Title
New Methods in Engineering Adeno-Associated Virus (AAV) for Improved Gene Delivery

Author
Weinstein, John Ryan

Publication Date
2013

Peer reviewed|Thesis/dissertation

New Methods in Engineering Adeno-Associated Virus (AAV) for Improved Gene Delivery

By

John Ryan Weinstein

A dissertation in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor David V. Schaffer, Co-chair

Professor Matthew Francis, Co-chair

Professor Christopher Chang

Professor John Flannery

Spring 2013

Abstract

New Methods in Engineering Adeno-Associated Virus (AAV) for

Improved Gene Delivery

by

John Ryan Weinstein

Doctor of Philosophy in Chemistry

University of California, Berkeley

Professor David V. Schaffer, Co-chair

Professor Matthew Francis, Co-chair

Gene delivery vehicles, or vectors, based on adeno-associated viruses (AAV) have demonstrated success in both preclinical disease models and recently in human clinical trials for several disease targets, including muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis, and macular degeneration. AAV has multiple characteristics that make it an effective gene therapy vector: the parent virus is nonpathogenic in humans, they can transduce both dividing and non-dividing cells, and they efficiently transduce some important cell and tissue types. The AAV genome contains three open reading frames, which encode the nonstructural proteins needed for viral replication and virus assembly (*rep* and *aap*) and the three structural proteins that assemble to form a 60-mer viral capsid (*cap*). To create a gene therapy vector, a therapeutic gene of interest is inserted in place of the viral open reading frames to be packaged during vector production. Despite its considerable promise and emerging clinical success, several challenges impede the broader implementation of AAV gene therapy, including the prevalence of anti-AAV neutralizing antibodies in the human population due to natural exposure to the parent virus, low transduction of a number of therapeutically relevant cell types, and an inability to overcome physical transport barriers in the body. These challenges arise since the demands we place on AAV vectors are often different from or even at odds with the properties nature bestowed on their parent viruses. Viral directed evolution – the iterative generation of large, diverse libraries of viral mutants and selection for variants with specific properties of interest – offers a promising means to address these problems.

Directed evolution is a high-throughput, molecular engineering approach that our group has adapted and implemented to create AAV variants with novel properties, such as altered receptor binding, altered cell transduction, and altered tissue transduction in the body. In general, the method emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic

modifications that progressively improve a molecule's function, even without knowledge of the underlying mechanistic basis for the problem. For AAV, this process has involved mutating wild-type AAV *cap* genes to create large genetic libraries, which can be packaged to generate libraries of viral particles, each of which is composed of a variant capsid surrounding a viral genome encoding that capsid. A selective pressure – such as the ability to selectively infect HIV-infected T-cells – is then applied to promote the emergence of variants able to surmount these barriers. After each such selection step, the successful variants can be recovered and used as the starting material for the next selection step to further enrich for improved variants. After several such selection steps, the resulting *cap* gene pool is subjected to additional mutagenesis and selection. After several rounds of mutagenesis and selection, the resulting variants can be analyzed individually for the desired property.

I have tried to improve the efficiency of directed evolution of AAV, to create new novel types of AAV libraries, and to utilize these libraries for novel functionality. A thermostable AAV was engineered to compensate beneficial mutations that destabilize the viral capsid, however, there was a trade-off between high and low temperature stability. From these studies, it was discovered that there is variability in the stabilities of natural AAV serotypes that could be exploited to study the mechanism of improved capsid stability as well as compensating destabilizing mutational effects. Alternatively, by allowing AAV to evolve along a neutral network, accumulating mutations that do not have a detrimental effect to natural AAV function but could allow new promiscuous functions, allowed the production of a small but highly useful AAV library. A novel, highly diverse library based on ancestral reconstruction was created with variants inaccessible by standard mutagenesis-based approaches. Finally, utilizing directed evolution of AAV, viral variants were isolated that could selectively infect HIV-infected T-cells for potential use in gene therapy treatments against HIV.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ACKNOWLEDGMENTS

There are so many people who have contributed to this work in a variety of ways and they deserve be acknowledged and thanked for enabling my happy and successful time in graduate school. My advisor, Professor David Schaffer, has been an amazing scientific mentor. I have learned so much from his advice and suggestions and I am very thankful for the opportunity to work with him during my graduate career.

All members of the Schaffer lab, past and present, have made my time here amazing. Each and every one of you is an intelligent, talented individual from whom I have learned so much. The members of "Team AAV," especially Jamie Bergen, Daniel Stone, Bum-Yeol Hwang, Prasanth Asuri and Melissa Bartel, were immensely helpful in discussions about data, papers, presentations, and problems of any sort. Thanks to Jonathan Foley, Lukasz Bugaj, Dawn Spelke, Jorge Santiago-Ortiz and Prajit Limsirichai for help with new techniques, fruitful laboratory discussions and being great friends outside of lab as well. Wanichaya Ramey has been the most amazing lab manager that I could have imagined. I appreciate everything she has done to keep the lab operating efficiently but I am even more thankful for her being such a caring person and amazing friend. We joke about being brother and sister but she has truly been like family. On the topic of "sisters," I need to acknowledge the important role my lab bff, Ashley Fritz, played in my time here. Together we have been through so much, starting in lab together and navigating the trials and tribulations of graduate school. I cannot think of anyone with whom I'd much rather have taken this journey.

I was lucky enough to have the most amazing set of friends outside of lab. A group of us came together during orientation and have been friends ever since. Jessica Herrick, Heidi Ferguson, David Unruh and Leah Witus, we've road tripped together, graded together, been graded together, cut it on the dance floor together. At times it seemed like we did everything together. We've all moved to different cities but I cherish the time we've spent together and look forward to the many good times ahead. A list of acknowledgements would be incomplete if I did not mention the rock climbing crew (Russell Bradley, Kristen Potter, Michael and Stephen McKinley, and especially Lea Dulatas, Jocelyn Scheintaub, and Julian Bigi). I am so grateful to have had such a great group of friends to let off some steam on the rock walls and to get into silly highjinks with.

My family has been so inspirational in this entire process. My mother, Ellen Weinstein, has been an immense source of support (as well as the subject of many funny stories and impressions). She is the most caring person on the planet and I am glad she is my mother. My brother, Lenard, has been my international travelling companion and friend over the years and for that I am thankful. Finally, my deceased grandfather was my personal hero and without whom I would not be the man I am today. I miss him everyday and I try to live my life in a way that would make him proud. I dedicate this dissertation in his memory.

Chapter 1

Engineering Adeno-Associated Virus for Therapeutic Gene Delivery

Portions of this chapter are a postprint of a paper submitted to and accepted for publication as

Bartel MA, Weinstein JR, Schaffer DV. (2012) Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. Gene Ther 19: 694-700.

The copy of record is available at
http://www.nature.com/gt/journal/v19/n6/full/gt201220a.html

AAV Biology

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome within a non-enveloped, icosahedral capsid.[1] The genome contains three open reading frames (ORF) flanked by inverted terminal repeats (ITR) that function as the viral origin of replication and packaging signal.[1] The rep ORF encodes four nonstructural proteins that play roles in viral replication, transcriptional regulation, genomic integration, and virion assembly.[1] The cap ORF encodes three structural proteins (VP1-3) that assemble to form a 60-mer viral capsid.[1] Finally, an ORF present as an alternate reading frame within the cap gene produces the assembly-activating protein (AAP),[2,3] a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process.[2]

The virus's capsid governs its ability to transduce cells, from initial cell surface receptor binding to gaining entry into the nucleus. Briefly, AAV2 – the variant most broadly studied to date – is internalized via receptor-mediated endocytosis, with evidence supporting a role for both the clathrin-coated pit pathway[4,5] and the clathrin-independent carriers/GPI-anchored-protein-enriched endosomal compartment pathway.[6] Following cellular entry, the virion escapes from early endosomes and traffics to the perinuclear area. There is evidence supporting both AAV trafficking into the nucleus prior to uncoating[7-9] and AAV uncoating prior to viral DNA entry into the nucleus.[10] Upon nuclear entry, second-strand synthesis – i.e. conversion of its single-stranded genome into double-stranded, transcriptionally available DNA – must occur for viral gene expression.[11] Finally, in the absence of helper virus co-infection, AAV enters a latent life-cycle in which viral genomes can integrate selectively into the AAV1S locus on human chromosome 19 (for replication competent, wild-type AAV) or persist as extrachromosomal episomes (for both wild-type and recombinant AAV).[12-14]

In recombinant versions of AAV, a gene of interest is inserted between the ITRs in place of rep and cap, and the latter are provided in trans, along with helper viral genes,

during vector production.[15] The resulting vector can transduce both dividing and non-dividing cells with stable transgene expression in the absence of helper virus for years in post-mitotic tissue. There are eleven naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino acid sequence, particularly within the hypervariable regions of the capsid proteins, and thus in their gene delivery properties.[16,17] Importantly, no AAV has been associated with any human disease, making recombinant AAV attractive for clinical translation.[1]

AAV has yielded promising results in an increasing number of clinical trials. As a prominent example, during Phase I clinical trials for Leber's congenital amaurosis (LCA), numerous patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measurements of vision.[18-20] Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the trial.[18-20] As a second recent example, AAV8-mediated delivery of cDNA encoding factor IX to the liver of hemophilia B patients resulted in sufficient levels of secreted protein to alleviate the patients' bleeding phenotype.[21] AAV vectors are also being clinically explored for muscular dystrophy, Parkinson's disease, and Alzheimer's disease.[22] AAV thus has considerable promise. Nevertheless, there are impediments that may limit its utility, such as anti-capsid immune responses, low transduction of certain tissues, an inability for targeted delivery to specific cell types, and a relatively low carrying capacity. Rational design has made progress in creating AAV variants with enhanced properties.[23,24]

Rational Engineering of AAV

Targeting AAV through Linkers

In 2002, Ponnazhagan et al. described a method by which to target AAV to non-permissive tissues through the use of the biotin-streptavidin binding affinity.[25] The group conjugated biotin to the exterior surface of the AAV serotype 2 viral capsid by reacting surface lysines with an NHS-ester containing biotin. Incubation of AAV viral capsid with streptavidin-growth factor fusion proteins yielded AAV-biotin-streptavidin-growth factor conjugates. While the conjugation of biotin and biotin-strepavidin-EGF to AAV did not ablate infection through its native receptor, the EGF-modified virus had a 100-fold increase infectivity in cells expressing the EGF receptor. Similarly, AAV conjugated to FGF through this system was able to infect cells expressing the FGF receptor in a heparan-sulfate proteoglycan (HSPG)-independent manner; HSPG serves as the primary cellular receptor for AAV2. Due to the nature by which biotin is conjugated to AAV, the resulting virus population is heterogeneous and potentially contains multiple biotins conjugated to each capsid monomer. This, however, has been remedied by Arnold et al. (2006) who showed that biotin can be incorporated site-specifically by the addition of a biotin acceptor peptide to the AAV capsid.[26] Incorporation of the biotin accepting peptide, however, decreased the infectivity of AAV 1000-fold. Utilizing avidin-linked peptides suffers from the need to have intimate knowledge of both receptor and ligands for the cell of targeting interest. This technology has been expanded to allow the metabolic conjugation of ketone-bearing biotin analogues, allowing bioconjugation with ketone reactive groups and subsequent retargeting through these groups.[27]

Alternatively, in 2002, Ried et al. incorporated a Z34C protein A immunoglobulin IgG-binding domain into the AAV2 capsid.[28] This domain binds the Fc region of immunoglobululins, allowing the Fab region to target the virus to the cell through the antibody-antigen interaction. Viruses containing the Z34C insertion bound to antibody were successfully retargeted to cells through the antigen for which the antibody was raised. While this targeted the insertional mutant in the presence of antibody, infection levels were less than that of wild-type AAV. The infection, however, was independent of HSPG and was maintained in the presence of soluble heparin. This method has promise, though, in order to be utilized, antibodies must exist against a receptor expressed at high levels in the cell line of interest. This method also suffers from the need to insert a peptide fragment into the AAV viral capsid, ablating normal tissue tropism, which upon conjugation to an antibody only allows rescue to wild-type infectivity levels. Due to the fact that insertion binds all IgG this method could not work in an in vivo setting. Any circulating antibody could displace antibodies bound to AAV thus removing the AAV targeting ability.

In comparison, Bartlett et al. (1998) targeted AAV utilizing a bispecific F(ab'γ)2 antibody whereby one F(ab'γ) arm bound a cellular receptor and the other F(ab'γ) arm bound the AAV viral capsid.[29] This allowed efficient transduction of non-permissive cell lines through the receptor the antibody was raised against. Vector mediated gene expression, however, was significantly reduced, potentially due to the antibody interfering with nuclear targeting steps following viral entry. This method, like the use of monoclonal antibodies for AAV targeting, also suffers from the need for antibodies already raised against a cellular receptor, which requires intimate knowledge of the cell biology of the cell of interest.

Chemical modification has also been utilized to reduce antibody neutralization of AAV. Lee et al (2005) conjugated polyethylene glycol (PEG) chains to the AAV2 viral capsid.[30] PEG was conjugated to AAV through reaction of electrophilic succinimidyl propionic acid (SPA) and NHS-ester groups to reactive lysine residues on the viral surface. Conjugation of PEG showed a moderate protection (up to 2.3 fold) against neutralizing serum at varying serum concentrations. Similarly, Carlisle et al (2008) modified AAV5 and AAV8 with amine reactive HPMA polymers and viral conjugates reduced neutralization by anti-AAV antibodies.[31] This work relied on conjugation through amine reactive functionalities of which there are eighteen per VP3 protein. Therefore, with over 1000 lysines per viral particle, this method yields a heterogeneous mixture of AAV-PEG particles with no control over which lysines are PEGylated and only mild control on the number of PEG molecules conjugated to the viral surface.

Targeting AAV through Genetic Modifications

Several groups inserted peptide groups into the AAV viral capsid for both targeting purposes as well as for immune evasion. In 2003, Huttner et al. described the incorporation of ligands for common cellular receptors (L14 and MecA) into the AAV2 viral capsid.[32] In comparison to wild-type, transduction by the L14 insertion mutant was significantly less reduced in the presence of neutralizing antibodies; this increase in immune evasion was also shown to be independent of the peptide sequence inserted into the capsid. This insertion also successfully targeted the virus to non-permissive B16F10

cells and the MecA insertion allowed retargeting to Mec1 cells. While this method has been shown to be effective at retargeting AAV vectors and evading host antibodies, it still requires knowledge of the binding interaction on the cell of interest, which is not possible for all cells of interest in targeting.

Combinations of phage display and genetic modification allows retargeting of AAV through previously unknown targeting peptides. Work et al. (2006) selected for targeting peptides against rat tissues and incorporated these peptides into the AAV capsid to retarget the viral capsid.[33] *In vivo* biodistribution studies after AAV vector administration, showed increased viral accumulation in the tissues against which the targeting peptide was selected one hour (2.5-11 fold increase) and 28 days following infusion. While the modified AAV showed increased accumulation in target tissues, it was significantly reduced from those seen during phage display selection. The differing 3-dimensional environments between the two viruses could display the targeting peptide differently on AAV, thus leading to differences in retargeting efficiency.

Directed Evolution of AAV for Novel Gene Delivery Properties

In many situations, however, there is insufficient mechanistic knowledge to effectively empower rational design with the capacity to improve AAV. As an alternative, directed evolution has been emerging as a strategy to create novel AAV variants that meet specific biomedical needs.

Directed evolution is a high-throughput molecular engineering approach that has been successfully utilized to generate protein pharmaceuticals with enhanced biological activities, antibodies with enhanced binding affinity, and enzymes with new specificities.[34] The method emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule's function, even without knowledge of the underlying mechanistic basis for the problem. For AAV (Figure 1a), this process has involved mutating wild-type AAV cap genes to create large genetic libraries (described below), which can be packaged to generate libraries of viral particles, each of which is composed of a variant capsid surrounding a viral genome encoding that capsid. A selective pressure is then applied – such as high-affinity antibodies against the AAV capsid (Figure 1b), the need to bind new cell surface receptors or circumvent intracellular barriers (Figure 1c), or tissue structures that bar the virus from accessing target cells in vivo (Figure 1d) – to promote the emergence of variants able to surmount these barriers. After each such selection step, the successful variants can be recovered (e.g. by superinfection with a helper virus or PCR amplification) and used as the starting material for the next cycle of selection (Figure 1a, step 6) to further enrich for improved variants. If the process is halted after a single library diversification and selection step, it is referred to as library selection. However, after several such selection steps, directed evolution can be conducted by subjecting the resulting cap gene pool (Figure 1a, step 7) to additional mutagenesis and selection (Figure 1a, step 8). After library selection or directed evolution, the resulting variants can be analyzed clonally for the desired property.

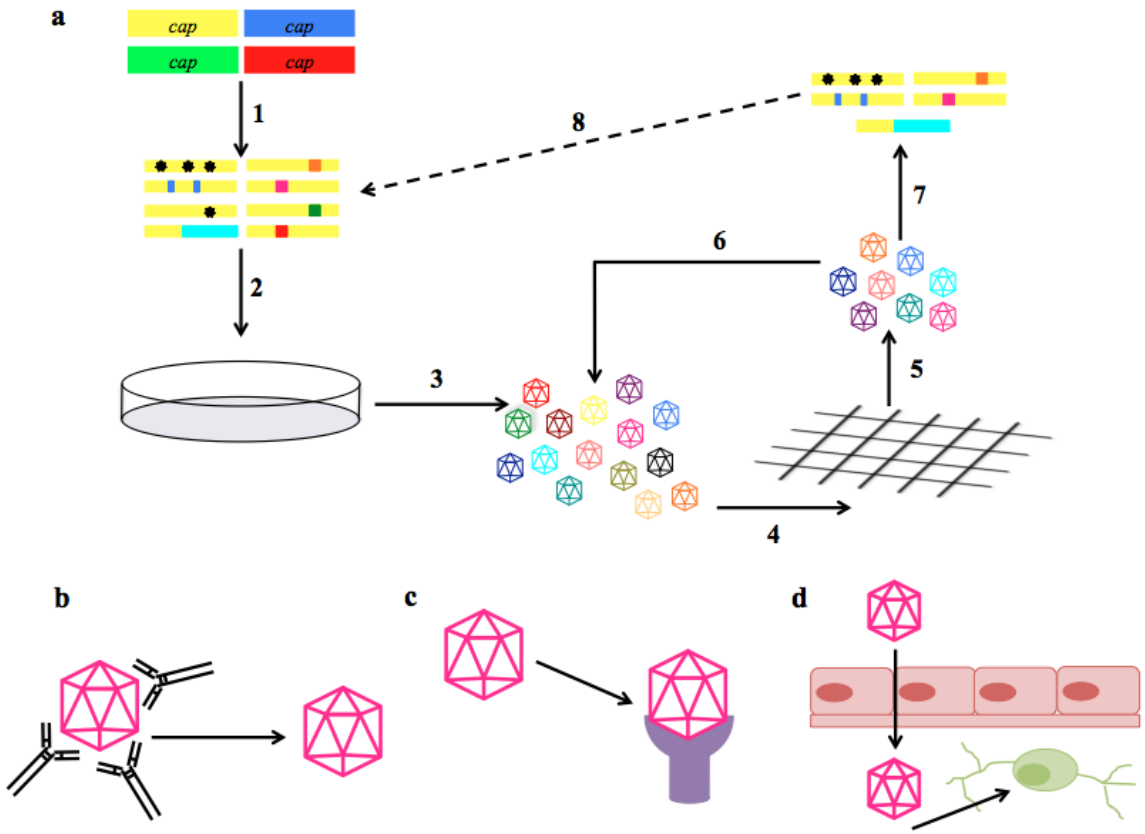


Figure 1. Directed evolution of AAV. (a) In a schematic of the process, 1) a viral library is created by mutating the cap gene. 2) Viruses are packaged (typically in HEK293T cells using plasmid transfection), such that each particle is composed of a mutant capsid surrounding the cap gene encoding that protein capsid. 3) Viruses are harvested and purified. 4) The viral library is placed under selective pressure. 5) Successful viruses are amplified and recovered. 6) Successful clones are enriched through repeated selection steps. 7) Isolated viral DNA reveals selected cap genes. 8) Selected cap genes are again mutated to serve as a new starting point for further selection steps to iteratively increase viral fitness. (b) – (d) Examples of selective pressure for directed evolution. (b) Evasion of antibody neutralization. (c) Altered receptor binding. (d) Cell specificity within complex tissue structures.

Successful library selection or directed evolution begins with the creation of high quality, high diversity libraries. In the last few years, a variety of library types have been created using several in vitro and in vivo techniques for viral DNA mutagenesis. The resulting libraries can be used individually or in combination to isolate novel variants. In addition, the techniques used to create the libraries can be used singly or in tandem for additional genetic diversification midway through the evolution process.

Library Classes

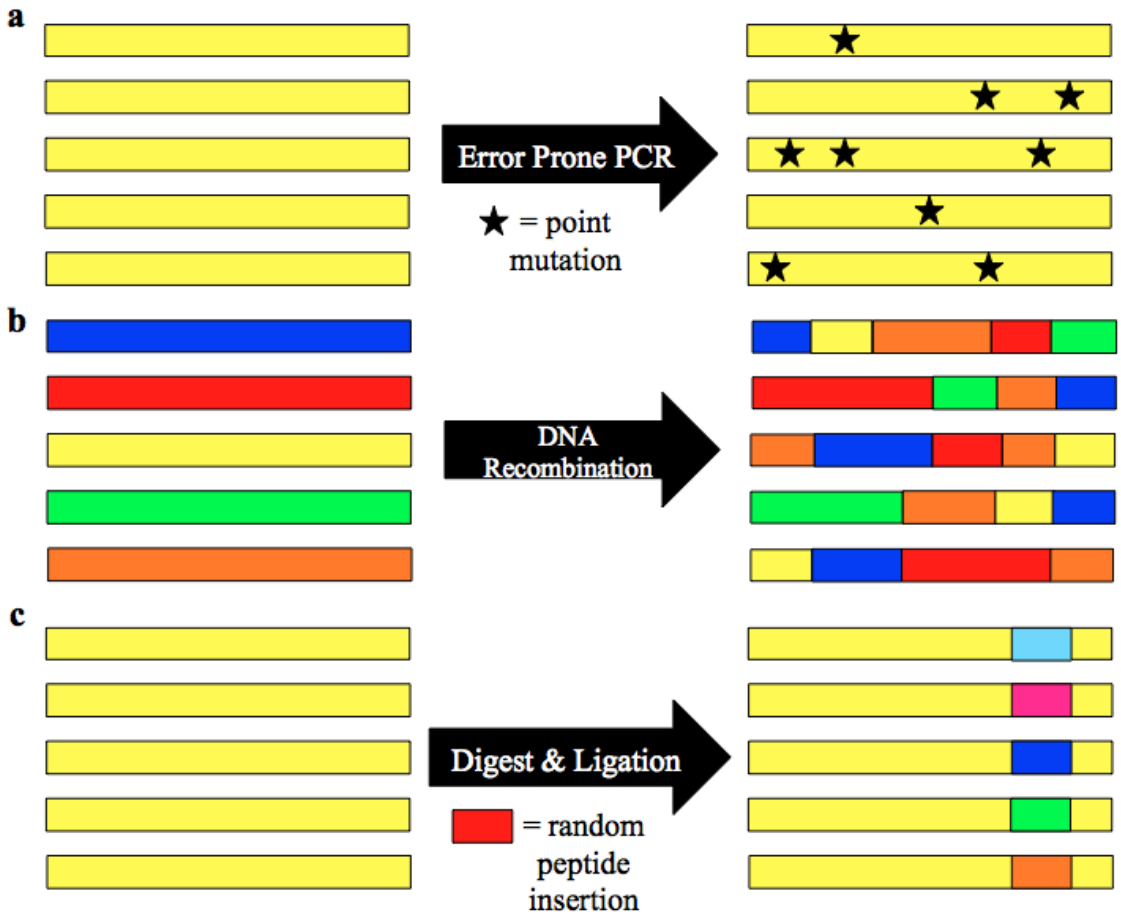


Figure 2. Schematic of library AAV capsid protein engineering strategies. Methods for generation of highly diverse viral libraries include (a) random point mutagenesis (error-prone PCR), (b) in vitro recombination (e.g. DNA shuffling), and (c) insertion of random peptides. Directed evolution strategies use these approaches as part of an iterative strategy to increase AAV's fitness for various applications.

As the most straightforward library, sub-optimal polymerase chain reaction (PCR), i.e. error-prone PCR, can be used to amplify and introduce random point mutations into the AAV cap ORF at a defined and tunable rate (Figure 2a).[35] This approach has been used to introduce mutations into either single[35,36] or multiple[37] AAV serotypes for subsequent selection.

In addition to point mutation, genetic recombination has been used to generate chimeric capsids. The earliest report of random chimeras of AAV cap genes was an in vivo viral rescue method, in which cellular co-transfection of a defective AAV2 genome with PCR fragments of the cap gene of another serotype (AAV3) led to rescued viral

chimeras capable of replication.[38] DNA shuffling, an in vitro PCR-based method, has subsequently been implemented to create large chimeric cap gene libraries composed of multiple serotypes (Figure 2b).[39-42]

Furthermore, random peptide sequences have been inserted into defined sites of the viral capsid, such as in the heparin binding domain of the AAV2 capsid (at capsid residue R588), via ligation of degenerate oligonucleotides into the cap ORF (Figure 2c).[43] This technique has recently been extended to AAV9.[44] Defined peptide-encoding sequences can also be inserted into random locations of the AAV2 cap ORF via transposon mutagenesis.[45] This approach was used to incorporate hexahistadine tags randomly throughout the AAV2 capsid to explore AAV clones capable of immobilized metal affinity chromatography purification.[46]

Finally, diversity can be focused on several hypervariable regions of the AAV capsid, which lie on surface-exposed loops. One of the first studies to conduct DNA shuffling of the AAV capsid noted that many of the functionally selected variants were composed primarily of a single serotype (AAV1 and AAV6) with surface loops exchanged with other serotypes.[40] This motivated the development of a "loop swap" library in which four loops of AAV2 were replaced with a library of peptide sequences designed based on the level of conservation of each amino acid position among natural AAV serotypes and variants.[47]

In Vitro Selection and Evolution

Alternate Receptor Targeting

AAV mutant libraries are being utilized in an increasing number of selection strategies to isolate novel variants, both in vitro and in vivo. For example, there is a strong biomedical motivation to generate AAV variants capable of transducing previously non-permissive cell types and/or to target gene delivery to specific, therapeutically relevant cell types. The important finding that insertion of small (seven amino acid) peptides into the heparin binding domain of the AAV2 capsid could alter viral tropism without disrupting capsid stability lay the groundwork for initial library selection approaches.[43,48] In particular, Perabo et al. inserted random peptide sequences into AAV2's heparin binding domain and selected the resulting library for the capacity to infect a human megakaryocytic cell line and a B-cell lymphocytic leukemia cell line.[48] Variants isolated from the human megakaryocytic cell selections shared an RGD motif and were capable of up to a 100-fold increase in transduction versus AAV2.[48] Müller et al. also generated a random peptide insertion library, selected for the capacity to infect primary human coronary artery endothelial cells, and thereby created variants capable of 4- to 40-fold increased gene transfer to endothelial cells in vitro compared to AAV2. These mutants also showed increased accumulation in heart tissue and decreased localization to liver in vivo, demonstrating that in vitro selections can produce variants with improved in vivo transduction properties.[43]

AAV2 random peptide library selections have subsequently been applied to improve transduction of primary human venous endothelial cells, lung carcinoma cells, prostate carcinoma cells,[49] acute myeloid leukemia cells (and other hematopoietic cancer cell lines),[50] and primary human hematopoietic progenitor cells.[51] Recent selection of an AAV9 random peptide insertion library for the capacity to transduce human coronary

artery endothelial cells yielded a variant with a 200-fold improved infection efficiency compared to AAV9 on postnatal human umbilical vein endothelial cells.[44]

In addition to AAV peptide display, other forms of mutagenesis can alter receptor binding. As a proof-of-concept, Maheshri et al. showed that directed evolution can be applied to modulate the affinity of AAV for its primary receptor. Specifically, they evolved AAV2 variants with both increased and decreased affinity for heparan sulfate proteoglycans by repeatedly selecting an error prone AAV2 library for elution from a heparin affinity chromatography column at salt concentrations either higher or lower than those needed to elute wild-type AAV2.[36] In addition, shuffling the cap genes of several AAV serotypes can enable a shift in viral tropism, potentially modulating affinity for existing viral receptors, pairing different primary and secondary receptors, or creating binding domains for new receptors. Li et al. used a shuffled library of AAV1-9 (excluding AAV7) to isolate a variant capable of melanoma cell transduction. A chimeric variant of AAV1, 2, 8, and 9 was more efficient at infecting hamster, mouse, and human melanoma cell lines in vitro, as well as hamster melanoma cell-derived tumors in vivo following direct injection.[41] Mutational studies to map the melanoma specific tropism identified residues 705 to 735 contributed by AAV9 as playing a critical but not sufficient role in the new tropism, demonstrating that these variants can contribute to knowledge of the structure-function relationships of capsid regions.[41]

Adult and Pluripotent Stem Cells

AAV can potentially be applied as an in vitro tool to improve the biomedical utility of stem cells. It is now well-recognized that AAV vector genomes carrying gene targeting constructs can mediate homologous recombination with target loci in a cellular genome at efficiencies 103-104-fold higher than corresponding plasmid constructs.[52] AAV-mediated gene targeting could thus aid in creating stem cell lines harboring mutations involved in human disease for basic investigation of disease mechanisms or high throughput in vitro small molecule drug discovery and toxicity studies. Gene targeting can also mediate the safe harbor integration of transgenes that guide differentiation into specific lineages, mediate secretion of therapeutic products, or enable cells to better resist the toxic effects of a diseased tissue. Finally, in the long term, gene correction of disease-causing mutations may enhance the therapeutic potential of individualized cell replacement therapies, such as ones based on pluripotent stem cells. However, while AAV-mediated gene targeting has been successfully applied to cells that AAV can effectively transduce, naturally occurring AAV variants are typically highly inefficient at infecting a number of stem cell types.

Directed evolution was first applied to stem cells by Jang et al. to isolate a variant capable of efficient transduction of neural stem cells (NSCs).[53] Selections using an error-prone AAV2 library, an AAV2 with random peptide insert library, and an AAV2 pairwise shuffled library on NSCs (from the adult hippocampus) yielded an AAV2 variant containing a peptide insertion that mediated 50-fold increased transduction of rat NSCs, as well as increased transduction of murine NSCs, human fetal NSCs, and human embryonic stem cell (hESC) derived neural progenitor cells.[53] Presumably as a result of the increased transduction, the variant also exhibited a 5-fold increased rate of targeted gene correction compared to natural serotypes in NSCs.[53]

The most successful report of gene targeting to human pluripotent stem cells using wild-type AAV demonstrated correct targeting of 1.3% of all colony forming units, which corresponds to an overall gene targeting frequency for the originally infected hESCs of approximately 0.03%.[54] To build upon this result, Asuri et al. applied directed evolution to create an AAV variant capable of enhanced gene delivery and gene targeting in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs).[37] Selecting an AAV2 and AAV6 error-prone library, an AAV2 loop-swap library, a shuffled library (containing AAV 1, 2, 4-6, 8, and 9), and a random peptide insert library for enhanced infection of hESCs yielded a simple AAV2 variant harboring a single R459G mutation. This mutant, which exhibited higher heparin affinity, was capable of increased transduction of the hESCs used in the selection, as well as other hESC and human iPSC lines.[37] In addition to increased transduction, the variant exhibited a 0.1% gene targeting efficiency, already substantially higher than plasmid-mediated gene targeting, which increased to over 1% in the presence of zinc-finger nuclease-induced double-stranded breaks at the target locus.[37] This increase in gene targeting efficiency was most likely due to an increase in both the total number of cells transduced by the variant, as well as an increase in the number of viral copies that infected each cell.[37]

Antibody Evasion

AAV has been successful in clinical studies involving delivery to immune privileged regions,[18-20,55] and immunosuppression is a promising approach for reducing antiviral cellular immune responses in general.[21,56-58] However, humoral immunity – for example anti-AAV antibodies resulting from childhood exposure to one or more serotypes, or from prior administration of an AAV vector – poses a significant challenge, particularly for intrahepatic and intravascular administration.[59,60] Recent analysis indicated that the prevalence of anti-AAV IgG antibodies in humans was highest for AAV2 (72%) and AAV1 (67%), but AAV9 (47%), AAV6 (46%), AAV5 (40%), and AAV8 (38%) antibodies were also present in a large portion of the population studied.[61] Other studies have shown a lower but significant prevalence of anti-AAV antibodies in the population against multiple AAV serotypes.[62]

Directed evolution can create AAV variants that evade such neutralizing antibodies. Using potent serum from rabbits immunized with AAV2 vector, Maheshri et al. evolved an antibody-resistant AAV2 variant using error-prone PCR and staggered extension process (StEP) mutagenesis over two rounds of evolution (i.e. two mutagenesis and six selection steps).[36] An AAV2-based variant containing E12A, K258N, T567S, N5871, and T716A point mutations emerged from the selections. Compared to vector with wild-type AAV2 capsid, a 96-fold higher neutralizing antibody concentration was required to neutralize the variant in vitro, and 100-1000 fold higher levels of antiserum were required in vivo.[36]

Perabo et al. selected an error-prone AAV2 library for variants capable of transduction in the presence of human serum. Mutations to amino acids 459 and 551 were dominant among the analyzed clones, and a variant with R459K and N551D substitutions withstood 5.5-fold higher neutralizing antibody levels compared to wild-type AAV2 in vitro.[35] Grimm et al. selected a library of shuffled cap genes from wild-type AAV serotypes of primate (AAV2, AAV4, AAV5, AAV8, AAV9) and nonprimate (caprine,

bovine, avian) origin for variants that could more efficiently transduce HepG2 liver cells in the presence of intravenous immunoglobulin (IVIG), the pooled IgG fraction from over 1000 human donors.[39] A single clone with sequence contributions from AAV2, AAV8, and AAV9 emerged from the selection and showed higher transduction efficiency compared to wild-type AAV1, 2, 3, 4, 5, 6, 8, and 9 in liver, kidney, fibroblast, and lung cell lines.[39] In addition, the variant exhibited antibody resistance levels comparable to AAV8 and AAV9, but much higher than AAV2 in vivo.[39]

Library selection can be guided by some aspects of rational design to analyze the effects of comprehensive amino acid substitutions at certain sites of interest. For example, Maersch et al. performed saturation mutagenesis at amino acid positions 449, 458, 459, 493, and 551 of the AAV2 capsid,[63] positions previously implicated in key antibody epitopes.[35,36] Though the resulting variants did not reach the antibody resistance levels of some natural serotypes, the mutants isolated from selections in the presence of human serum importantly reduced the antibody susceptibility of AAV2 while conserving tropism.[63]

In addition to selections designed specifically to reduce antibody neutralization, modulating other AAV properties (such as tissue or cell tropism) can serendipitously yield variants that also exhibit anti-AAV antibody resistance. For example, Koerber et al. selected functional AAV chimeras from a DNA shuffled library of cap genes from AAV serotypes 1, 2, 4-6, 8, and 9.[40] Though the selections did not involve antibodies, four out of the seven clones analyzed withstood higher neutralizing antibody concentrations than their parent serotypes, potentially through the loss of key epitopes at the junctures of capsid regions from different serotypes.[40] As another example, a chimeric variant isolated by Li et al. during selections on hamster melanoma cells had no cross-reactivity to antisera of mice immunized with AAV1, AAV8, or AAV9, and low cross-reactivity with AAV2.[41] In addition, a chimeric variant isolated by Yang et al. from in vivo selection to identify a muscle-targeting variant (described in more detail below) exhibited a similar level of in vitro resistance to IVIG as compared to AAV8 and a higher level of resistance compared to AAV2.[42] Finally, Varadi et al. demonstrated that random peptide insertions can alter AAV immunoreactivity, as AAV9-SLRSPPS and AAV9-RDVRAVS vectors exhibited enhanced in vitro transduction in the presence of IVIG compared to wild-type AAV2 and AAV9.[44] Furthermore, the anti-AAV9 antibody ADK9 did not neutralize the variants.[44] These results indicate that it may be possible to simultaneously select for two independent properties: antibody resistance and maintenance of existing or engineering of novel tropism.

In Vitro Models of Human Disease

Similar to the selections to alter the receptor binding of AAV, in vitro selections can be performed to increase transduction of specific cell types used in culture models of human disease. Excoffon et al. selected several libraries for efficient transduction of human airway epithelial cell cultures from the apical surface, a property that is critical for cystic fibrosis (CF) gene therapy and that natural AAV variants lack.[64] A chimera of the VP1-unique domain of AAV2 with the remainder of the AAV5 capsid, along with a key A581T point mutation in the sialic acid binding domain, emerged from two rounds of evolution. This evolved variant, AAV2.5T, bound to the apical surface of human airway epithelial cells at 100-fold higher levels than AAV5, transduced human airway nearly

100-fold more efficiently than AAV2 and AAV5, and as a result was able to correct the chloride ion transport defect of human cystic fibrosis airway epithelia upon delivery of the CFTR gene.[64] Li et al. created a library of shuffled cap genes from AAV1-6, 8, and 9 and selected for transduction of primary differentiated ciliated airway epithelium. The selections yielded two chimeras composed of AAV1, 6, and/or 9.[65] These variants were able to increase CFTR mRNA 25-fold over endogenous CF levels and rescue up to 31% of the normal CFTR response to forskolin in cystic fibrosis airway epithelia.[65]
Neurodegenerative diseases, in both the central and peripheral nervous systems, are also clinical targets that could significantly benefit from improved vectors. As one example, AAV typically has strong tropism for neurons; however, for a number of reasons it would be beneficial to develop variants that transduce glia. Astrocytes outnumber neurons in some regions of the nervous system, often play natural neuroprotective roles that can be further enhanced, and have even been shown to contribute to disease pathology in Alzheimer's Disease and Amyotrophic Lateral Sclerosis.[66,67] Using an AAV2 error-prone library, an AAV2 loop-swap library, a shuffled library containing AAV 1, 2, and 4-9, and AAV2 with random peptide insertions, Koerber et al. evolved variants for the ability to infect primary human astrocytes.[47] In addition to enhanced transduction of astrocytes in vitro, two AAV2-based variants from the shuffled and loop-swap libraries transduced 5.5- and 3.3-fold more astrocytes than AAV2 within the striatum following intracranial injection in rats.[47] As described below, one of these variants had highly advantageous properties in another neural tissue, the retina. Finally, Maguire et al. created a library of shuffled cap genes from AAV1, 2, 5, 8, 9, and 10 and selected for infection of glioblastoma multiforme cells.[68] The isolated variant was capable of efficient transduction of these cells in vitro and performed as well as or better than AAV2 on a panel of other glioma cells.[68]

In Vivo Selection and Evolution

Tissue-Specific Transduction

A number of variants selected in vitro have exhibited correspondingly promising properties in vivo. However, in numerous situations cell culture models cannot adequately emulate the properties of some complex tissues, such as those with complex physical and cellular barriers that can impede viral gene delivery, delicate cells that cannot be cultured, or situations in which targeted delivery to one cell type within a heterogeneous tissue is desired. In one of the first examples of in vivo selection, Michelfelder et al. isolated variants from an AAV peptide insertion library for the potential to transduce either the lung or a tumor cell graft.[69] The resulting clones mediated much higher gene expression in the target tissue; however, isolated vectors also transduced heart tissue (both tumor and lung variants) and other tissues (lung variants),[69] indicating that targeted in vivo delivery can be challenging.
Similarly, Yang et al. isolated a chimeric variant composed of AAV1, 6, 7, and 8 through in vivo biopanning for variants that infect muscle. Compared to AAV9 (the most efficient muscle-transducing serotype), the variant showed nearly equal cardiac infectivity and significantly decreased localization to the liver.[42] Interestingly, analysis of the variant's in vitro transduction of cardiomyocytes and in vivo transduction after direct muscle injection showed that its increased infectivity of muscle tissue was due to more efficient

crossing of tight endothelial barriers, an example where library selection provided both high infectivity of target cells and the ability to overcome a cellular barrier within the tissue.[42]

Crossing Physical and Cellular Barriers

Cellular and extracellular matrix barriers significantly impede the transport of macromolecules to target sites within tissues,[70] and AAV can be evolved for the ability to overcome these limitations. For example, the majority of retinal diseases afflict photoreceptors (PR) and retinal pigment epithelia (RPE), cells that lie deep within the retina. Numerous AAV serotypes can infect PR and RPE when administered subretinally (injected underneath the retina), which contributed to success in three recent clinical trials for Leber's congenital amaurosis.[18-20] However, unlike LCA, in the majority of retinal diseases the retina undergoes comparatively rapid cell death and tissue degeneration and can be further damaged by the retinal detachment that accompanies subretinal injection. This concern motivates the need for gene delivery approaches that can transduce or otherwise rescue PRs upon non-invasive administration to the vitreous humor of the eye. One type of retinal cell, the Müller glia, spans the entire length of the retina and contacts all retinal neuronal cell types. Klimczak et al. tested wither AAV variants previously selected for the ability to infect central nervous system (CNS) glia were capable of Müller cell transduction.[71] In vivo analysis revealed an AAV6-based mutant (ShH10) capable of highly specific (94%) and efficient infection of Müller cells compared to AAV2 and AAV6.[71] In subsequent work using a rat model of retinitis pigmentosa, infection of Müller glia with ShH10 encoding glial-derived neurotrophic factor (GDNF) slowed the progression of retinal degeneration and enhanced retinal electrophysiological responses for five months.[72]

While using Müller cells to secrete factors within the retina is a useful strategy, in other situations – such as dominant disorders directly affecting the outer retina – it would be advantageous to directly transduce photoreceptors. Dalkara et al. used a randomly mutagenized AAV2 cap library, a library of chimeric AAV cap genes from serotypes 1, 2, and 4-9, and a library with randomized peptides inserted near the three-fold axis of symmetry on the AAV2 capsid to evolve variants capable of transducing PRs from the vitreous humor of the eye.[73] A resulting variant mediated strong, pan-retinal expression in the photoreceptors with both ubiquitous and photoreceptor-specific promoters,[73] work with implications for the treatment of a range of retinal degenerative diseases.
Recent promising clinical studies have investigated CNS gene therapy to treat Canavan's disease [55,74] and Parkinson's Disease.[75] However, in some situations the target cells are surgically inaccessible, delicate, or span large regions of a tissue that would require multiple direct vector injections. Gray et al. selected a library of shuffled cap genes from wild-type AAV serotypes 1-6, 8, 9, and AAV8 with an E531K mutation for the ability to gain access to regions of the brain in which seizure had compromised the blood-brain barrier.[76] Two clones composed primarily of AAV1, 8, and 9 could transduce either the piriform cortex or both the piriform cortex and ventral hippocampus upon tail vein administration of the vectors after induction of CNS seizure,[76] and no transduction occurred in brain areas where the blood-brain barrier was not compromised. Within the targeted brain areas, the variants efficiently transduced oligodendrocytes and neurons, but not astrocytes or microglia, demonstrating a tropism consistent with that of AAV8.[76] This

approach highlights the potential for targeting specific regions of the CNS upon systemic administration.

Future Directions

Directed evolution is a powerful approach that enables relatively rapid selection and isolation of AAV variants with novel and therapeutically valuable properties. Numerous reports have now demonstrated the utility of this methodology to create AAV mutants capable of utilizing alternate cell-surface receptors for transduction, transducing specific cells and tissues *in vitro* and *in vivo*, and evading neutralizing antibodies. There are considerable additional opportunities to further improve the AAV vector repertoire and in turn, through investigation of the structure-function relationships of the resulting variants, to progressively enhance our understanding of AAV biology. For example, evolution could be employed as a forward genetic screen to identify and investigate functionally important regions of the AAV capsid. In addition, the discovery of an alternative reading frame within the *cap* gene encoding the assembly activating protein (AAP) potentially complicates the analysis of variants isolated from directed evolution.[2,3] Mutations that are silent in the *cap* ORF could constitute non-synonymous, advantageous mutations in AAP that affect viral assembly. In addition, while *in vitro* selections have generated vectors with properties that are useful *in vivo*,[36,39,47] it is likely that increasingly complex challenges for AAV vector engineering will require *in vivo* selection.[42,69,72]

Finally, as novel AAV variants enter into the therapeutic pipeline, they will likely progress closer to the clinic. Phase I and Phase I/II clinical trials involving wild-type AAV1, 2, 5, 6, and 8 serotypes have been approved by the U.S. Food and Drug Administration, and they are increasingly yielding promising results.[18-21,77] Recently, the first clinical trial involving an engineered AAV variant established that this rationally designed AAV1/2 chimera was safe and well-tolerated for treatment of Duchenne muscular dystrophy, laying the foundation for future trials involving additional AAV variants designed to suit a given clinical objective.[78] Engineered and evolved AAV vectors are therefore highly promising for a range of applications from the lab to the clinic.

References

1 Knipe, D. M. *et al. Field's Virology*. Fifth Edition edn, (Lippincott Williams & Wilkins, 2006).

2 Sonntag, F., Schmidt, K. & Kleinschmidt, J. A. A Viral Assembly Factor Promotes AAV2 Capsid Formation in the Nucleolus. *Proceedings of the National Academy of Science* **107**, 10220-10225 (2010).

3 Sonntag, F. *et al.* The Assembly-Activating Protein Promotes Capsid Assembly of Different Adeno-Associated Virus Serotypes. *Journal of Virology* **85**, 12686-12697 (2011).

4 Bartlett, J. S., Wilcher, R. & Samulski, R. J. Infectious Entry Pathway of Adeno-Associated Virus and Adeno-Associated Virus Vectors. *Journal of Virology* **74**, 2777-2785 (2000).
5 Duan, D. *et al.* Dynamin is Required for Recombinant Adeno-Associated Virus Type 2 Infection. *Journal of Virology* **73**, 10371-10376 (1999).
6 Nonnenmacher, M. & Weber, T. Adeno-Associated Virus 2 Infection Requires Endocytosis through the CLIC/GEEC Pathway. *Cell Host and Microbe* **10**, 563-576 (2011).
7 Sonntag, F., Bleker, S., Leuchs, B., Fischer, R. & Kleinschmidt, J. A. Adeno-associated Virus Type 2 Capsids with Externalized VP1/VP2 Trafficking Domains Are Generated Prior to Passage Through the Cytoplasm and Are Maintained Until Uncoating Occurs in the Nucleus. *Journal of Virology* **80**, 11040-11054 (2006).
8 Johnson, J. S. & Samulski, R. J. Enhancement of Adeno-Associated Virus Infection by Mobilizing Capsids into and out of the Nucleolus. *Journal of Virology* **83**, 2632-2644 (2009).
9 Johnson, J. S. *et al.* Mutagenesis of Adeno-Associated Virus Type 2 Capsid Protein VP1 Uncovers New Roles for Basic Amino Acids in Trafficking and Cell-Specific Transduction. *Journal of Virology* **84**, 8888-8902 (2010).
10 Lux, K. *et al.* Green Fluorescent Protein-Tagged Adeno-Associated Virus Particles Allow the Study of Cytosolic and Nuclear Trafficking. *Journal of Virology* **79**, 11776-11787 (2005).
11 Ferrari, F. K., Samulski, T., Shenk, T. & Samulski, R. J. Second-Strand Synthesis is a Rate-Limiting Step for Efficient Transduction by Recombinant Adeno-Associated Virus Vectors. *Journal of Virology* **70**, 3227-3234 (1996).
12 Kotin, R. M. *et al.* Site-specific Integration by Adeno-associated Virus. *Proceedings of the National Academy of Sciences* **87**, 2211-2215 (1990).
13 Duan, D. *et al.* Circular Intermediates of Recombinant Adeno-associated Virus have Defined Structural Characteristics Responsible for Long-term Episomal Persistence in Muscle Tissue. *Journal of Virology* **72**, 8568-8577 (1998).
14 Nakai, H., Storm, T. A. & Kay, M. A. Recruitment of Single-stranded Recombinant Adeno-associated Virus Vector Genomes and Intermolecular Recombination Are Responsible for Stable Transduction of Liver In Vivo. *Journal of Virology* **74**, 9451-9463 (2000).
15 Flotte, T. R. Gene Therapy Progress and Prospects: Recombinant Adeno-Associated Virus (rAAV) Vectors. *Gene Therapy* **11**, 805-810 (2004).
16 Schaffer, D. V., Koerber, J. T. & Lim, K.-i. Molecular Engineering of Viral Gene Delivery Vehicles. *Annual Reviews of Biomedical Engineering* **10**, 169-194 (2008).
17 Wu, Z., Asokan, A. & Samulski, R. J. Adeno-associated Virus Serotypes: Vector Toolkit for Human Gene Therapy. *Molecular Therapy* **14**, 316-327 (2006).
18 Maguire, A. M. *et al.* Age-dependent Effects of RPE65 Gene Therapy for Leber's Congenital Amaurosis: A Phase 1 Dose-Escalation Trial. *The Lancet* **374**, 1597-1605 (2009).
19 Maguire, A. M. *et al.* Safety and Efficacy of Gene Transfer for Leber's Congenital Amaurosis. *New England Journal of Medicine* **358**, 2240-2248 (2008).

20 Bainbridge, J. W. B. *et al.* Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis. *New England Journal of Medicine* **358**, 2231-2239 (2008).
21 Nathwani, A. C. *et al.* Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B. *New England Journal of Medicine* **365**, 2357-2365 (2011).
22 Edelstein, M. in *Journal of Gene Medicine* (John Wiley and Sons, 2011).
23 Girod, A. *et al.* Genetic Capsid Modifications Allow Efficient Re-Targeting of Adeno-Associated Virus Type 2. *Nature Medicine* **5**, 1052 - 1056 (1999).
24 Zhong, L. *et al.* Next Generation of Adeno-associated Virus 2 Vectors: Point Mutations in Tyrosines Lead to High-Efficiency Transduction at Lower Doses. *Proceedings of the National Academy of Science* **105**, 7827-7832 (2008).
25 Ponnazhagan, S., Mahendra, G., Kumar, S., Thompson, J. A. & Castillas, M., Jr. Conjugate-based targeting of recombinant adeno-associated virus type 2 vectors by using avidin-linked ligands. *J Virol* **76**, 12900-12907 (2002).
26 Arnold, G. S., Sasser, A. K., Stachler, M. D. & Bartlett, J. S. Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypes. *Mol Ther* **14**, 97-106 (2006).
27 Stachler, M. D., Chen, I., Ting, A. Y. & Bartlett, J. S. Site-specific modification of AAV vector particles with biophysical probes and targeting ligands using biotin ligase. *Mol Ther* **16**, 1467-1473 (2008).
28 Ried, M. U., Girod, A., Leike, K., Buning, H. & Hallek, M. Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptors. *J Virol* **76**, 4559-4566 (2002).
29 Bartlett, J. S., Kleinschmidt, J., Boucher, R. C. & Samulski, R. J. Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat Biotechnol* **17**, 181-186 (1999).
30 Lee, G. K., Maheshri, N., Kaspar, B. & Schaffer, D. V. PEG conjugation moderately protects adeno-associated viral vectors against antibody neutralization. *Biotechnol Bioeng* **92**, 24-34, doi:10.1002/bit.20562 (2005).
31 Carlisle, R. C. *et al.* Coating of adeno-associated virus with reactive polymers can ablate virus tropism, enable retargeting and provide resistance to neutralising antisera. *J Gene Med* **10**, 400-411 (2008).
32 Huttner, N. A. *et al.* Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies. *Gene Ther* **10**, 2139-2147 (2003).
33 Work, L. M. *et al.* Vascular bed-targeted in vivo gene delivery using tropism-modified adeno-associated viruses. *Mol Ther* **13**, 683-693 (2006).
34 Romero, P. A. & Arnold, F. H. Exploring Protein Fitness Landscapes by Directed Evolution. *Nature Reviews Molecular Cell Biology* **10**, 866-876 (2009).
35 Perabo, L. *et al.* Combinatorial Engineering of a Gene Therapy Vector: Directed Evolution of Adeno-Associated Virus. *Journal of Gene Medicine* **8**, 155-162 (2006).
36 Maheshri, N., Koerber, J. T., Kaspar, B. K. & Schaffer, D. V. Directed Evolution of Adeno-associated Virus Yields Enhanced Gene Delivery Vectors. *Nature Biotechnology* **24**, 198-204 (2006).

37 Asuri, P. *et al.* Directed Evolution of Adeno-associated Virus for Enhanced Gene Delivery and Gene Targeting in Human Pluripotent Stem Cells. *Molecular Therapy* **20**, 329-338 (2012).
38 Bowles, D. E., Rabinowitz, J. E. & Samulski, R. J. Marker Rescue of Adeno-Associated Virus (AAV) Capsid Mutants: a Novel Approach for Chimeric AAV Production. *Journal of Virology* **77**, 423-432 (2003).
39 Grimm, D. *et al.* In Vitro and In Vivo Gene Therapy Vector Evolution via Multispecies Interbreeding and Retargeting of Adeno-Associated Viruses. *Journal of Virology* **82**, 5887-5911 (2008).
40 Koerber, J. T., Jang, J.-H. & Schaffer, D. V. DNA Shuffling of Adeno-associated Virus Yields Functionally Diverse Viral Progeny. *Molecular Therapy* **16**, 1703-1709 (2008).
41 Li, W. *et al.* Engineering and Selection of Shuffled AAV Genomes: A New Strategy for Producing Targeted Biological Nanoparticles. *Molecular Therapy* **16**, 1252-1260 (2008).
42 Yang, L. *et al.* A Myocardium Tropic Adeno-Associated Virus (AAV) Evolved by DNA Shuffling and In Vivo Selection. *Proceedings of the National Academy of Sciences* **106**, 3946-3951 (2009).
43 Müller, O. J. *et al.* Random Peptide Libraries Displayed on Adeno-associated Virus to Select for Targeted Gene Therapy Vectors. *Nature Biotechnology* **21**, 1040-1046 (2003).
44 Bridgham, J. T., Ortlund, E. A. & Thornton, J. W. An epistatic ratchet constrains the direction of glucocorticoid receptor evolution. *Nature* **461**, 515-519, doi:10.1038/nature08249 (2009).
45 Koerber, J. T. & Schaffer, D. V. Transposon-Based Mutagenesis Generates Diverse Adeno-Associated Viral Libraries with Novel Gene Delivery Properties. *Methods in Molecular Biology* **434**, 161-170 (2008).
46 Koerber, J. T., Jang, J.-H., Yu, J. H., Kane, R. S. & Schaffer, D. V. Engineering Adeno-Associated Virus for One-Step Purification via Immobilized Metal Affinity Chromatography. *Human Gene Therapy* **18**, 367-378 (2007).
47 Koerber, J. T. *et al.* Molecular Evolution of Adeno-associated Virus for Enhanced Glial Gene Delivery. *Molecular Therapy* **17**, 2088-2095 (2009).
48 Perabo, L. *et al.* In Vitro Selection of Viral Vectors with Modified Tropism: The Adeno-associated Virus Display. *Molecular Therapy* **8**, 151-157 (2003).
49 Waterkamp, D. A., Muller, O. J., Ying, Y., Trepel, M. & Kleinschmidt, J. A. Isolation of Targeted AAV2 Vectors from Novel Virus Display Libraries. *Journal of Gene Medicine* **8**, 1307-1319 (2006).
50 Michelfelder, S. *et al.* Vectors Selected from Adeno-associated Viral Display Peptide Libraries for Leukemia Cell–targeted Cytotoxic Gene Therapy. *Experimental Hematology* **35**, 1766-1776 (2007).
51 Sellner, L. *et al.* Generation of Efficient Human Blood Progenitor–targeted Recombinant Adeno-associated Viral Vectors (AAV) by Applying an AAV Random Peptide Library on Primary Human Hematopoietic Progenitor Cells. *Experimental Hematology* **36**, 957-964 (2008).
52 Russell, D. W. & Hirata, R. K. Human Gene Targeting by Viral Vectors. *Nature Genetics* **18**, 325-330 (1998).

53 Jang, J.-H. *et al.* An Evolved Adeno-associated Viral Variant Enhances Gene Delivery and Gene Targeting in Neural Stem Cells. *Molecular Therapy* **19**, 667-675 (2011).
54 Khan, I. F. *et al.* Engineering of Human Pluripotent Stem Cells by AAV-mediated Gene Targeting. *Molecular Therapy* **18**, 1192-1199 (2010).
55 McPhee, S. W. J. *et al.* Immune Responses to AAV in a Phase I Study for Canavan Disease. *Journal of Gene Medicine* **8**, 577-588 (2006).
56 Halbert, C. L., Standaert, T. A., Wilson, C. B. & Miller, A. D. Successful Readministration of Adeno-Associated Virus Vectors to the Mouse Lung Requires Transient Immunosuppression during the Initial Exposure. *Journal of Virology* **72**, 9795-9805 (1998).
57 Jiang, H. *et al.* Effects of Transient Immunosuppression on Adeno-Associated, Virus-Mediated, Liver-Directed Gene Transfer in Rhesus Macaques and Implications for Human Gene Therapy. *Blood* **108**, 3321-3328 (2006).
58 Manning, W. C., Zhou, S., Bland, M. P., Escobedo, J. A. & Dwarki, V. Transient Immunosuppression Allows Transgene Expression Following Readministration of Adeno-Associated Viral Vectors. *Human Gene Therapy* **9**, 477-485 (1998).
59 Manno, C. S. *et al.* Successful Transduction of Liver in Hemophilia by AAV-Factor IX and Limitations Imposed by the Host Immune Response. *Nature Medicine* **12**, 342-347 (2006).
60 Stroes, E. S. *et al.* Intramuscular Administration of AAV1-Lipoprotein LipaseS447X Lowers Triglycerides in Lipoprotein Lipase–Deficient Patients. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28**, 2303 (2008).
61 Boutin, S. *et al.* Prevalence of Serum IgG and Neutralizing Factors Against Adeno-Associated Virus (AAV) Types 1, 2, 5, 6, 8, and 9 in the Healthy Population: Implications for Gene Therapy Using AAV Vectors. *Human Gene Therapy* **21**, 704-712 (2010).
62 Calcedo, R., Vandenberghe, L. H., Gao, G., Lin, J. & Wilson, J. M. Worldwide Epidemiology of Neutralizing Antibodies to Adeno-associated Viruses. *Journal of Infectious Diseases* **199**, 381-390 (2009).
63 Maersch, S., Huber, A., Büning, H., Hallek, M. & Perabo, L. Optimization of Stealth Adeno-associated Virus Vectors by Randomization of Immunogenic Epitopes. *Virology*, 1-9 (2009).
64 Excoffon, K. J. D. A. *et al.* Directed Evolution of Adeno-associated Virus to an Infectious Respiratory Virus. *Proceedings of the National Academy of Science* **106**, 3865-3870 (2009).
65 Li, W. *et al.* Generation of Novel AAV Variants by Directed Evolution for Improved CFTR Delivery to Human Ciliated Airway Epithelium. *Molecular Therapy* **17**, 2067-2077 (2009).
66 Nagele, R. G., Wegiel, J., Venkataraman, V., Imaki, H. & Wang, K. C. Contribution of Glial Cells to the Development of Amyloid Plaques in Alzheimer's Disease. *Neurobiology of Aging* **25**, 663-674 (2004).
67 Nagai, M. *et al.* Astrocytes Expressing ALS-linked Mutated SOD1 Release Factors Selectively Toxic to Motor Neurons *Nature Neuroscience* **10**, 615-622 (2007).

68 Maguire, C. A. *et al.* Directed Evolution of Adeno-associated Virus for Glioma Cell Transduction. *Journal of Neurooncology* **96**, 337-347 (2010).
69 Michelfelder, S. *et al.* Successful Expansion but Not Complete Restriction of Tropism of Adeno-Associated Virus by In Vivo Biopanning of Random Virus Display Peptide Libraries. *PLoS One* **4**, e5122 (5121-5113) (2009).
70 Perrault, S. D., Walkey, C., Jennings, T., Fischer, H. C. & Chan, W. C. Mediating Tumor Targeting Efficiency of Nanoparticles Through Design. *Nanoletters* **9**, 1909-1915 (2009).
71 Klimczak, R. R., Koerber, J. T., Dalkara, D., Flannery, J. G. & Schaffer, D. V. A Novel Adeno-Associated Viral Variant for Efficient and Selective Intravitreal Transduction of Rat Muller Cells. *PLoS One* **4**, e7467 (2009).
72 Dalkara, D. *et al.* AAV Mediated GDNF Secretion From Retinal Glia Slows Down Retinal Degeneration in a Rat Model of Retinitis Pigmentosa. *Molecular Therapy* **19**, 1602-1608 (2011).
73 Dalkara, D., Klimczak, R., Visel, M., Schaffer, D. & Flannery, J. in *American Society of Gene and Cell Therapy's 14th Annual Meeting* (Seattle, WA, 2011).
74 Cabrera-Salazar, M. A. *et al.* Timing of Therapeutic Intervention Determines Functional and Survival Outcomes in a Mouse Model of Late Infantile Batten Disease. *Molecular Therapy* **15**, 1782-1788 (2007).
75 Muramatsu, S.-i. *et al.* A Phase I Study of Aromatic L-Amino Acid Decarboxylase Gene Therapy for Parkinson's Disease. *Molecular Therapy* **18**, 1731-1735 (2010).
76 Gray, S. J. *et al.* Directed Evolution of a Novel Adeno-associated Virus (AAV) Vector That Crosses the Seizure-compromised Blood-Brain Barrier (BBB). *Molecular Therapy* **18**, 570-578 (2010).
77 Mueller, C. & Flotte, T. R. Clinical Gene Therapy Using Recombinant Adeno-Associated Virus Vectors. *Gene Therapy* **15**, 858-863 (2008).
78 Bowles, D. E. *et al.* Phase 1 Gene Therapy for Duchenne Muscular Dystrophy Using a Translational Optimized AAV Vector. *Molecular Therapy* **Advanced Online Publication** (2011).

Chapter 2

Evolving AAV for Increased Stability

Abstract

Mutations, especially those beneficial to new functions, are frequently destabilizing to protein structure. Therefore, a large percentage of library variation is lost due to protein instability. Stabilizing the protein template has been shown to increase the both the total number and number of functional mutations that are successfully accommodated. To extend this work to AAV, directed evolution was performed to stabilize the viral capsid and thereby increase its mutational robustness as well as improve the efficiency of selections for novel function. Mutational analysis was performed to determine the mechanism by which mutations contributed to increased thermostability. Four rounds of selection were performed. However, while more stable at higher temperatures, we found a trade-off between stability at higher versus lower temperatures.

Introduction

The majority of mutations are destabilizing to protein structure.[1,2] To mitigate such destabilization, beneficial de-stabilizing mutations often necessitate silent compensatory mutations.[3] This frequent necessity of beneficial and compensatory mutations can greatly decrease the rate of protein evolution.[4] This is paralleled in directed evolution studies that often require multiple rounds of selection in order to accumulate both stabilizing mutations and the cognate beneficial mutations.

It has been shown that stabilizing a protein of interest improves its evolvability.[5] Simulations indicated that extra stability promotes protein evolution by compensating for mutations beneficial for novel function, yet deleterious to protein folding. Stabilizing a protein substrate of directed evolution compensates for destabilizing mutations, thereby allowing a greater percentage of library variants to fold properly[5]. In addition, it has been shown that, aside from compensating for all mutations, stabilized proteins have the ability to compensate a far greater percentage of mutations that alter protein phenotype.[5] Utilizing stabilized template proteins to improve evolution of new function has been utilized for lactonase,[6] xylose isomerase,[7] and a Kemp eliminase.[8]

Here I describe my efforts to improve AAV evolvability by making it more thermostable. Four rounds of directed evolution were performed with clonal analysis after the first and fourth rounds. Mutational analysis allowed investigation into the residues within the AAV capsid protein that contributes to assembly and inter-subunit interactions. Unfortunately, one round of evolution did not improve mutational robustness and four rounds led to unexpected changes in capsid stability.

Results

Correlation of thermostability and thermodynamic stability

Few methods exist to directly select for increases in thermodynamic stability. Those that do rely on bacterial expression and do not select for maintenance of function[9]. To select for mutations that increase the thermodynamic stability of a protein, the authors expressed the protein library within E. coli as a fusion with a split β–lactamase; protein variants with higher stabilities led to a lower sensitivity to cellular proteases and higher levels of resistance to β-lactam antibiotics by the cell. Such a selection scheme is not available for stabilizing AAV as it must be expressed in mammalian cells for proper capsid assembly. Capid assembly could also be disrupted by the addition of a fusion protein to capsid subunits further preventing the use of this selection scheme.

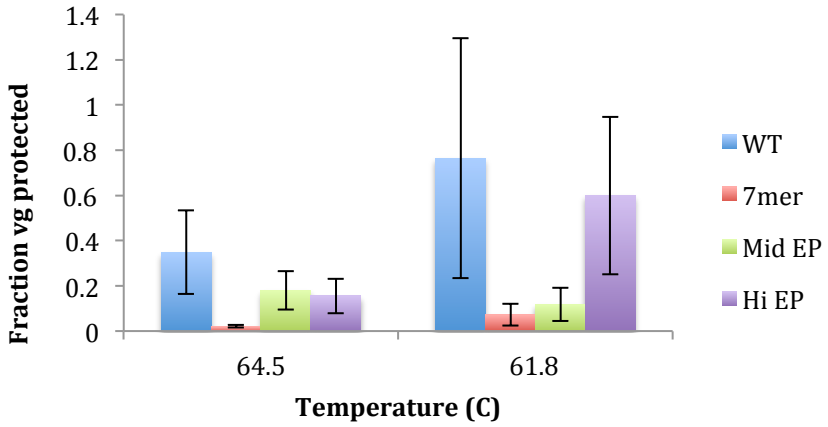


Figure 1. Thermostability of AAV2 libraries compared to wild-type. The amount of intact capsid at elevated temperatures was determined for an AAV2 peptide insert library and two error-prone libraries. Replication competent virus was heat-treated for 10 minutes and immediately cooled to 4°C. Genomes released from denatured capsids were digested with DNAse and protected genomes determined in triplicate by qPCR and standardized to non-heat treated controls. There appears to be a trend between destabilizing mutations and thermostability, however, this is not statistically significant. (error bars: standard deviation of the mean)

More commonly, proteins are selected for increased thermostability, the temperature at which a protein denatures and/or loses function.[10-12] To determine whether there was a correlation between thermostability of the AAV capsid and thermodynamically destabilizing mutations, wild-type AAV2 and multiple AAV2-based libraries, two mutagenized through error-prone PCR[13] and one containing random seven amino acid peptide inserts,[14] were heat-treated and the amount of viable virus was determined by qPCR, standardizing to a non-heat treated control (Fig. 1). Samples were treated with DNAse prior to qPCR analysis with intact capsids protecting the viral

genome against enzymatic degradation. Viral titers determined by qPCR, therefore, represent the concentration of intact capsid. Though not statistically significant there appears to be a trend between thermodynamic stability and thermostability for the AAV capsid. Perhaps if the experiment was performed with more replicates than the triplicate reported here, statisitical significance could be achieved. This suggested that increasing the thermostability of the viral capsid, a selectable quality, could allow for a wider range of mutations to be sampled in directed evolution experiments.

Thermostability of natural AAV serotypes

To determine the natural variability of thermostability within natural AAV serotypes, AAV1-3, 5, 6, 8, and 9 were heated at various temperatures, and the percent functional capsids were determined by infecting 293T cells (Fig 2). The natural serotypes exhibited a range of thermostabilities over a 10°C range, with the most commonly used serotype, AAV2, having one of the lowest thermostabilities. Interestingly, while only differing by six amino acids, AAVs 1 and 6 had the lowest and highest thermostabilities, respectively. This natural difference could be used to investigate thermostability differences within the viral capsid, the modularity of thermostabilizing mutations, and their effect on mutational robustness (Chapter 3).

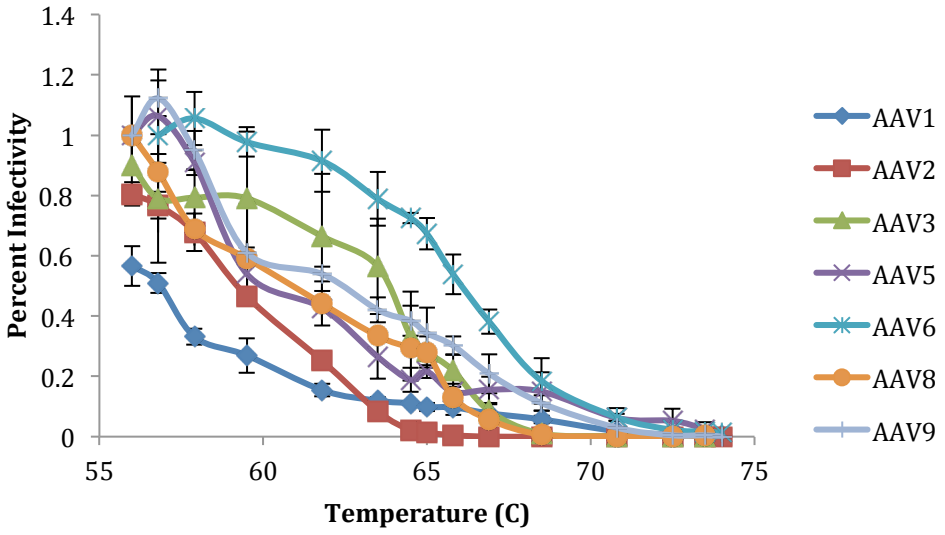


Figure 2. Thermostability comparison of AAV serotypes. The relationship between temperature and capsid integrity were determined for the natural AAV serotypes 1-3, 5, 6, 8, and 9. Recombinant virus was heat-treated for 10 minutes and immediately cooled to 4°C. Capsid integrity was measured by determining the remaining infectious MOI in triplicate and standardizing to non-heat treated standard. AAV2, the most commonly utilized serotype exhibited comparably low thermostability. (error bars: standard deviation of the mean)

Directed evolution to improve the thermostability of AAV2

To evolve an AAV with improved thermostability, an error-prone AAV2,[15] an AAV2-based 7mer-peptide insert,[14] and shuffled library[16] were packaged and selected for the ability to maintain viral activity after heat treatment (Fig. 3). Viral variants were heat-treated at temperatures at which 1 and 10% of wild-type virus infectivity remained. After heat treatment, the viable variants were amplified by infection on 293Ts and adenovirus-5 super infection. These rescued variants were then used for subsequent rounds of heat treatment and rescue. Three rounds of heat-treatment and rescue constituted one round of evolution. After each round of evolution, isolated variants were subjected to error-prone mutagenesis to introduce new, potentially stabilizing mutations and selection utilizing increasing temperatures for heat-treatment. A total of four rounds of evolution were performed. An analysis of the sequence and thermostability of individual variants was performed after the first and fourth rounds.

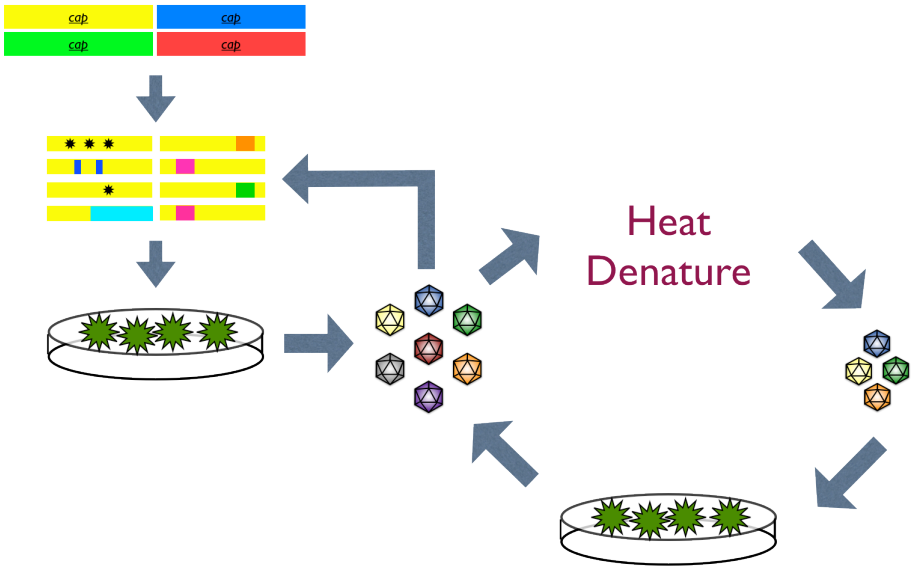


Figure 3. Selection scheme for AAV variants with improved thermostability. A combined library of AAV2-error prone, AAV2 7mer insert, and AAV shuffled libraries were packaged and selected for improved thermostability over AAV2. Selection for AAV mutants with increased thermostability was performed by heat-treating virus and precipitating denatured capsid proteins. Thernostabilized mutants were amplified through infection on 293T cells and adenovirus rescue. Rescued virus was then subjected to two more cycles of heat-denaturation and viral infection, constituting one round of selection. Viral *cap* genes were PCR-amplified and cloned into pXX2 for clonal analysis or subjected to subsequent rounds of error-prone mutagenesis and selection.

First round of directed evolution

After one round of evolution for improved thermostability, two dominant clones were present in the viral population, one containing an A593E mutation and one a Q598L mutation (Table 1). Both mutations are located at the interface of three capsid subunits

(three-fold axis of symmetry of the viral capsid) with residue 593 on the capsid exterior and 598 buried at the interface (Fig. 4)

Mutation	Frequency Round 1	Frequency Round 4	Interaction Energy[a]
A593E	4/8	14/14	-
Q598L	4/8	14/14	3-fold
N215Y	-	14/14	-
A218T	-	13/14	-
T491I	-	2/14	-
D514N	-	14/14	-
T597S	-	14/14	3-fold
F666V	-	3/14	5-fold

a. Residue's contribution to interaction energy between capsid monomers as indicated for the crystal structure on the Viper Database (http://viperdb.scripps.edu/)

Table 1. Capsid mutations identified from clones after one and four rounds of selection for increased thermostability. After one and four rounds of selection individual clones were sequenced to determine sequence convergence (eight clones for round one; fourteen for round four). Multiple amino acids are at residues determined to contribute to the interaction energy at either the three-fold or five-fold axis of symmetry.

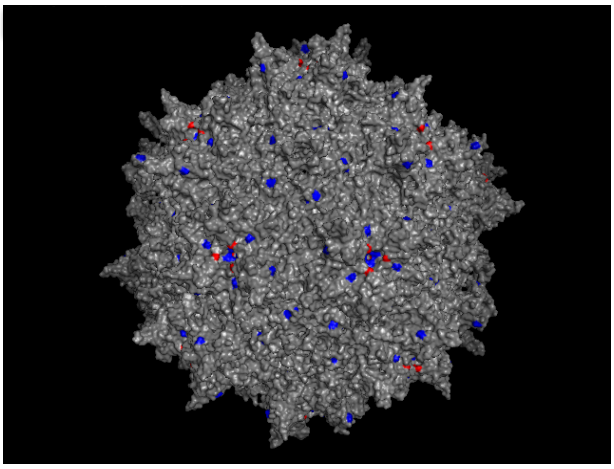


Figure 4. Location of mutations isolated from selection for increased thermostability. Mutations identified after one and four rounds of selection are highlighted on the AAV2 viral capsid. Mutations seen after the first round are shown in red, while those after the fourth round are shown in blue. Most mutations are isolated around the three-fold axis of symmetry.

To determine whether such mutations stabilized the AAV capsid, the thermostability of each clone was determined and compared to wild-type. Virus was heat-treated for 10 minutes and immediately cooled to 4°C. Capsid integrity was measured by determining the remaining infectious titer and standardizing to a non-heat treated control. Each mutation increased the thermostability of the capsid by 2°C (Fig. 5).

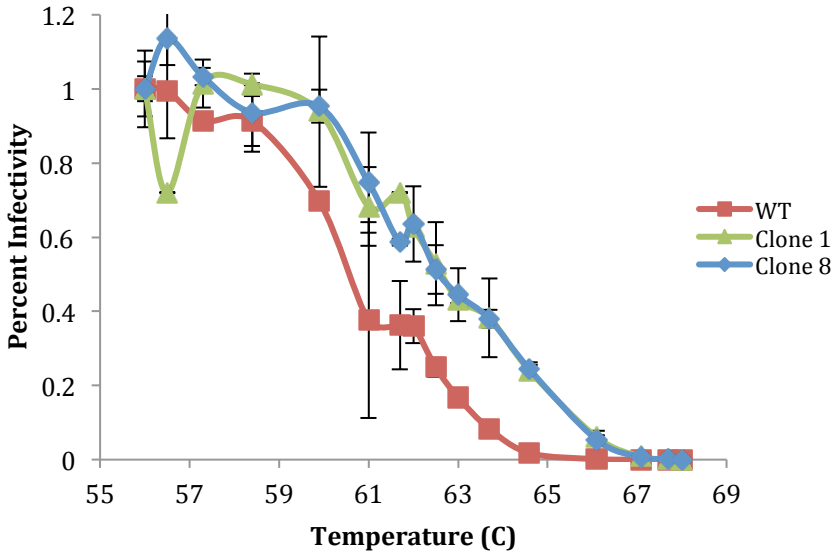


Figure 5. **Temperature dependence of capsid stability for wild-type AAV2 and clones isolated after one round of selection**. After one round of selection for improved thermostability, the relationship between temperature and capsid integrity for representative clones from the selection was determined. Virus was heat-treated and capsid integrity was measured by determining the remaining infectious MOI standardized to non-heat treated standard. Selected clones showed an improvement in thermostability of 2°C.

It has been shown that increased van der Waal's interactions within the protein core can improve thermostability of an enzyme.[17] Mutating the residues identified from selection on the crystal structure and determining the van der Waal interaction energy of the mutants indicated that the observed mutations improve thermostability of the capsid by a similar method. Specifically, both mutations increase van der Waal overlaps with surrounding residues by over 0.5 Å, increasing the interaction at the three-fold axis. Similarly, studies investigating the thermostability of minute virus of mouse, another parvovirus, showed an improvement of thermostability with increased interactions at the capsid three fold axis of symmetry, whereas foot-and-mouth disease virus exhibited greater thermostability with stabilizing interactions at the five-fold axis of symmetry.[18,19] This suggests that the intersubunit interaction energies of AAV and possibly mechanism of assembly of the viral capsid may differ from other viruses, especially those in other viral families.

As an alternative to such an increase in van der Waal's interactions, increased hydrophobicity within a protein core has been shown to increase the thermostability of enzymes[20]. Similar to those results seen on globular proteins, residue 598 buried at the subunit interface was mutated from the H-bonding glutamine to the hydrophobic leucine. This is in contrast, however, to residue 593 where it is mutated to an acidic glutamate, far less hydrophobic than the native alanine. This may suggest that this mutation increases thermostability through electrostatic effects, thereby altering the capsid surface charge density (Fig. 6).

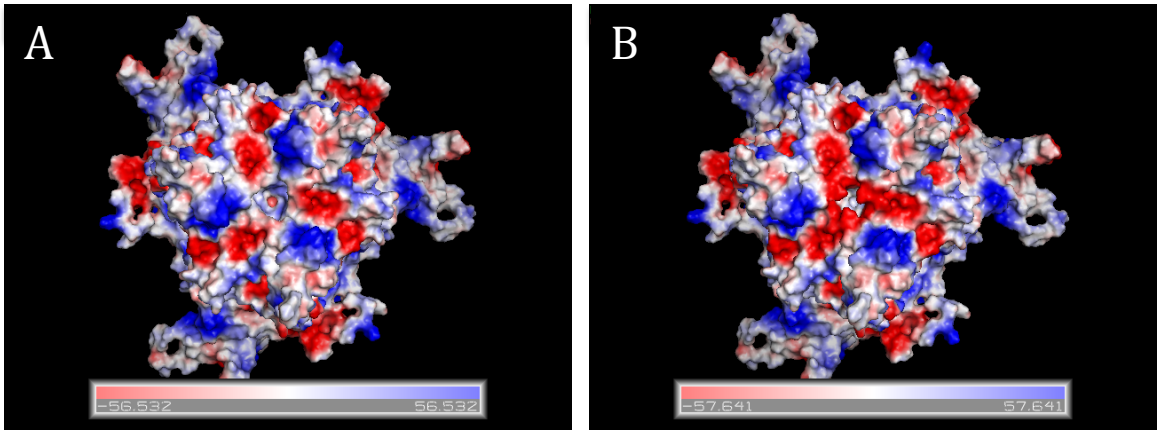


Figure 6. **Surface charge density of AAV2-WT and AAV2-A593E**. The wild-type AAV2 crystal structure was mutated at residue 593 to reflect the selected A593E mutation. The surface charge density of (A) wild-type capsid and (B) A593E mutant were then calculated within Pymol.

To determine to what extent the selected mutations improve mutational robustness, each was used as a template for error-prone mutagenesis. The titer from each library of mutagenized *cap* genes was used as read-out of mutational robustness; a higher titer indicates a greater proportion of variants that successfully package and, thus, a more robust template against mutation. Unfortunately, the isolated mutants showed no increase in mutational robustness compared to wild-type (data not shown).

Fourth round of directed evolution

Because the mutations selected from one round of directed evolution did not have a significant compensatory effect on other mutations, three additional rounds of selection were performed with the final selection at nearly 10°C higher than the first. After the fourth round of selection, one dominant clone was present, which included an additional mutation at the three-fold axis of symmetry (Table 1). The thermostability of this clone in concert with other selected point mutations, including one at the five-fold axis, was determined and compared to wild-type. Unfortunately, while the mutants displayed a higher percentage of functional capsid at the highest range of temperatures tested (Fig 7B), they appeared to be less stable at lower temperatures (Fig. 7A). Thus, while the

evolution yielded variants with the phenotype for which it was selected, it resulted in undesired side effects.

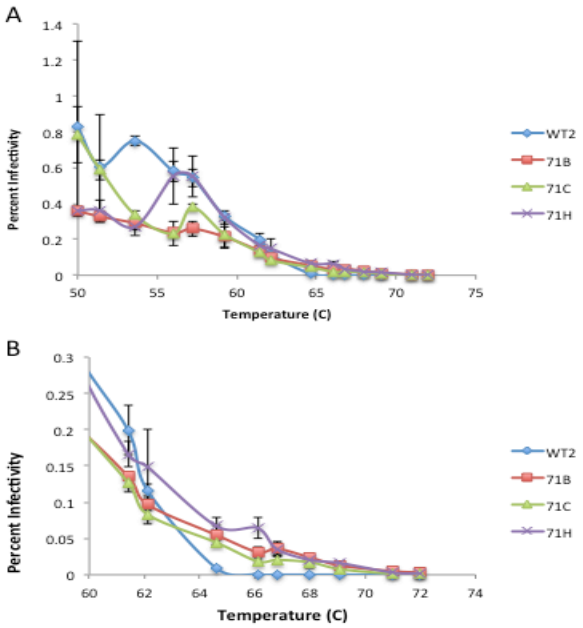


Figure 7. **Temperature dependence of capsid stability for wild-type AAV2 and clones isolated after four rounds of selection**. After four rounds of selection for improved thermostability, the relationship between temperature and capsid integrity for representative clones from the selection was determined. Virus was heat-treated for 10 minutes and immediately cooled to 4°C. (A) Capsid integrity was measured by determining the remaining infectious MOI in triplicate and standardizing to non-heat treated standard over a wide range of temperatures. At lower temperatures, selected clones showed either no improvement or a decrease in thermostability. (B) At higher temperatures, selected clones exhibited an improvement over wild-type. (error bars: standard deviation of the mean)

Discussion

The change in shape of the thermostability curve of the fourth-round mutants in comparison to wild-type could indicate a change in mechanism of denaturation. Furthermore, selection could have super-stabilized the three-fold axis of symmetry against denaturation, revealing or at the expense of a less stable capsid interaction. Alternatively, it has been shown that hepatitis B protein stability is optimized for viral replication kinetics, not protein stability[21]. Therefore, the selection for improved thermostability could have led to sub-optimal viral replication, requiring compensatory mutations, and resulting in unforeseen changes in thermostability.

Intersubunit interactions contribute to the stability of the viral capsid. Analysis of mutations contributing to improved thermostability provided insight into the residues of the AAV capsid important in intersubunit interactions. Many of the selected mutations

occurred at intersubunit interfaces, increasing hydrophobic and van der Waal's interactions, paralleling results seen from globular proteins.[17,20] As I (Chapter Three) and published work[22,23] have shown, mutations can stabilize the capsid through electrostatic effects, which can contribute to the thermostabilizing effect of the isolated A583E mutation.

While other viruses have been evolved to be more thermostable,[18,19] there is no literature precedent for a cognate improvement in mutational robustness. Viral proteins tend to increase robustness through partially disordered and loosely packed cores[24]. In this manner, improving capsid thermostability may have proven deleterious to mutational robustness, despite the correlation seen between mutation and thermostability.

While the ultimate goal of increasing the mutational robustness of AAV was not achieved, valuable insights into capsid stability, subunit interactions, thermostability and its effect on mutational robustness were gained. The viral capsid is a highly multi-functional multimeric protein complex. While structurally well defined and, therefore, not compensating for mutations in an identical fashion as non-structured viral proteins, it might have thermodynamics optimized for proper maintenance of its various functions such as externalization of the protein N-terminus and capsid disassembly. Thus, while the selected mutations might have led to an improvement in the desired quality of improved thermostability, it could have led to a loss of viral infectivity. Continued mutation through the multiple rounds of viral evolution might have led to compensatory, infectious mutations, allowing improved thermostability but not at the detriment of natural viral capsid function. This may have been seen as the radical change in the capsid thermostability profile and should be of note for studies trying to improve the stability of multi-functional and multimeric protein complexes.

Materials and Methods

Cell lines

Cell lines were cultured at 37°C and 5% CO_2. 293T cells were obtained from the American type culture collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Construction of AAV libraries

Error-prone mutagenesis of AAV2 was performed as described.[13] Briefly *cap* gene was mutagenized by $MnCl_2$ error-prone mutagenesis. Multiple PCR reactions of varying concentrations of manganese chloride were performed to provide a range of mutation frequency. Successful reactions were combined, purified with a commercial PCR clean up kit (Promega) to remove excess $MnCl_2$, and cloned into pSub2. Peptide insert and shuffled AAV libraries were also prepared as described.[14,16] Recombinant and replication competent AAV was then produced, as previously described.[13]

Determination of AAV thermostability profile

A method adapted from Mani et al [25], was utilized to determine the temperature dependence of capsid denaturation. Recombinant virus was heated for ten minutes in a thermocycler and immediately cooled to 4°C to precipitate denatured capsids. The percentage of intact capsids was determined by infecting 293Ts ($2.5 \times x10^5$) and standardizing to a non-heat treated sample. For replication-competent libraries, the percentage of intact capsids was determined by qPCR, whereby DNase resistant viral genomes of samples after heat-treatment was standardized to that of a non-heat treated sample.

Selection for thermostable AAV variants

An error-prone AAV2 library, AAV2-based 7mer-peptide insert library and an AAV shuffled library were heated in a thermocycler for ten minutes and denatured capsids precicipated from solution by cooling at 4°C for an additional ten minutes. Thermostable variants were rescued by infecting 293T cells (2.5×10^5) at varying genomic multiplicities of infection. Twenty-four hours after infection, media was changed and wild-type adenovirus added to amplify infectious variants. After forty-eight hours, viral particles were harvested and tittered by quantitative PCR. This viral pool was utilized for the next round of infection. After three heat treatment-rescue steps, viral genomes were harvested from which library *cap* genes were amplified by PCR and subject to additional mutagenesis to generate a library for additional rounds of selection.[15] Selection for a thermostable AAV variant as described here consisted of a total of four rounds of evolution by mutagenesis and selection.

Determination of van der Waal overlaps

The crystal structure of the AAV capsid protein was changed to reflect isolated mutations with the mutagenesis function in PyMol. Mutant crystal structures were saved and van der Waal's radii for each mutated residue were determined in MolProbity (http://molprobity.biochem.duke.edu/) and compared to that in wild-type.

Determination of mutational robustness

Mutational robustness was determined by subjecting a *cap* gene of interest to a high mutational load (10-15 nucleic acid mutations/*cap*) by error-prone mutagenesis and determining the fraction of viable mutants. The fraction of viable mutants was calculated as the titer of the mutagenized library over that of unmutagenized capsid.

References

1 Camps, M., Herman, A., Loh, E. & Loeb, L. A. Genetic constraints on protein evolution. *Critical reviews in biochemistry and molecular biology* **42**, 313-326, doi:10.1080/10409230701597642 (2007).

2 Tokuriki, N., Stricher, F., Schymkowitz, J., Serrano, L. & Tawfik, D. S. The stability effects of protein mutations appear to be universally distributed. *Journal of molecular biology* **369**, 1318-1332, doi:10.1016/j.jmb.2007.03.069 (2007).

3 Tokuriki, N., Stricher, F., Serrano, L. & Tawfik, D. S. How protein stability and new functions trade off. *PLoS computational biology* **4**, e1000002, doi:10.1371/journal.pcbi.1000002 (2008).

4 Tokuriki, N. & Tawfik, D. S. Stability effects of mutations and protein evolvability. *Current opinion in structural biology* **19**, 596-604, doi:10.1016/j.sbi.2009.08.003 (2009).

5 Bloom, J. D., Labthavikul, S. T., Otey, C. R. & Arnold, F. H. Protein stability promotes evolvability. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5869-5874, doi:10.1073/pnas.0510098103 (2006).

6 Zhang, Y. *et al.* Enhancing the promiscuous phosphotriesterase activity of a thermostable lactonase (GkaP) for the efficient degradation of organophosphate pesticides. *Applied and environmental microbiology* **78**, 6647-6655, doi:10.1128/AEM.01122-12 (2012).

7 Patel, D. H., Cho, E. J., Kim, H. M., Choi, I. S. & Bae, H. J. Engineering of the catalytic site of xylose isomerase to enhance bioconversion of a non-preferential substrate. *Protein engineering, design & selection : PEDS* **25**, 331-336, doi:10.1093/protein/gzs022 (2012).

8 Khersonsky, O. *et al.* Bridging the gaps in design methodologies by evolutionary optimization of the stability and proficiency of designed Kemp eliminase KE59. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 10358-10363, doi:10.1073/pnas.1121063109 (2012).

9 Foit, L. *et al.* Optimizing protein stability in vivo. *Mol Cell* **36**, 861-871, doi:10.1016/j.molcel.2009.11.022 (2009).

10 Salazar, O., Cirino, P. C. & Arnold, F. H. Thermostabilization of a cytochrome p450 peroxygenase. *Chembiochem : a European journal of chemical biology* **4**, 891-893, doi:10.1002/cbic.200300660 (2003).

11 Wu, I. & Arnold, F. H. Engineered thermostable fungal Cel6A and Cel7A cellobiohydrolases hydrolyze cellulose efficiently at elevated temperatures. *Biotechnol Bioeng*, doi:10.1002/bit.24864 (2013).

12 Kumar, R., Sharma, M., Singh, R. & Kaur, J. Characterization and evolution of a metagenome-derived lipase towards enhanced enzyme activity and thermostability. *Molecular and cellular biochemistry* **373**, 149-159, doi:10.1007/s11010-012-1483-8 (2013).

13 Koerber, J. T., Maheshri, N., Kaspar, B. K. & Schaffer, D. V. Construction of diverse adeno-associated viral libraries for directed evolution of enhanced gene delivery vehicles. *Nat Protoc* **1**, 701-706, doi:10.1038/nprot.2006.93 (2006).

14 Koerber, J. T. *et al.* Molecular Evolution of Adeno-associated Virus for Enhanced Glial Gene Delivery. *Molecular Therapy* **17**, 2088-2095 (2009).

15 Maheshri, N., Koerber, J. T., Kaspar, B. K. & Schaffer, D. V. Directed Evolution of Adeno-associated Virus Yields Enhanced Gene Delivery Vectors. *Nature Biotechnology* **24**, 198-204 (2006).
16 Koerber, J. T., Jang, J.-H. & Schaffer, D. V. DNA Shuffling of Adeno-associated Virus Yields Functionally Diverse Viral Progeny. *Molecular Therapy* **16**, 1703-1709 (2008).
17 LeMaster, D. M. & Hernandez, G. Residue cluster additivity of thermodynamic stability in the hydrophobic core of mesophile vs. hyperthermophile rubredoxins. *Biophysical chemistry* **125**, 483-489, doi:10.1016/j.bpc.2006.10.013 (2007).
18 Mateo, R., Luna, E., Rincon, V. & Mateu, M. G. Engineering viable foot-and-mouth disease viruses with increased thermostability as a step in the development of improved vaccines. *J Virol* **82**, 12232-12240, doi:10.1128/JVI.01553-08 (2008).
19 Reguera, J., Carreira, A., Riolobos, L., Almendral, J. M. & Mateu, M. G. Role of interfacial amino acid residues in assembly, stability, and conformation of a spherical virus capsid. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2724-2729, doi:10.1073/pnas.0307748101 (2004).
20 Clark, A. T., McCrary, B. S., Edmondson, S. P. & Shriver, J. W. Thermodynamics of core hydrophobicity and packing in the hyperthermophile proteins Sac7d and Sso7d. *Biochemistry* **43**, 2840-2853, doi:10.1021/bi0358263 (2004).
21 Tan, Z., Maguire, M. L., Loeb, D. D. & Zlotnick, A. Genetically altering the thermodynamics and kinetics of hepatitis B virus capsid assembly has profound effects on virus replication in cell culture. *J Virol* **87**, 3208-3216, doi:10.1128/JVI.03014-12 (2013).
22 Ding, Y., Cai, Y., Han, Y., Zhao, B. & Zhu, L. Application of principal component analysis to determine the key structural features contributing to iron superoxide dismutase thermostability. *Biopolymers* **97**, 864-872, doi:10.1002/bip.22093 (2012).
23 Di Venere, A. *et al.* Role of Arg403 for thermostability and catalytic activity of rabbit 12/15-lipoxygenase. *Biochimica et biophysica acta*, doi:10.1016/j.bbalip.2013.02.006 (2013).
24 Tokuriki, N., Oldfield, C. J., Uversky, V. N., Berezovsky, I. N. & Tawfik, D. S. Do viral proteins possess unique biophysical features? *Trends in biochemical sciences* **34**, 53-59, doi:10.1016/j.tibs.2008.10.009 (2009).
25 Mani, B. *et al.* Molecular mechanism underlying B19 virus inactivation and comparison to other parvoviruses. *Transfusion* **47**, 1765-1774, doi:10.1111/j.1537-2995.2007.01393.x (2007).

Chapter 3

Investigating Natural Stable AAV Serotypes for Gene Therapy

Abstract

Mutations, especially those beneficial to new functions, are frequently destabilizing to protein structure. Therefore, a large percentage of library variation is lost due to protein instability. Stabilizing the protein template has been shown to increase both the total number and number of functional mutations that are successfully accommodated. Differences in stability between the natural AAV serotypes were found to exist and could potentially be exploited to investigate both the mechanism by which improved capsid stability can arise and its effect on mutational robustness. Despite differing by only six amino acids, AAV serotypes 1 and 6 exhibit a greater than 10°C difference in thermostability. This difference was directly attributable to the E531K mutation within AAV6. The region surrounding this residue is homologous amongst most natural serotypes and the E531K mutation was enriched upon selection with shuffled libraries. Despite this, similar improvements were not seen upon mutation to other serotypes, allowing insight into the mechanism of this improved stability. Finally, investigation into the mutational robustness of the natural serotypes and stabilized mutant showed a trend between thermostability and mutational robustness, though this relationship was not statistically significant.

Introduction

As described earlier (Chapter 2), improved protein stability promotes evolvability. Here I describe my efforts to investigate and exploit stability differences between AAV serotypes. Viral serotypes represent distinct genotypic and phenotypic variations of a viral species. There are eleven naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino acid sequence and, thus, in their gene delivery properties.[1,2] Aside from differing tissue tropisms, AAV serotypes display unique thermostabilities.

AAV1 was one of the first AAV serotypes isolated as a viral contaminant in preparations of adenovirus in the mid-1960s.[3-5] A genetically similar serotype that differs from AAV1 by only six amino acids, AAV6, was discovered in 1998.[6] Despite so few amino acid differences AAV1 and AAV6 have distinct phenotypic properties, such as the ability of AAV6 to utilize heparan sulfate as a viral receptor.[7] Additionally, these two serotypes were shown to have a large difference in thermostability (Chapter 2). The two serotypes differ at residues 129, 482, 531, 584, 598, and 642 located at various regions of the AAV capsid. Such few amino acid differences allowed studies into the molecular basis of the difference in thermostability on a single amino acid level and whether thermostabilizing mutations can be transferred from one serotype to another in a modular

fashion. The difference in thermostabilities also allowed the investigation into differences in mutational robustness between AAV serotypes and stabilized mutants.

Results

Thermostability difference between AAV1 and AAV6

AAV serotypes 1 and 6, though differing in only six amino acids, exhibit a large difference in thermostabilities (Fig. 1). One residue (129) lies in the VP1 unique region of the capsid protein. Two of these residues (418 and 642) lie in the capsid interior (Fig. 2B), and two others (584 and 598) lie on the capsid exterior on inter-subunit interface of the three-fold axis of symmetry (Fig 2A). One (residue 531) lies on the capsid surface near the two-fold axis of symmetry (Fig 2A) and has been shown to be responsible for AAV6's ability to bind heparin.[8] Some mutation or combination of mutations was responsible for such a large shift, making this system valuable to investigate individual residue contributions to thermostability.

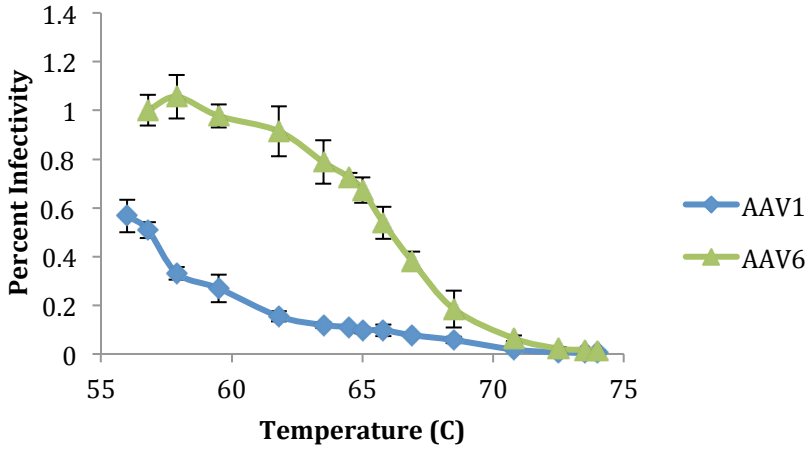


Figure 1. **Thermostability comparison of AAV serotypes 1 and 6**. The relationship between temperature and capsid integrity was determined for the natural AAV serotypes AAV1 and AAV6. Recombinant virus was heat-treated for 10 minutes and immediately cooled to 4°C. Capsid integrity was measured by determining the remaining infectious MOI in triplicate and standardizing to non-heat treated standard. Though differing in only six amino acids, AAV6 exhibited a thermostability far greater than that of AAV1. (error bars: standard deviation of the mean)

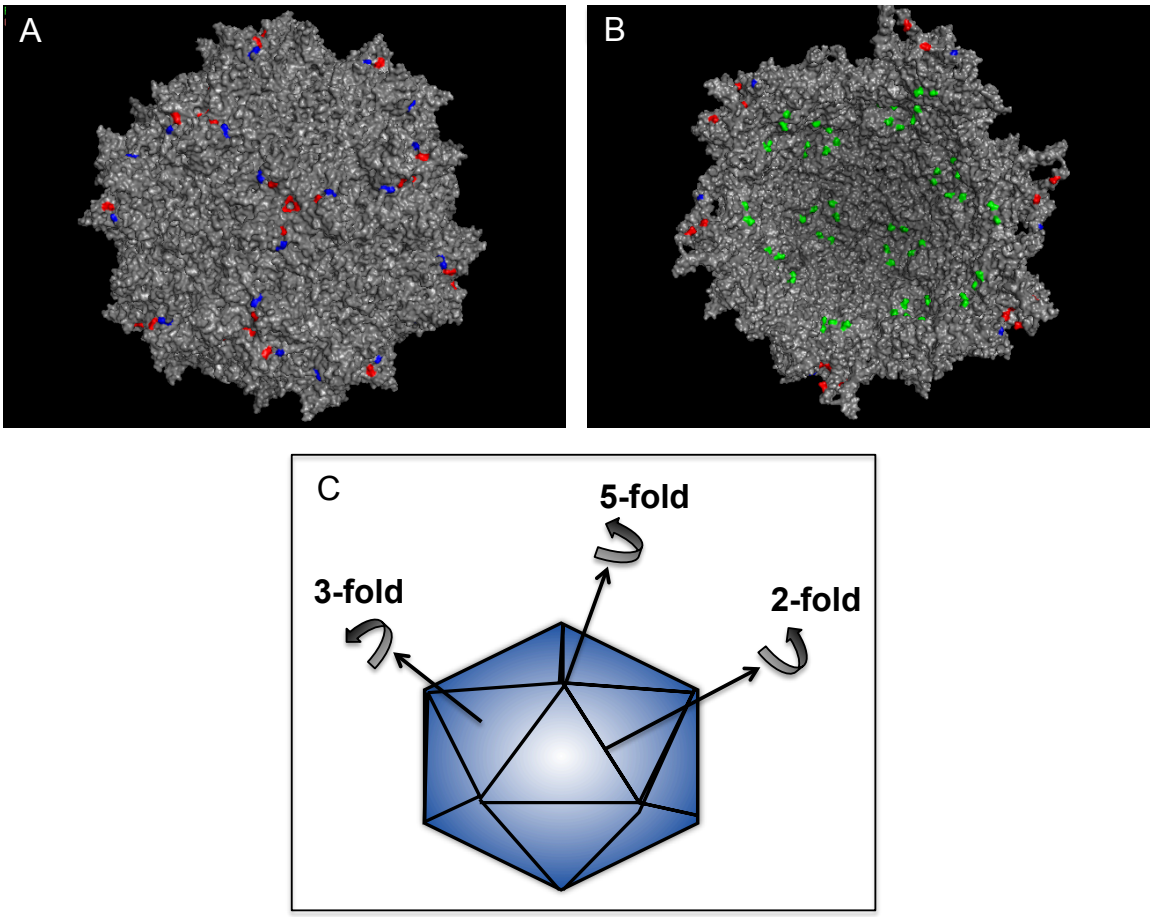


Figure 2. **Amino acid differences between AAV serotypes 1 and 6.** AAV1 and AAV6 differ from each other by six amino acids. Amino acids differences are shown on the AAV6 capsid (A) exterior and (B) interior. One (residue 129) lies in the VP1 unique region of the capsid protein, a region not included in the crystal structure. Two residues differing between the serotypes (418 and 642, shown in green) lie in the capsid interior and three others (584 and 598 in red and 531 in blue) lie on the capsid exterior on inter-subunit interfaces. (C) Representative diagram showing the different axes of symmetry within the icosahedral capsid.

Mutational analysis of thermostability difference

To determine which mutations contribute to the difference in thermostability between AAV1 and AAV6, individual residues on AAV1 were mutated to those of AAV6 through site-directed mutagenesis. The thermostability of each AAV1 mutant was then determined and compared to wild-type AAV1 and AAV6. Though mutations at the three-fold axis have been shown to improve the thermostability of AAV2 (see Chapter 2) and the crystal contacts between serotypes in this region are conserved, mutation of residues 584 and 598 had no appreciable effect on AAV1 thermostability (Fig. 3). Similarly, mutation of the interior residues, 418 and 642, had little effect on thermostability (Fig. 3). Mutation of residue 531, however, increased the thermostability of AAV1 almost to that of AAV6 (Fig. 3).

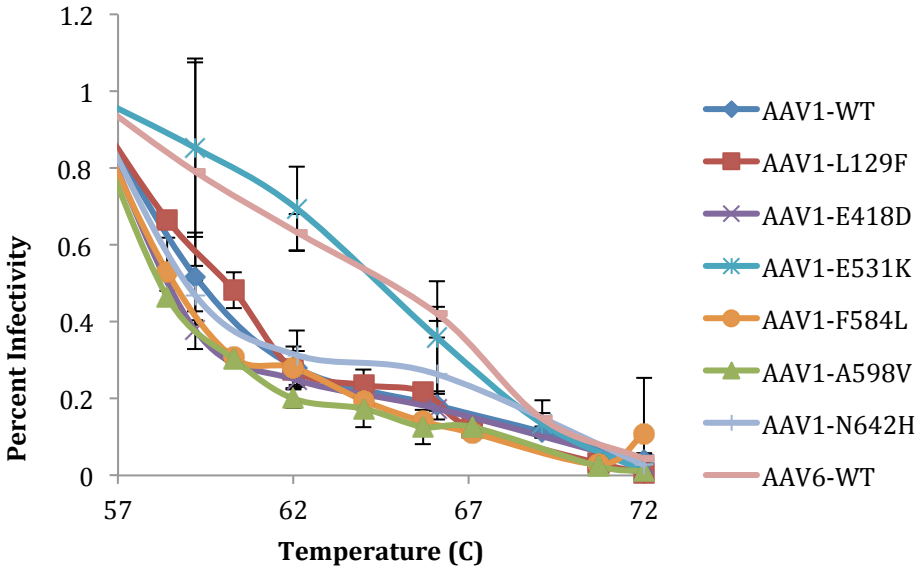
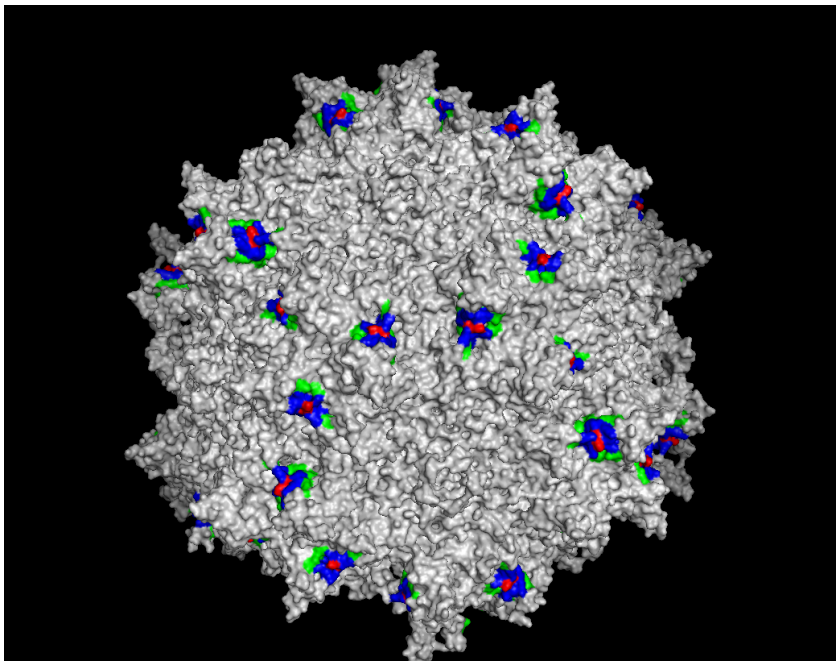


Figure 3. **Individual AAV6 residue's effect on thermostability**. To determine which amino acid contributed to the significant improvement of thermostability of AAV6 over AAV1, each differing AAV1 residue was mutated to that of AAV6. Virus was heat-treated for 10 minutes and immediately cooled to 4°C. Capsid integrity was measured by determining the remaining infectious MOI in triplicate and standardizing to non-heat treated standard. AAV1-E531K exhibited a thermostability very near that of AAV6 while all other single point mutations had a negligible effect. (error bars: standard deviation of the mean)

Effect of E531K mutation on the thermostability of other AAV serotypes

As the intention of raising thermostability was to increase mutational robustness, a modular mutation would prove useful for multiple serotypes. To determine whether the E531K mutation could be modular, the homology for the region surrounding the residue was determined; if the mutation improves thermostability by short-range interactions (< 8 Å), modularity would require that interaction partners be conserved. The residues within 6 and 8 Å of residue 531 on the AAV6 crystal structure were determined within PyMol (Fig. 4A), and those residues were aligned between the serotypes (Fig. 4B). Those residues close to residue 531 were conserved and, therefore, if the stabilizing effect of 531K acted over the short-range, the mutation may be modular.

A



B

AAV1 translation:	~^{383}N ~^{488}RVSK ~^{526}SHKDDEDKFF ~^{562}DEEEIK ~^{574}TE ~^{584}F
AAV2 translation:	~^{382}N ~^{487}RVSK ~^{525}SHKDDEEKFF ~^{561}DEEEIR ~^{573}TE ~^{583}L
AAV3 translation:	~^{382}N ~^{488}RVSK ~^{526}SHKDDEEKFF ~^{562}DEEEIR ~^{574}TE ~^{584}L
AAV6 translation:	~^{383}N ~^{488}RVSK ~^{526}SHKDDKDKFF ~^{562}DEEEIK ~^{574}TE ~^{584}L
AAV7 translation:	~^{384}N ~^{490}RVSK ~^{528}THKDDEDRFF ~^{563}NEEEIR ~^{575}TE ~^{585}L
AAV8 translation:	~^{385}N ~^{490}RVST ~^{528}THKDDEERFF ~^{564}SEEEIK ~^{576}TE ~^{586}L
AAV9 translation:	~^{384}D ~^{488}RVST ~^{526}SHKEGEDRFF ~^{562}NEEEIK ~^{574}TE ~^{584}H

Figure 4. **AAV homology in region surrounding AAAV1/6 residue 531**. (A) Amino acids within six (blue) and eight (green) angstroms of residue 531 (red) on the AAV6 viral capsid were determined and are shown. (B) Those residues within six (blue) and eight (green) angstroms to residue 531 on the AAV1/6 capsid were aligned to serotypes 2, 3, 7, 8, and 9. All given serotypes were homologous at those residues.

Further support of possible modularity would be evidence of the 531K mutation stabilizing shuffled clones, viral variants created by the shuffling of *cap* genes from multiple serotypes;[9] taken out of the context of the full AAV6 capsid, if the mutation was still stabilizing, it would indicate a potential for modularity. Stabilizing mutations increase the likelihood that a given variant is viable[10] and, thus, there should be a purifying selection towards its presence upon viral packaging. Library members from a shuffled library[9] were sequenced before and after viral packaging to determine if such an enrichment occurred for the AAV6 unique residue, K531. After packaging, a greater proportion of library members contained K531 (Fig. 5), indicating that the residue might stabilize shuffled library members. It has been shown that while compensating for all mutations, stabilizing variants compensate functional variants to a higher degree.[10]

Therefore, the frequency of K531 amongst published shuffled variants was determined. The AAV6 unique K531 appeared in published variants isolated with new phenotypes at an even greater frequency than packaged shuffled library members (Fig. 5), indicating a further enrichment of this mutation and providing additional evidence towards its stabilizing effect and modularity. Interestingly, in a shuffled library in which AAV6 was not included, an isolated variant had a spontaneous E531K mutation,[11] providing further evidence that this mutation could be stabilizing to other serotypes.

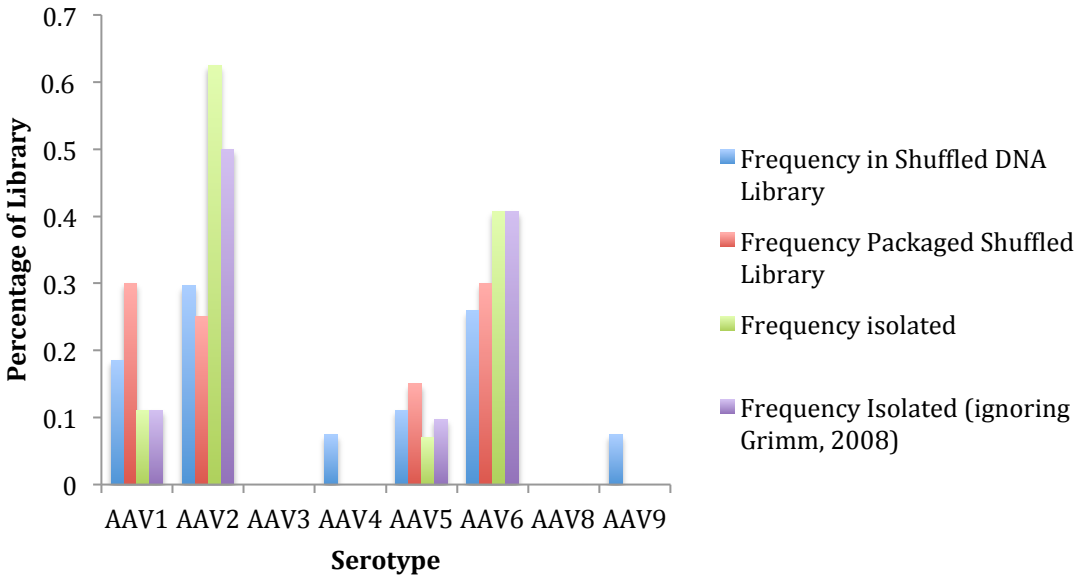


Figure 5. **Frequency of E531K mutation in shuffled libraries.** To determine whether the E531K mutation was stabilizing for shuffled AAV variants, its frequency within a shuffled library before and after packaging was determined. As stabilizing mutations not only increase folded mutants but improve the frequency of functional mutants, the frequency of the E531K mutation was determined within all published, isolated AAV shuffled clones. In addition to the enrichment for the E531K mutation after packaging and selection, one published clone had a spontaneous E531K mutation despite no AAV6 *cap* used as template in the shuffling reaction.[11]

To directly determine whether an E531K mutation could stabilize alternative serotypes, the corresponding residue within AAV2 and AAV9 was mutated through site-directed mutagenesis. The inclusion of the E531K mutation had no effect on viral titers (data not shown), however, the mutation ablated viral infectivity (data not shown) and lowered capsid thermostability (Fig 6). Therefore, the stabilizing effect of the E531K mutation on AAV6 arises from an interaction greater than the 8 Å for which homology was investigated.

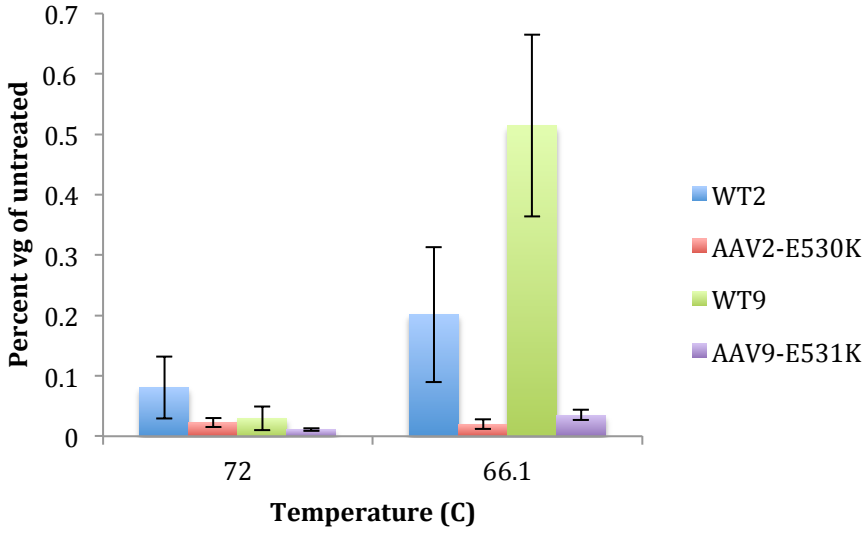


Figure 6. **E531K mutation on AAV2/9 thermostability**. To determine whether the stabilizing effect of the E531K mutation is transferrable to other serotypes, residue 531 was mutated to lysine on AAV2 and AAV9. To quantify any thermostabilizing effect, virus was heat-treated for 10 minutes and immediately cooled to 4°C. Genomes released from denatured capsids were digested with DNAse, and protected genomes quantified in triplicate by qPCR and standardized to non-heat treated controls. For both serotypes, the E531K mutation decreased the thermostability of the capsid and greatly reduced infectivity (data not shown). (error bars: standard deviation of the mean)

The E531K mutation could contribute to improved capsid stability by a long-distance electrostatic interaction. This residue is responsible for the affinity of AAV6 toward heparan sulfate,[8] through a largely electrostatic interaction. On the AAV6 capsid, residue K531 creates a basic patch with residues R488, K528, and K533 (Fig. 7) that is stabilized by an interaction of D532 with H527 and D562.[8] Aside from serotypes 4 and 5, most of these residues are relatively homologous amongst the naturally occurring serotypes; if not sharing identical amino acids at each residue, serotypes share similar amino acid chemistries. The one exception is D562, where serotypes 7-9 lack an acidic residue. The disruption of this D532-H527-D562 interaction in AAV9 could disrupt the stabilizing interaction between the peptide loops, destabilizing the capsid upon incorporation of the E531K mutation. Alternatively, loss of the negatively charged acidic residue could eliminate a direct stabilizing electrostatic interaction for K531. Mutation of one of these residues dramatically altered peptidoglycan interactions,[12] which could contribute to the loss of infectivity of the AAV9-E531K mutant. Comparing crystal structures, residue R488 is surface exposed on AAV6, whereas it is not in AAV2;[8] this could disrupt the basic patch to which K531 contributes and, thus, prevent its stabilizing effect. This indicates that, despite sequence homology, conformation-shifting interactions elsewhere within the viral capsid can shift the described residues' physical locations and, therefore, interactions within the viral capsid. Therefore, long-range electrostatic interactions and altered capsid tertiary structure might influence interactions within a

apparently homologous region of the capsid, precluding the modularity of stabilizing mutations.

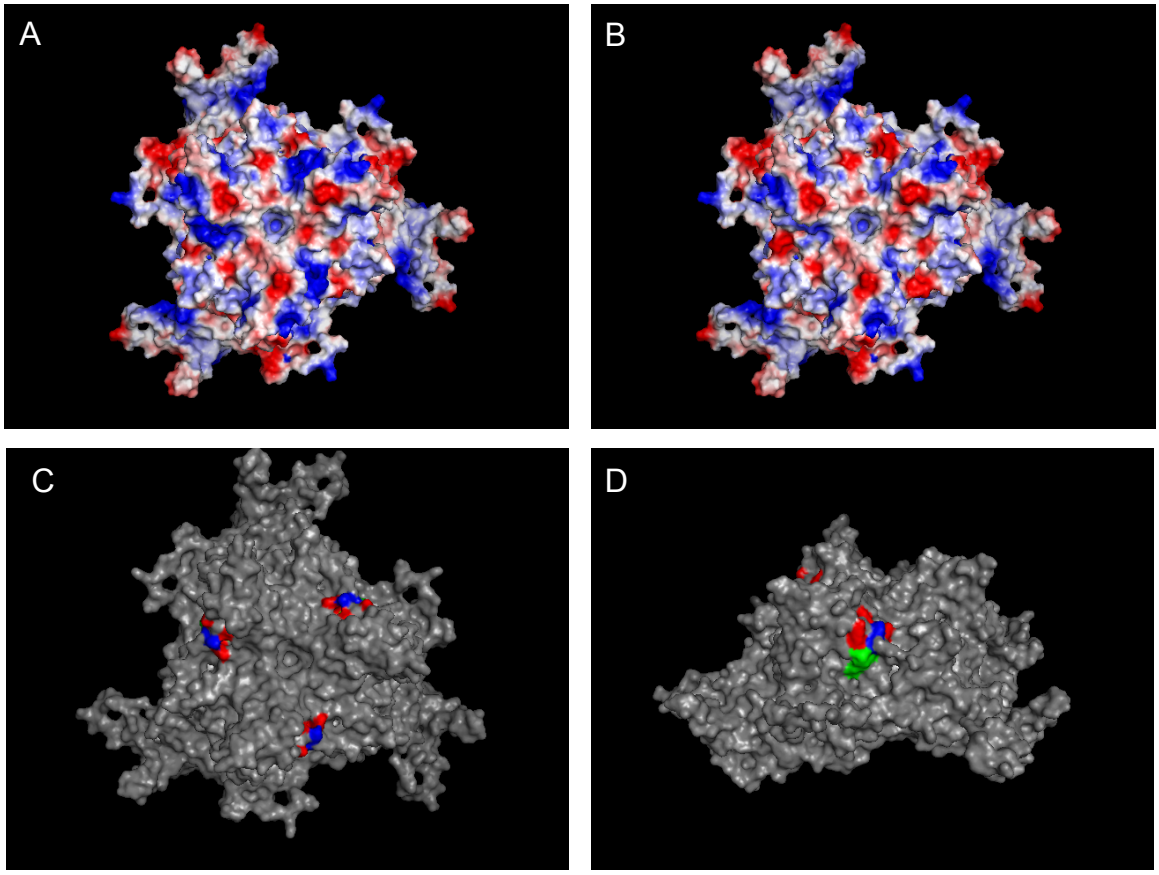


Figure 7. **Surface charge density of AAV6-WT and AAV6-K531E**. The wild-type AAV6 crystal structure was mutated at residue 531 to reflect the AAV1-K531E mutation. The surface charge density of (A) wild-type capsid and the (B) K531E mutant were then calculated within Pymol. (C and D) Locations of residues important to the formation and stabilization of the AAV6 basic patch. Residue 531 (shown in blue) forms a basic patch with residues 488, 528, and 533 (shown in red) on the AAV6 capsid surface. This basic patch is stabilized by an electrostatic interaction of residue 532 with residues 527 and 562 (shown in green). Contributing amino acids are viewed on a trimer of AAV6 capsid proteins (C) from above and (D) side on.

Determination of the effect of the E531K mutation on mutational robustness

To determine whether the change in stability with mutation at residue 531 led to a concomitant improvement in mutational robustness, AAV1, AAV-E531K, and AAV6 *cap* genes were subjected to a high mutational load and the fraction of viable mutants was determined. There appears to be an improvement in mutational robustness with the inclusion of the E531K (Fig. 8); however, large errors precluded statistical significance.

Viral titer as a metric inherently has much error due to the lack of reproducibility within transfections and standardizing library titers to unmutagenized capsid (the titer of which has similar issues with reproducibility) increases error to an even greater degree. Therefore, increased replicates beyond the triplicate presently described or a more accurate method to measure mutational robustness could provide a statistically significant difference between the serotypes and point mutant.

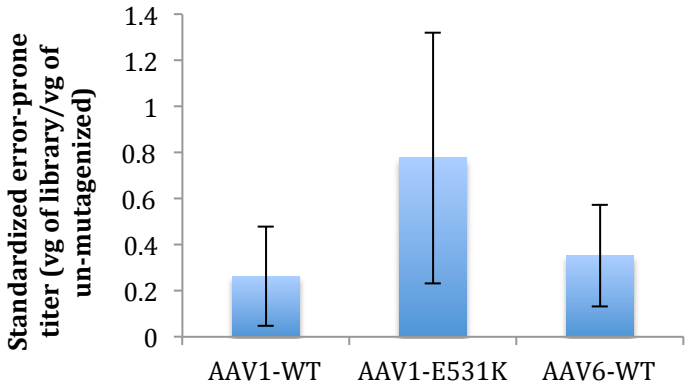


Figure 8. **Effect of E531K mutation on mutational robustness**. To determine whether the E531K mutation can compensate for destabilizing mutation AAV1-WT, AAV-E531K, and AAV6-WT *cap* genes were subject to high mutational loads by error-prone PCR and utilized to package virus. The titers for each error-prone library were standardized to the titer for unmutagenized capsid.

Discussion

Mutational analysis into the difference in stabilities between the natural serotypes AAV1 and AAV6 provided a greater understanding of the mechanisms of capsid stabilization. The predominant residue contributing to the improved thermostability of AAV6 in comparison to AAV1 was K531, a residue unique to AAV6 in a region relatively conserved amongst the serotypes 1-3 and 6-9. It has been shown that electrostatic interactions can contribute to the improved stability of an enzyme[13,14] and evidence suggests its possible contribution to improving the thermostability of AAV (see Chapter 2). In contrast to the mutations isolated from directed evolution, K531 does not lie at either the three or five-fold axes of symmetry, appears to exert its affect through a strictly electrostatic mechanism, and improves capsid stability to a larger extent. This mutation creates a continuous basic patch, likely improving capsid stability through a network of electrostatic interactions. This could explain the greater improvement in stability as compared to those isolated from directed evolution. This network of stabilizing electrostatic interactions might extend beyond the region that is directly homologous amongst the serotypes, precluding its modularity. Mutating the corresponding residue on AAV2 and AAV9 provided more evidence into those residues that might help contribute to the improved stability. AAV9 lacks an important residue to stabilizing the loop on which residue 531 lies,[8] which could have prevented improved stability upon the E531K mutation. Alternatively, a necessary residue within the AAV6

basic patch, while homologous amongst AAV serotypes, is not exposed on the AAV2 capsid,[8] which could indicate the contribution of capsid tertiary structure to capsid stability. Together this illustrates the fortuitous evolution of improved stability with a functional mutation.

While the E531K mutation greatly improved the thermostability of AAV1, it did not exhibit a similar trade-off between high and low temperature stability as seen with those clones isolated from directed evolution (Chapter 2). While this trade-off was attributed to the requirement that capsid stability is optimized for the simultaneous selective pressure of mutational robustness and maintenance of function,[15] the hypothesis does not appear to be valid for this difference. Electrostatic interactions within a protein can be shielded by salt concentrations and thereby modulate protein stability.[16] As the improvement to AAV6 stability is mostly through electrostatic interactions, proper capsid function might be maintained through shielding of the stabilizing electrostatic interactions within the crowded intracellular environment due to the significant increase in protein concentration. The concentration of proteins, highly charged macromolecules, is higher intracellularly than the experimental buffer and might shield the electrostatic interaction suggested to stabilize the AAV6 capsid. Alternatively, this interaction might be directly shielded by interactions between the viral capsid and accessory proteins necessary for intracellular trafficking. In this way, the investigation into the stability differences between natural serotypes provided an increased understanding of complex interplay between viral capsid stability and maintenance of function.

Materials and Methods

Cell lines

Cell lines were cultured at 37°C and 5% CO_2. 293T cells were obtained from the American type culture collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Construction of AAV variants

Individual AAV1 point mutants were made by site-directed mutagenesis with an Agilent Quikchange II Site-Directed Mutagenesis Kit (Santa Clara, CA). Recombinant AAV was then produced, as previously described.[17]

Determination of AAV thermostability profile

A method adapted from Mani et al [18], was utilized to determine the temperature dependence of capsid denaturation. Recombinant virus was heated for ten minutes in a thermocycler and immediately cooled to 4°C to precipitate denatured capsids. The percentage of intact capsids was determined by infecting 293Ts ($2.5 \times x10^5$) and standardizing to a non-heat treated sample. For those variants with undetectable infectivities, the percentage of intact capsids was determined by qPCR, whereby the

number of DNase resistant viral genomes after heat-treatment was standardized to that of a non-heat treated sample.

Determination of mutational robustness

Mutational robustness was determined by subjecting a *cap* gene of interest to a high mutational load (10-15 nucleic acid mutations/*cap*) by error-prone mutagenesis and determining the fraction of viable mutants. The fraction of viable mutants was determined as the titer of the mutagenized library over that of unmutagenized capsid.

References

1 Schaffer, D. V., Koerber, J. T. & Lim, K.-i. Molecular Engineering of Viral Gene Delivery Vehicles. *Annual Reviews of Biomedical Engineering* **10**, 169-194 (2008).

2 Wu, Z., Asokan, A. & Samulski, R. J. Adeno-associated Virus Serotypes: Vector Toolkit for Human Gene Therapy. *Molecular Therapy* **14**, 316-327 (2006).

3 Atchison, R. W., Casto, B. C. & Hammon, W. M. Adenovirus-Associated Defective Virus Particles. *Science* **149**, 754-756 (1965).

4 Hoggan, M. D., Blacklow, N. R. & Rowe, W. P. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proceedings of the National Academy of Sciences of the United States of America* **55**, 1467-1474 (1966).

5 Parks, W. P., Melnick, J. L., Rongey, R. & Mayor, H. D. Physical assay and growth cycle studies of a defective adeno-satellite virus. *J Virol* **1**, 171-180 (1967).

6 Rutledge, E. A., Halbert, C. L. & Russell, D. W. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol* **72**, 309-319 (1998).

7 Wu, Z. *et al.* Single amino acid changes can influence titer, heparin binding, and tissue tropism in different adeno-associated virus serotypes. *J Virol* **80**, 11393-11397, doi:10.1128/JVI.01288-06 (2006).

8 Ng, R. *et al.* Structural characterization of the dual glycan binding adeno-associated virus serotype 6. *J Virol* **84**, 12945-12957, doi:10.1128/JVI.01235-10 (2010).

9 Koerber, J. T., Jang, J.-H. & Schaffer, D. V. DNA Shuffling of Adeno-associated Virus Yields Functionally Diverse Viral Progeny. *Molecular Therapy* **16**, 1703-1709 (2008).

10 Bloom, J. D., Labthavikul, S. T., Otey, C. R. & Arnold, F. H. Protein stability promotes evolvability. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5869-5874, doi:10.1073/pnas.0510098103 (2006).

11 Grimm, D. *et al.* In Vitro and In Vivo Gene Therapy Vector Evolution via Multispecies Interbreeding and Retargeting of Adeno-Associated Viruses. *Journal of Virology* **82**, 5887-5911 (2008).

12 Klimczak, R. R., Koerber, J. T., Dalkara, D., Flannery, J. G. & Schaffer, D. V. A Novel Adeno-Associated Viral Variant for Efficient and Selective Intravitreal Transduction of Rat Muller Cells. *PLoS One* **4**, e7467 (2009).
13 Di Venere, A. *et al.* Role of Arg403 for thermostability and catalytic activity of rabbit 12/15-lipoxygenase. *Biochimica et biophysica acta*, doi:10.1016/j.bbalip.2013.02.006 (2013).
14 Ding, Y., Cai, Y., Han, Y., Zhao, B. & Zhu, L. Application of principal component analysis to determine the key structural features contributing to iron superoxide dismutase thermostability. *Biopolymers* **97**, 864-872, doi:10.1002/bip.22093 (2012).
15 Tokuriki, N., Oldfield, C. J., Uversky, V. N., Berezovsky, I. N. & Tawfik, D. S. Do viral proteins possess unique biophysical features? *Trends in biochemical sciences* **34**, 53-59, doi:10.1016/j.tibs.2008.10.009 (2009).
16 Zhang, N., Pan, X. M. & Ge, M. Without salt, the 'thermophilic' protein Mth10b is just mesophilic. *PLoS One* **7**, e53125, doi:10.1371/journal.pone.0053125 (2012).
17 Koerber, J. T., Maheshri, N., Kaspar, B. K. & Schaffer, D. V. Construction of diverse adeno-associated viral libraries for directed evolution of enhanced gene delivery vehicles. *Nat Protoc* **1**, 701-706, doi:10.1038/nprot.2006.93 (2006).
18 Mani, B. *et al.* Molecular mechanism underlying B19 virus inactivation and comparison to other parvoviruses. *Transfusion* **47**, 1765-1774, doi:10.1111/j.1537-2995.2007.01393.x (2007).

Chapter Four

Evolution of Small Polymorphic AAV Libraries

Abstract

Most commonly, directed evolution is performed by mutagenizing a protein of interest and selecting for a novel function. This procedure requires libraries of large sequence diversity and multiple rounds of evolution; its efficiency could thus be improved, such as by improving the quality of the library. Neutral drift provides a method that more closely parallels natural protein evolution, potentially yielding highly functionally diverse variants with a much smaller library size. Neutral drift has been applied to AAV2 in an attempt to yield a library of functionally diverse variants. After three rounds of selection for neutral mutations, a library that contained on average two non-silent mutations per variant remained. Mutations included within the library lie within every functional domain, greatly alter residue chemistry, and have interesting phylogenetic correlations.

Introduction

Directed evolution of proteins is commonly performed by subjecting a gene of interest to mutagenesis and selecting for those variants with a novel function, oftentimes repeating the protocol until the desired activity level is obtained. Unfortunately, the majority of mutations are destabilizing,[1,2] and mutations that do not ablate protein structure are rarely beneficial. Destabilizing beneficial mutations often require functionally-silent, stabilizing mutations to compensate.[3] Thus, large libraries are required for selection in order to increase the likelihood of isolating advantageous mutations, and often require multiple rounds of evolution and selection to achieve the desired levels of functionality.

However, this method strongly differs from how proteins naturally evolve. The majority of genetic diversity occurs through neutral drift, defined as gradual sequence diversity obtained through selection to maintain existing function and structure.[4] Viral quasi-species provide an example, whereby highly diverse populations of sequences are maintained under selection for maintenance of function.[5-7] It has even been shown that novel viral phenotypes can arise through neutral drift.[7] According to the theory of neutral evolution positive selection is a comparably rare event with the majority of mutations accumulating under purifying selection.[8,9] Thus, molecular evolution, rather than survival of the fittest, can be survival of the flattest;[10,11] variants with flatter fitness surfaces can tolerate more mutations and thus, are selected for over time. Alternatively, those with flat fitness surfaces sample more phenotypic diversity and, thus, show a fitness advantage under positive selection.[12]

An alternative method of directed evolution that more closely parallels this natural protein evolution involves the continuous neutral drift of a protein of interest to create a small library of highly polymorphic variants, variants that maintain native function but can be functionally promiscuous. Subjecting a protein of interest to neutral drift serves as a purifying selection, removing mutations that render it inactive, while

simultaneously leading to mutational robustness and evolvability greater than that of wild-type.[13] The subsequent library may contain divergent sequences that maintain wild-type folding and function with large changes in latent, promiscuous functions.[14,15] After applying neutral drift to serum paraoxonase (PON1)[14] and cytochrome P450 enzymes,[15] the enzymes maintained wild-type or near wild-type reactivity with the native substrate but were able to catalyze substrates for which the enzyme was not evolved to catalyze. Under high mutational rates, the population can evolve toward increased thermodynamic stability and mutational robustness,[16,17] highly desirable qualities in directed evolution studies. Additionally response to these higher mutation rates can drive the sequence of resulting variants toward the family consensus sequence and inferred ancestor.[16] Selections utilizing these methods have been utilized to engineer PON1,[13] β-glucuronidase,[18] and TEM-1[16] for novel functions. Variants shuffled from selected variants to combine mutations demonstrated further improvements in novel functionality.[13]

Here I describe the creation of a novel AAV2 library derived from neutral drift for use in future directed evolution studies. Each library variant contains on average two amino acid mutations and four silent mutations compared to the parent sequence. Mutations range from those that do not alter surface chemistry to those that remove or reverse charge density. Compared to previous studies, mutations frequently drove sequence away from consensus or toward an alternative highly divergent serotype.[16] Sampled mutations occur in nearly every functional domain within the AAV capsid, potentially indicating a library of highly functionally diverse variants.

Results

Creation of a small polymorphic AAV library

Adapting previous published methods,[13] adeno-associated virus serotype 2 was subject to three rounds of neutral drift. Specifically, AAV2 *cap* gene was subject to error-prone PCR with a high error rate (5-7 nucleic acid mutations/*cap*) and selected for its ability to maintain infection on a permissive cell line, 293T, at wild-type levels (Fig.1). Infectious virus was rescued by adenovirus-5 super infection and used to repeat the infection. Three infection-rescue steps constituted one round of evolution. After the first round of evolution isolated mutants contained only one to two mutations. Therefore, subsequent mutagenesis steps were performed with an increased error rate (10-15 nucleic acid mutations/*cap*). In total, AAV was subjected to three rounds of evolution to select for neutral mutations.

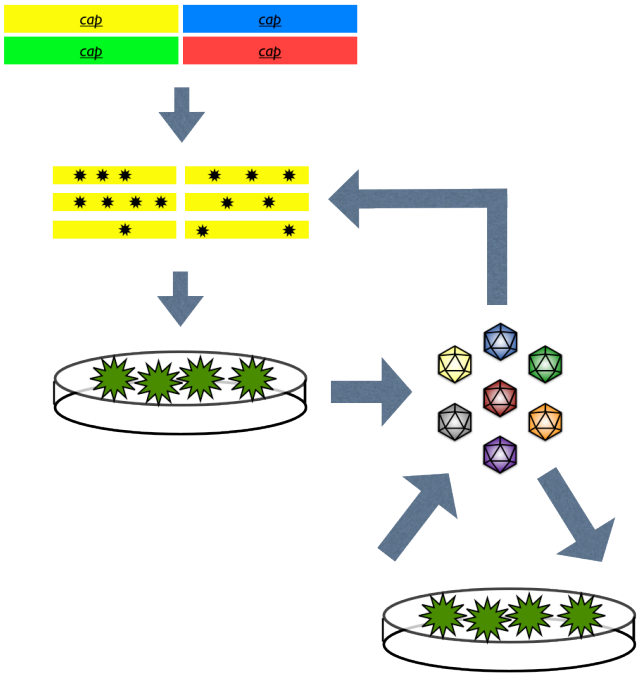


Figure 1. **Selection scheme for neutral drift of AAV2**. An AAV2 error-prone was packaged and selected for improved thermostability over AAV2. Selection for neutral mutations was performed by infecting 293T cells at a wild-type MOI followed by adenovirus super-infection rescue. Rescued virus was then subject to two more cycles of viral infection, constituting one round of selection. Viral *cap* genes were PCR-amplified and subjected to subsequent rounds of error-prone mutagenesis and selection.

Sequence analysis of neutral drift library

After three rounds of neutral drift, individual clones from the selection were sequenced. No two sequenced clones were identical, indicating sequence diversity remains within the library. Some mutations, notably an S452G, were found in multiple clones, indicating the presence of a bottleneck at some stage within the selection or a stabilizing effect of those mutations.[16] Despite the high mutational loads applied during mutagenesis, library members only contain 2.3 ± 1.1 amino acid changes and 3.8 ± 1.8 silent mutations versus the wild-type AAV2 sequence. It is possible the high mutational loads surpassed that which the AAV capsid could tolerate, so those containing fewer mutations were strongly selected for. Alternatively, requiring AAV variants to infect 293T cells might have biased the library for this infections step, thus limiting library diversity, both of note for any subsequent neutral drift studies on AAV. Alternatively, increasing concentrations of manganese chloride has been shown to increase the frequency of stop codons, insertions, and deletions within the error prone PCR mutagenesis reaction (data not shown). Alternative error-based mutagenic methods that reduce these unwanted side effects have been described.[19] These methods should be strongly considered in subsequent studies to ensure library members with high numbers

of mutations are lost due to surpassing the mutational load the capsid can tolerate, rather than premature stop codons or frame shift mutations.

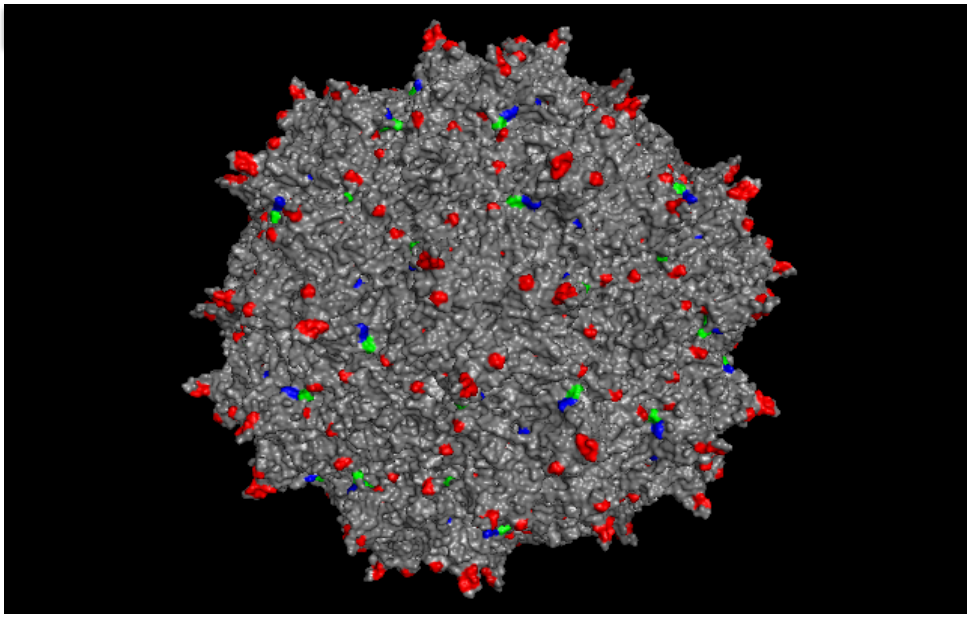


Figure 2: **Location of mutations isolated from neutral drift selection.** Mutations identified after three rounds of neutral drift are highlighted on the AAV2 viral capsid. Many mutations lie on or near the three-fold spike, which has been shown to be important in receptor binding. Mutations were also found on nuclear localization sequences and a phospholipase A2 domain on the uncrystallized VP1/2 unique regions. Mutations that lead to a loss of charge are shown in blue and those that mutate residues conserved amongst AAV serotypes are shown in green. All others are shown in red.

While many individual mutations were minimally perturbing to amino acid chemistry (polar to polar or hydrophobic to hydrophobic), multiple instances of significant changes to residue chemistry are present within the library. There are several instances of loss of charge mutations (K544M and R566G). This is particularly interesting in the context of the significant Coulombic interaction of AAV2 with heparan sulfate.[20] More dramatically, mutations that reversed the polarity of a given residue (D152K) were isolated.

Although the number of mutations per isolated clone was low, mutations were identified in regions implicated in every step of viral infection. Many identified mutations clustered around the three-fold spike, a region of the AAV capsid crucial in receptor binding (Fig. 2). Mutations were identified in or near regions necessary for binding to the viral primary receptor, heparan sulfate,[20,21] as well as binding to identified co-receptors, the laminin receptor[22] and integrin α5β1.[23] Mutations were also found in regions important in intracellular trafficking and endosomal escape (Table 1). Mutations were isolated in three of the four basic patches required for nuclear trafficking[24,25] as well as

within the phospholipase A2 domain, important in endosomal escape.[26,27] New evidence suggests the presence of other important motifs within the viral capsid necessary for intracellular trafficking and nuclear entry.[28] Mutations within the library lie within many of these motifs including one found to be necessary for nuclear uptake.

Mutation	Region
T14S	
E36Q	
S42I	Near PLA2
E63Q	PLA2
E106G	Near PLA2
F119Y	
P145Q	BR2
H148L	Near BR2
E152K	
R170K	BR3
A194S	
N201D	
M203I	
W247L	
R310S	BR4

Table 1. **N-terminal mutations isolated from neutral drift selection.** Mutations identified in regions of the AAV2 capsid not crystallized are given. Also, noted are their location within and in proximity to nuclear localization sequences and the phospholipase A2 domain.

A study has shown neutral drift mutations converge toward the inferred ancestral sequence under high mutational loads.[16] For a few of the identified mutations, this appears to be true; for instance E36 is mutated to the conserved glutamine (Fig. 3). However, mutations were more likely to mutate residues that are conserved amongst AAV serotypes 1-9 away from the consensus. Examples of such mutations are residues E106, F119, W247, K544, T624, and H641 (Fig. 3). Residue 544 is the only one of these

located on the capsid surface (Fig. 2); the other mutations are in the capsid interior, at intersubunit interfaces, or buried within the core of the capsid protein. While many mutations neither alter conserved residues nor revert back to consensus, they frequently are shared amongst other serotypes. Interestingly, the serotypes that share the majority of mutations isolated from this study are AAV4, 5, and 9, serotypes phylogenetically distant from AAV2.

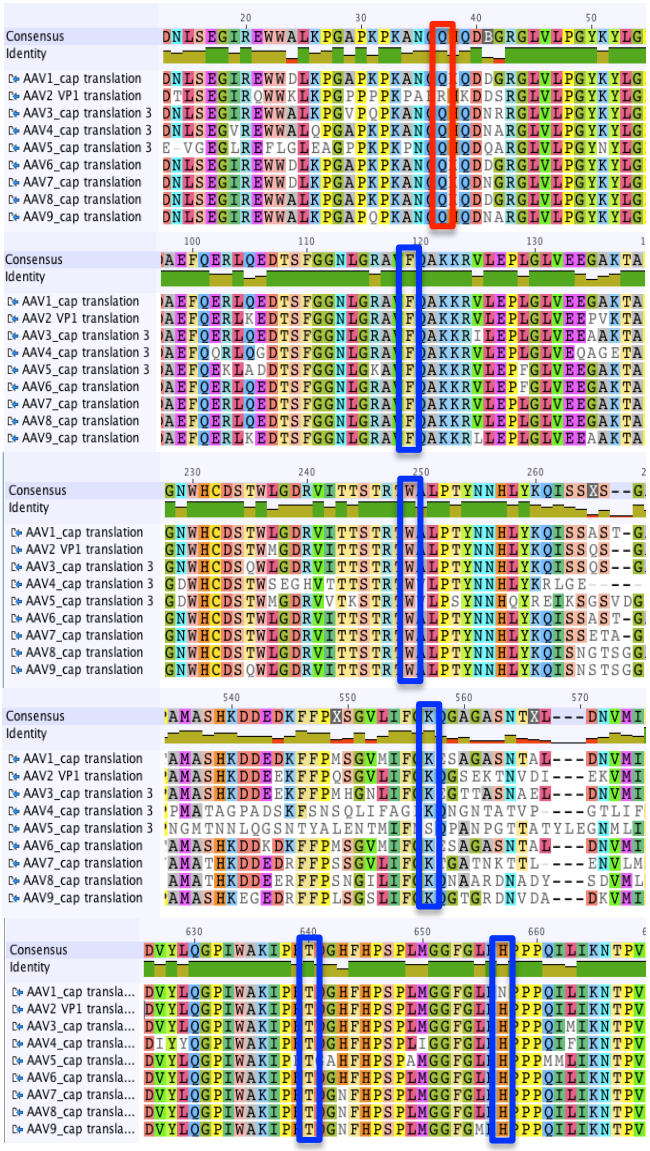


Figure 3. **Sequence alignments at residues where isolated mutations differ from or return to AAV consensus.** Alignments of AAV serotypes 1-9 at residues where mutations identified from the neutral drift of AAV2 either return the mutant to the consensus sequence or mutate conserved residues. Residues that are mutated toward the AAV consensus sequence are highlighted in red and those that mutate conserved residues are highlighted in blue.

Discussion

While my library sequence differs from the parental sequence to a lesser extent than published studies[13], this library should still prove valuable in selections for AAV infection on novel cell types. As I (Chapter 6) and published directed evolution studies have shown, AAV variants that differ from wild-type by as little as one to three mutations can have vastly altered phenotypes.[29] Though differing from AAV2 by few amino acids, library members contain mutations that retain the capacity for viral packaging and infectivity, making every library member viable and potentially useful in selections.

The mutations contained within the library should prove particularly useful in directed evolution studies. While many mutations are likely minimally perturbing, numerous library members contain mutations that potentially alter the charge state of the viral capsid. Heparan sulfate binding proteins often utilize charge-charge interactions with the multiple negative charges on the polymer to bind.[30] In this way, the binding of AAV2 to heparan occurs through a highly charged region on the three fold spike consisting of multiple basic residues.[20] Similarly, it has been shown that the ability of AAV6 to utilize heparan sulfate as a receptor is due to a basic patch created by its unique E531K mutation.[31] Disruptions and changes to the surface charge density of the viral capsid could alter the affinity to cellular proteoglycans allowing library variants to bind new cell types.

In addition to non-synonymous mutations, library members contain multiple silent mutations. There is evidence of viral codon bias tuning translation kinetics to ensure proper protein folding;[32,33] some viruses can use combinations of rare and common codons to tune the rate of translation allowing protein domains to fold properly. In this way, while silent on the amino acid level, these mutations ensure proper viral protein folding yet allow a larger portion of protein sequence space and, therefore, phenotypic space to be sampled upon further mutation. Viral codon usage is necessary for its mutational robustness.[34] If silent mutations negatively influenced mutational robustness, they would be selected against over multiple rounds of viral evolution. As virus containing these mutations underwent multiple rounds of mutagenesis, they have been proven to increase the accessible codon space without detrimentally affecting mutational robustness.

The library contains mutations in nearly every identified functional portion of the AAV2 viral capsid. Though viral receptor binding is an important step in cellular specificity, evidence suggests intracellular trafficking and endosomal release can act as a barrier to successful gene transfer.[35] Studies have even shown that altering intracellular trafficking can help contribute to a novel cellular specificity.[36] The library not only includes mutations that can alter receptor binding but also those that could alter intracellular trafficking and endosomal escape. Interestingly, many of these mutations are shared with serotypes highly divergent from AAV2. Thus, this library may sample multiple functionalities from multiple serotypes through shared mutations and, therefore, prove useful in new cell types of interest.

While not wholly successful in creating highly divergent sequences, the neutral drift library may prove advantageous in directed evolution studies in the future. Though differing by few mutations from wild-type, each mutation is permissive to viral packaging and in vitro infectivity and, thus, has the potential of exhibiting novel phenotype upon selection especially given the range of alterations to residue chemistry

and location of mutations. The library also has the potential of sampling functionality from divergent serotypes, as numerous library mutations in multiple regions are shared with AAV serotypes 1/6, 4, 5, 7, 8, and 9. Additionally, selected mutations from the library could prove to act synergistically when combined to further improve a desired function as in previous studies.[13] Thus, the described neutral drift library and similar future studies will prove an invaluable tool in AAV directed evolution.

Materials and Methods

Cell lines

Cell lines were cultured at 37°C and 5% CO_2. 293T cells were obtained from the American type culture collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Construction of AAV libraries

Error-prone mutagenesis of the AAV2 was performed as described.[37] Briefly *cap* gene was mutagenized by $MnCl_2$ error-prone mutagenesis. Multiple PCR reactions of varying concentrations of manganese chloride were performed to provide a range of mutation frequency. Successful reactions were combined, purified with a commercial PCR clean up kit (Promega) to remove excess $MnCl_2$, and cloned into pSub2. Replication competent AAV was then produced, as previously described.[37]

Neutral drift of AAV error-prone libraries

293T cells (2.5×10^5) were infected with the error-prone AAV2 library at varying genomic multiplicities of infection representative of a wild-type infectious MOI of one. Twenty-four hours after infection, media was changed and wild-type adenovirus added to rescue infectious variants. After forty-eight hours, viral particles were harvested and titered by quantitative PCR. This viral pool was utilized for the next round of infection. After three infection-rescue steps, viral genomes were harvested from which library *cap* genes were amplified by PCR and subject to additional mutagenesis to generate a library for additional rounds of selection.[38] Neutral drift of AAV2 as described here consisted of a total of three rounds of evolution by mutagenesis and selection.

References

1 Camps, M., Herman, A., Loh, E. & Loeb, L. A. Genetic constraints on protein evolution. *Critical reviews in biochemistry and molecular biology* **42**, 313-326, doi:10.1080/10409230701597642 (2007).

2 Tokuriki, N., Stricher, F., Schymkowitz, J., Serrano, L. & Tawfik, D. S. The stability effects of protein mutations appear to be universally distributed. *Journal of molecular biology* **369**, 1318-1332, doi:10.1016/j.jmb.2007.03.069 (2007).
3 Tokuriki, N., Stricher, F., Serrano, L. & Tawfik, D. S. How protein stability and new functions trade off. *PLoS computational biology* **4**, e1000002, doi:10.1371/journal.pcbi.1000002 (2008).
4 Hughes, A. L. Looking for Darwin in all the wrong places: the misguided quest for positive selection at the nucleotide sequence level. *Heredity* **99**, 364-373, doi:10.1038/sj.hdy.6801031 (2007).
5 van Nimwegen, E. Epidemiology. Influenza escapes immunity along neutral networks. *Science* **314**, 1884-1886, doi:10.1126/science.1137300 (2006).
6 Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E. & Andino, R. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* **439**, 344-348, doi:10.1038/nature04388 (2006).
7 Koelle, K., Cobey, S., Grenfell, B. & Pascual, M. Epochal evolution shapes the phylodynamics of interpandemic influenza A (H3N2) in humans. *Science* **314**, 1898-1903, doi:10.1126/science.1132745 (2006).
8 King, J. L. & Jukes, T. H. Non-Darwinian evolution. *Science* **164**, 788-798 (1969).
9 Kimura, M. *The neutral theory of molecular evolution*. 367 p. (Cambridge University Press, 1983).
10 Codoner, F. M., Daros, J. A., Sole, R. V. & Elena, S. F. The fittest versus the flattest: experimental confirmation of the quasispecies effect with subviral pathogens. *PLoS Pathog* **2**, e136, doi:10.1371/journal.ppat.0020136 (2006).
11 Wilke, C. O., Wang, J. L., Ofria, C., Lenski, R. E. & Adami, C. Evolution of digital organisms at high mutation rates leads to survival of the flattest. *Nature* **412**, 331-333, doi:10.1038/35085569 (2001).
12 Tawfik, D. S. Messy biology and the origins of evolutionary innovations. *Nature chemical biology* **6**, 692-696, doi:10.1038/nchembio.441 (2010).
13 Gupta, R. D. & Tawfik, D. S. Directed enzyme evolution via small and effective neutral drift libraries. *Nat Methods* **5**, 939-942, doi:10.1038/nmeth.1262 (2008).
14 Amitai, G., Gupta, R. D. & Tawfik, D. S. Latent evolutionary potentials under the neutral mutational drift of an enzyme. *HFSP journal* **1**, 67-78, doi:10.2976/1.2739115/10.2976/1 (2007).
15 Bloom, J. D., Romero, P. A., Lu, Z. & Arnold, F. H. Neutral genetic drift can alter promiscuous protein functions, potentially aiding functional evolution. *Biology direct* **2**, 17, doi:10.1186/1745-6150-2-17 (2007).
16 Bershtein, S., Goldin, K. & Tawfik, D. S. Intense neutral drifts yield robust and evolvable consensus proteins. *Journal of molecular biology* **379**, 1029-1044, doi:10.1016/j.jmb.2008.04.024 (2008).
17 Bornberg-Bauer, E. & Chan, H. S. Modeling evolutionary landscapes: mutational stability, topology, and superfunnels in sequence space. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 10689-10694 (1999).
18 Smith, W. S., Hale, J. R. & Neylon, C. Applying neutral drift to the directed molecular evolution of a beta-glucuronidase into a beta-galactosidase: Two

different evolutionary pathways lead to the same variant. *BMC research notes* **4**, 138, doi:10.1186/1756-0500-4-138 (2011).

19 Zaccolo, M., Williams, D. M., Brown, D. M. & Gherardi, E. An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. *Journal of molecular biology* **255**, 589-603, doi:10.1006/jmbi.1996.0049 (1996).

20 Xie, Q. *et al.* The Atomic Structure of Adeno-associated Virus (AAV-2), a Vector for Human Gene Therapy. *Proceedings of the National Academy of Science* **99**, 10405-10410 (2002).

21 Wu, P. *et al.* Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* **74**, 8635-8647 (2000).

22 Akache, B. *et al.* The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *J Virol* **80**, 9831-9836, doi:10.1128/JVI.00878-06 (2006).

23 Asokan, A., Hamra, J. B., Govindasamy, L., Agbandje-McKenna, M. & Samulski, R. J. Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. *J Virol* **80**, 8961-8969, doi:10.1128/JVI.00843-06 (2006).

24 Johnson, J. S. *et al.* Mutagenesis of adeno-associated virus type 2 capsid protein VP1 uncovers new roles for basic amino acids in trafficking and cell-specific transduction. *J Virol* **84**, 8888-8902, doi:10.1128/JVI.00687-10 (2010).

25 Grieger, J. C., Snowdy, S. & Samulski, R. J. Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly. *J Virol* **80**, 5199-5210, doi:10.1128/JVI.02723-05 (2006).

26 Girod, A. *et al.* The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *The Journal of general virology* **83**, 973-978 (2002).

27 Stahnke, S. *et al.* Intrinsic phospholipase A2 activity of adeno-associated virus is involved in endosomal escape of incoming particles. *Virology* **409**, 77-83, doi:10.1016/j.virol.2010.09.025 (2011).

28 Popa-Wagner, R. *et al.* Impact of VP1-specific protein sequence motifs on adeno-associated virus type 2 intracellular trafficking and nuclear entry. *J Virol* **86**, 9163-9174, doi:10.1128/JVI.00282-12 (2012).

29 Asuri, P. *et al.* Directed Evolution of Adeno-associated Virus for Enhanced Gene Delivery and Gene Targeting in Human Pluripotent Stem Cells. *Molecular Therapy* **20**, 329-338 (2012).

30 Kreuger, J., Spillmann, D., Li, J.-p. & Lindahl, U. Interactions between heparan sulfate and proteins: the concept of specificity. *The Journal of Cell Biology* **174**, 323-327, doi:10.1083/jcb.200604035 (2006).

31 Ng, R. *et al.* Structural characterization of the dual glycan binding adeno-associated virus serotype 6. *J Virol* **84**, 12945-12957, doi:10.1128/JVI.01235-10 (2010).

32 Zhou, J. H. *et al.* The effects of the synonymous codon usage and tRNA abundance on protein folding of the 3C protease of foot-and-mouth disease virus. *Infection, genetics and evolution : journal of molecular epidemiology and*

evolutionary genetics in infectious diseases **16C**, 270-274, doi:10.1016/j.meegid.2013.02.017 (2013).

33 Aragones, L., Guix, S., Ribes, E., Bosch, A. & Pinto, R. M. Fine-tuning translation kinetics selection as the driving force of codon usage bias in the hepatitis A virus capsid. *PLoS Pathog* **6**, e1000797, doi:10.1371/journal.ppat.1000797 (2010).

34 Lauring, A. S., Acevedo, A., Cooper, S. B. & Andino, R. Codon usage determines the mutational robustness, evolutionary capacity, and virulence of an RNA virus. *Cell host & microbe* **12**, 623-632, doi:10.1016/j.chom.2012.10.008 (2012).

35 Nonnenmacher, M. & Weber, T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther* **19**, 649-658, doi:10.1038/gt.2012.6 (2012).

36 Excoffon, K. J. D. A. *et al.* Directed Evolution of Adeno-associated Virus to an Infectious Respiratory Virus. *Proceedings of the National Academy of Science* **106**, 3865-3870 (2009).

37 Koerber, J. T., Maheshri, N., Kaspar, B. K. & Schaffer, D. V. Construction of diverse adeno-associated viral libraries for directed evolution of enhanced gene delivery vehicles. *Nat Protoc* **1**, 701-706, doi:10.1038/nprot.2006.93 (2006).

38 Maheshri, N., Koerber, J. T., Kaspar, B. K. & Schaffer, D. V. Directed Evolution of Adeno-associated Virus Yields Enhanced Gene Delivery Vectors. *Nature Biotechnology* **24**, 198-204 (2006).

Chapter 5

Reconstruction of an Ancestral AAV Variant

Abstract

Ancestral reconstruction holds great promise in improving protein directed evolution. The reconstructed sequence of an ancestral enzyme for a protein family of interest can be an ideal substrate with which to begin directed evolution studies. Previous studies have indicated that ancestral proteins can be more stable, can have promiscuous functions, and have already been shown to be evolvable, having given rise to all members within its protein family. Ancestral reconstructions have been performed for globular proteins and libraries based on these reconstructions have been utilized to engineer novel protein functions. To extend this work to adeno-associated virus, an ancestral AAV sequence was reconstructed utilizing a novel alignment program from which a library of evolutionarily relevant sequences was designed and synthesized.

Introduction

The majority of mutations destabilize protein stability and function,[1,2] a major impediment to engineering proteins with novel functions. Improving the stability of a protein substrate has been shown to make it more evolvable by compensating for these destabilizing mutations. One study has shown that reconstructed ancestral proteins were more stable than extant sequences[3] and, therefore, could be capable of tolerating more destabilizing mutations. The reconstructed proteins evolved during an era of increased temperature on Earth and, thus, required a higher stability in order to maintain function. However, whether this trend is general to all ancestral proteins or specific to environmental conditions has yet to be determined.

Ancestral sequences have already proven useful in evolution, as they by definition have evolved into an array of progeny that often exhibit varied functionalities. Studies have also suggested that ancestral proteins have more promiscuous functions;[4,5] reconstructed ancestral proteins were shown to catalyze a wide-range of substrates as opposed to specializing in the catalysis of one. Thus, ancestral proteins could potentially be more favorable substrates for directed evolution studies. Thus, recapitulation of the ancestral sequence could yield a protein substrate ideal for engineering novel properties.

Previously, ancestral sequences for multiple families of proteins have been reconstructed for studies on the evolution of modern function.[3,6-10] Inferred ancestral sequences have also been utilized to construct libraries of enzyme with hybrid modern-ancestral active sites.[11-13] Here we extend the work to AAV and describe the sequence reconstruction of an ancestral AAV. Due to uncertainty in the identity of multiple residues, an ancestral library containing multiple phylogenetically possible amino acids at each position was constructed. This represents the first construction of a library utilizing variability at evolutionarily relevant residues within an ancestral protein sequence.

Results and Discussion

Reconstruction of an AAV ancestor

In collaboration with the laboratory of Ian Holmes (Bioengineering, UC Berkeley), *handalign*[14] was utilized on all available AAV *cap* sequences from Genbank (n=156), including those from human, mammalian, and avian origin, to create a multiple sequence alignment and phylogenetic tree. From this tree, two reconstructions of ancestral nodes could be performed, one reconstructing the codon sequence and one the amino acid sequence. Due to the interesting gene delivery properties of viral progeny, the ancestral node (Node 27) for serotypes 1, 6, 7, and 8 and their phylogenetic neighbors were focused upon (Fig. 1). Both reconstructions yielded near identical sequences with some amino acid differences at those residues with low confidences and two extra residues in the codon reconstruction. Since, in general, the amino acid reconstruction had higher confidences, this reconstruction was utilized for subsequent analyses. The sequence for Node 27 is nearly equidistant from all viral progeny and differs by up to 11% from the most distant progeny (Table 1), which equates to over 70 amino acid differences, a number of mutations not accessible by standard error-prone mutagenesis methods. This difference reflects almost that of two distinct serotypes and could represent a serotype of its own.

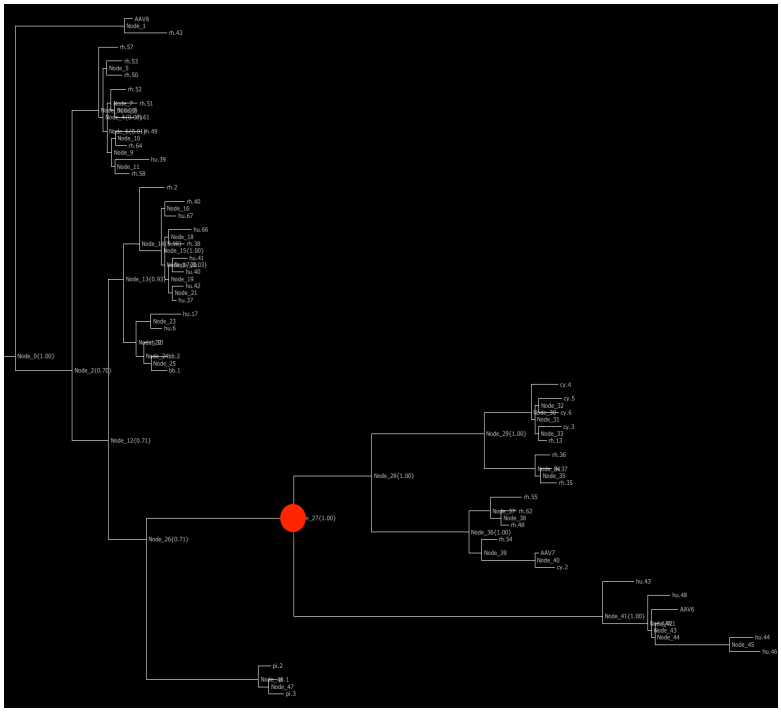


Figure 1. **Phylogeny of AAV variants similar to AAV1, 6, 7, and 8.** All AAV sequences similar to serotypes 1, 6, 7, and 8 available from GenBank were aligned from which a phylogenetic tree could be created. Sequence reconstruction could be performed for all nodes, however, due to its equidistance from all isolates, Node 27 (identified in red) was focused upon for future studies.

	AAV1	AAV6	AAV7	AAV8	Node 27
AAV1	100	-	-	-	-
AAV6	99.2	100	-	-	-
AAV7	85.1	85.3	100	-	-
AAV8	84	84.2	88	100	-
Node 27	89	89.1	92.5	90.1	100

Table 1. **Pairwise identities between reconstructed ancestral AAV and viral progeny**. The pairwise identities were determined between the ancestral AAV and its viral progeny. The ancestor is near equidistant genetically from all of its progeny and its sequence differs from each 7.5-11%.

Amino acid differences between progeny and the reconstructed ancestor lay mostly on the exterior of the AAV capsid (Fig. 2) in the variable loop regions. In particular, mutations clustered around the three-fold spike, a region important in viral receptor binding.[15,16] Interestingly, AAV8 also differed from the ancestral sequence near the pore at the five-fold axis of symmetry (Fig. 2C), whereas the other progeny were homologous at this region (Fig. 2A and B). This pore represents the capsid region through which DNA is likely packaged into the intact capsid[17] and the VP1/2 unique regions are externalized.[18] This region, while influencing DNA encapsidation and externalization of the VP1/2 terminus, has not been shown to influence cellular specificity. However, preliminary evidence indicates that mutation within this region can redirect AAV infection to non-permissive cell types (Chapter 6). An overlay of variations between all viral progeny and the reconstructed ancestor (Fig. 2D) indicates that on the ancestral capsid surface, the majority of residues differ from at least one of the viral progeny. The N-terminal regions unique to VP1 and VP2 contain multiple domains required for endosomal escape and nuclear trafficking.[19-23] While this region is more homologous between viral progeny and the ancestor than external capsid loop regions, the ancestor differs from at least one of its progeny at multiple positions, including those within the phospholipase A2 domain[19] and newly identified motifs required for intracellular targeting.[23]

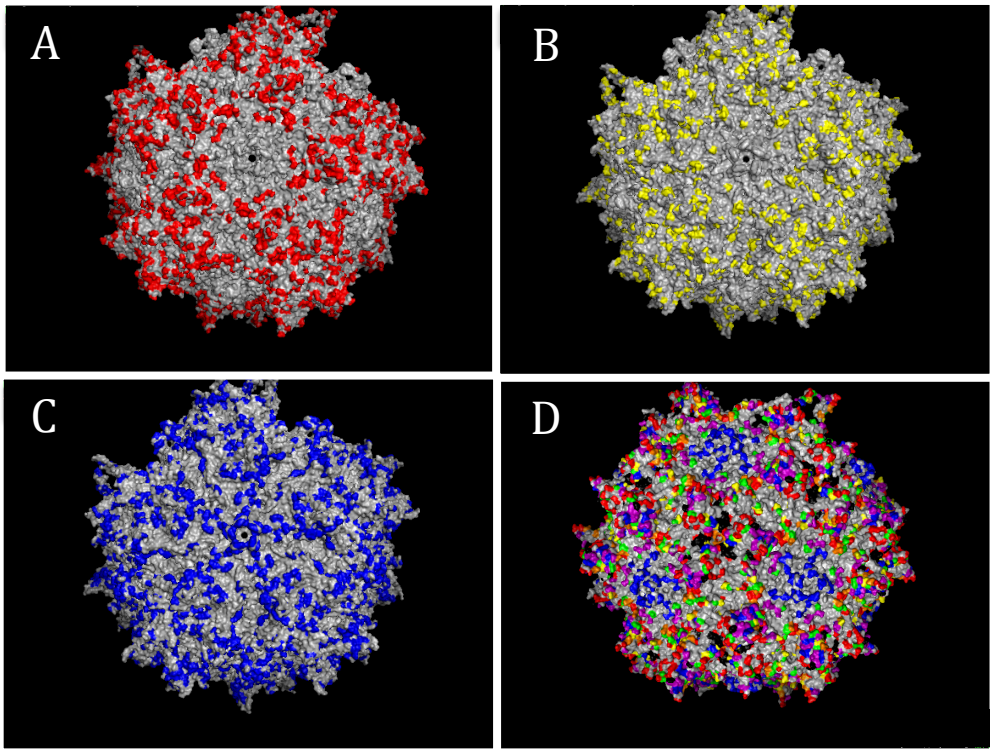


Figure 2. **Amino acid differences between reconstructed ancestor and viral progeny.** Residues that differ between the reconstructed ancestor and (A) AAV1/6, (B) AAV7, and (C) AAV8 are shown. (D) Compiled differences between all progeny and the reconstructed ancestor are given (Red: AAV1/6; Blue: AAV8; Yellow: AAV7; Purple: AAV1, 6 and 8; Orange: AAV1, 6, and 7; Green: AAV7 and 8; Black: AAV1, 6, 7, and 8).

Ancestral library sequence

There were several regions corresponding to external loop sequences in which the alignment was uncertain, which precluded an accurate sequence prediction in these regions. To incorporate this uncertainty into the ancestral sequence construction, an ancestral library was designed. In the amino acid reconstruction, residues in which confidence was below 0.90 were mutagenized in the library to those amino acids that had a confidence greater than 0.08. In this way, the library samples a majority of all the possible ancestral sequence variations ($>10^{11}$) of which 10^{6}-10^{7} can be experimentally sampled. The location on the viral capsid of those residues that are mutagenized within the library are given in Figure 3. To prevent sequence bias within the library toward those amino acids having low confidences within the reconstruction, the fraction of library members containing each amino acid reflect the confidence for that amino acid. For example, the confidence for asparagine at residue 718 is 0.60 and that for serine is 0.40. Therefore, by tuning the DNA synthesis strategy, 60% of library members will contain asparagine and 40% serine. As mentioned above, the codon and amino acid

reconstructions differed by multiple amino acids. All but two of these residues were mutagenized due to uncertainty. To sample the data from both reconstructions, the two remaining residues that differed were mutagenized such that the amino acid predicted by each reconstruction would appear in half of all library members. Including all the diversified residues and the amino acids possible at each position yields a library far larger than current technology can experimentally sample. By weighting amino acid composition by confidence within the reconstruction, viral variants not sampled are thus those least likely to have existed and, therefore, this issue is diminished. The described ancestral library is currently on order (GeneArt).

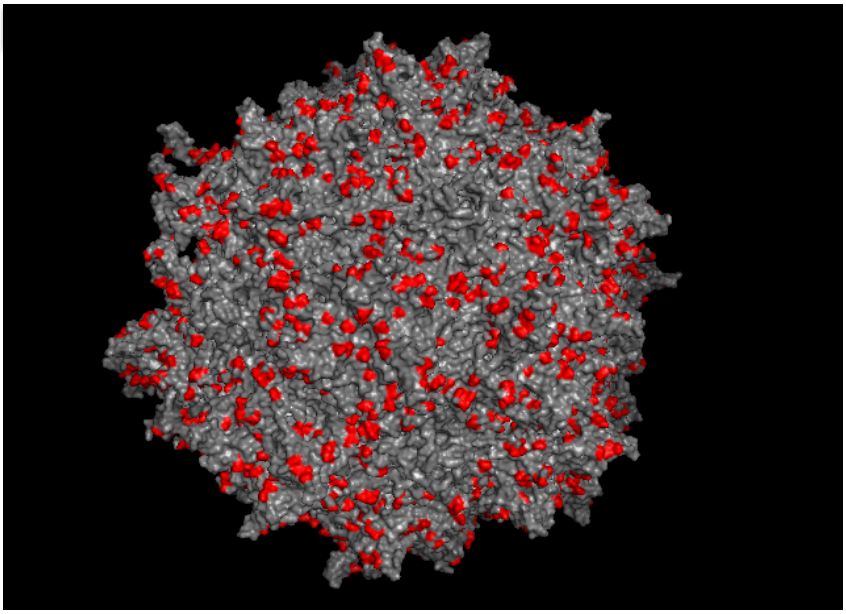


Figure 3. **Residues randomized in the ancestral library**. Residues that were randomized from the highest confidence sequence in the ancestral AAV library are shown.

Other attempts at creating libraries based on ancestral reconstructions have been reported to engineer polymerases with the ability to incorporate a new class of triphosphate substrates[13] and enzymes capable of utilizing new cofactors[11] or metabolizing new substrates.[12] The library described here, however, is the only one that utilizes the highest confidence ancestral sequence as template, a sequence that is more likely to be promiscuous and mutationally robust.[4,5] All other libraries based on ancestral reconstruction randomly incorporated ancestral amino acids into the active site of modern enzymes to create hybrid enzymes, whereas all amino acids within this library are based on ancestral sequence. In these regards, this approach to an ancestral library represents a better approach in sampling ancestral sequences for novel phenotypes and, due to the higher mutational robustness of ancestral enzymes, should serve as a better starting point for further mutagenesis and evolution.

The described library will be utilized in selections for AAV variants with highly desirable phenotypes, such as the ability to cross the blood-brain barrier or the ability to infect cancer stem cells.

Materials and Methods

Reconstruction of ancestral AAV and gene synthesis

Reconstruction of ancestral AAV sequences were performed as described.[14] Briefly, software designed by the Holmes group, *handalign*, was utilized to perform a multiple sequence alignment of AAV *cap* sequences from which a phylogenetic tree and ancestral reconstruction could be created. Ancestral library sequences were synthesized (GeneArt). Library sequences were synthesized such that degenerated positions represented the amino acids the reconstruction determined possible, the ratios of which reflected the confidence level for that amino acid.

Acknowledgements

This work was performed jointly with Ian Holmes and Oscar Westesson at the University of California, Berkeley.

References

1 Camps, M., Herman, A., Loh, E. & Loeb, L. A. Genetic constraints on protein evolution. *Critical reviews in biochemistry and molecular biology* **42**, 313-326, doi:10.1080/10409230701597642 (2007).

2 Tokuriki, N., Stricher, F., Schymkowitz, J., Serrano, L. & Tawfik, D. S. The stability effects of protein mutations appear to be universally distributed. *Journal of molecular biology* **369**, 1318-1332, doi:10.1016/j.jmb.2007.03.069 (2007).

3 Gaucher, E. A., Govindarajan, S. & Ganesh, O. K. Palaeotemperature trend for Precambrian life inferred from resurrected proteins. *Nature* **451**, 704-707, doi:10.1038/nature06510 (2008).

4 Bershtein, S., Goldin, K. & Tawfik, D. S. Intense neutral drifts yield robust and evolvable consensus proteins. *Journal of molecular biology* **379**, 1029-1044, doi:10.1016/j.jmb.2008.04.024 (2008).

5 Tokuriki, N. & Tawfik, D. S. Stability effects of mutations and protein evolvability. *Current opinion in structural biology* **19**, 596-604, doi:10.1016/j.sbi.2009.08.003 (2009).

6 Bridgham, J. T., Ortlund, E. A. & Thornton, J. W. An epistatic ratchet constrains the direction of glucocorticoid receptor evolution. *Nature* **461**, 515-519, doi:10.1038/nature08249 (2009).
7 Thomson, J. M. *et al.* Resurrecting ancestral alcohol dehydrogenases from yeast. *Nat Genet* **37**, 630-635, doi:10.1038/ng1553 (2005).
8 Ortlund, E. A., Bridgham, J. T., Redinbo, M. R. & Thornton, J. W. Crystal structure of an ancient protein: evolution by conformational epistasis. *Science* **317**, 1544-1548, doi:10.1126/science.1142819 (2007).
9 Ugalde, J. A., Chang, B. S. & Matz, M. V. Evolution of coral pigments recreated. *Science* **305**, 1433, doi:10.1126/science.1099597 (2004).
10 Skovgaard, M. *et al.* Using evolutionary information and ancestral sequences to understand the sequence-function relationship in GLP-1 agonists. *Journal of molecular biology* **363**, 977-988, doi:10.1016/j.jmb.2006.08.066 (2006).
11 Flores, H. & Ellington, A. D. A modified consensus approach to mutagenesis inverts the cofactor specificity of Bacillus stearothermophilus lactate dehydrogenase. *Protein engineering, design & selection : PEDS* **18**, 369-377, doi:10.1093/protein/gzi043 (2005).
12 Alcolombri, U., Elias, M. & Tawfik, D. S. Directed evolution of sulfotransferases and paraoxonases by ancestral libraries. *Journal of molecular biology* **411**, 837-853, doi:10.1016/j.jmb.2011.06.037 (2011).
13 Chen, F. *et al.* Reconstructed evolutionary adaptive paths give polymerases accepting reversible terminators for sequencing and SNP detection. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 1948-1953, doi:10.1073/pnas.0908463107 (2010).
14 Westesson, O. *Statistical phylogenetic methods with applications to virus evolution* PhD thesis, University of California, Berkeley/University of California, San Francisco, (2012).
15 Ng, R. *et al.* Structural characterization of the dual glycan binding adeno-associated virus serotype 6. *J Virol* **84**, 12945-12957, doi:10.1128/JVI.01235-10 (2010).
16 Nam, H. J. *et al.* Structure of adeno-associated virus serotype 8, a gene therapy vector. *J Virol* **81**, 12260-12271, doi:10.1128/jvi.01304-07 (2007).
17 Bleker, S., Pawlita, M. & Kleinschmidt, J. A. Impact of capsid conformation and Rep-capsid interactions on adeno-associated virus type 2 genome packaging. *J Virol* **80**, 810-820, doi:10.1128/jvi.80.2.810-820.2006 (2006).
18 Kronenberg, S., Bottcher, B., von der Lieth, C. W., Bleker, S. & Kleinschmidt, J. A. A conformational change in the adeno-associated virus type 2 capsid leads to the exposure of hidden VP1 N termini. *J Virol* **79**, 5296-5303, doi:10.1128/JVI.79.9.5296-5303.2005 (2005).
19 Girod, A. *et al.* The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *The Journal of general virology* **83**, 973-978 (2002).
20 Stahnke, S. *et al.* Intrinsic phospholipase A2 activity of adeno-associated virus is involved in endosomal escape of incoming particles. *Virology* **409**, 77-83, doi:10.1016/j.virol.2010.09.025 (2011).

21 Grieger, J. C., Snowdy, S. & Samulski, R. J. Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly. *J Virol* **80**, 5199-5210, doi:10.1128/JVI.02723-05 (2006).
22 Johnson, J. S. *et al.* Mutagenesis of Adeno-Associated Virus Type 2 Capsid Protein VP1 Uncovers New Roles for Basic Amino Acids in Trafficking and Cell-Specific Transduction. *Journal of Virology* **84**, 8888-8902 (2010).
23 Popa-Wagner, R. *et al.* Impact of VP1-specific protein sequence motifs on adeno-associated virus type 2 intracellular trafficking and nuclear entry. *J Virol* **86**, 9163-9174, doi:10.1128/JVI.00282-12 (2012).

Chapter Six

Engineering of AAV Variants Targeting HIV-infected T-cells

Abstract

There are serious shortcomings with current HIV therapies. Gene therapy holds promise in the treatment HIV; however, a selective vector is necessary for such an approach. Infection by HIV has been shown to dramatically alter the surface of T-cells; therefore, there is a possibility that viral vectors could be engineered to selectively infect HIV-infected T-cells. Directed evolution of AAV was performed to engineer variants with enhanced infectivity of T-cells and/or selectivity for HIV-infected T-cells. Several viral variants that more efficiently transduce T-cells were isolated, as well as cells actively and latently infected with HIV. Sequence analysis of viral variants provided insights into potential mechanisms of re-targeting and suggested possible cellular barriers to infection by natural AAV serotypes.

Introduction

HIV is one the world's most prominent infectious diseases. There were 35 million people (3.3 million children) infected with HIV worldwide in 2011, leading to 1.7 million deaths.[1] There is no successful vaccine and the conventional treatment, HAART, suffers from serious drawbacks in drug resistance, toxicity, and cost.[2] Novel therapies are thus necessary to prevent, treat, and possibly even cure infection.

The "Berlin patient" demonstrated the feasibility of gene therapy in the treatment of an active HIV infection. This HIV-positive patient underwent a bone marrow transplant to treat his leukemia.[3] The donor was naturally resistant to HIV infection due to a delta32/delta32 mutation in CCR5, the HIV primary receptor, and after tissue transplantation this genotype conferred resistance to the recipient, evidenced by the lack of HIV viral load after the suppression of HAART.[3] Subsequent studies have shown the possibility of conferring HIV resistance through CCR5 knockout utilizing zinc finger nucleases.[4] Alternatively, CRISPR-mediated genome editing could be used in treatment and prevention.[5] Additionally, RNA-interference has been utilized to prevent HIV-replication *in vitro.*[6] A vector specific to T-cells for preventative therapies or infected T-cells for treatment would be an invaluable tool in the treatment of HIV by delivering any of these cargoes.

Significant changes to the T-cell surface occur upon HIV infection. Several proteins, including the HIV viral receptors, MHC class I and class II molecules, and clusters of differentiation antigens (CD), are down-regulated,[7-11] while others are upregulated.[10,12] Latently infected T-cells, cells in which HIV has entered but is not actively replicating, exhibit changes in cell surface proteins as well.[13] Glycosylation changes on the cell surface have also been noted.[14] These changes have been utilized to rationally engineer viruses to selectively bind and infect HIV-infected T-cells;[15-17] however, the parental viruses are pathogenic. Thus, a safe and selective alternative is necessary for *in vivo* clinical efficacy.

AAV is a non-pathogenic virus not associated with any human disease. Unfortunately, no AAV variant that efficiently transduces T-cells exists.[18-23] Barriers to AAV transduction can occur at any of the multiple steps of AAV infection, including cellular binding, internalization, endosomal escape, and intracellular trafficking. Directed evolution of AAV provides a facile approach to create vectors with novel phenotypes without knowledge of the mechanism preventing viral transduction. This method has been utilized previously in engineering AAV to have unique gene delivery properties.[24-28] The work outlined here describes the use of directed evolution of AAV to engineer a viral variant capable of efficiently infecting HIV-infected T cells for use in HIV gene therapy.

Results

Selection of AAV variants specific to HIV-infected T-cells

To evolve an AAV variant that can selectively infect HIV-infected T-cells, an error-prone AAV2,[29] an AAV2-based 7mer-peptide insert,[28] and a shuffled library[30] were packaged and selected for the ability to infect HIV-infected H9-cells (Fig 1). In collaboration with the laboratories of Prof. Kate Excoffon and Dawn Wooley (Wright State University), cells were treated with the pooled viral libraries and infectious variants were amplified with adenovirus super-infection (Fig 1A). Viral genomes were harvested, *cap* gene was PCR-amplified, and the selected viral pool was re-packaged for subsequent rounds of infection at decreasing genomic MOIs (Fig. 1A). To monitor sequence convergence, viral *cap* genes were sequenced after every infection (Fig. 1A). After four rounds of infection viral, genomes were subjected to error-prone mutagenesis to add additional diversity and thus additionally potentially advantageous mutations. To improve selectivity of viral variants to HIV-infected T cells, subsequent infections were performed with a negative selection prior to positive selection (Fig. 1B). Viral library was added to H9 cells not infected with HIV and non-specific variants were allowed to bind. The supernatant was then added to HIV-infected H9s (Fig. 1B). Infectious variants were amplified in both cell-types with adenovirus superinfection, viral genomes harvested, and *cap* PCR-amplified (Fig 1B). Variants were sequenced to assess sequence convergence and clonal cellular specificity. After four rounds of positive selection followed by two rounds of alternating negative and positive selection, sequence convergence was reached. A parallel selection was performed for a viral variant selective to uninfected T-cells.

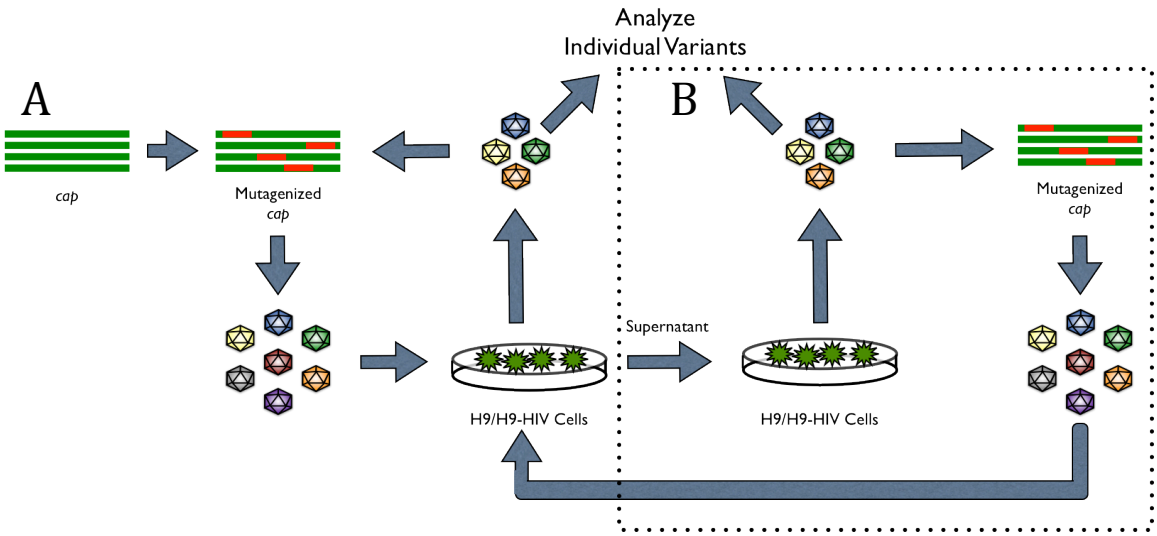


Figure 1. **Selection scheme for T-cell and HIV-infected T-cell specific AAV variants**. (A) A combined library of AAV2-error prone, AAV2 7mer insert, and AAV shuffled libraries were packaged and selected for specificity toward either H9 or HIV-infected H9 cells by infecting the cell-type of interest. Infectious clones were rescued with adenovirus super-infection and viral genomes isolated. Viral *cap* genes were then re-cloned and packaged for subsequent infections. Sequence analysis of isolated cap genes was performed after every selection to determine sequence convergence. (B) After three selections, selected viral *cap* genes were subjected to error-prone mutagenesis. Subsequent infections were performed with a negative selection, whereby virus was added to the other cell-type, non-specific clones were allowed to bind, and supernatant then transferred to the cell-type of interest for viral infection.

Sequence analysis of selected variants

After selection for AAV variants capable of selectively infecting either HIV-infected or uninfected T-cells, four families of variants emerged. All variants isolated from selections for uninfected T-cell specificity contained a peptide insert, LANKTQTINA, and differed in their point mutations. Three dominant families emerged from selection for HIV-infected T-cell specificity, two containing peptide inserts and one based on error-prone mutagenesis. The family containing the peptide insert LAKNKTAENA represented 18% of the clones isolated and those that contained the peptide insert LAKNPTATTA represented 64% of the isolated clones. The remaining 16% of clones isolated lacked any peptide insert and differed from wild-type by two to three point mutations. The members of each family differed from each other in the number and location of point mutations (Fig. 2). One representative clone from each family was selected for use in further studies; the sequence of each is given in Table 1.

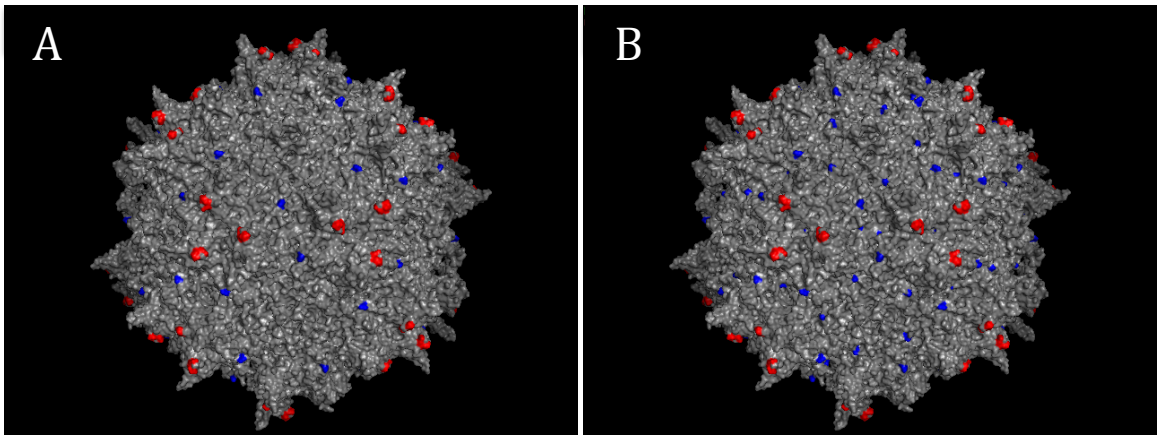


Figure 2. **Location of mutations isolated for T-cell and HIV-infected T-cell specificity**. Mutations identified after five selections for AAV variants specific to (A) T-cells and (B) HIV-infected T-cells. The location for the 7mer mutation is shown in red and point mutations are in blue.

Clone	Peptide Insert	Mutations
H9-5C	^{587}N~ LANKTQTINA ~^{588}R	V708I
HIV-2B	^{587}N~ LAKNKTAENA ~^{588}R	E67A, I698V
HIV-2C	None	E67A, S207G, Q598L
HIV-4C	^{587}N~ LAKNPTATTA ~^{588}R	S207G, I648V, V708I

Table 1. **Clones isolated for T-cell and HIV-infected T-cell specificity.** Sequences for select T-cell and HIV-infected T-cell targeted clones are given. Most clones isolated after five rounds of selection contained a 7mer peptide insertion with several point mutations. The E67A mutation lies near the active site of the phospholipase A2 domain of the AAV capsid.

Infectivity of evolved variants on T-cells

To determine the infectivity and specificity of the isolated viral variants to HIV-infected and uninfected T-cells, H9 or HIV-infected H9 cells were infected with recombinant AAV expressing green fluorescent protein under the control of the ubiquitin

promoter. Preliminary evidence suggests that clone HIV-4C isolated from the selection for HIV-infected T-cell specificity exhibited a high transduction level on uninfected H9 cells, whereas H9 cells were not transduced by wild-type AAV2 (data not shown). Other selected variants (H9-5C and HIV-2C) transduced the cells to a larger extent than wild-type but not as efficiently as HIV-4C (data not shown). Preliminarily, HIV-2C showed some selectivity for HIV-infected H9 cells with an improvement in transduction upon HIV infection (data not shown), whereas transduction by clone HIV-4C was not significantly improved on this cell type as compared to uninfected cells (data not shown). These results are currently being verified.

Latently infected T-cells represent an important target for HIV gene therapy. The latently-HIV infected population of T-cells presents the biggest barrier to the elimination of HIV from a patient, since suspending HAART allows reactivation of this population and the re-emergence of viral load. Though variants were not selected for this cell-type, specificity would be a valuable phenotype in selected clones. The J-Lat cell lines serve as a model of these latently infected T-cell populations.[31] To determine whether selected clones exhibited any specificity to latently infected T-cells, several J-Lat cell lines were infected with self-complementary recombinant AAV expressing mCherry under the control of the CAG promoter and compared to the infectivity on parental Jurkat cells. All clones exhibited a higher infectivity than wild-type on Jurkat cells and J-Lat 8.4; however, clone HIV-2C exhibited a greater infectivity on all cell lines tested (Fig. 3). This clone also exhibited a greater infectivity on J-Lat lines than the parental Jurkats, indicating some selectivity for these latently infected T-cells. Interestingly, there is a correlation between the infectivity of clone HIV-2C on latently infected T-cell line and the basal level of HIV gene expression within that line. In general, those cell lines with higher basal levels of HIV gene expression[32] are more susceptible to infection by HIV-2C. This could indicate that, while not actively replicating, latent HIV can still change protein expression levels within the cell or proteoglycan and lipid composition on the cell membrane, a change that can be exploited by HIV-2C for improved cellular transduction. Even in cell lines with low basal levels of HIV activation, HIV-2C was rather infectious indicating an ability to target the latent population regardless of how transcriptionally active the HIV genome.

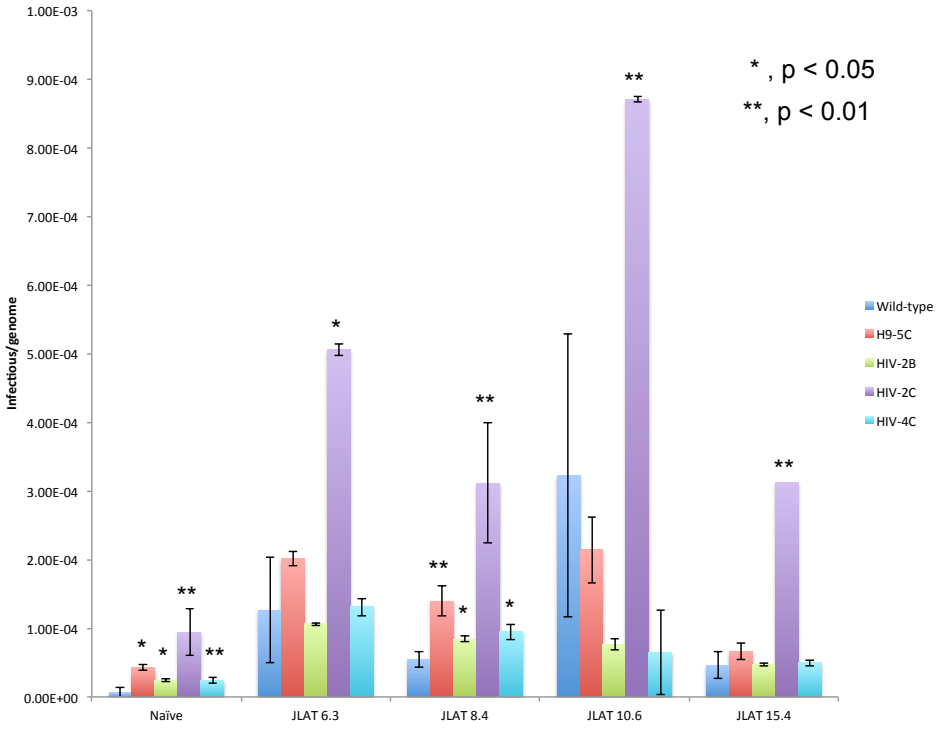


Figure 3. **Infectivity of selected clones on Jurkat and J-Lat cell lines.** Several latently infected T-cell lines, J-Lat, were infected by isolated AAV variants and compared to the transduction on the parental cell line, Jurkat. Statistics indicate a significant difference in infectivity of the viral variant in comparison to wild-type on that cell line as calculated by student t-test. (error-bars: standard deviation of the mean)

Mechanistic insight into HIV-infected T cell specificity

To provide initial insights into possible mechanisms by which select variants selectively infect T-cells, peptide inserts were compared to published protein and peptide sequences. The peptide from clone H9-5C is homologous to Serpin B12 (Table 2), a member of the human ov-serpin family of serpins expressed in thymus tissue.[33] Interestingly, previous experiments successfully retargeted AAV to new cell-types by incorporating a serpin ligand into the viral capsid, though this work did not investigate any infectivity on T cells.[34] The ov-serpin family of serpins has been implicated in regulating immune responses.[35] While the homologous region lies outside of the reactive site loop (RSL), it lies at a fairly conserved C-terminal segment.[33] This serpin has been shown to interact with the nuclear protein, sister chromatid cohesion protein PDS5 homolog A,[36] an interaction the capsid could utilize to successfully target to the nucleus. This could contribute to the increased infectivity on HIV-infected H9 cells. Alternatively, the peptide is homologous to one identified from phage display as interacting with chromatin high mobility group protein 1 (Table 2), a protein that interacts with numerous nuclear proteins,[37] any of which could be utilized by the viral capsid to target to the nucleus. Quantification of intact internalized genomes by qPCR analysis showed an increase of viral genomes within cells upon HIV-infection (data not shown). This could

indicate an improvement in viral binding or internalization, both extracellular events, as well as a reduced proteolysis within the cell due to improved intracellular targeting. The virus, therefore, might alternatively bind to or is internalized more efficiently in HIV-infected T-cells through an as yet unidentified peptide-protein interaction. Further mechanistic studies are necessary to provide additional evidence as to the mechanism of improved transduction.

The peptide from clone HIV-2B is homologous to ErbB3-binding protein (Table 2) in a region immediately C-terminal to a putative RNA interaction domain and within a region necessary for nucleolar transport.[38] This peptide, therefore, could direct capsid intracellular and nuclear trafficking through a similar interaction. This is particularly interesting given the accumulation of intact virus within the nucleolus during cellular infection.[39] Additionally, searching a database of small peptides, the peptide was found to be homologous to a peptide utilized in a study of epidermal growth factor receptor (EGFR) kinetics[40] (Table 2). It has been shown that EGFR signaling can negatively influence AAV transduction efficiency through receptor-tyrosine kinase phosphorylation of tyrosine residues on the exterior of the capsid and subsequent ubiquitination and proteolysis.[41,42] Mutagenesis of these tyrosine residues has been shown to improve transduction efficiency of viral vectors by eliminating phosphorylation and proteolysis.[43] This peptide could operate by similar mechanism; the peptide, while lacking a tyrosine residue, could bind and interact with EGFR preventing its phosphorylation of capsid tyrosine residues.

Interestingly, one of the most infectious and selective viral variants, HIV-2C, differs from wild-type AAV2 by only three point mutations. One mutation, Q598L, has been shown to improve viral thermostability (Chapter Two) but has no effect on heparin binding (Fig. 4). However, the mutation could subtly alter capsid tertiary structure, affecting binding to viral secondary receptors. One mutation, E67A, lies on the VP1 unique region near the active site of the phospholipase A2 domain,[44] a region important in endosomal escape of AAV.[45] Other studies have shown the importance of endosomal escape in determining AAV cellular specificity.[46] The final mutation, S207G, lies at the N-terminus of the VP3 protein near the pore at the five fold-axis of symmetry through which the VP1/VP2 N-terminus is externalized.[47] While this region of the viral capsid has not been shown to affect cellular transduction, this mutation might alter the kinetics of externalization of the VP1/2 unique domains to improve cell type specificity of the viral vector. Both HIV-4C and HIV-2B share one of these latter mutations suggesting it is a synergistic combination that contributes to its improved selectivity, rather than either individual mutation.

A BLAST search of the HIV-4C peptide indicated it is most highly homologous to α-ketoacid dehydrogenases (Table 2), which would not explain its improved transduction efficiency. It is, however, homologous to a peptide isolated from phage display that bound prostate carcinoma cells[48] (Table 2). It is possible that a similar protein is overexpressed in T-cells and prostate carcinoma cells, a protein to which HIV-4C can bind or utilize for internalization, increasing its transduction efficiency. The use of an alternative receptor would reduce the virus's dependence on heparan-sulfate and, thus, explain why the reduced affinity (Fig. 4) did not abrogate infectivity. This hypothesis of improved transduction by alternative cellular receptor binding is supported

by qPCR, which indicated that a greater number of HIV-4C viral particles were internalized by T-cells than wild-type (data not shown).

A	Homologous peptide	Homologous protein	E value	Genbank accession
LANKTQTINA	NKTQTI	Serpin B12	25	AAI03885.1
LAKNPTATTA	LAKDPTA	BCKDHB protein	47	AAH3448.1
LAKNKTAENA	KTAENA	ErbB3-binding protein (EBP1)	66	AAD00646.1
B	**Homologous peptide**	**Interaction**	**E()**	**Pubmed Accession**
LANKTQTINA	NYTQTVP	Chromatin high mobility group protein 1 (HMGB1)	6.3	11748221
LAKNPTATTA	YPRATTAT	LnCaP prostate carcinoma cells	13	11398171
LAKNKTAENA	KGSTAENAEYLRV	EGFR	0.78	3181128

Table 2. **HIV selection peptide homology results**. (A) A BLAST search was performed to compare the peptides isolated from the T-cell and HIV-selected T-cell selections to known human proteins. (B) To identify whether peptides were homologous to any small peptides identified in multiple studies, the peptides were subject to a Smith-Waterman search on PepBank (http://pepbank.mgh.harvard.edu/).

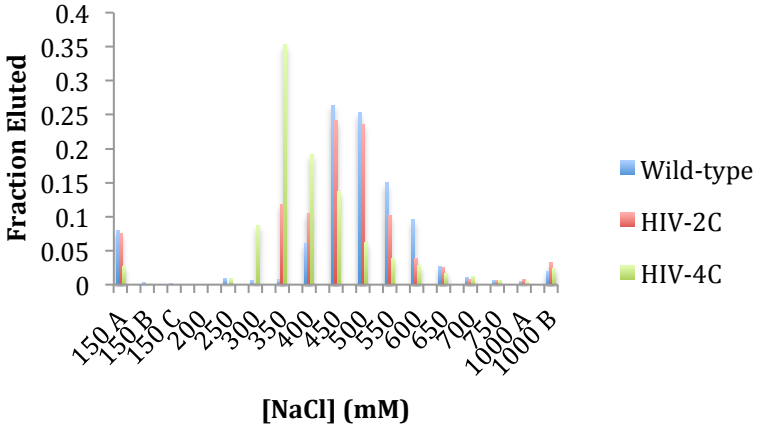


Figure 4. **Heparan dependence of HIV selected clones**. Wild-type AAV2 and clones HIV-2C and HIV-4C were bound to a heparin-affinity column and eluted with increasing concentrations of NaCl. Clone HIV-2C showed an affinity to heparin similar to wild-type, whereas HIV-4C showed a reduced affinity.

Ongoing work

To determine whether the cellular specificity of identified clones is specific to the system within which it was selected or whether it has general T-cell specificity, viral transduction is being determined in several T-cell lines and primary human T-cells. Gene therapy approaches utilizing RNA-interference to prevent HIV replication *in vitro* have been explored.[6] shRNAs shown to successfully knockdown HIV replication are currently being tested in concert with the isolated viral variants to determine whether infection by AAV carrying an anti-HIV gene construct can prevent its transmission *in vitro*.

Discussion

A vector selective for HIV-infected or uninfected T-cells would provide a valuable tool for gene therapy against HIV. Selective infection of uninfected T-cells within a seropositive individual could provide a treatment in a parallel method to that of the "Berlin patient." Alternatively, vector selectivity toward HIV-infected T-cells, especially the latent population, could be utilized to deliver gene constructs that prevent HIV replication. Viral vectors isolated from directed evolution of AAV for T-cell specificity appear to have the requisite phenotypes for use in such therapies.

Of particular interest, gene therapy to the latently infected T-cells would be invaluable as this cell-type is the most difficult target cell to identify and eliminate. One identified clone, HIV-2C, appears to preferentially infect a cellular model of this population. More importantly, while it has improved transduction on cells with higher levels of baseline HIV activation, it maintains a preferential infectivity on those with the lowest levels compared to uninfected cells. This indicates the virus can recognize a phenotypic change within the cell upon HIV infection that it can exploit for infection. Future mechanistic studies into this selectivity could identify biomarkers by which to identify this cellular population.

Sequence analysis suggests multiple possible mechanisms by which AAV can alter its transduction to T-cells. Future experiments can further explore these possible mechanisms and potentially provide information on HIV biology. Thus, directed evolution allowed the engineering of AAV with novel cellular specificity capable of overcoming multiple cellular barriers to infection.

Materials and Methods

Cell lines

Cell lines were cultured at 37°C and 5% CO_2. Jurkat, 293T and H9 cells were obtained from the American type culture collection (Manassas, VA). J-Lat clones[31] were obtained from the lab of Dr. Eric Verdin through the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH. 293T cells were cultured in Dulbecco's modified Eagle's medium. Jurkats, H9 cells and J-Lat clones were cultured in RPMI-1640. All media was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Construction of AAV libraries

Error-prone mutagenesis of AAV2 was performed as described.[49] Briefly *cap* gene was mutagenized by $MnCl_2$ error-prone mutagenesis. Multiple PCR reactions of varying concentrations of manganese chloride were performed to provide a range of mutation frequency. Successful reactions were combined, purified with a commercial PCR clean up kit (Promega) to remove excess $MnCl_2$, and cloned into pSub2. Peptide insert and shuffled AAV libraries were also prepared as described.[28,30] Recombinant and replication competent AAV was then produced, as previously described.[49]

Selection for HIV-infected T-cell specific AAV variants

Cells (stably HIV-infected H9) were incubated with a mixed library consisting of a shuffled AAV library, an AAV2 random 7mer peptide-insert library, and an AAV2 error-prone library. Infectious variants were amplified with adenovirus super-infection and viral genomes isolated. The *cap* genes were PCR-amplified and used to produce virus for subsequent infections. To increase selective pressure, continued selections utilized reduced genomic MOIs of library and decreased cellular incubation times. For rounds of evolution including negative selection, uninfected cells were incubated with the viral library to allow non-specific variants to bind. The supernatant was then transferred to stably HIV-infected cells and infectious clones isolated as above. In total, four rounds of positive selection followed by error-prone mutagenesis and two rounds of negative and positive selection were performed. In parallel, an identical scheme utilizing uninfected H9 cells was performed to isolate a viral variant specific to uninfected T-cells.

T-cell transduction of AAV

H9 and HIV-infected H9 cells (2.5×10^5) in complete media were incubated with virus in an equal volume of media containing recombinant AAV expressing green fluorescent protein under the control of the ubiquitin promoter. Flow cytometry was used to determine the percentage of GFP-positive cells. The relative number of internalized genomes for each virus was determined through harvesting cellular DNA and analysis of the GFP gene by qPCR.

Jurkat and J-Lat cells (5.0×10^4) in complete media were treated with recombinant self-complimentary AAV expressing mCherry under the control of the CAG promoter. Flow cytometry was performed 48 hours after infection to determine the percentage of mCherry-positive cells.

Heparin column chromatography of AAV vectors

Heparin column chromatography was used to determine the affinity of viral vectors to heparin as described.[29] Briefly, recombinant virus was loaded onto a pre-equilibrated 1-mL HiTrap heparin column (Amersham). Washes were performed using Tris buffer containing increasing concentrations of NaCl (up to 750 mM) with one final wash with 1M NaCl. The percentage of virus eluted in each fraction was calculated by harvesting viral genomes and determining the concentration in each by qPCR.

Acknowlegements

This work was performed in collaboration with the research groups of Kate Excoffon and Dawn Wooley at Wright State University.

References

1 WHO. *WHO HIV/AIDS Factsheet*, <http://www.who.int/mediacentre/factsheets/fs360/en/index.htm> (2012).

2 Trono, D. *et al.* HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals. *Science* **329**, 174-180, doi:10.1126/science.1191047 (2010).

3 Hutter, G. *et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *The New England journal of medicine* **360**, 692-698, doi:10.1056/NEJMoa0802905 (2009).

4 Voit, R. A., McMahon, M. A., Sawyer, S. L. & Porteus, M. H. Generation of an HIV Resistant T-cell Line by Targeted "Stacking" of Restriction Factors. *Mol Ther* **21**, 786-795, doi:10.1038/mt.2012.284 (2013).

5 Damian, M. & Porteus, M. H. A Crisper Look at Genome Editing: RNA-guided Genome Modification. *Mol Ther* **21**, 720-722, doi:10.1038/mt.2013.46 (2013).

6 Shah, P. S., Pham, N. P. & Schaffer, D. V. HIV develops indirect cross-resistance to combinatorial RNAi targeting two distinct and spatially distant sites. *Mol Ther* **20**, 840-848, doi:10.1038/mt.2012.3 (2012).

7 Chenine, A. L., Sattentau, Q. & Moulard, M. Selective HIV-1-induced downmodulation of CD4 and coreceptors. *Archives of virology* **145**, 455-471 (2000).

8 Choi, B. *et al.* Down-regulation of cell surface CXCR4 by HIV-1. *Virology journal* **5**, 6, doi:10.1186/1743-422X-5-6 (2008).

9 Hoxie, J. A. *et al.* Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. *Science* **234**, 1123-1127 (1986).

10 Meerloo, T., Parmentier, H. K., Osterhaus, A. D., Goudsmit, J. & Schuurman, H. J. Modulation of cell surface molecules during HIV-1 infection of H9 cells. An immunoelectron microscopic study. *Aids* **6**, 1105-1116 (1992).

11 Stevenson, M., Zhang, X. H. & Volsky, D. J. Downregulation of cell surface molecules during noncytopathic infection of T cells with human immunodeficiency virus. *J Virol* **61**, 3741-3748 (1987).

12 Meerloo, T. *et al.* Host cell membrane proteins on human immunodeficiency virus type 1 after in vitro infection of H9 cells and blood mononuclear cells. An immuno-electron microscopic study. *The Journal of general virology* **74 (Pt 1)**, 129-135 (1993).

13 Berro, R. *et al.* Identifying the membrane proteome of HIV-1 latently infected cells. *The Journal of biological chemistry* **282**, 8207-8218, doi:10.1074/jbc.M606324200 (2007).

14 Lanteri, M. *et al.* Altered T cell surface glycosylation in HIV-1 infection results in increased susceptibility to galectin-1-induced cell death. *Glycobiology* **13**, 909-918, doi:10.1093/glycob/cwg110 (2003).
15 Mebatsion, T., Finke, S., Weiland, F. & Conzelmann, K. K. A CXCR4/CD4 pseudotype rhabdovirus that selectively infects HIV-1 envelope protein-expressing cells. *Cell* **90**, 841-847 (1997).
16 Schnell, M. J., Johnson, J. E., Buonocore, L. & Rose, J. K. Construction of a novel virus that targets HIV-1-infected cells and controls HIV-1 infection. *Cell* **90**, 849-857 (1997).
17 Somia, N. V., Miyoshi, H., Schmitt, M. J. & Verma, I. M. Retroviral vector targeting to human immunodeficiency virus type 1-infected cells by receptor pseudotyping. *J Virol* **74**, 4420-4424 (2000).
18 Horster, A. *et al.* Recombinant AAV-2 harboring gfp-antisense/ribozyme fusion sequences monitor transduction, gene expression, and show anti-HIV-1 efficacy. *Gene Ther* **6**, 1231-1238, doi:10.1038/sj.gt.3300955 (1999).
19 Srivastava, A. Adeno-associated virus-mediated gene transfer. *Journal of cellular biochemistry* **105**, 17-24, doi:10.1002/jcb.21819 (2008).
20 Ponnazhagan, S. *et al.* Adeno-associated virus type 2-mediated transduction in primary human bone marrow-derived CD34+ hematopoietic progenitor cells: donor variation and correlation of transgene expression with cellular differentiation. *J Virol* **71**, 8262-8267 (1997).
21 Gardner, J. P., Zhu, H., Colosi, P. C., Kurtzman, G. J. & Scadden, D. T. Robust, but transient expression of adeno-associated virus-transduced genes during human T lymphopoiesis. *Blood* **90**, 4854-4864 (1997).
22 Mendelson, E., Grossman, Z., Mileguir, F., Rechavi, G. & Carter, B. J. Replication of adeno-associated virus type 2 in human lymphocytic cells and interaction with HIV-1. *Virology* **187**, 453-463 (1992).
23 Zhang, P. X. & Fuleihan, R. L. Transfer of activation-dependent gene expression into T cell lines by recombinant adeno-associated virus. *Gene Ther* **6**, 182-189, doi:10.1038/sj.gt.3300803 (1999).
24 Asuri, P. *et al.* Directed Evolution of Adeno-associated Virus for Enhanced Gene Delivery and Gene Targeting in Human Pluripotent Stem Cells. *Molecular Therapy* **20**, 329-338 (2012).
25 Excoffon, K. J. D. A. *et al.* Directed Evolution of Adeno-associated Virus to an Infectious Respiratory Virus. *Proceedings of the National Academy of Science* **106**, 3865-3870 (2009).
26 Jang, J.-H. *et al.* An Evolved Adeno-associated Viral Variant Enhances Gene Delivery and Gene Targeting in Neural Stem Cells. *Molecular Therapy* **19**, 667-675 (2011).
27 Klimczak, R. R., Koerber, J. T., Dalkara, D., Flannery, J. G. & Schaffer, D. V. A Novel Adeno-Associated Viral Variant for Efficient and Selective Intravitreal Transduction of Rat Muller Cells. *PLoS One* **4**, e7467 (2009).
28 Koerber, J. T. *et al.* Molecular Evolution of Adeno-associated Virus for Enhanced Glial Gene Delivery. *Molecular Therapy* **17**, 2088-2095 (2009).

29 Maheshri, N., Koerber, J. T., Kaspar, B. K. & Schaffer, D. V. Directed Evolution of Adeno-associated Virus Yields Enhanced Gene Delivery Vectors. *Nature Biotechnology* **24**, 198-204 (2006).
30 Koerber, J. T., Jang, J.-H. & Schaffer, D. V. DNA Shuffling of Adeno-associated Virus Yields Functionally Diverse Viral Progeny. *Molecular Therapy* **16**, 1703-1709 (2008).
31 Jordan, A., Bisgrove, D. & Verdin, E. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *The EMBO journal* **22**, 1868-1877, doi:10.1093/emboj/cdg188 (2003).
32 Miller-Jensen, K. *et al.* Chromatin accessibility at the HIV LTR promoter sets a threshold for NF-kappaB mediated viral gene expression. *Integrative biology : quantitative biosciences from nano to macro* **4**, 661-671, doi:10.1039/c2ib20009k (2012).
33 Askew, Y. S. *et al.* SERPINB12 is a novel member of the human ov-serpin family that is widely expressed and inhibits trypsin-like serine proteinases. *The Journal of biological chemistry* **276**, 49320-49330, doi:10.1074/jbc.M108879200 (2001).
34 Wu, P. *et al.* Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* **74**, 8635-8647 (2000).
35 Bots, M. & Medema, J. P. Serpins in T cell immunity. *Journal of leukocyte biology* **84**, 1238-1247, doi:10.1189/jlb.0208140 (2008).
36 Hutchins, J. R. *et al.* Systematic analysis of human protein complexes identifies chromosome segregation proteins. *Science* **328**, 593-599, doi:10.1126/science.1181348 (2010).
37 Dintilhac, A. & Bernues, J. HMGB1 interacts with many apparently unrelated proteins by recognizing short amino acid sequences. *The Journal of biological chemistry* **277**, 7021-7028, doi:10.1074/jbc.M108417200 (2002).
38 Kowalinski, E. *et al.* The crystal structure of Ebp1 reveals a methionine aminopeptidase fold as binding platform for multiple interactions. *FEBS letters* **581**, 4450-4454, doi:10.1016/j.febslet.2007.08.024 (2007).
39 Johnson, J. S. & Samulski, R. J. Enhancement of Adeno-Associated Virus Infection by Mobilizing Capsids into and out of the Nucleolus. *Journal of Virology* **83**, 2632-2644 (2009).
40 Honegger, A. *et al.* Kinetic parameters of the protein tyrosine kinase activity of EGF-receptor mutants with individually altered autophosphorylation sites. *The EMBO journal* **7**, 3053-3060 (1988).
41 Zhong, L. *et al.* Tyrosine-Phosphorylation of AAV2 Vectors and Its Consequences on Viral Intracellular Trafficking and Transgene Expression. *Virology*, 1-9 (2008).
42 Zhong, L. *et al.* A Dual Role of EGFR Protein Tyrosine Kinase Signaling in Ubiquitination of AAV2 Capsids and Viral Second-strand DNA Synthesis. *Molecular Therapy* **15**, 1323-1330 (2007).
43 Zhong, L. *et al.* Next Generation of Adeno-associated Virus 2 Vectors: Point Mutations in Tyrosines Lead to High-Efficiency Transduction at Lower Doses. *Proceedings of the National Academy of Science* **105**, 7827-7832 (2008).

44 Girod, A. *et al.* The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *The Journal of general virology* **83**, 973-978 (2002).
45 Stahnke, S. *et al.* Intrinsic phospholipase A2 activity of adeno-associated virus is involved in endosomal escape of incoming particles. *Virology* **409**, 77-83, doi:10.1016/j.virol.2010.09.025 (2011).
46 Nonnenmacher, M. & Weber, T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther* **19**, 649-658, doi:10.1038/gt.2012.6 (2012).
47 Bleker, S., Sonntag, F. & Kleinschmidt, J. A. Mutational analysis of narrow pores at the fivefold symmetry axes of adeno-associated virus type 2 capsids reveals a dual role in genome packaging and activation of phospholipase A2 activity. *J Virol* **79**, 2528-2540, doi:10.1128/JVI.79.4.2528-2540.2005 (2005).
48 Romanov, V. I., Durand, D. B. & Petrenko, V. A. Phage display selection of peptides that affect prostate carcinoma cells attachment and invasion. *The Prostate* **47**, 239-251, doi:10.1002/pros.1068 (2001).
49 Koerber, J. T., Maheshri, N., Kaspar, B. K. & Schaffer, D. V. Construction of diverse adeno-associated viral libraries for directed evolution of enhanced gene delivery vehicles. *Nat Protoc* **1**, 701-706, doi:10.1038/nprot.2006.93 (2006).