Published in final edited form as:

Curr Opin Virol. 2014 October ; 0: 90–97. doi:10.1016/j.coviro.2014.07.008.

Adeno-associated Virus: Fit to serve

Eric Zinn¹ and Luk H. Vandenberghe^{1,*}

¹Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA 02114

Abstract

Adeno-associated virus (AAV) is a helper-dependent parvovirus which has not been linked with human disease. This aspect, in combination with its broad cell and tissue tropism, and limited viral host response has made it an attractive vector system for gene therapy. The viral protein capsid, the primary interface with the host, is the main determinant for these phenotypes, is highly variable, and is most subject to pressures during replication. Here, we explore the evolutionary path of AAV and other parvoviruses in respect to these phenotypes, as well as directed evolution and engineering strategies that have exploited the lessons learned from natural selection in order to address remaining limitations of AAV as a therapeutic gene transfer platform.

Keywords

evolution; directed evolution; biopanning; parvovirus; adeno-associated virus; AAV; gene therapy; gene transfer; tropism; immunity

Introduction

Originally discovered as a contaminant in a preparation of simian adenovirus[1], adeno-associated virus (AAV) has come to prominence as an attractive candidate to serve as a vector system for therapeutic gene transfer. A member of the family Parvoviridae, AAVs carry their 4.7 kb single-stranded genomes within non-enveloped T=1 icosahedral capsids[2]. AAVs have been isolated from a wide range of animal samples including human, non-human primate, caprine, bovine, and avian samples. However, a defining characteristic of AAV is its apparent dependence on a helper virus co-infection (such as adenoviruses or herpesviruses) for productive replication. Helper-dependent replication also distinguishes AAVs from other members of the family Parvoviridae and delineates the subfamily, dependoparvovirus (formerly known as dependovirus).

Due to a number of features inherent to their viral biology, AAVs have also become widely used as gene transfer vectors. AAV has not been associated with disease, has a wide and

© 2014 Elsevier B.V. All rights reserved.

*Corresponding author: 20 Staniford St., Boston MA 02114, +1-617-573-6993, luk_vandenberghe@meei.harvard.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

promiscuous tropism, is minimally immunogenic, and can achieve efficient and long-lived gene transfer. Eliminating all viral open-reading frames from the viral genome, replication defective AAV viral-like particles (also known as recombinant AAV or rAAV) containing heterologous genetic information can be assembled and packaged to high vector yields for gene transfer applications. When loaded with a therapeutic transgene, clinical safety and efficacy of AAV has been shown for inherited forms of blindness[3,4] and hemophilia B[5]. At the preclinical stage many approaches have been explored using AAV ranging from therapeutic paradigms for single gene inherited disorders, complex acquired disease (reviewed in Weinberg et al. [6]), and infectious disease (e.g. for influenza and HIV[7–10]).

While AAV has evolved several desirable phenotypes for therapeutic gene transfer, its biology also poses certain important limitations to its application for gene therapy. AAV is only capable of efficiently packaging about 5 kb of DNA, excluding many therapeutic genes and approaches from development. Moreover, the fact that many AAV serotypes appear to be endemic results in extensive anti-viral immunity in human populations[11,12], complicating future AAV gene transfer in pre-immune subjects. Furthermore, although the natural diversity of AAVs is vast, and host tropism differs among AAV species, several important cell types and tissues for gene therapy remain to be unlocked for targeting. The study of AAVs natural evolution and exploitation of laboratory based directed evolution and engineering has been creatively used to influence its biological properties and phenotypes to address some of these remaining limitations.

Parvoviridae evolution

A sister clade to dependoparvovirus, protoparvovirus (formerly parvovirinae) is a subfamily of Parvoviridae encompassing some of the first members to be isolated and characterized including Kilham rat virus and canine parvovirus[13] (Figure 1). These viruses bear many similarities to AAVs with a notable exception that they are all autonomous viruses capable of efficient replication without coinfection of a helper virus. An additional difference to keep in mind is that unlike AAV these viruses can also be pathogenic to their hosts. Despite these differences, interest surrounding the emergence of novel protoparvovirus members and the evolutionary drivers of other member viruses has motivated phylogenetic studies which may serve as a model for similar work in other Parvoviridae subfamilies including dependoparvovirus.

One feature of protoparvoviruses which may apply to other subfamilies is the relatively high rate of observed nucleotide substitution relative to other DNA viruses. While studying canine parvovirus (CPV), it was observed that the rate of nucleotide substitutions in the CPV genome more closely resembled those of RNA viruses rather than those of double-stranded DNA viruses[14]. This high rate of variation has also been seen in another protoparvovirus, the minute virus of mice (MVM) where it has been attributed to being involved in immune escape [15]. Furthermore, similar substitution rates have also been observed in human parvovirus B19 [16], a member of another subfamily of Parvoviridae (erythroparvovirus) suggesting that this high rate of evolution may be a general characteristic of the Parvoviridae family. Additional studies have begun to explore the extent to which these high mutational rates affect evolution of parvoviruses.

Extensive phylogenetic studies on the emergence of CPV have helped to clarify and redefine a story of viral evolution. It had long been understood that CPV emerged from a feline parvovirus-like ancestor, primarily under the influence of adaptation to a canine transferrin receptor (TfR)[17]. However, recent phylogenetic studies have suggested a role for antigenicity and cross-species transmission to play in host adaptation[18,19]. Moreover, a recent phylogenetic analysis of caniniform TfR genes has revealed evidence of an evolutionary arms race between virus and host[20]. Taken together, these studies of the evolution of protoparvoviruses have revealed a new evolutionary framework wherein a myriad of viral and host factors act in concert to shape the history of CPV and related viruses. Studies like these in protoparvovirus and other subfamilies of Parvoviridae may serve as a template to understand the evolution of AAVs, however certain considerations may need to be taken into account when studying the evolution of AAV due to the distinct aspects of their biology.

Although phylogenetic analysis suggests that dependoparvoviruses shared a common ancestor with other members of Parvoviridae, they are a distinct outgroup more closely related to autonomous avian parvoviruses than other viruses which also infect humans and human primates[21]. The helper-dependent phenotype is unique to this subfamily and the origin of this phenotype and the impact it has had on the lineage of dependoparvoviridae remains largely unstudied. In addition, the lack of pathogenicity in its host might alter the impact of evolutionary determinants relative to other parvoviruses. Furthermore, unlike other Parvoviridae members, AAVs have an additional recently discovered short, overlapping gene which encodes an assembly-activating protein (AAP) which appears to be necessary for production of AAV VLPs[22,23]. A recent computational model developed from data acquired from experiments performed on AAV2 suggest that AAP restricts diversity at some positions along the AAV capsid gene[24]. These factors and others may need to be considered when performing evolutionary studies on AAV.

Natural Diversity and Selection of AAVs

One consequent of the evolution of AAVs is the large genetic diversity of the over 100 isolates characterized at this time. Most of these isolates were found in biological samples from primates with the intent of finding orthologous AAV capsids with desirable biological characteristics for gene transfer purposes [25–27]. However, viruses have also been isolated from pigs[28], sea lions[29], bats[30], and snakes[31]. Even when limited to viruses isolated from primates, capsids of these different viruses demonstrate 51–87% identity between clades (Table 1) with most variability being located in surface exposed regions of the capsid (Figure 2).

In addition to a diversity of host species, AAVs appear to have some diversity with regards to the molecular receptors they exploit to bind to host cells as assessed through experiments performed on recombinant AAV vectors. While many AAVs bind glycans, there is some diversity across and even within clades. Furthermore, different AAVs don't necessarily use the same structural areas of the capsid to bind these glycans. As an example, while it has long been known that AAV2 exploits heparan sulfate proteoglycan as a receptor[32], recent studies have found that N-linked galactose is likely the molecular receptor of AAV9[33,34]

and galactose binding takes place in a region of the AAV9 capsid distinct from the HSPG binding region in AAV2[35]. This fact was recently used to engineer new vectors by engrafting the galactose binding footprint of AAV9 onto the capsid of AAV2 to create a vector capable of binding both glycans[36]. The fact that these viruses use structurally distinct regions and distinct substrates to achieve the same biological goal (i.e. glycan mediated receptor binding) may suggest divergent evolution and study of the selective pressures which may have contributed to this diversity of receptor binding phenotypes may provide insight into the biology of AAV. Although the receptors for many AAVs remain unknown, a recently developed technique to rapidly screen AAVs for their abilities to bind different glycans[37] may help to refine our understanding of the evolution of glycan binding in AAVs.

In order to understand more clearly the evolutionary landscape surrounding AAV, it will likely be helpful or even necessary to study the relationship of AAV with their hosts. A study of viral integration events into a wide spectrum of organism genomes found an abundance of dependoparvovirus and protoparvovirus integrations which the authors date back to over 40 million years ago[38]. The implication is that these viruses and their hosts have had many opportunities to influence each other's evolutionary lineage over timescales which include many speciation events. A recent study of the Rep proteins of AAV2 found that they act to repress the activity of the host-promoter of the *PPP1R12C* gene within which the wild-type virus prefers to integrate[39]. The fact that AAV Rep proteins have evolved to acquire multifunctional roles to control the transcription of its host suggests an interconnected evolutionary history.

An additional possible factor to consider when studying the evolution of AAV is the role played by the helper virus. Some evidence that helper viruses and AAVs have coevolved comes in the form of studies of helper virus genomes. Human herpes virus 6 (HHV-6), as an example, possesses a gene (*U94/Rep*) which was likely captured from an adeno-associated virus[2,40]. Within the context of this helper virus, this homologue of AAV Rep has been demonstrated to inhibit the replication of HHV-6 and other betaherpesviruses[41] while repressing some steps of angiogenesis[42]. Additionally, a recent review of mutualistic viruses suggested that AAV may play a role in mitigating adenoviral induced oncogenesis[43] which may suggest a complex three way evolutionary network between a host and two viruses. In short, there is some evidence which raises the possibility that AAV might cooperate or compete with its helper viruses within the context of an infected host cell.

While the mechanism of AAV transmission between hosts is currently unknown, analysis of the phylogenetic relationship of AAV isolates has suggested a role for cross-species transmission between humans and non-human primates[26]. Furthermore, the same studies revealed a clade of hybrid viruses which appeared to have originated as a result of a single recombination event between members of two different clades of AAV[26]. Additionally, when a group endeavored to study porcine AAVs, it was discovered that pigs from privately owned farms had much higher incidence of AAV than those from slaughterhouses, a fact which the authors speculated may have arisen from higher frequencies of zoonotic transmission on private farms due to closer contact between humans and pigs[28]. Taken

together, the evidence for zoonotic transmission and recombination might help to explain the high rate of viral evolution broadly observed in AAV.

Hosts may also contribute to the evolution of AAV through the immune responses they mount against these viruses. Analysis of seroprevalence of AAV in healthy populations across the globe has indicated that, depending on the serotype of the virus, a majority of the population may have circulating neutralizing antibodies against AAV[11,12]. Recent efforts to map murine monoclonal fragment antibodies and their binding footprints onto the structures of different AAV capsids suggests that much of this neutralization may result from the binding of antibodies to certain common regions[44]. These data suggest a potential for host adaptive immunity to influence AAV evolution.

Directed Evolution of AAV

One useful tool in the toolbox of AAV vector engineering has been the use of directed evolution in which a heterologous library of AAVs is subjected to an experimentally controlled selective pressure for the purposes of screening for desirable phenotypes. Intended to simulate many of the processes of natural evolution, this technique has been applied to engineer a variety of AAV phenotypes. While these techniques have been extensively reviewed elsewhere[45], what follows is a brief discussion of components of directed evolution, some of its recent successes, and some of its challenges.

Directed evolution of AAV presupposes the existence of a relevant and diverse library of AAV genes, most often variants of the AAV capsid gene. The first AAV libraries created for the purposes of directed evolution were made using error-prone PCR[46] which to some extent mimics a likely mechanism by which diversity naturally arises in AAV populations. The next generation of libraries also incorporated a technique now referred to as capsid-shuffling, in which many AAV capsid genes are fragmented and randomly reassembled to form chimeras[47,48]. The net result mimics another mechanism suspected to contribute to natural AAV evolution, recombination within cells. These two methods have also recently been combined with the insertion of random peptides in certain regions of the AAV capsid and replacement of loop regions with random peptides to yield highly diverse pools of AAV for directed evolution[49].

After generating a sufficiently diverse pool of AAVs, these viruses are then subjected to some kind of controlled selection either *in vitro* or *in vivo*. A recent *in vitro* directed evolution experiment resulted in the discovery of an AAV capsid capable of more efficiently transducing human pluripotent stem cells (hPSCs)[50]. One recent *in vivo* study made use of a xenograft model in which human hepatocellular carcinomas were grafted onto murine liver to form chimaeric human-murine livers which were then used to select for variants in an AAV library which efficiently transduced human liver cells[51]. Another *in vivo* directed evolution experiment succeeding in improving AAVs ability to transduce murine and primate retina following intravitreal injection[52]. These studies and others illustrate the diversity of selective pressures that can be applied to a library of AAV capsids.

Study of genetically diverse libraries under an experimentally controlled selective pressure may also provide additional insight into the evolutionary dynamism of AAV not previously

possible through natural data alone. A recently developed technique to study phenotypes of many AAVs concurrently dubbed “AAV Barcode-Seq” demonstrates that next-gen sequencing can be used to inform our understanding of genotype-phenotype correlations[53]. By applying this technique or similar next-generation sequencing based techniques in the context of selective pressure, it may be possible to observe AAV evolution in real time.

Conclusion

Despite almost fifty years since the isolation of the first AAV, research into the evolution of AAV and how natural selection has contributed to the current biological diversity is only beginning. Although the power of selection to influence the phenotype of AAV has been embraced by the academic community through its continued use of directed evolution, the natural evolution of the virus remains largely understudied. By highlighting the utility that phylogenetics and evolutionary approaches have demonstrated in the parallel field of protoparvovirus study, examining the diversity of natural AAVs, and reviewing how various aspects of AAV biology can influence its evolution, we believe we have demonstrated that study of the evolution of AAV has an enormous amount of potential. Research along these avenues will have a dual benefit by enhancing our ability to discover and engineer better vectors for gene therapy as well as improving our understanding of viral evolution as a whole and thus should be pursued with renewed enthusiasm.

Acknowledgments

Research was supported by NIH 1DP1OD008267-01, The Curing Kids Fund, Foundation for Retinal Research, Foundation Fighting Blindness (Individual Investigator Award and Center Grant), Research to Prevent Blindness and Institutional Funds. EZ and LHV are inventors on patents related to AAV gene therapy. LHV has served as a consultant and is inventor on technologies licensed to biotechnology and pharmaceutical industries. LHV is co-founder and consultant to GenSight Biologics.

References

1. Atchison RW, Casto BC, Hammon WM. Adenovirus-associated defective virus particles. *Science*. 1965; 149:754–756. [PubMed: 14325163]
2. Knipe, DM.; Howley, P. *Fields Virology*. Philadelphia: Lippincott Williams & Wilkins; 2013.
3. MacLaren RE, Groppe M, Barnard AR, Cottrill CL, Tolmachova T, Seymour L, Clark KR, During MJ, Cremers FP, Black GC, et al. Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial. *Lancet*. 2014; 383:1129–1137. [PubMed: 24439297]
4. Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, et al. Safety and efficacy of gene transfer for Leber’s congenital amaurosis. *N Engl J Med*. 2008; 358:2240–2248. [PubMed: 18441370]
5. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, Chowdary P, Riddell A, Pie AJ, Harrington C, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*. 2011; 365:2357–2365. [PubMed: 22149959]
6. Weinberg MS, Samulski RJ, McCown TJ. Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology*. 2013; 69:82–88. [PubMed: 22465202]
7. Johnson PR, Schnepf BC, Zhang J, Connell MJ, Greene SM, Yuste E, Desrosiers RC, Clark KR. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nat Med*. 2009; 15:901–906. [PubMed: 19448633]
- 8**. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature*. 2012; 481:81–84. AAV is used here to

provide protection to HIV infection in a humanized mouse HIV infection model by constitutively expressing high levels of broadly neutralizing anti-HIV antibodies. [PubMed: 22139420]

9. Balazs AB, Bloom JD, Hong CM, Rao DS, Baltimore D. Broad protection against influenza infection by vectored immunoprophylaxis in mice. *Nat Biotechnol.* 2013; 31:647–652. [PubMed: 23728362]
10. Limberis MP, Adam VS, Wong G, Gren J, Kobasa D, Ross TM, Kobinger GP, Tretiakova A, Wilson JM. Intranasal antibody gene transfer in mice and ferrets elicits broad protection against pandemic influenza. *Sci Transl Med.* 2013; 5:187ra172.
11. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis.* 2009; 199:381–390. [PubMed: 19133809]
12. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, Masurier C. 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. *Hum Gene Ther.* 2010; 21:704–712. [PubMed: 20095819]
13. Cotmore SF, Agbandje-McKenna M, Chiorini JA, Mukha DV, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen P, Gatherer D, et al. The family Parvoviridae. *Arch Virol.* 2014; 159:1239–1247. [PubMed: 24212889]
14. Shackelton LA, Parrish CR, Truyen U, Holmes EC. High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proc Natl Acad Sci U S A.* 2005; 102:379–384. [PubMed: 15626758]
15. Lopez-Bueno A, Mateu MG, Almendral JM. High mutant frequency in populations of a DNA virus allows evasion from antibody therapy in an immunodeficient host. *J Virol.* 2003; 77:2701–2708. [PubMed: 12552010]
16. Shackelton LA, Holmes EC. Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. *J Virol.* 2006; 80:3666–3669. [PubMed: 16537636]
17. Hueffer K, Parker JS, Weichert WS, Geisel RE, Sgro JY, Parrish CR. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. *J Virol.* 2003; 77:1718–1726. [PubMed: 12525605]
18. Stucker KM, Pagan I, Cifuentes JO, Kaelber JT, Lillie TD, Hafenstein S, Holmes EC, Parrish CR. The role of evolutionary intermediates in the host adaptation of canine parvovirus. *J Virol.* 2012; 86:1514–1521. [PubMed: 22114336]
19. Allison AB, Kohler DJ, Fox KA, Brown JD, Gerhold RW, Shearn-Bochsler VI, Dubovi EJ, Parrish CR, Holmes EC. Frequent cross-species transmission of parvoviruses among diverse carnivore hosts. *J Virol.* 2013; 87:2342–2347. [PubMed: 23221559]
20. Kaelber JT, Demogines A, Harbison CE, Allison AB, Goodman LB, Ortega AN, Sawyer SL, Parrish CR. Evolutionary reconstructions of the transferrin receptor of Caniforms supports canine parvovirus being a re-emerged and not a novel pathogen in dogs. *PLoS Pathog.* 2012; 8:e1002666. [PubMed: 22570610]
21. Lukashov VV, Goudsmit J. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. *J Virol.* 2001; 75:2729–2740. [PubMed: 11222696]
22. Sonntag F, Kother K, Schmidt K, Weghofer M, Raupp C, Nieto K, Kuck A, Gerlach B, Bottcher B, Muller OJ, et al. The assembly-activating protein promotes capsid assembly of different adeno-associated virus serotypes. *J Virol.* 2011; 85:12686–12697. [PubMed: 21917944]
23. Naumer M, Sonntag F, Schmidt K, Nieto K, Panke C, Davey NE, Popa-Wagner R, Kleinschmidt JA. Properties of the adeno-associated virus assembly-activating protein. *J Virol.* 2012; 86:13038–13048. [PubMed: 23015698]
24. Kawano Y, Neeley S, Adachi K, Nakai H. An experimental and computational evolution-based method to study a mode of co-evolution of overlapping open reading frames in the AAV2 viral genome. *PLoS One.* 2013; 8:e66211. [PubMed: 23826091]
25. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, Calcedo R, Sanmiguel J, Abbas Z, Wilson JM. Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci U S A.* 2003; 100:6081–6086. [PubMed: 12716974]

26. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM. Clades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol.* 2004; 78:6381–6388. [PubMed: 15163731]
27. Gao G, Vandenberghe LH, Wilson JM. New recombinant serotypes of AAV vectors. *Curr Gene Ther.* 2005; 5:285–297. [PubMed: 15975006]
28. Bello A, Tran K, Chand A, Doria M, Allocca M, Hildinger M, Beniac D, Kranendonk C, Auricchio A, Kobinger GP. Isolation and evaluation of novel adeno-associated virus sequences from porcine tissues. *Gene Ther.* 2009; 16:1320–1328. [PubMed: 19626054]
29. Li L, Shan T, Wang C, Cote C, Kolman J, Onions D, Gulland FM, Delwart E. The fecal viral flora of California sea lions. *J Virol.* 2011; 85:9909–9917. [PubMed: 21795334]
30. Li L, Victoria JG, Wang C, Jones M, Fellers GM, Kunz TH, Delwart E. Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. *J Virol.* 2010; 84:6955–6965. [PubMed: 20463061]
31. Farkas SL, Zadori Z, Benko M, Essbauer S, Harrach B, Tijssen P. A parvovirus isolated from royal python (*Python regius*) is a member of the genus *Dependovirus*. *J Gen Virol.* 2004; 85:555–561. [PubMed: 14993638]
32. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol.* 1998; 72:1438–1445. [PubMed: 9445046]
33. Bell CL, Vandenberghe LH, Bell P, Limberis MP, Gao GP, Van Vliet K, Agbandje-McKenna M, Wilson JM. The AAV9 receptor and its modification to improve in vivo lung gene transfer in mice. *J Clin Invest.* 2011; 121:2427–2435. [PubMed: 21576824]
- 34*. Shen S, Bryant KD, Brown SM, Randell SH, Asokan A. Terminal N-linked galactose is the primary receptor for adeno-associated virus 9. *J Biol Chem.* 2011; 286:13532–13540. The authors identify a novel cellular receptor for AAV9 through biochemical studies in vitro and demonstrate that pre-treatment of mouse-airway epithelia with an enzyme which exposes additional receptors can increase transduction by over an order of magnitude. [PubMed: 21330365]
35. Bell CL, Gurda BL, Van Vliet K, Agbandje-McKenna M, Wilson JM. Identification of the galactose binding domain of the adeno-associated virus serotype 9 capsid. *J Virol.* 2012; 86:7326–7333. [PubMed: 22514350]
- 36*. Shen S, Horowitz ED, Troupes AN, Brown SM, Pulicherla N, Samulski RJ, Agbandje-McKenna M, Asokan A. Engraftment of a galactose receptor footprint onto adeno-associated viral capsids improves transduction efficiency. *J Biol Chem.* 2013; 288:28814–28823. A new AAV vector is engineered by engrafting a galactose receptor domain into an AAV2 background, resulting in a vector which the authors demonstrate can utilize both heparan sulfate as well as galactose for infection. [PubMed: 23940044]
37. Mietzsch M, Broecker F, Reinhardt A, Seeberger PH, Heilbronn R. Differential adeno-associated virus serotype-specific interaction patterns with synthetic heparins and other glycans. *J Virol.* 2014; 88:2991–3003. [PubMed: 24371066]
38. Belyi VA, Levine AJ, Skalka AM. Sequences from ancestral single-stranded DNA viruses in vertebrate genomes: the parvoviridae and circoviridae are more than 40 to 50 million years old. *J Virol.* 2010; 84:12458–12462. [PubMed: 20861255]
39. Dutheil N, Smith SC, Agundez L, Vincent-Mistiaen ZI, Escalante CR, Linden RM, Henckaerts E. Adeno-associated virus Rep represses the human integration site promoter by two pathways that are similar to those required for the regulation of the viral p5 promoter. *J Virol.* 2014
40. Thomson BJ, Efstathiou S, Honess RW. Acquisition of the human adeno-associated virus type-2 rep gene by human herpesvirus type-6. *Nature.* 1991; 351:78–80. [PubMed: 1851252]
41. Caselli E, Bracci A, Galvan M, Boni M, Rotola A, Bergamini C, Cermelli C, Dal Monte P, Gompels UA, Cassai E, et al. Human herpesvirus 6 (HHV-6) U94/REP protein inhibits betaherpesvirus replication. *Virology.* 2006; 346:402–414. [PubMed: 16368124]
42. Caruso A, Caselli E, Fiorentini S, Rotola A, Prandini A, Garrafa E, Saba E, Alessandri G, Cassai E, Di Luca D. U94 of human herpesvirus 6 inhibits in vitro angiogenesis and lymphangiogenesis. *Proc Natl Acad Sci U S A.* 2009; 106:20446–20451. [PubMed: 19918067]

43. Roossinck MJ. The good viruses: viral mutualistic symbioses. *Nat Rev Microbiol.* 2011; 9:99–108. [PubMed: 21200397]
- 44**. Gurda BL, DiMattia MA, Miller EB, Bennett A, McKenna R, Weichert WS, Nelson CD, Chen WJ, Muzyczka N, Olson NH, et al. Capsid antibodies to different adeno-associated virus serotypes bind common regions. *J Virol.* 2013; 87:9111–9124. Through cryo-electron microscopy, several different monoclonal fragment antibodies (Fabs) were mapped to the surface of four different AAV serotypes, revealing that Fabs frequently bind common regions of the viral capsid near the 3-fold axes. [PubMed: 23760240]
45. Bartel MA, Weinstein JR, Schaffer DV. Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. *Gene Ther.* 2012; 19:694–700. [PubMed: 22402323]
46. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat Biotechnol.* 2006; 24:198–204. [PubMed: 16429148]
47. Li W, Asokan A, Wu Z, Van Dyke T, DiPrimio N, Johnson JS, Govindaswamy L, Agbandje-McKenna M, Leichtle S, Redmond DE Jr, et al. Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. *Mol Ther.* 2008; 16:1252–1260. [PubMed: 18500254]
48. Koerber JT, Jang JH, Schaffer DV. DNA shuffling of adeno-associated virus yields functionally diverse viral progeny. *Mol Ther.* 2008; 16:1703–1709. [PubMed: 18728640]
49. Koerber JT, Klimczak R, Jang JH, Dalkara D, Flannery JG, Schaffer DV. Molecular evolution of adeno-associated virus for enhanced glial gene delivery. *Mol Ther.* 2009; 17:2088–2095. [PubMed: 19672246]
50. Asuri P, Bartel MA, Vazin T, Jang JH, Wong TB, Schaffer DV. Directed evolution of adeno-associated virus for enhanced gene delivery and gene targeting in human pluripotent stem cells. *Mol Ther.* 2012; 20:329–338. [PubMed: 22108859]
- 51**. Lisowski L, Dane AP, Chu K, Zhang Y, Cunningham SC, Wilson EM, Nygaard S, Grompe M, Alexander IE, Kay MA. Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature.* 2014; 506:382–386. The authors utilize a chimaeric human-murine liver model to compare the ability of different AAVs to transduce human hepatocytes and further use the model to select for an engineered virus which efficiently transduces human hepatocytes. They propose the model as a more precise tool for evaluating AAV serotypes in a human context. [PubMed: 24390344]
52. Dalkara D, Byrne LC, Klimczak RR, Visel M, Yin L, Merigan WH, Flannery JG, Schaffer DV. In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. *Sci Transl Med.* 2013; 5:189ra176.
53. Adachi K, Enoki T, Kawano Y, Veraz M, Nakai H. Drawing a high-resolution functional map of adeno-associated virus capsid by massively parallel sequencing. *Nat Commun.* 2014; 5:3075. [PubMed: 24435020]

Highlights

- The adeno-associated virus (AAV) is attractive for use as a gene therapy vector
- The parvoviral capsid evolves to modify receptor binding and host immunity
- AAV relies on a helper-virus infection to go through replication
- AAV is under selective pressure to alter receptor binding and evade host immunity
- Laboratory-based directed evolution is guiding gene therapy vector development

Figure 1. Phylogenetic relationship of representative members of the family Parvoviridae. Maximum likelihood phylogeny of viruses based on a full-genome alignment. Subfamilies are labeled with brackets.

Figure 2.

Natural diversity of adeno-associated virus capsids. A) Maximum likelihood phylogeny of AAVs based on a multiple sequence alignment of capsids. Muscovy Duck Parvovirus was included as an out-group. B) Variability plotted on the structure of the AAV2 capsid monomer (PDB: 1LP3). Blue end of the spectrum represents more conserved positions while the red end represents more variable positions. Variability was calculated using the Shannon-entropy statistic on a multiple sequence alignment of the sequences depicted in the phylogeny, excluding MDPV. C) Variability plotted as before on the AAV2 full capsid.

Sequence identity matrix of representative primate AAVs. Percentage sequence identity based on alignments of the capsid amino acid sequences.

Table 1

	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9
AAV1	ID	83%	86%	63%	57%	99%	84%	84%	81%
AAV2		ID	87%	60%	57%	83%	81%	82%	81%
AAV3			ID	62%	57%	87%	83%	85%	83%
AAV4				ID	51%	63%	63%	63%	61%
AAV5					ID	57%	56%	57%	55%
AAV6						ID	84%	84%	81%
AAV7							ID	87%	80%
AAV8								ID	84%
AAV9									ID