptible to antigen presentation and host cell

immunity. Therefore, understanding the mechanism of nuclear entry by AAV would create a foundation to design improved capsids that can overcome this barrier.

The majority of nuclear-targeted cellular proteins utilize the canonical nuclear import pathway (for a review, see (215, 216)). Proteins with a nuclear localization signal (NLS) are bound by karyopherins, most typically a member of the Importin-β family or an Importin-α adapter/Importin-β heterodimer. The importin proteins serve as chaperones to bring the nuclear-bound "cargo" through the nuclear pore complex (NPC), a multi-protein complex that can accommodate import of proteins up to 39 nm (217). This process is dependent on the small GTPase Ran, which provides the energy required for the translocation and mediates the dissociation of the Importin-β complex once inside the nucleus (218-221).

Many viruses take advantage of all or parts of this pathway (for reviews, see (222, 223). For example, the DNA-bound Protein VII of Adenovirus enters the nucleus through the NPC through interactions with Importin-β, Importin-α, and Importin 7 (224). Herpes simplex virus 1 (HSV-1) mediates nuclear entry of its nucleic acid through directly binding to the cytoplasmic filaments of the NPC in an Importin-β-dependent manner (225, 226). The hepadnavirus Hepatitis B (HBV) has been shown to bind to the NPC in a manner mediated by the phosphorylation of its capsid and the exposure of two NLSs which bind to Importin-α and Importin-β (227-229). Interestingly, the autonomous parvovirus MVM has been shown to utilize an alternative nuclear entry mechanism involving direct disruption of the nuclear lamina in a caspase-dependent manner (230, 231).

Several studies have yielded insight regarding how AAV translocates into the nucleus; however, the mechanism of nuclear entry remains ill-defined and could differ between wild type (wt) virions and AAV vectors. With a capsid diameter of 26 nm, AAV particles are within the

size limit to traverse the NPC. The unique N termini of AAV2 VP1 and VP2 contain three basic regions (BRs) that resemble classic NLSs and can support nuclear entry of exogenous proteins (92, 232). Mutations to these basic regions inhibit nuclear entry of rAAV2 and subsequent transduction (95, 96). These results suggest that the mechanism of nuclear entry by rAAV2 involves components of the canonical nuclear import pathway such as importin proteins and the NPC. In contrast, a report using wtAAV2 has shown that inhibition of the nuclear pore by wheat germ agglutinin (WGA) or an antibody against nucleoporin p62 in purified nuclei did not prevent nuclear accumulation of intact virions (208). In a study utilizing wtAAV2 in the presence of Adenovirus, inhibition of nuclear entry via the NPC through use of the calcium channel inhibitor thapsigargin did not prevent the appearance of viral genomes in the nucleus but did affect the level of nuclear accumulation and replication (20). Recently, a PDZ-binding domain was discovered on the N-terminal of VP1 that was shown to be important for nuclear entry of wtAAV2 (233). While the current study was under review, a recent study revealed that AAV2 that had been acidified and then neutralized could cause nuclear envelope breakdown in permeabilized HeLa cells (234). As in vitro assays become more harmonized with respect to physiological infection, these results taken together may suggest that AAV may utilize multiple import mechanisms to gain access to the nucleus, or that the mechanism of nuclear entry might vary for wtAAV2 and AAV vectors.

Therefore, we sought to determine the mechanism of nuclear entry for rAAV in the absence of a helper virus as applicable to gene therapy applications. Here we show that rAAV2 utilizes the host cell canonical nuclear entry pathway to enter host cell nuclei. Unlike the autonomous parvovirus MVM, rAAV does not appear to disrupt host cell nuclear lamina and is not dependent on caspase activity for nuclear entry. Rather, inhibition of the nuclear pore

through microinjection of WGA partially inhibited the nuclear translocation of viral particles. Moreover, nuclear import of rAAV2 was dependent on Importin-β and may also be facilitated by several Importin-α proteins as well as Importin 7. Interactions between rAAV2 capsid proteins and Importin-β were mediated by VP1 and VP2, specifically the BR domains therein. Interestingly, the extent of interaction with Importin-β varied among the rAAV serotypes tested. Taken together, our results suggest that entry through the canonical pathway plays a role in rAAV2 nuclear translocation.

Materials and Methods

Cell culture. HeLa and HEK-293 cells were maintained at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 g/ml streptomycin. For imaging experiments, HeLa cells were maintained in phenol red free DMEM for at least 2 passages prior to plating.

Drug Treatment. HeLa cells (3×10^4 cells/well) were treated with either z-vad-fmk (200 μM, Tocris) or DMSO 2h prior to infection with rAAV2. To confirm the functionality of z-vad-fmk, HeLa cells were treated with etoposide (200 μM, Tocris) following a 2h pre-treatment with z-vad-fmk or DMSO. Prevention of apoptosis was visually inspected 48h after etoposide treatment and quantitatively confirmed through 7AAD (Life Technologies) analysis using flow cytometry.

PCR site-directed mutagenesis. Capsid mutations were generated in the pACG2 backbone as previously described (27), with minor modifications. PCR was performed with the QuickChange

Lightning site-directed mutagenesis kit (Stratagene). PCR products were digested with DpnI and transformed into DH10B bacteria, which were selected on ampicillin-containing agar plates. Single colonies were picked and grown in liquid cultures overnight, and plasmids were isolated by column purification (Qiagen). Mutations were verified by sequencing of the plasmids utilizing the Eton Bioscience Automated DNA Sequencing Facility.

Virus production. Virus was produced in HEK-293 cells as previously described (47). Briefly, polyethylenimine max was used for the triple transfection of the pXR2 cap and rep plasmid, the pXX6-80 helper plasmid, and a TR-Luciferase reporter plasmid containing the firefly luciferase (Luc) transgene flanked by inverted terminal repeats. Cells were harvested between 48 and 72h post transfection, and virus was purified by iodixanol gradient centrifugation followed by ion exchange chromatography. After identifying peak fractions by dot blot hybridization, virus was dialyzed into phosphate-buffered saline (PBS) containing 5% sorbitol, $MgCl_2$ (1 mM), and $CaCl_2$ (.5 mM). Titers were calculated by quantitative PCR (qPCR) according to established procedures (235) using a LightCycler 480 instrument with Sybr green (Roche) and primers designed against the Luc transgene, 5_ AAA AGC ACT CTG ATT GAC AAA TAC 3_ (forward) and 5_ CCT TCG CTT CAA AAA ATG GAA C 3_ (reverse). Conditions used for the reaction were as follows: 1 cycle at 95°C for 10 min; 45 cycles at 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s for acquisition; and 1 cycle at 95°C for 30 s, 65°C for 1 min, and 99°C for melting curve analysis. For Cy5-labeled virus, purified rAAV2, VP3-only particles, and BR mutants were labeled with Cy5 dye (G.E. Amersham) as previously described (236).

Confocal immunofluorescence microscopy. Similar to what we have previously described (26), HeLa cells (3×10^4 cells/well) were plated onto poly-L-lysine coated 12-mm glass coverslips (thickness, 1.5) 16 h before infection. 30 minutes before infection, HEPES was added to the media (10 mM total concentration). Cy5-labeled recombinant virions were added to cell media (100,000 vg/cell) for 1 hour. No virus was added to control wells. Media was then replaced with pre-warmed media and cells were placed at 37°. At the indicated time points, cells were washed three times with PBS and then fixed with 2% paraformaldehyde for 15 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Following four washes with PBS, the permeabilized cells were blocked with immunofluorescence buffer (IFB) (20 mM Tris [pH 7.5], 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl2, 5 mg/ml bovine serum albumin, 0.05% Tween) for 30 min at room temperature. Where indicated, the cells were incubated with Importin-β primary antibody diluted in IFB overnight at 4°C. The cells were then incubated in secondary antibody diluted 1:5,000 in IFB (anti-mouse Dylight 488 or anti-rabbit Dylight 488; Abcam) for 1 h at 37°C. After six washes in PBS, coverslips were mounted cell side down onto glass slides with mounting medium (Prolong Antifade gold with DAPI [4_,6_-diamidino-2-phenylindole]; Molecular Probes). Images were captured on a Zeiss 710 upright laser scanning confocal microscope. 3D rendering and image processing of confocal z stack images were performed using AutoQuant and Imaris software, respectively (UNC Microscopy Services Laboratory).

Microinjections with pulse infection. HeLa cells (7.5×10^4) were plated on fibronectin-coated 35mm glass bottom microwell dishes, no. 1.5 coverglass (MatTek). Cells were microinjected using the FemptoJet system with Femptotips (Eppendorf). For virus studies, 30 minutes before

infection, cells were cooled to 4°C. Cy5-labeled AAV2 (100,000 vg/cell) was added to each dish for 1h. Where indicated, microinjection mixes (10 μL) included FITC-dextran (0.8 mg/mL, Life Technologies), wheat germ agglutinin (2 mg/mL, Calbiochem), and phosphate-buffered saline. For rhodamine B-labeled, NLS-conjugated BSA co-microinjections, mixtures included rhodamine B-BSA (1.33 mg/mL, Sigma). Following microinjection, media was replaced with fresh, prewarmed media. 2h post-microinjection, cells were fixed and processed for microscopy as described.

Transduction assays. For siRNA studies, HeLa cells were plated in 24-well plates 18h prior to transfection at densities to approximate 30% confluence (3×10^4 cells/well) in antibiotic-free DMEM. siRNA (total concentration, 25 nM, ON-TARGETplus SMARTpool, Dharmacon) to KPNB1 (Importin-β), KPNA2 (Importin-α1), KPNA4 (Importin-α3), KPNA1 (Importin-α5), and IPO7 (Importin 7) was utilized in combination with 1 uL DharmaFECT 1 (Dharmacon) as per the manufacturer's instructions. 24h post-transfection (for Importin-β) or 48h post-transfection (for Importin-α1, α3, α5, and Importin 7), cells were infected with purified rAAV2 at the designated number of vector genomes per cell and typically harvested after 24 h unless otherwise noted. For drug studies, $5X10^4$ HeLa cells were plated in 24-well plates 18h prior to drug treatment. For treatment with z-vad-fmk (Tocris Bioscience,200 nM in DMSO), cells were treated 2h prior to infection; for treatment with chloroquine (Sigma, 100 uM in PBS), cells were treated at the same time as infection. Luciferase activity was measured in accordance with the manufacturer's instructions (Promega) using a Perkin Elmer 1420 VICTOR3 automated plate reader. Error bars represent standard deviations from samples scored in triplicate. Graphs are representative of data sets from at least three independent assays.

Co-immunoprecipitation. Protein G Dynabead:antibody complexes were prepared by washing the beads 2 times in PBS and resuspending in PBS with 0.02% Tween 20 and antibody (30 µL beads, 3 µg antibody per reaction). The Dynabeads were rocked at 4° C overnight. For rAAV2 and mutant studies, HeLa cells ($1x10^7$ per co-immunoprecipitation) were washed 2 times with ice cold PBS and harvested in lysis buffer (Tris, pH 7.4, 50 mM; $MgCl_2$, 10 mM; NaCl, 150 mM; Triton X-100, 0.1%; deoxycholate, 0.1%; protease inhibitor cocktail (Pierce), 1x). Lysate was incubated for 30 minutes at 4°C and clarified by centrifugation. Lysate was pre-cleared by incubating with beads for 30 minutes at 4°C. rAAV2 or mutants (2.5×10^{11}) were boiled in lysis buffer for 10 minutes. Lysate was then added to virus and incubated for 2h at 4°C. 10% of each co-IP reaction was collected to serve as input amount. Dynabeads were washed 2 times with lysis buffer and then added to the virus/lysate mix for 2h at 4°C. Dynabeads were then washed 3 times with lysis buffer and transferred to a new tube. Eluates were collected by resuspending the Dynabeads in 50 µL 2x NuPage LDS sample buffer (Life Technologies) and boiling samples for 10 minutes. Samples were then subjected to SDS-PAGE and immunoblot analysis using the B1 antibody (a kind gift from Dr. Jürgen A. Kleinschmidt). For RanQ69L studies, HEK-293 cells were used. Cells were plated 24 hours prior to transfection (2.5×10^4 cells per 10 cm plate, 2 plates per co-IP). Polyethylenimine max was used to transfect pmCherry-C1-RanQ69L (a kind gift from Dr. Jay Brenman) or pmCherry-C1. 40 hours post-transfection, cells were harvested in lysis buffer and processed as described. 1 mg of total lysate was used for each co-IP.

Statistics. All statistical analyses were performed using the Student's t-test. Asterisks represent statistical significance ($p<.05$).

Results

Previous studies have shown that the majority of rAAV2 virions traffick to the perinuclear region of cells but never enter the nucleus. These virions likely become degraded by proteases or are subject to proteasomal degradation and antigen presentation by host cells (214). Current opinion suggests that if the barrier to nuclear entry could be overcome, more viruses could translocate to the nucleus for subsequent uncoating and transgene expression. To date, the mechanism of nuclear entry by rAAV is largely unknown. However, a few reports have suggested potential mechanisms by which rAAV might enter the nucleus, including entry via the NPC through the classic nuclear entry pathway (95, 96), entry through NPC-independent mechanisms (20, 208), and direct lamina disruption like that of the autonomous parvoviruses (231). Focusing our studies on AAV in the context of gene therapy vectors, we aimed to determine the mechanism for nuclear entry by rAAV2 in the absence of a helper virus.

Analysis of host cell nuclear lamina during rAAV2 infection.

Previous studies have described a unique nuclear entry mechanism for the autonomous parvovirus Minute Virus of Mice (MVM), whereby MVM enters the nucleus through physical disruption of the nuclear lamina in a caspase-dependent mechanism (231). Since AAV is a parvovirus, we sought to determine whether rAAV2 utilized a similar mechanism. To investigate the integrity of the nuclear lamina upon rAAV2 infection, we utilized confocal microscopy to visualize Lamin A/C and Lamin B1 during infection with Cy5-labeled rAAV2 **(Figure 3.1A)**. Immunofluorescence analysis revealed no detectable changes in the appearance of Lamin A/C or Lamin B1 2h post-infection, a time that has been reported to capture 50% of total viral nuclear entry (236). We next utilized quantitative imaging analysis to measure the

percent of nuclear Cy5 fluorescence within HeLa cells treated with caspase inhibitor z-vad-fmk, Our lab has established that confocal analysis of Cy5-labeled rAAV2 particles can be used to quantify the percentage of nuclear rAAV2 (236) and our quantification of the percentage of Cy5 fluorescence within nuclei of vehicle-treated cells was consistent with what has been previously reported (126, 155). Treatment with z-vad-fmk, which was shown to inhibit lamina disruption and nuclear entry of MVM, resulted in no change in nuclear Cy5 fluorescence compared with vehicle-treated cells **(Figure 3.1B, C)**. In fact, pre-treatment of HeLa cells with z-vad-fmk followed by infection with rAAV2-Luc led to a small but non-significant increase in transduction as compared to vehicle control **(Figure 3.1D)**. We confirmed the functionality of the z-vad-fmk used in these studies through 7-AAD analysis of apoptosis prevention in etoposide treated cells **(Supplementary Figure S3.1)**. Taken together, these results suggest that unlike the autonomous parvovirus MVM, rAAV2does not overtly disrupt host cell nuclear lamina nor rely on caspase activation for nuclear entry or transduction.

<u>rAAV2 can utilize the Nuclear Pore Complex to enter the nucleus.</u>

Many viruses utilize the nuclear pore complex to traffic intact particles, viral proteins or DNA into the nucleus (222, 223). The nuclear pore has been shown to accommodate proteins of sizes up to 39 nm in diameter (217). Because the diameter of the AAV capsid is approximately 26 nm, and it has been shown that rAAV2 can enter the nucleus as an intact particle (87), we investigated whether rAAV2 could enter the nucleus using the NPC. A common method to determine if the NPC is necessary to facilitate nuclear entry is to physically block the outer pore with the lectin wheat germ agglutinin (WGA) (237). WGA binds to O-linked N-acetylglucosamine residues on NPC proteins, thereby blocking any incoming cargo. We confirmed this observation by microinjecting HeLa cells with rhodamineB-labeled, NLS-

conjugated BSA with either FITC-labeled dextran or dextran and WGA **(Figure 3.2A)**. In cells that were injected with dextran alone, the NLS-BSA can be visualized inside the nucleus 2h post-microinjection. However, in cells that were injected with dextran and WGA, the NLS-BSA was mostly exluded from the nucleus. We then applied this technique to HeLa cells subjected to viral infection with Cy5-labeled rAAV2. We pre-bound HeLa cells with Cy5-labeled rAAV2 and performed microinjections with either dextran alone or dextran and and WGA. 2h post-microinjection, we processed the cells for confocal microscopy to assess nuclear localization through visualization of the Cy5-labeled particles. In cells that were microinjected with dextran, viral particles could be seen inside of the nucleus **(Figure 3.2B, top)**. However, in cells that were microinjected with WGA, fewer viral particles could be seen inside the nucleus, suggesting that nuclear entry was limited **(Figure 3.2B, bottom)**. We quantified the percent of nuclear Cy5 fluorescence in microinjected cells and found an average of 15% nuclear Cy5 fluorescence in dextran injected cells, while dextran + WGA injected cells showed an average of 8% nuclear Cy5 fluorescence **(Figure 3.2C)**. Taken together, these results suggest that rAAV2 can utilize the NPC to enter the nucleus.

<u>rAAV2 shares spatial distribution with Importin-β during later trafficking events.</u>

Importin-β plays a key role in canonical nuclear entry by either directly binding to NLS-harboring cargo or interacting with cargo through an adapter Importin-α protein. Importin-β has been shown to be involved in nuclear import for several viral infectious pathways, including facilitating the binding of HSV-1 capsids (226), HBV core particles (227), HIV-1 preintegration complexes (238), and Influenza A nucleoprotein (239) to host nuclei. Importantly, Importin-β has been shown to interact with a DNA-bound protein of the AAV helper virus Adenovirus (224). Therefore, we aimed to determine if rAAV2 would localize near cellular regions of high

Importin-β concentration during the course of infection. We utilized Cy5-labeled rAAV2 and immunofluorescence confocal microscopy to acquire z-stack images of HeLa cells infected with rAAV2 at various time points. These images were rendered in three dimensions using volume imaging software. During the course of infection, rAAV2 trafficks from the cell periphery to the MTOC and nearby cellular compartments before translocating to the nucleus (85, 86, 121). As expected, 30 minutes post-infection, when the majority of rAAV2 was detected dispersedly throughout the cytoplasm , there appears to be little to no detectable co-distribution between rAAV2 and Importin-β **(Figure 3.3A)**. However, as early as 1h post infection and through 9h post infection, Cy5-rAAV2 was localized in cellular regions of high Importin-β concentration. These results suggested that rAAV2 and Importin-β may interact once AAV2 has accumulated near the MTOC.

Previous reports have suggested that endosomal escape precedes nuclear entry of rAAV2 (82, 92). We therefore investigated whether the co-distribution of rAAV2 and Importin-β was dependent on later trafficking events that could involve escape from the endosome by acquiring 2D images of cells in the z plane harboring the center of the nucleus to represent the center of the cell. Endosomal escape has been shown to be inhibited by pharmacological agents that block vesicle acidification (82, 214). We therefore utilized chloroquine, a small molecule that blocks the acidification of endosomes, to inhibit later trafficking events and endosomal escape of rAAV2. Treatment of cells with chloroquine resulted in the loss of co-distribution between rAAV2 and Importin-β **(Figure 3.3b)**. These results suggested that rAAV2 and Importin-β interact in later trafficking steps, presumably once rAAV2 has exposed the unique N-terminal of VP1 and escaped the endosome.

rAAV2 interacts with Importin-β in a Ran-sensitive manner.

Because we observed a co-distribution pattern between Cy5-rAAV2 and Importin-β in later viral trafficking steps, we sought to determine if rAAV2 and Importin-β formed a physical interaction. We hypothesized that because rAAV2 contains putative NLSs on VP1 and VP2, an interaction could occur in late trafficking events, perhaps once rAAV2 had undergone conformational changes to expose these regions of the capsid. Previous studies have shown that classical Importin-β/cargo complexes are mediated through an NLS on the cargo and can be dissociated by the small GTPase Ran in its GTP bound form (220, 221). Therefore, we performed co-immunoprecipitation (IP) experiments using dissociated capsid proteins (to expose VP1 and VP2) and HEK 293 lysate transfected with a permanently GTP-bound form of Ran (Q69L) or a control plasmid **(Figure 3.4)**. rAAV2 capsid proteins were able to co-IP with Importin-β; however, this interaction was completely inhibited in the presence of RanQ69L. This result suggested that, rAAV2 capsid proteins could form a specific interaction with Importin-β which could be disrupted by RanGTP.

Knockdown of Importin-β inhibits nuclear entry and transduction of rAAV2

Because we observed similar spatial distribution between rAAV2 and Importin-β,we next investigated whether Importin-β played a physiological role in rAAV2 trafficking and transduction. We utilized an siRNA approach to transiently silence Importin-β expression in HeLa cells. Previous studies have shown that long-term inhibition of Importin-β expression can disrupt cellular homeostasis, since Importin-β plays a vital role in the nuclear import of essential nuclear proteins. Therefore, we chose to examine the effects of Importin-β knockdown on rAAV2 transduction within the first 24 hours post siRNA treatment, resulting in approximately

50% knockdown of Importin-β at the time of rAAV2 infection **(Figure 3.5A)**. While we cannot rule out inhibition of rAAV-specific transcription factor import by Importin-β knockdown, we verified that global gene transcription was not altered by utilizing qRT-PCR to measure mRNA levels of housekeeping gene GAPDH and determined that these levels were similar between Importin-β knockdown and treatment with a scrambled siRNA **(Supplementary Figure S3.2)**. To assess effects on transduction, we utilized rAAV2 carrying a CBA-luciferase transgene. rAAV2 transduction was inhibited when luciferase activity was measured at either early (12h) or late (24h) time points, with the greatest inhibition (approximately 70%) at 24h post infection **(Figure 3.5B)**. We next assessed whether this inhibition was maintained at various viral doses. Consistent with our initial results, knockdown of Importin-β inhibited rAAV2 transduction between 50-70% at all vector doses higher than 500 vg/cell **(Figure 3.5C)**. Finally, we knocked down Importin-β and investigated changes in the nuclear localization of Cy5-labeled rAAV2. Consistent with the changes in transduction, siRNA knockdown of Importin-β inhibited Cy5-rAAV2 nuclear localization **(Figure 3.5D)**. To quantify these observations, we obtained a value for the percent of nuclear Cy5 fluorescence in cells treated with siRNA to Importin-β or a scramble control. In control cells, nuclear Cy5 fluorescence increased from 21% at 2h post-infection to 27% at 8h post-infection. Nuclear Cy5 fluorescence in Importin-β knock-down cells remained lower and increased from 14% to 18% at 8h post-infection. Complete inhibition of nuclear entry and transduction was not observed, either reflecting the activity of the remaining Importin-β in knock-down cells or the utilization of alternative, Importin-β-independent nuclear entry pathways by rAAV2. These results do suggest that Importin-β plays a role in rAAV2 transduction as a mediator of nuclear entry.

Determination of Unique Regions of the AAV2 capsid important for interaction with Importin-β.

Since we were able to detect a functional role for Importin-β in the rAAV2 infectious pathway, we sought to determine which region of the AAV capsid was mediating this interaction. Importin-β binds to nucleus-bound cargo via classical NLSs (cNLS) but has also been shown to interact with proteins with non-conventional NLSs. Becaue the unique N-terminal regions of VP1 and VP2 contain basic stretches of amino acids resembling cNLSs **(Figure 3.6A)**, we hypothesized that one or both of these capsid proteins were mediating the interaction with Importin-β. To determine which capsid proteins were important for binding to Importin-β, we performed co-IPs using dissociated capsids from particles consisting of all three capsid proteins, particles with only VP2/VP3, and VP3 only particles, produced as previously described (240). Dissociated rAAV2 particles with all three capsid proteins or VP2/VP3-only particles were able to co-IP with Importin-β; however, VP3-only particles resulted in limited to non-detectable co-IP **(Figure 3.6B)**. Co-IP utilizing an isotype control antibody also yielded non-detectable product. Interestingly, while co-IP seemed to only require the presence of VP1 and/or VP2, we were able to detect capsid protein VP3 in the IP product. We believe this is due to incomplete capsid dissociation or oligomerization between NLS-containing capsid proteins and VP3 that may occur after capsid dissociation.

AAV2 harbors 4 putative NLSs: BR1, located exclusively in VP1, BR2 and BR3, located in VP1 and VP2, and BR4, located in all 3 capsid proteins **(Figure 3.6A)**. The 4 BR domains have been studied and their implications in AAV infectivity have been characterized in detail (92, 95, 96, 232). Mutations to BR1, BR2, and BR3 have been shown to hinder rAAV2 transduction, with mutations to BR2 and BR3 having the greatest effect on transduction. Since mutations to BR4 result in defects in capsid assembly (96), we only investigated mutations to

BR1, BR2, and BR3. Therefore, to assess the contributions of each BR domain in the interaction with Importin-β, we produced virions with mutations in each of these BR domains. Consistent with the results presented by Grieger et al and Johnson et al (95, 96), mutations to BR1 alone had a modest effect on transduction, while mutations to BR2 or BR3 hindered transduction by over 10-fold in both HeLa cells and HEK 293 cells (**Figure 3.6C**, data not shown).

We next wanted to determine whether the transduction profile of these mutants would be consistent with their capabilities to interact with Importin-β. Co-IP analysis revealed that while the capsid proteins of rAAV2 interacted with Importin-β, mutations toBR2 and BR3 limited this interaction, while mutations to BR1 had no effect on co-IP **(Figure 3.6D)**. The higher density band seen in the BR1- co-IP is likely due to higher capsid protein input. Taken together, our data supports that the BR domains, especially BR2 and BR3, are necessary for transduction through mediating interactions with Importin-β.

Interaction with Importin-β is varied among rAAV serotypes.

The majority of information about the infectious pathway of AAV has been determined for AAV2, with limited understanding of the subcellular trafficking of other serotypes. Comparison of VP1up regions of several common AAV serotypes revealed that most serotypes harbor similar, if not identical, BR domains **(Figure 3.7A)**. The only exception is AAV5, which has a bipartite-like NLS in between BR2 and BR3 rather than two monopartite-like NLSs. Because these serotypes share similar BR domains, we investigated whether they could interact with Importin-β. Co-IP analysis between Importin-β and dissociated capsid proteins from each serotype revealed that all viral particles tested could interact with Importin-β, but to varying extents **(Figure 3.7B)**. We measured our co-IP analysis by quantifying the ratio of capsid

protein product in the co-IP over the capsid protein applied to the lysate. We found that rAAV2 had the most robust interaction with Importin-β, followed by rAAV1. rAAV5, rAAV6, and rAAV8 were similarly able to interact with Importin-β, although to a lesser extent than rAAV2 and rAAV1. Finally, we detected interaction, but to a lesser extent, between rAAV9 and Importin-β. Interestingly, while rAAV8 and rAAV9 vectors do not typically transduce HeLa cells, we were still able to detect interactions between these capsid proteins and Importin-β. Since our co-IP assay utilizes HeLa cell lysate and denatured capsid proteins, our experimental set-up bypasses cellular barriers such as receptor-mediated endocytosis and endosomal escape. Thus, it is possible that the use of import proteins such as Importin-β is a shared feature of rAAV vectors but occurs downstream of initial cellular entry and subcellular trafficking. Our results suggest that different AAV serotypes may utilize alternative import proteins in addition to Importin-β for nuclear entry.

Analysis of the role of other import proteins in rAAV2 nuclear localization

The import protein superfamily consists of the Importin-β proteins, Importin-α adapters, transportins, and several other importins such as Importin 7 (241, 242). Importin-α proteins link nuclear-bound cargo to Importin-β through an Importin-β binding domain. Importin 7 and transportins can mediate nuclear import independently of Importin-β (243-245). It has also been shown that Importin 7 can form a cooperative dimer with Importin-β (244). Studies have shown that Importin-α proteins recognize both mono- and bi-partite NLSs and can have redundant functions in host cells (246-249). Because we established an interaction between rAAV2 and Importin-β, we investigated if this interaction was mediated by the Importin-α adaptor proteins. Additionally, we wanted to identify any potential interactions with the alternative import proteins Importin 7, Transportin 1, or Transportin 2. Co-immunoprecipitation analysis revealed strong

interactions between dissociated rAAV2 capsid proteins and Importin-α1, Importin-α3, and Importin-α5 **(Figure 3.8A)**. Under these conditions, we did not detect any co-immunoprecipitation between rAAV2 capsid proteins and Importin 7, Transportin 1, or Transportin 2 (data not shown). Interestingly, siRNA knockdown of Importin-α1, Importin-α3, or Importin-α5 had no effect on rAAV2 transduction 24 hours post-infection **(Figure 3.8b)**. Similarly, knockdown of all three Importin proteins, to eliminate any compensatory function among these proteins, also resulted in no change in transduction of rAAV2 (data not shown). The Importin-α superfamily consists of at least 7 known members; therefore, it is possible that other Importin-α proteins that were not identified in this study may also mediate nuclear import of rAAV2. In contrast to our co-immunoprecipitation data, knockdown of Importin 7 reduced transduction by approximately 60% **(Figure 3.8B)**. Taken together, our results suggest that multiple Importin-α proteins may play a role in the import of AAV2, and may be able to compensate for the depletion of specific Importin-α proteins. Additionally, Importin 7 appears to be important for transduction, but may only weakly interact with rAAV2.

Discussion

Mammalian viruses have been shown to utilize components of the classical nuclear import pathway, including the NPC, nucleoporins, and various import proteins. Herpes simplex virus 1 (HSV-1) and Adenovirus both directly bind the cytoplasmic side of the NPC via nucleoporins (225, 226, 250-253). The adenoviral core protein Protein VII has been shown to bind to several import proteins, including Importin-α, Importin-β, Importin 7, and transportin, which are thought to mediate entry of the nucleoprotein through the NPC (224). Previous studies have suggested that some viruses, such as Hepatitis B virus (HBV) and SV40, may enter the

nucleus intact. Two NLSs on the core protein of HBV become exposed and mediate movement into the NPC in an Importin-α and Importin-β dependent manner (227-229, 254). Our data suggesting that rAAV2 utilizes components of the classical nuclear import machinery to enter the nucleus is consistent with the mechanism of how other viruses achieve nuclear entry. While the current study is limited to characterization and mechanistic insights utilizing an established cell line, it provides a foundation for further studies in primary cells and *in vivo*. A growing interest in the AAV vector field has focused on understanding vector trafficking through host cells with the notion that understanding the viral trafficking pathway can lead to the design of novel vectors that can overcome cellular barriers to transduction. Our study provides insight into a rate-limiting step of AAV vector transduction, nuclear entry, and thus the foundation for novel vector design aimed to expedite this step in subcellular trafficking.

We have shown that blockade of the NPC through microinjection of WGA followed by infection with rAAV2 inhibits the nuclear entry of rAAV2, thus supporting a role for the NPC in rAAV2 nuclear entry. We did not observe complete inhibition of nuclear entry at this time point, suggesting either reversible inhibition of nuclear import by WGA which has previously been reported (255) or an alternative, NPC-independent pathway utilized by rAAV2. The classical import pathway described herein for rAAV2 differs from the mechanism of nuclear entry proposed by Cohen et al for a related parvovirus, MVM (230) and recent findings describing nuclear envelope breakdown of permeabilized HeLa cells by AAV2 that had been acidified and then neutralized (234). Future work will be required to understand how the acidification and neutralization of rAAV2 mediates nuclear entry under physiological subcellular trafficking conditions. It is possible that wtAAV2 and/or rAAV2 can cause nuclear envelope breakdown during infection, but the effects on the nuclear lamina might be more subtle than what can be

detected by fluorescence microscopy. Since the infectious pathway of rAAV2 differs from other autonomous parvoviruses, it is not surprising that the mechanisms of nuclear entry may include both shared and disparate features between autonomous parvoviruses and non-autonomous parvoviruses. For instance, while proteosome inhibitors have been shown to inhibit the infectivity of MVM and canine parvovirus (CPV) and have no affect on bovine parvovirus (BPV) (256), it is well established that proteasome inhibition greatly enhances the nuclear entry and transduction of rAAV2 (87, 113). Our results also differ from a previous report showing that intact AAV2 could enter purified nuclei, despite blocking the pore with WGA or an antibody to an NPC protein (208), suggesting the nuclear pore was dispensable for nuclear entry of AAV2. It is important to note, however, that the previous study utilized AAV2 virions that had not gone through physiological endosomal processing, allowing for the conformational change that exposes the basic regions along with the PLA2 domain required for transduction. A recent study by Salganik et al showed that purified AAV2 virions have external protease activity at physiological pH that is ablated at low pH (257). It is possible that virions that traffick to the nucleus using alternative pathways can exert alternative functions, such as protease-mediated, NPC-independent nuclear entry under certain conditions. Xiao et al showed that treatment with thapsigargan, an inhibitor of cellular calcium flux and modulator of nuclear pore structure, had no effect on the nuclear entry and subsequent replication of wtAAV2 in the presence of Adenovirus, suggesting that the nuclear pore was not necessary for AAV2 nuclear entry in a wild-type context (20). The role of calcium depletion in relation to nuclear entry through the NPC remains controversial (258), and could vary depending on the protein (or virus) being investigated (259). Furthermore, in addition to inhibiting calcium flux, thapsigargin has many off-target effects, which could have offset any effects on the nuclear entry of AAV2. Finally, it

is possible that the mechanism of nuclear entry could differ between wild-type AAV2 in the presence of Adenovirus and rAAV2 independent of any helper viruses.

Import proteins, also known as karyopherins, are generally thought of as chaperones aiding in the translocation of nuclear-bound cargo. Karyopherins have been implicated in the import of other viruses, including Adenovirus (224), HIV-1 (238, 260-262), Influenza A (239, 263-265), HBV (227), and HSV-1 (226). We have shown that knockdown of Importin-β inhibits the nuclear translocation and transduction of rAAV2, suggesting that Importin-β facilitates nuclear import. Furthermore, we have shown that rAAV2 can co-distribute with Importin-β within cells and form a Ran-sensitive complex, supporting an interaction that may facilitate nuclear entry. Interestingly, while we saw co-IP with Importin-α1, -α3, and –α5, knockdown of these proteins did not affect transduction. Currently, seven human Importin-α proteins have been identified(266), and it is known that these karyopherins can have both distinct substrate recognitions as well as redundant NLS recognition (267). It is likely that despite knockdown, import of rAAV2 is compensated for through the presence of other Importin-α proteins and Importin-β. Furthermore, while we did not observe co-IP between rAAV2 and Importin 7 in the conditions tested, transduction was inhibited in cells that had been subjected to Importin 7 knockdown. Finally, while a third class of karyopherins, known as the transportins, have been shown to be involved with the import of Adenovirus, we saw no co-immunoprecipitation with Transportin-1 or Transportin-2 under the conditions tested.

Previous reports have shown that AAV2 contains putative NLS domains within the unique N terminal regions of VP1 and VP2, and that mutations to these domains inhibits nuclear entry and transduction (95, 96). Incorporating one of the BR domains (BR3) into rAAV2 particles lacking VP1up has been shown to partially rescue the infectivity of these mutant virions

(268). Additionally, conjugation of BR domains to exogenous GFP has been shown to be sufficient to direct this protein to the nucleus (92, 232). Taken together, these results provide evidence for the role of the BR domains in rAAV2 nuclear entry. Indeed, we have shown that while rAAV2 capsid proteins interact with Importin-β through co-IP, this interaction is ablated when VP1 or VP2 is not available or when BR2 or BR3 is mutated. Intriguingly, even though at least one BR domain was still present in the case of the BR2- and BR3- mutant (i.e. BR3 is available in the BR2- mutant), we were not able to detect an interaction with Importin-β through co-IP analysis. This observation suggests that the interaction between Importin-β and rAAV2 capsid proteins may be conformation dependent. Indeed, while the other rAAV vectors we tested share fairly conserved BR domains, their ability to co-IP with Importin-β varied. Further understanding of the contributions of serotype-specific domains will be required to understand the differential interactions between Importin-β and other AAV serotypes.

Questions still remain about the nuclear translocation efficiency of rAAV2. Import mediated by Importin-β has been historically regarded as highly efficient and rapid (269). Since our results indicated that the interaction between Importin-β and rAAV2 was the most robust compared to the other serotypes tested, one would assume that rAAV2 nuclear entry would be the most rapid and efficient as well. However, Keiser et al showed that in HeLa cells, rAAV1 and rAAV5 nuclear entry was initially more efficient than rAAV2 (90). This same study revealed that rAAV1 and rAAV5 likely utilize different subcellular trafficking pathways in order to gain access to the nucleus. Indeed, recent work has demonstrated that while the microtubule network acts to direct rAAV2 particles to the perinuclear region in cells (17), microtubules within the MTOC may also act as a net, physically trapping virions in this region. This assertion is supported by the fact that applying microtubule disrupting drugs after rAAV2 has accumulated

in the MTOC actually increases transduction (P.J. Xiao and R. J. Samulski, unpublished data). Therefore, it is possible that despite efficient interaction with Importin-β, rAAV2 nuclear translocation might be physically impeded by other cellular factors such as microtubules in the MTOC. Consistent with this notion, we observed similar cellular distribution between AAV2 and Importin-β at the nuclear periphery up to 9h post-infection, suggesting that while interactions may be occurring between AAV2 and Importin-β, translocation might be inhibited by another cellular factor that does not impede translocation of other AAV serotypes. While our data suggests a mechanism for rAAV2 nuclear import through the NPC mediated by Importin-α and Importin-β proteins, our findings may only describe one of several translocation pathways utilized by rAAV vectors. In fact, recent work by Popa-Wagner et al identified a PDZ-binding motif on VP1 that, when mutated, rendered AAV2 defective in nuclear entry. They concluded this AAV2 PDZ binding domain might initiate signalling pathways within the cell to facilitate nuclear entry (233). Moreover, previous work has shown that AAV2 can interact with the nucleolar proteins nucleophosmin and nucleolin (87, 270, 271), suggesting that rAAV2 could translocate into the nucleus through interaction with these proteins. In addition to the observations of nuclear envelope break down by parvoviruses including AAV2, Porwal et al has shown that AAV2 can directly interact with nucleoporins that comprise the nuclear pore complex (234). Finally, given that the abundance of karyopherins differs among cell types, it is possible that rAAV serotypes could utilize different karyopherins for nuclear entry, which would further contribute to differences in cellular tropism historically observed among the rAAV serotypes.

Based on our results, we have constructed a working model for the mechanism of nuclear import by rAAV2 **(Figure 3.9)**. Upon acidification of the late endosomal compartment and the conformational change that facilitates the exposure of VP1up, the BR2 and BR3 domains interact

with Importin-β or an Importin-α/β complex. rAAV2 could also interact with Importin 7 or an Importin-β/Importin 7 complex. Once rAAV2 escapes the endosomal compartment or the MTOC, the virus/importin complex translocates to the nucleus via the NPC, where subsequent uncoating and gene expression can occur. Improving the transduction efficiency of AAV vectors has become paramount to successful gene therapy applications. While much work has been devoted to discerning the infectious pathway of AAV vectors, further understanding of specific trafficking events within host cells is necessary to define cellular barriers to transduction. Ultimately, understanding the details of viral trafficking and host cell interactions will facilitate the rational design of AAV vectors that can overcome some of the trafficking inefficiencies currently observed. Vectors that are more efficient at navigating the subcellular space in order to deliver their genetic payload should improve overall gene therapy applications and clinical outcomes.

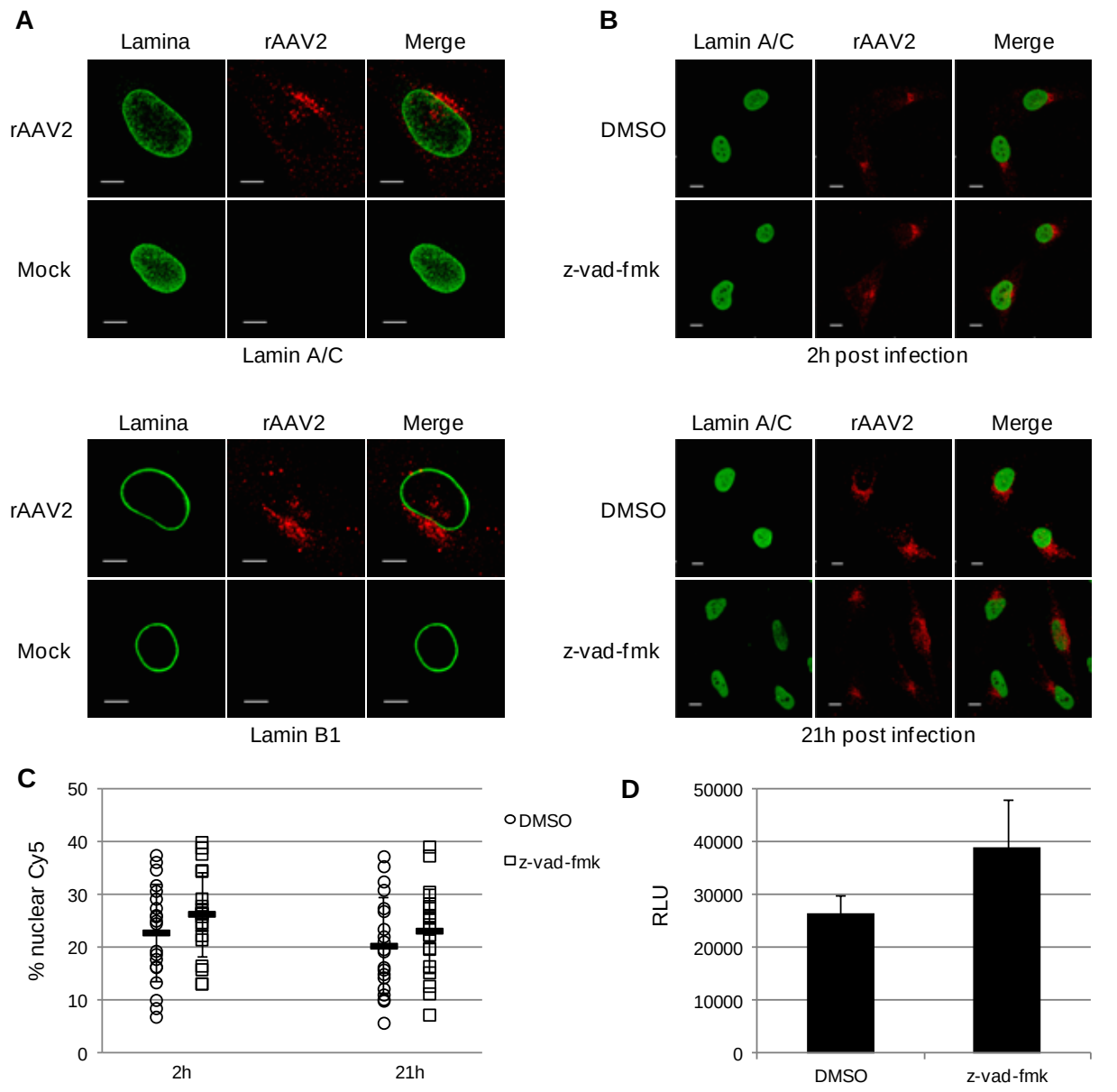


Figure 3.1. Nuclear lamina integrity and caspase involvement during rAAV2 infection. A. Immunofluorescence of Cy5-labeled rAAV2 and intact nuclear lamina 2h post infection. Capsids (red) are shown juxtaposed to the nuclear membrane, composed of Lamin A/C (top, green) and Lamin B1 (bottom, green). Scale bar, 10 µm. B. Nuclear entry of Cy5-labeled rAAV2 in cells treated with vehicle or z-vad-fmk. HeLa cells were treated with z-vad-fmk or vehicle (DMSO) 1h prior to and throughout infection. Cells were fixed 2h and 21h post-infection. Red, Cy5-labeled rAAV2; green, Lamin A/C. Scale bar, 10 µm. C. Quantification of nuclear Cy5 fluorescence in z-vad-fmk or vehicle treated cells. Confocal z-stack images for 20 cells per treatment group were acquired. Images were rendered in 3D and isosurfaces were created for nuclei and Cy5 fluorescence within each cell. Percent Cy5 fluorescence within nuclei

compared to total Cy5 fluorescence within the cell was calculated. D. Luciferase assay of transduction in Hela cells. Cells were treated with either z-vad fmk or vehicle 1h prior to infection and then infected with rAAV2-CBA-Luc (1000 vg/cell). Luciferase activity was measured 24h post infection. Error bars represent standard deviations from a representative experiment performed in triplicate.

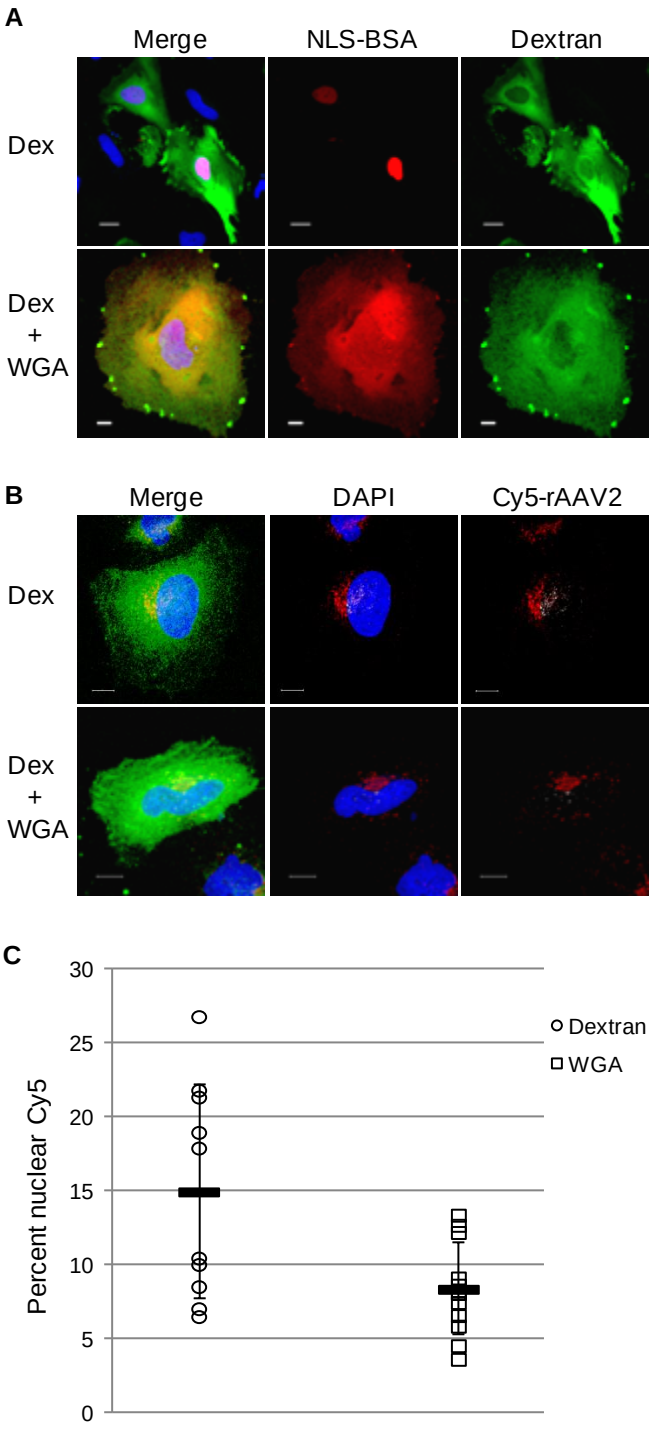


Figure 3.2. rAAV2 can enter HeLa cell nuclei through the NPC. A. Rhodamine-labeled, NLS-conjugated BSA (red) was co-microinjected with dextran alone (top, green) or dextran + WGA (bottom, green) into HeLa cells. Cells were fixed and imaged 2h after microinjection to assess NLS-BSA localization. Scale bar, 10 µm. B. Cells were cooled to 4° C and infected with Cy5-labeled rAAV2 (red). 1h later, cells were microinjected with dextran (green, top), or

dextran + WGA (green, bottom). Immunofluorescence microscopy was used to assess Cy5-rAAV2 nuclear entry 2h post-infection. Shown are maximum intensity projections of 3D reconstructed cells, where red depicts Cy5 fluorescence within cytoplasm and white depicts nuclear Cy5 fluorescence. Scale bar, 10 µm. C. Quantification of microinjected cells in B. Confocal z-stack images of microinjected cells were rendered in 3D and isosurfaces were created for nuclei and Cy5 fluorescence within the cell. Percent Cy5 fluorescence within nuclei compared to total Cy5 fluorescence within the cell was calculated (n = 10 for Dextran-injected cells; n = 12 for WGA-injected cells).

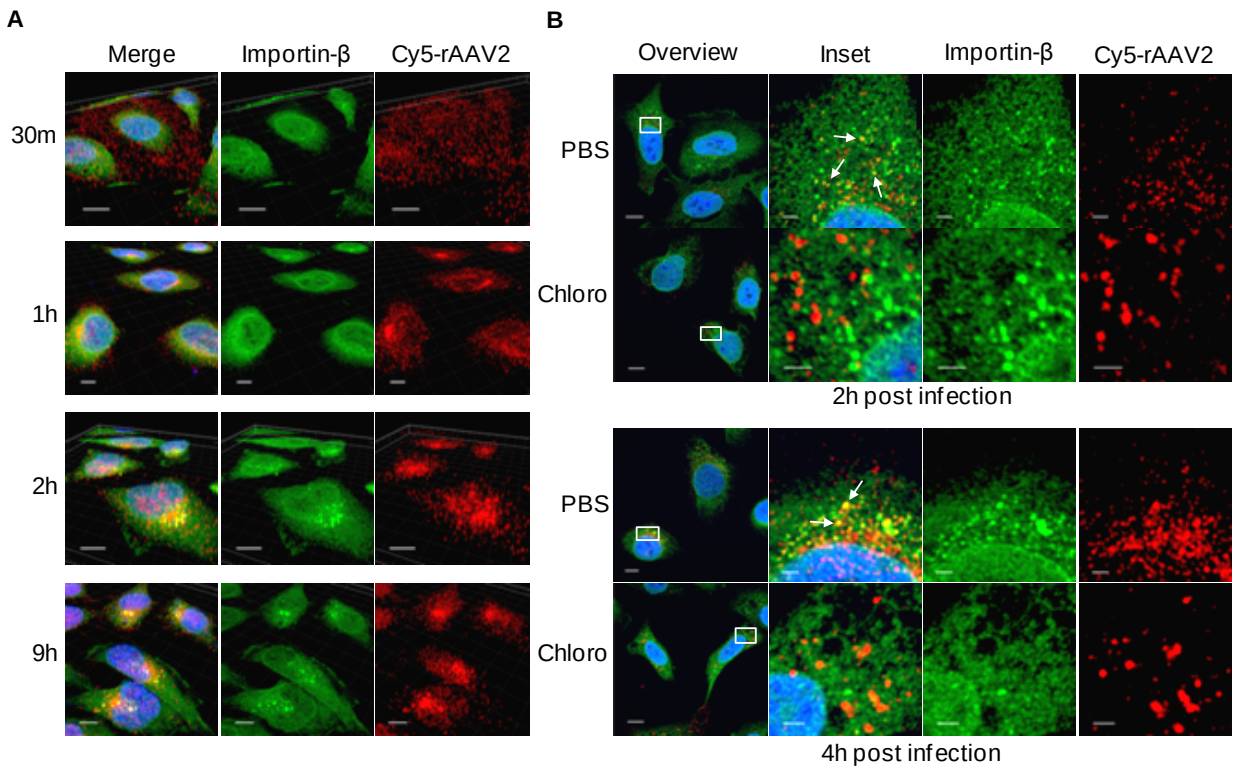


Figure 3.3. rAAV2 co-distributes with Importin-β in cells in later trafficking steps. A. HeLa cells were pulse-infected with Cy5-labeled rAAV2 for the indicated times. Confocal z-stack images were acquired and rendered in three dimensions to assess the localization of rAAV2 (red) and Importin-β (green) in relation to the nucleus (blue). Scale bar, 10 µm. B. Hela cells were treated with Chloroquine (100 uM) or vehicle (PBS) 2 hours prior to and throughout the duration of infection. Cells were pulse-infected with Cy5-rAAV2 for the indicated times. Confocal images were acquired in the z plane that harbored the center of the nucleus of the to assess the 2D localization of Cy5-rAAV2 (red) and Importin-β (green). Arrows indicate regions of extremely similar spatial distribution. Scale bar, 10 µm (field), 2 µm (zoom).

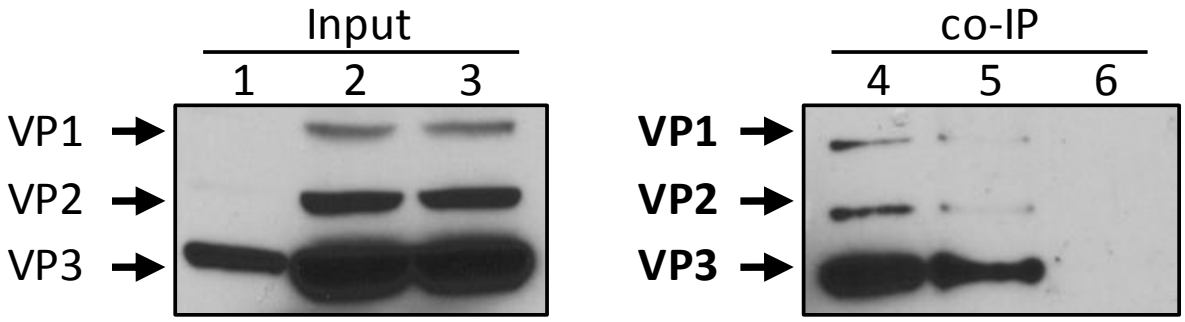


Figure 3.4. rAAV2 capsid proteins form an interaction with Importin-β that can be dissociated by RanGTP. rAAV2 particles were dissociated by heat and incubated with HEK 293 cell lysate previously transfected with control plasmid or RanQ69L. Co-IPs were performed using Dynabeads and an anti-Importin-β antibody, followed by immunblot with B1. rAAV2 capsid protein marker, lanes 1 and 4; control, lanes 2 and 5; RanQ69L, lanes 3 and 6. Relevant bands are highlighted in bold.

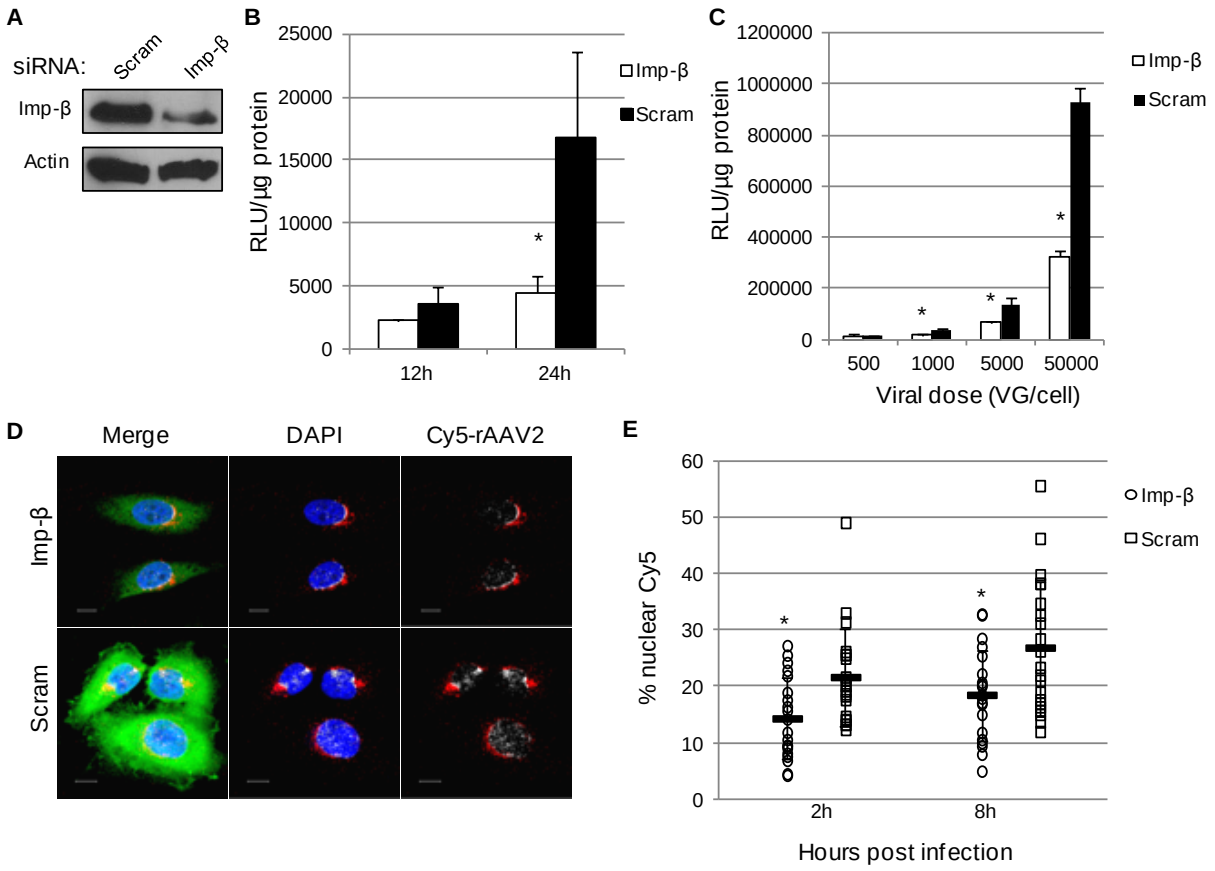


Figure 3.5. Importin-β is important for rAAV2 nuclear entry and transduction. A. HeLa cells were treated with siRNA to Importin-β or a scrambled siRNA control. Cell lysate was collected 24 hours post-transfection and immunoblotted for Importin-β levels. B. HeLa cells were treated with siRNA to KPNB1 (Importin-β) and infected with rAAV2-CBA-luc (1000 vg/cell) at the indicated times. Luciferase activity was measured 12 h and 24 h post infection. C. HeLa cells were treated with siRNA to KPNB1 and infected with rAAV2-luc at the indicated doses. Luciferase activity was measured 24 h post infection. D. HeLa cells were treated with either scrambled siRNA or siRNA to KPNB1 for 24h. Cells were pulse-infected with Cy5-labeled rAAV2. 8h post-infection, cells were fixed and confocal images were acquired to assess nuclear entry of virions. Red, Cy5 fluorescence within cytoplasm; white, nuclear Cy5 fluorescence, DAPI, nucleus; Green,Importin-β. Scale bar, 10 µm. E. HeLa cells were treated with siRNA to KPNB1 or a scrambled siRNA and pulse-infected with Cy5-rAAV2 for either 2 or 8 hours. Confocal z-stack images for at least 20 cells per treatment group were acquired. Images were rendered in 3D and isosurfaces were created for nuclei and Cy5 fluorescence within each cell. Percent Cy5 fluorescence within nuclei compared to total Cy5 fluorescence within the cell was calculated.

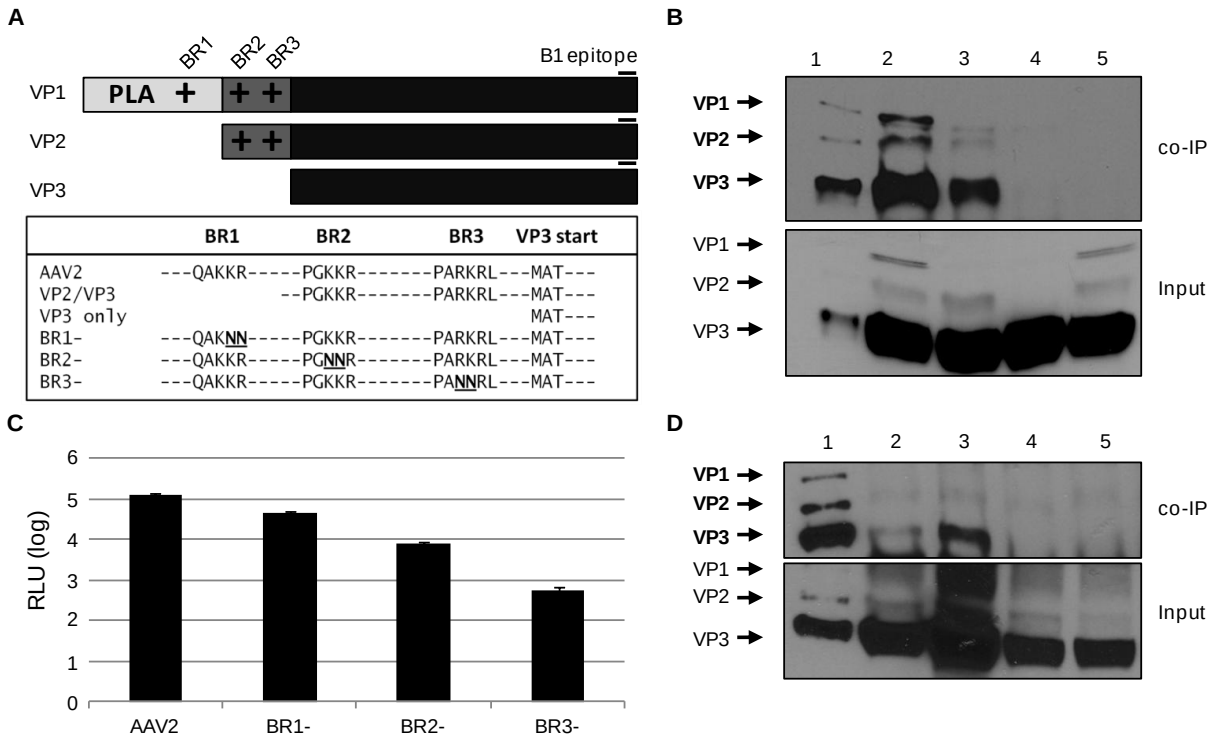


Figure 3.6. BR domains on VP1/VP2 mediate interactions with Importin-beta. A. Schematic of basic regions located in rAAV2 VPs and mutations made to ablate NLS function. B1 epitope is highlighted. B. rAAV2 (lane 2), VP2/VP3 (lane 3), and VP3-only (lane 4) particles were dissociated by heat and incubated with HeLa cell lysate. Co-IPs were performed using Dynabeads and an anti-Importin-β antibody, followed by immunoblot with B1. Lane 1, capsid protein marker; lane 5, mouse IgG2a isotype control. C. HeLa cells were infected with rAAV2-CBA-Luc and mutants (1000 vg/cell). Luciferase activity was measured 24 h post infection. Error bars represent standard deviations from three independent samples. D. Co-IPs were performed with rAAV2 (lane 2), BR1- (lane 3), BR2- (lane 4), and BR3- (lane 5) followed by immunoblot with B1. Lane 1, capsid protein marker. Relevant bands are highlighted in bold.

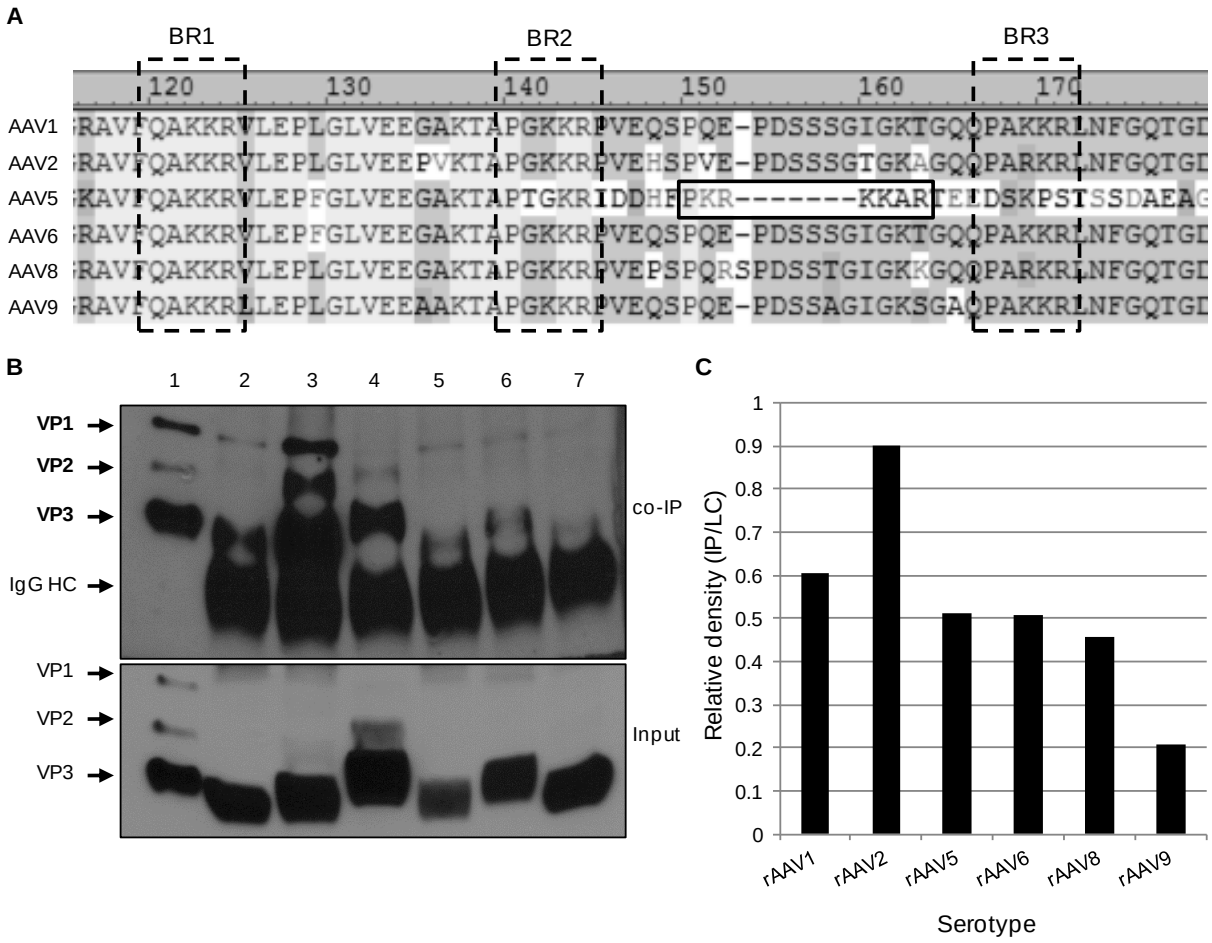


Figure 3.7. Interaction with Importin-β varies among rAAV serotypes. A. Alignment of N-terminal region of VP1 in AAV serotypes. BR domains are outlined in dashed lines, and putative bipartite NLS of AAV5 is highlighted in solid lines. B. rAAV1 (lane 2), 2 (lane 3), 5 (lane 4), 6 (lane 5), 8 (lane 6), and 9 (lane 7) particles were dissociated by heat and incubated with HeLa cell lysate. Co-IP was performed using Dynabeads and an anti-Importin-β antibody, followed by immunblot with B1. Lane 1, capsid protein marker. Relevant bands are highlighted in bold. C. Co-IPs were quantified by densitometric calculation of the co-IP product over initial input.

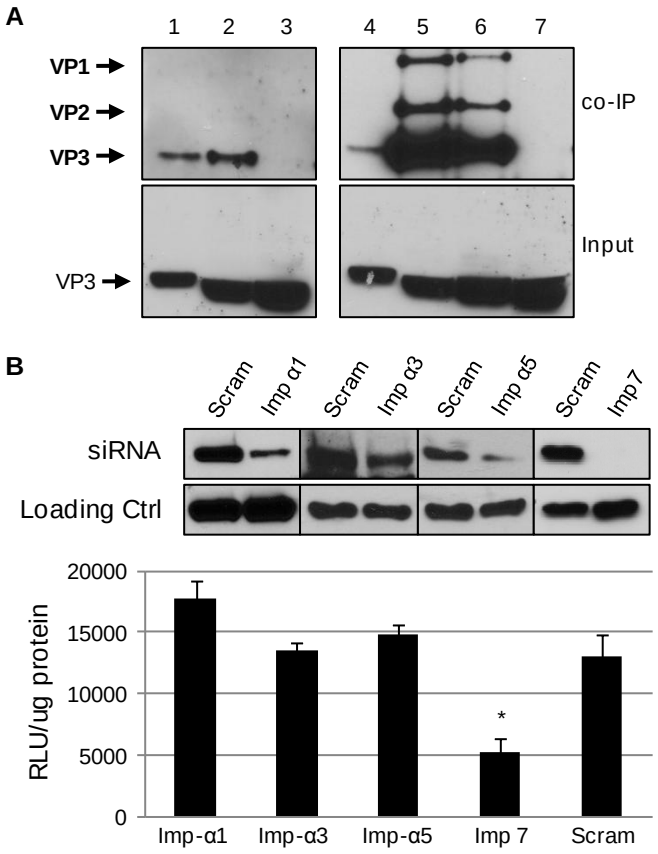


Figure 3.8. Analysis of additional Importin proteins in rAAV2 nuclear entry. A. rAAV2 particles were dissociated by heat and incubated with HeLa cell lysate. Co-IPs were performed using antibodies to Importin-α1, -3, or -5. Immunoblots were performed using the B1 antibody. Lanes 1 and 4, capsid protein marker; lane2, Importin-α1 co-IP; lane 3, mouse IgG1 isotype control; lane 5, Importin-α3 co-IP, lane 6, Importin-α5 co-IP; lane 7, rabbit pAb isotype control. B. Relevant bands are highlighted in bold. HeLa cells were treated with scrambled siRNA (blot, lanes 1, 3, 5, 7) or to KPNA2 (Importin alpha 1,lane 2), KPNA4 (Importin alpha 3, lane 4), KPNA1 (Importin alpha 5, lane 6) or IPO7 (Importin 7, lane 8). Luciferase activity was measured 24 h post infection. Error bars represent standard deviations from a representative experiment performed in triplicate.

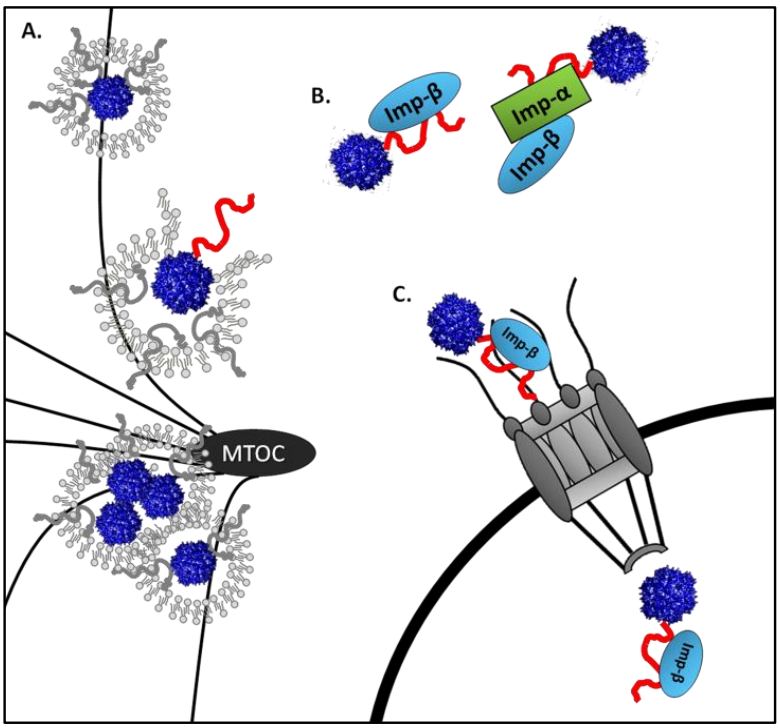
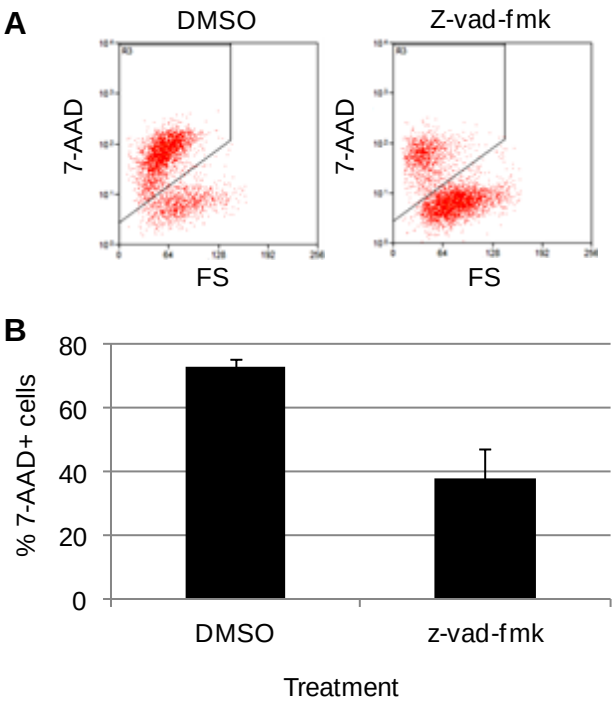
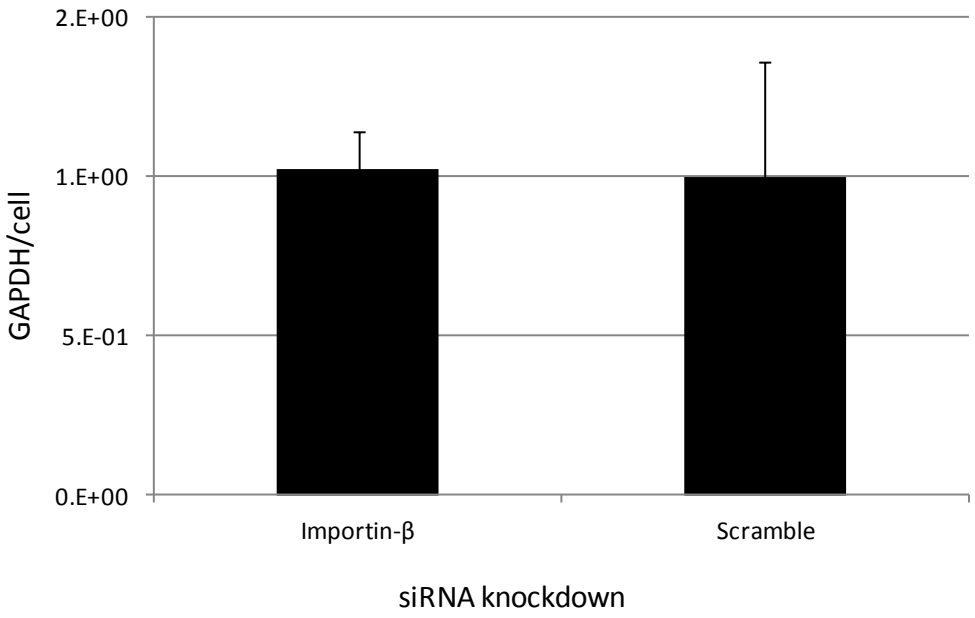


Figure 3.9. Model of nuclear import. rAAV2 trafficks to the MTOC in late endosomal or lysosomal compartments. A. rAAV2 undergoes a conformational change, exposing VP1up (shown in red), which harbors the three NLSs. B. rAAV2 interacts with Importin-β or an Importin-α/Importin-β heterodimer. rAAV2 could also interact with Importin 7 or an Importin 7/Importin-β heterodimer. C. rAAV2 translocates to the nucleus through the NPC, assisted by cellular karyopherins.



Supplementary figure S3.1. Confirmation of functionality of z-vad-fmk. A. Representative dot plot from flow cytometry analysis assessing 7-AAD positive cells after treatment with etoposide in conjunction with vehicle or z-vad-fmk. B. Quantification of flow cytometry, performed in triplicate.



Supplementary figure S3.2. Quantification of housekeeping mRNA (GAPDH) upon siRNA knockdown of Importin-β. HeLa cells were transfected with siRNA to either GAPDH or a scrambled control. 48h post-transfection, cells were harvested for qRT-PCR. Briefly, RNA was extracted from an equivalent number of cells. cDNA was produced from this RNA, and qRT-PCR was performed using primers for GAPDH.

CHAPTER 4

Conclusions and Further Explorations

Summary of findings

The attractive qualities possessed by AAV for gene therapy have transformed its initial perception as a "defective virus" into one of the most promising gene delivery systems for clinical applications. Owing to its modularity and clonability, rAAV systems allow for relatively easy development of vectors with continually expanding applications. The recent successes that rAAV-mediated gene therapy has seen in the clinic have potentiated its expansion into a growing number of indications. Likely due to its consistently demonstrated safety profile, explorations into rAAV-mediated gene therapy have expanded from conservative uses in orphan diseases to proof-of-concept studies designed to poise rAAV-mediated gene therapy as a competitive therapeutic for more widespread indications, including dyslipidemia (272, 273) obesity and diabetes (157, 274), and even color blindness (275). Reinvigoration of this field, as well as the growing number of results from clinical trials, has provided essential information to define current barriers that limit widespread clinical applicability in the rAAV field. Thus, research to overcome these obstacles is now being directed into three areas: i) strategies to increase the packaging size of rAAV vectors or facilitate efficient transduction of oversized vectors, ii) development of "stealth" rAAV vectors that can bypass pre-existing immunity that would otherwise thwart efficient transduction in certain individuals, and iii) approaches to enhance transduction efficiency in general in order to avoid triggering an immune response to capsid

antigens that has been shown to limit widespread, long term transduction. The overall goal of this work was explore strategies to enhance transduction. In Chapter 2, we describe a method for screening small molecules and assessing their utility in potentiating transduction. We report the identification of several novel compounds, in addition to previously characterized pharmaceuticals, that were shown to enhance transduction efficiency both *in vitro* and *in vivo* and have wide applicability for several vector types. In relating the mechanism of enhanced transduction to the overall transduction pathway of rAAV, we have delineated areas where barriers to transduction can be manipulated in order to increase rAAV transduction. One area includes a pathway to the proteasome, that, when inhibited, likely facilitates rerouting of vector particles to a more favorable pathway (i.e. nuclear translocation and nucleolar accumulation). The other area includes processing of uncoated genomes, likely either through second-strand synthesis, de-repression by genome-sequestering repair factors, or utilization of these repair factors for circularization and/or duplex formation. Where our screen fell short was in the discovery of compounds that enhanced transduction by means other than what we have previously identified. In particular, it has long been speculated that endosomal escape and nuclear entry are barriers to enhanced transduction; however, we did not uncover any compounds that specifically facilitate this process. Therefore, we took another approach to explore this event in transduction in order to develop ways to overcome this limitation. In Chapter 3, we define a mechanism of nuclear entry for rAAV2, a step in the transduction pathway that has remained largely undefined. Using biochemical, fluorescence, and genetic approaches, we described a mechanism involving host cell nuclear import machinery, including Importin-β, Importin-α1, -α3, and –α5, as well as the NPC and RanGTPase. Our results provide a foundation whereby both

pharmacological approaches, as well as rational capsid design, might increase the magnitude and rate of nuclear entry that might lead to more efficient transduction.

Synopsis and Future Directions Stemming from Small Molecule Screen.

Co-treatment of rAAV vectors with pharmacologicals is not a new concept and has co-evolved with understanding the biological mechanisms behind rAAV transduction. The earliest example of pharmaco-gene therapy (111) resulted from the understanding that DNA repair pathways were important for efficient transduction. While it is clear that host DNA repair factors are implicated more in second-strand synthesis and gene expression of the single-stranded rAAV DNA, the exact mechanisms seem to be multi-faceted. We utilized three forms of rAAV transgenes- single-stranded, self-complementary, and fragmented- in order to demonstrate the utility of novel DNA-damaging agents. These molecules, in general, enhanced transduction of single-stranded vectors better than self-complementary vectors, suggesting their role in conversion of the single-stranded genome into the duplex molecule. Interestingly, fragmented vectors were enhanced by a much larger magnitude, emphasizing the perhaps more important role of DNA repair, which would be implicated in the production of stable concatemers for any rAAV transgene.

Even more striking was the effect of bortezomib, a proteasome inhibitor, on the transduction improvement of fragment rAAV. Proteasome inhibitors have typically been thought to exert their effects on transduction by eliminating unfavorable vector trafficking to the proteasome, where degradation ensues. As fewer vectors are being degraded in the cytoplasm, they become more available to translocate to the nucleus for downstream events leading to transduction. This mechanism, in theory, is capsid-mediated; whereby capsid ubiquitination by

the cell is inconsequential since the proteasome no longer functions to degrade proteins. The fact that fragment AAV, which in theory shares the same capsid as the wild type vectors, show such remarkable improvement in transduction upon application of bortezomib compared to single-stranded vectors, suggests that this compound exerts pleiotropic effects in cells, which could include certain DNA-related effects. Alternatively, bortezomib may facilitate nuclear accumulation of an abundant level of fragment vectors, thus allowing for efficient re-annealing based on a higher likelihood of stochastic interactions within the nucleus. Finally, perhaps proteasome inhibition allows for the accumulation of more host DNA damage repair factors, which could be favorable for the annealing of fragment genomes. Indeed, the epipodophyllotoxins and anthracyclines are thought to exert their transduction-enhancing effects at the DNA level, not on the viral capsid. The impressive enhancement seen with these compounds suggests an equally important role of the DNA damage response proteins in addition to any additional enhancement by a sheer increase in levels of fAAV DNA in the nucleus facilitated by bortezomib. Understanding the mechanisms of action behind these drug targets is a priority in order to identify and optimize pharmacological targets that will maximize rAAV transduction.

Improving transduction of these fragmented vectors has huge implications for the implementation of widespread applicability of gene therapy. To date, the clinical use of rAAV is limited by the packaging size of the vector; i.e., the incorporated transgene must be no larger than 4.7 kb. To combat this limitation, attempts have been made to create “minigenes” by removing defined intronic sequences. One example of this approach involved the creation of minidystrophin for applications in Duchenne muscular dystrophy (DMD). The dystrophin cDNA is approximately 14 kb, almost three times the size of the allowable packaged genome of

rAAV. Based on the observations that large deletions in the dystrophin gene still resulted in functional products, a minidystrophin gene that was within the packaging capacity of rAAV was developed (276). This construct was shown to be functional and ablated dystrophic pathology in a mouse model of DMD. Due to the impressive results, this construct was utilized in a clinical trial for the same indication (277). Unfortunately, long-term expression was not achieved due to a previously unidentified autoimmune complication involved in the pathology of DMD. While minigenes are conceptually an effective approach to overcome the packaging capacity of rAAV, development and optimization are costly and time-consuming. Additionally, exonic material may not be redundant in other genes to a level that would significantly cut down on gene size.

Fragment rAAV provides an advantage over minigenes in that it can achieve expression of large, intact genomes. The underlying mechanism is thought to be mediated by 3' packaging of the single-stranded molecule to the point at which the capsid reaches capacity, whereby the remaining 5' end is truncated. Once uncoated in cell nuclei, annealing of opposite polarity genome fragments followed by 3' extension is thought to complete the construction of the full length transgene. This process is thought to rely on homologous recombination and is dependent on the repair protein Rad51C (186). So far, oversized genomes have shown therapeutic benefit in mouse models of Stargart's Disease (144) and disferlinopathies (149), as well as positive results in large animal models of Hemophilia A (119). However, the transduction efficiency of fragment rAAV is even lower than single-stranded vectors, which makes widespread use improbable at this point. Therefore, it is highly encouraging that the transduction efficiency of these vectors can be improved by orders of magnitude using small molecules.

Another challenge that could possibly be overcome with pharmaco-gene therapy is vector silencing over time. The convergence of evidence from clinical trials has demonstrated that

therapeutic transgene expression wanes over time; which indicates that a second dose might be needed to achieve benefit. Due to the production of neutralizing antibodies against the capsid after vector administration, any type of repeat dosing would need to occur with a different serotype. This poses many challenges, including sufficient transduction efficiency with the secondary vector, as well as gene expression in the desired tissue types. While it is known that a capsid-mediated CTL response contributes to the clearing of transduced cells and therefore a reduction in gene expression (14, 278) rAAV genomes have been shown to persist in host nuclei regardless of transcriptional status (101). This data suggests that the rAAV genomes are somehow being silenced by an undefined mechanism. A recent trial for α-1 antitrypsin (AAT) deficiency demonstrated that rAAV1 capsids persisted in the perinuclear region in muscle cells out to 12 months post vector administration (279), suggesting that a percentage of capsids can avoid proteasomal degradation. Whether or not these capsids contain genomes and could represent additional infectious particles remains to be determined. From our data, we have shown that several clinically relevant small molecules enhance transduction of rAAV when co-administered with the virus. It is unknown whether application of these small molecules months or even years after administration could "reactivate" rAAV expression from these silenced genomes or mobilize perinuclear vectors into the nucleus to facilitate uncoating and gene expression. One of these mechanisms appears to be possible, at least to some extent, with the proteasome inhibitor MG132 (115). When MG132 was applied 13, 21, and 28 days after vector administration, transgene levels became elevated for a certain amount of time before dropping off again. It would be interesting to determine the fate of vector translocation and gene expression upon repeat administration of teniposide or daunorubicin.

The compound library obtained from the Developmental Therapeutics Program provided a foundation to create a robust screening assay to find novel transduction-enhancing small molecules. Understandably, this library is limited to donations from other laboratories and a limited selection of current FDA-approved chemotherapeutics. Since repurposing an already approved FDA therapeutic would be the fastest way to incorporate pharmaco-gene therapy into a treatment protocol, we were particularly interested in the hits that are already in use in the clinic. Since the majority of clinically available chemotherapeutics capitalize on the difference in cell division rates, they mostly target DNA integrity, microtubule assembly, and proteasomal function. Factors involved in maintaining the functionality of these targets have been shown to be important for rAAV transduction. In order to circumvent other trafficking barriers to transduction with clinically relevant therapeutics, alternative compound libraries should be employed. There are several commercially available small molecule libraries that include drugs that affect endocytosis, endosomal trafficking, and organelle function. Evaluating these alternative libraries may yield novel compounds that enhance transduction through mechanisms differing from proteasomal inhibition or DNA repair factors, resulting in the possibility of utilizing combinations of small molecules to achieve an even larger increase in transduction. Indeed, previous studies have shown that the use of the anthracycline doxorubicin in conjunction with MG132 or a similar compound, LLnL, enhanced transduction 17-fold over doxorubicin alone and 758-fold over either proteasome inhibitor alone (162). Our preliminary studies show that combining MG132 with two compounds isolated from our screen, nanaomycin and physalin B, results in an additive increase in transduction over any compound alone, without altering the change in number of cells transduced (Figure 4.1). Ideally, combinations of these small

molecules would provide a “two-hit” approach to boost rAAV transduction levels up to or beyond what is required for beneficial therapy.

To provide proof-of-concept evaluation and maintain simplicity, we carried out our screen and development of hits with rAAV2. However, this approach could, and should, be applied to other rAAV serotypes in other cell lines, as mechanisms of subcellular trafficking and transduction have been shown to vary among serotypes and cell types. For example, rAAV1 and rAAV5 have been shown to exhibit faster entry and nuclear import than rAAV2, in addition to differential trafficking patterns demonstrated by fluorescence microscopy (90). Furthermore, vectors comprised of the capsids from other serotypes, such as rAAV1, rAAV8, and rAAV9, are being targeted for clinical use because they display greater transduction capabilities and fewer neutralizing antibody complications than rAAV2. To evaluate the use of a compound library to identify compounds that enhanced a more clinically relevant vector, we carried out a third screen using the DTP library and rAAV8 in a human hepatocyte line, HepG2. Since we had evaluated the hits from our rAAV2 screen in HepG2 cells, these compounds were not included in the new screen. We identified several compounds that differed from those in the rAAV2 screen. The initial results of the screen are shown in **Table 4.1**. Novel compounds included the topoisomerase II inhibitor mitoxantrone, the topoisomerase I inhibitors topotecan hydrochloride and camptothecin, DNA crosslinkers mechlorethamine hydrochloride, mitomycin, and cyclophosphamide, DNA-damaging agent streptonigrin, the MTOR inhibitor rapamycin, and several molecules affecting microtubule dynamics including ergocristine dihydrochloride, vincristine sulfate, vinorelbine tartrate, docetaxel, and baccatin III. In addition, a few compounds with less defined functions appeared, including medicarpin, photobarbatusin, and streptoval C. While the known cellular mechanisms of action for some of these compounds

share similarity to what was previously discovered with rAAV2, the identification of additional hits suggests that screens utilizing different serotypes for other target cell lines will add to the compound repertoire that can be used in conjunction with rAAV.

Synopsis and future perspectives on characterizing and improving the nuclear entry of rAAV

Through decades of research from multiple investigators, a general description of how rAAV2 trafficks through the subcellular space has been defined. Slowly, studies have expanded into other serotypes, including rAAV5 (85) and rAAV8 (280). In general, rAAVs seems to engage in early endosomal trafficking. From this point, several routes can ensue and can differ within the same cell, between cell lines, and between serotypes. Possible trafficking includes movement to the perinuclear recycling endosome or the late endosome, which then leads to movement to either the Golgi apparatus or the lysosome. Endosomal escape could potentially occur in any of these locations based on the previous studies and the present understanding of vector trafficking. While these routes have been characterized, what remains undefined is whether a subset of these trafficking patterns leads to more or less efficient transduction. For example, trafficking from the late endosome to the lysosome might result in particle degradation rather than subsequent nuclear entry. Similarly, since several groups have described the persistent, long term perinuclear accumulation of viral particles, perhaps the trafficking route that leads to this localization is inefficient for transduction. Further work is required to delineate which of these pathways is the most effective for overall transduction in order to design vectors or utilize pharmacological approaches to take advantage of these "preferred" pathways.

One area of rAAV trafficking that has remained largely undefined has been how rAAV moves from the cytoplasm into the nucleus. Previous studies have shown that rAAV enters the nucleus as an intact particle. Basic regions, located on VP1up and sharing sequence homology to classical NLSs, have been shown to be important for nuclear entry and subsequent transduction by rAAV2, suggesting that rAAV2 enters the nucleus through an Importin-mediated mechanism. However, other studies have shown that blocking the NPCs of purified nuclei did not inhibit nuclear entry of unprocessed vector particles, suggesting an NPC-independent form of nuclear entry. Studies with MVM have identified a novel mechanism for autonomous parvoviral nuclear entry involving direct disruption of the nuclear membrane in a caspase-dependent manner. We have attempted to provide further insight into the mechanism of nuclear entry by rAAV2 in Chapter 3. We have shown that rAAV2 does not appear to overtly disrupt the nuclear lamina nor depend on caspases for nuclear entry or transduction under physiological conditions. Our results demonstrate that rAAV2 appears to utilize the nuclear pore complex, and nuclear entry is dependent on nuclear cargo carrier Importin-β. Other importin proteins, such as Importin -α1, -α3, and –α5 may play a role in nuclear entry as well; however, we could not demonstrate their individual requirement through genetic knockdown. This suggests that the import proteins that mediate rAAV2 nuclear entry might have some redundant functions.

Overall, we have characterized one of what might be several nuclear import pathways for rAAV. Indeed, capsid proteins of other rAAV serotypes were shown to interact with Importin-β; and the basic regions among known rAAV serotypes appear to be quite similar. However, differences among serotypes might exist, including differential involvement of other importin proteins or efficiency of nuclear translocation. Not limited to differences among serotypes, there could be alternative mechanisms for nuclear entry. This would not be surprising, as viruses in

general tend to hijack several routes of entry and subcellular trafficking in order to propagate efficiently. It has been shown that rAAV2 and rAAV5 can utilize multiple routes of endocytosis and subcellular trafficking; therefore, nuclear translocation could occur through multiple mechanisms as well. Indeed, a recent study has shown that rAAV2 that had been acidified and then neutralized could disrupt host cell nuclear lamina in permeabilized HeLa cells (234). While this study was not conducted under physiological conditions, it does pose an alternative mechanism of nuclear lamina disruption that might be more subtle during a more natural infection, as we observed. Similarly, in defining potential interactions between importin proteins and AAV capsid proteins, we utilized an *in vitro* approach with denatured capsid proteins. It will be important to validate these interactions in a more physiological setting (i.e. a pulldown assay) if i) interactions between rAAV2 and importin proteins are not transient, and ii) interactions within the cell are prevalent at a given time point where an effective pulldown could be performed. Importantly, neither our study nor the studies carried out by Hansen et al and Porwal et al were performed in the presence of Adenovirus. It is well established that Adenovirus potentiates AAV infection and rAAV transduction at almost every step in the infectious pathway. Therefore, it is possible that the mechanism of nuclear entry is either different or more efficient in the presence of Adenovirus. Future investigations will be required to understand the role of Adenovirus in rAAV nuclear entry.

All analyses of the nuclear entry mechanism by rAAV to date have occurred *in vitro*. Our results, demonstrating a mechanism utilizing components of the classical nuclear import pathway, will need to be validated *in vivo*. A possible avenue to explore is the use of Importin α knockout mice, as was demonstrated for the influenza virus (281). In this study, mice lacking Importin-α4 ($\alpha4^{-/-}$), Importin-α5 ($\alpha5^{-/-}$), Importin-α7 ($\alpha7^{-/-}$), or retaining a mutated form of

Importin-α7 that lacked the Importin-β binding domain ($\alpha7^{\Delta IBB/\Delta IBB}$) were challenged with influenza virus strain SC35M. Whereas all of the mice died in the wild type, $\alpha4^{-/-}$, and $\alpha5^{-/-}$ cohorts, one third of the $\alpha7^{-/-}$ and $\alpha7^{\Delta IBB/\Delta IBB}$ cohorts survived. Additionally, wild type and $\alpha7^{-/-}$ and $\alpha7^{\Delta IBB/\Delta IBB}$ mice were challenged with the H1N1v strain. While all of the wild type mice died at day 9 post challenge, none of the $\alpha7^{-/-}$ or $\alpha7^{\Delta IBB/\Delta IBB}$ died. Finally, after challenge with the H5N1 strain, mice in all of the cohorts died, however viral titers were significantly lower in the $\alpha7^{-/-}$ and $\alpha7^{\Delta IBB/\Delta IBB}$ animals. These results suggest that Importin-α7 plays an important role in the nuclear entry and infectivity of the influenza virus. Similar studies could be performed with different AAV serotypes to understand *in vivo* the involvement of specific import proteins in AAV nuclear entry.

The ultimate goal for studying the biology of rAAV vectors is to utilize the information in optimizing vector design or identifying cellular targets for pharmaco-gene therapy. In Chapter 3, we established that nuclear import, thought to be a barrier to efficient transduction, can occur through the classical nuclear import pathway. Therefore, the goal moving forward is to try to design rAAV vectors that can take better advantage of this pathway. Nuclear entry through the classical pathway involves a protein (in this case, the capsid) with an NLS being directed through the NPC via Importin proteins, which recognize the NLS. Previous research has shown that VP1up contains basic regions that resemble NLSs, and mutations to these regions inhibit nuclear entry of viral particles and attenuate transduction by log orders of magnitude (95, 96). Utilizing this information, additional positively charged residues were incorporated into VP1up, in particular to BR2 and BR3, in an attempt to make these basic regions more effective NLSs (95). Unfortunately, these mutations had no enhancing effect, and in fact the addition to BR3 resulted in decreased transduction in certain cell lines. The authors concluded that, at least in the case of

the BR3 mutant, additional positive charges to the VP1up region could result in enhanced and perhaps sustained nucleolar localization. Since it is thought that uncoating and downstream gene expression occur in the nucleoplasm, retention in the nucleolus is likely meant to be a transient event, whereby incoming particles localize until cellular conditions become favorable for uncoating and gene expression. While the concept of improving nuclear entry via enhancing NLS activity seems like a reasonable approach, modifications within the existing signaling architecture have yet to gain traction.

In an attempt to build upon these results, we have examined the capsid architecture on the surface of the capsid in search of locations where we could incorporate an NLS. The rationale behind this approach was three-fold. First, an NLS on the capsid surface would not interfere with the already established basic residues or the PLA2 domain located on VP1up. Second, the additional NLS could have additive effects over the VP1up basic regions; that is, the additional NLS would allow for more importin protein interaction and therefore more nuclear entry. Finally, adding an NLS to the outside of the capsid could allow for a mechanism whereby incoming particles would not rely on the conformational change dependent upon endosomal acidification in order to initiate nuclear translocation. Previous studies have shown that this conformational change is required for efficient transduction, as microinjection of intact particles results in virtually no transduction (92). Likewise, virus-like particles devoid of VP1up, or empty particles which do not readily undergo the conformational change, will not transit to the nucleus (87). The importance of the NLS-like sequence of BR3, demonstrated through mutational analysis, has also been substantiated through its addition to NLS-null virus-like particles (268). In this study, the PLA2 domain, as well as the PLA2 domain with BR3, were incorporated into VP2/VP3-only virus-like particles so that these domains would extrude from

the capsid surface. Transduction was negligible for the VP2/VP3-only mutants. Transduction improved when PLA2 was incorporated, but was improved even further when BR3 was available as well, confirming an important role for the NLS-like activity of this region.

Upon investigation of the capsid surface, we identified regions that would likely tolerate the incorporation of an NLS based on 3 criteria: secondary structure (i.e. flexible loops rather than underlying beta sheets or alpha helices), prior history of tolerating substitutions or additions, and access to the cellular environment (and therefore importins). Four regions were targeted for substitutions: the HI loop, located at the base of the 5-fold axis of symmetry; the GH loop, topping the 3-fold axis of symmetry peaks; and VR1 and VR7, both located on the sides of the 3-fold peaks **(Figure 4.2A)**. We chose to use the classical NLS from the Simian Virus 40 large T antigen, PKKRKV, as the substitution in these areas. Upon production of these vectors, we were not able to achieve preparations to levels suitable for transduction analysis **(Figure 4.2B)**. In fact, the viral titers resulting from these constructs were several orders of magnitude below a typical viral preparation, suggesting defects in particle assembly. It is currently unknown whether particles were assembled and failed to package genomes, or if viral proteins were indeed translated but could not converge into a properly structured capsid. We hypothesize that the latter case is most likely, since such a high concentration of positive charge might have occluded the proper convergence required for capsid assembly. An alternative mechanism to circumvent assembly difficulties could be the incorporation of an NLS post-assembly. This could be achieved through chemical crosslinking, as has been demonstrated for the Cowpea mosaic virus (282) or through oxidative coupling using unnatural amino acids, as has been demonstrated with the bacteriophage MS2 (283). However, it remains to be determined if this could be achieved on

an AAV capsid, or whether an NLS tethered to the surface would have any influence on overall transduction efficiency.

A final strategy to enhance nuclear entry would be the use of small molecules that created conditions for more permissive nuclear transit. Currently, there are no known pharmaceuticals that achieve this effect; however, the amphipathic molecule trans-cyclohexane-1,2-diol (TCHD) has been investigated for its role in facilitating the nuclear uptake of macromolecules and plasmid DNA (284, 285). Application of this chemical takes advantage of the natural properties of the NPC, where the inner nuclear pore is innately hydrophobic, which allows for selective passage of importin-bound cargo. The addition of TCHD disrupts these hydrophobic interactions, thus collapsing the gating mechanism and allowing passage of molecules that would normally be excluded. While we have shown that rAAV2 can utilize the NPC to enter host cell nuclei, the process might not be efficient given the size of the capsid (~25 nm) and the maximum allowable diameter accommodated by the pore (~39 nm). We speculate that relaxing the inner pore to allow for non-selective passage might facilitate more rapid and more abundant nuclear entry by vector particles. While this strategy has been successful in several cell lines and air liquid interface cultures, results have not shown efficacy *in vivo* (286). Therefore, the utility of this strategy may be an interesting approach but distant actuality for gene therapy applications.

Concluding Remarks

In this dissertation, we have underscored the utility of using small molecules to overcome cellular barriers to efficient transduction. We have also attempted to characterize an ill-defined aspect of rAAV transduction, nuclear entry, with the future goal of utilizing this information to rationally design vectors or identify small molecules that can more efficiently navigate this step

in transduction. Previous studies with HU and proteasome inhibitors seem to indicate that moving vector cargo into the nucleus can potentiate transduction; therefore, making this process more efficient could enhance transduction to levels that would be beneficial in the clinic. While nuclear entry seems to be a barrier to efficient transduction, it remains unclear whether this is actually because of inefficient nuclear translocation or if the steps preceding nuclear entry (i.e endosomal escape) limit the ability of vectors to transit to the nucleus. It is tempting to think that mobilizing vector particles out of the perinuclear region would facilitate more abundant nuclear entry, but this remains to be determined. Likewise, the subcellular trafficking pathway that leads to the most efficient transduction has yet to be defined; future work in this area will likely provide the foundation for the next generation of vectors that preferentially utilize this pathway.

Keeping all of this in mind, it is important to remember the origins of this simple, yet elegant parvovirus. AAV co-evolved with Adenovirus, and therefore many of the details behind its infectious pathway must be considered in the presence of Adenoviral functions, even if this helper virus is not present in host cells or gene therapy applications. AAV has likely retained its innate capabilities to avoid causing pathogenicity on its own at a cost to efficiency without Adenovirus present. Thus, the perceived inefficiencies of AAV are likely due to mechanisms that have evolved over time.

Thus far, viral gene delivery has been the most successful strategy for achieving gene transfer in the clinic. rAAV vectors are garnering promise because of their impressive safety profile and amenability for a wide range of applications. As more and more results emerge from the clinic, yielding important information about the shortcomings of vector delivery, more attention will be focused on overcoming these obstacles. As AAV has transformed from a

“defective virus” to an effective gene therapy vehicle, decades of development are beginning to translate into clinical success.

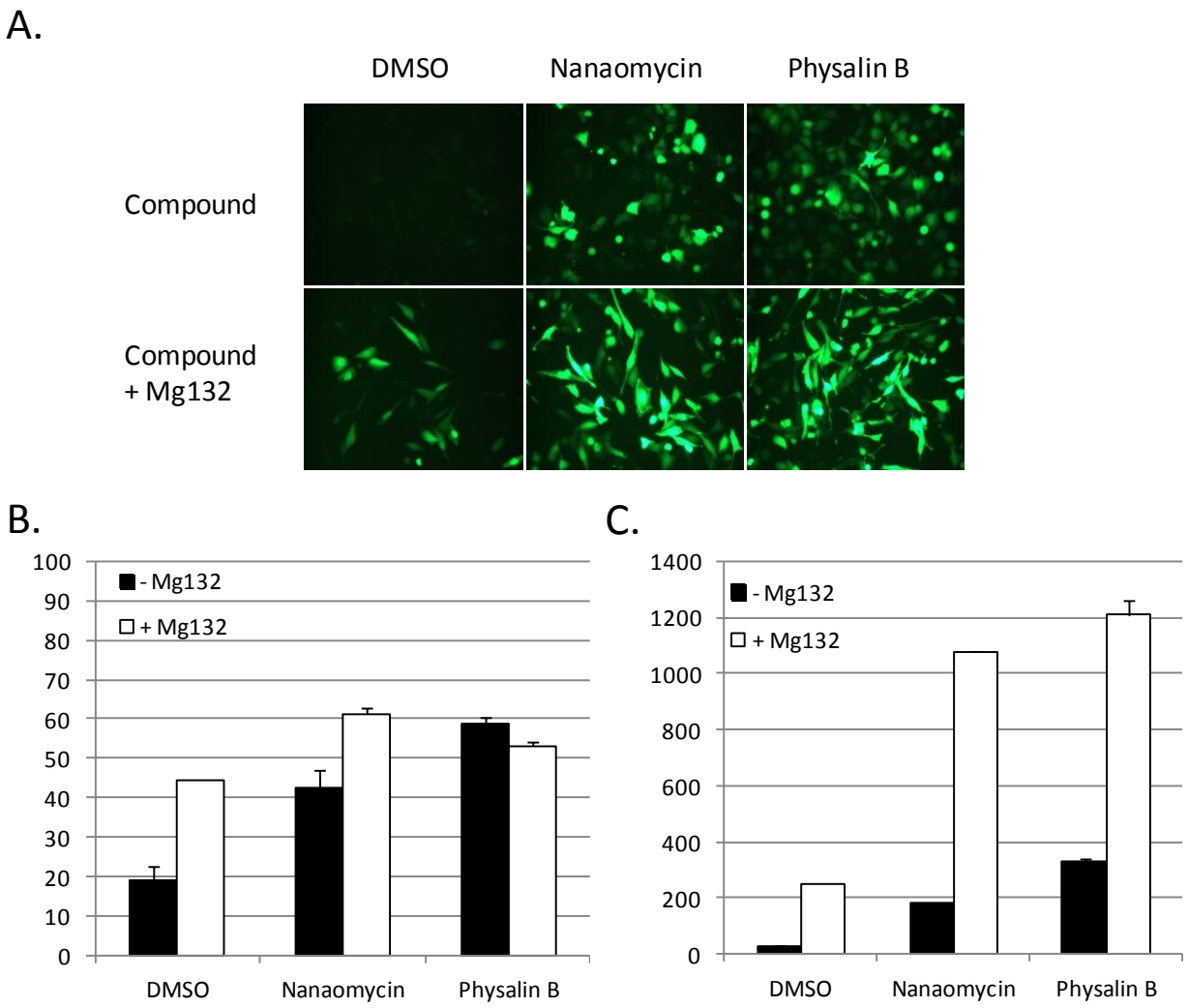
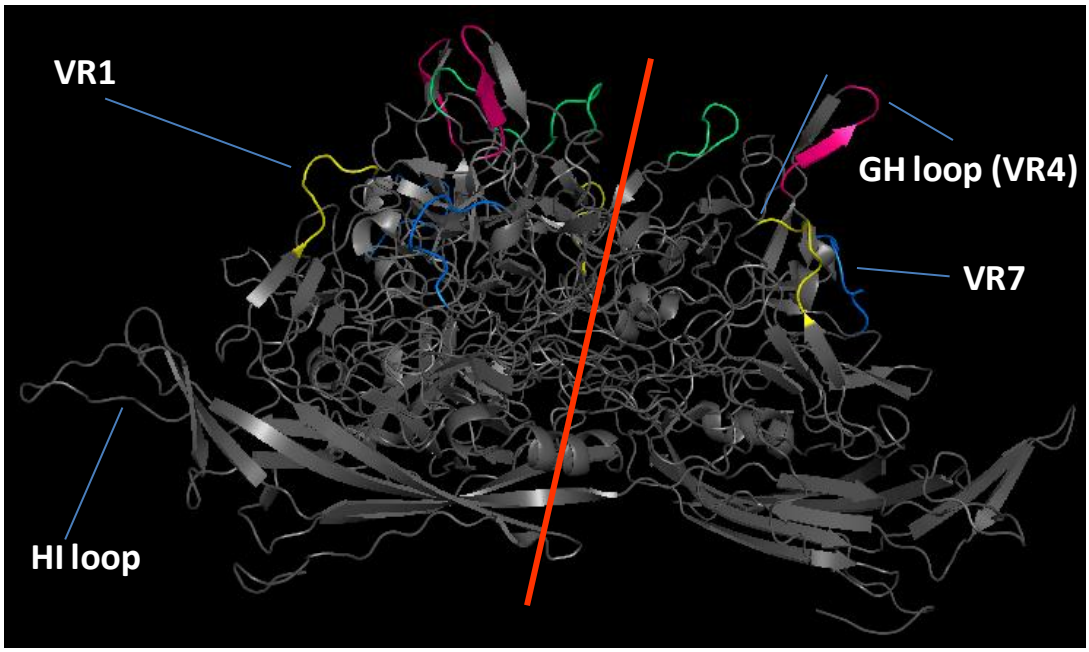


Figure 4.1. Additive effects of the proteasome inhibitor MG132 with Nanaomycin and Physalin B. A. HeLa cells were treated with either compound alone or in combination with 1 µM MG132 in addition to rAAV2_CMV_eGFP. 48h post transduction, GFP expression was visualized. Cells were then processed for flow cytometry, where the percent of GFP positive cells (B) and the mean fluorescence (C) was assessed.

NSC id	Name (if known)	Cellular function	Fold enhancement in screen
122819	Teniposide	topoisomerase inhib	153.00
279836	Mitoxantrone	topoisomerase inhib	35.59
762	Mechlorethamine hydrochloride	mustard gas (dna crosslinking, no mitosis)	24.23
330753	Baccatin III		17.34
609699	Topotecan hydrochloride	topoisomerase inhibitor	14.11
94600	Camptothecin	topoisomerase inhibitor	13.33
409663	Ergocristine, dihydro		11.87
26980	Mitomycin	DNA crosslinking	9.98
45383	Streptonigrin		9.60
67574	Vincristine sulfate	Inhibits microtubule formation, arrests cells in metaphase	7.94
26271	Cyclophosphamide	DNA crosslinking	6.92
350085	Medicarpin		6.78
67574	Vincristine sulfate	Inhibits microtubule assembly	6.68
608210	Vinorelbine tartrate	antimitotic	6.27
226080	Rapamycin	mTOR inhibitor, protein synthesis inhib	5.63
270914	Photobarbatusin I		5.44
169627	Streptoval C		5.39
49842	Vinblastine sulfate	microtubule inhibitor	5.23
628503	Docetaxel	microtubule stabilizer	5.21
369397	Qinghaosu		5.01

Table 4.1. List of hits and known function (if any) for compounds initially identified in 96-well screen using rAAV8 on HepG2 cells (credit: Dr. Marc Weinberg).

A.



B.

Mutant	Titer (vg/µL)
HI	5.52×10^6
VR1	**N/A**
$VR4_i$	1.21×10^6
$VR7_{sub}$	1.93×10^5

Figure 4.2. Target capsid regions for NLS substitution. A. Capsid architecture at 3-fold axis of symmetry with locations of regions for NLS insertion highlighted. The orange line represents the center of the axis. The green loop is the integrin binding domain, for reference. B. Mutants were produced via transfection with PEI and purified by continuous CsCl centrifugation. Resulting titers are shown.

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