Published in final edited form as:

J Struct Biol. 2020 February 01; 209(2): 107433. doi:10.1016/j.jsb.2019.107433.

Structure comparison of the chimeric AAV2.7m8 vector with parental AAV2

Antonette Bennett^{a,†}, Annahita Keravala^{b,†}, Victoria Makal^a, Justin Kurian^a, Brahim Belbellaa^b, Rangoli Aeraan^b, Yu-Shan Tseng^b, Duncan Sousa^c, John Spear^c, Mehdi Gasmi^{b,*}, Mavis Agbandje-McKenna^{a,*}

^aDepartment of Biochemistry and Molecular Biology, Center for Structural Biology, McKnight Brain Institute, College of Medicine, University of Florida, 1200 Newell Drive, Gainesville, FL, 32610, USA

^bAdverum Biotechnologies, 1035 O'Brien Dr., Menlo Park, CA 94025, USA

^cBiological Science Imaging Resource, Department of Biological Sciences, The Florida State University, 89 Chieftan Way, Rm 119, Tallahassee, FL 32306, USA

Abstract

The AAV2.7m8 vector is an engineered capsid with a 10-amino acid insertion in Adeno-associated virus (AAV) surface variable region VIII (VR-VIII) resulting in the alteration of an antigenic region of AAV2 and the ability to efficiently transduce retina cells following intravitreal administration. Directed evolution and *in vivo* screening in the mouse retina isolated this vector. In the present study, we sought to identify the structural differences between a recombinant AAV2.7m8 (rAAV2.7m8) vector packaging a GFP genome and its parental serotype, AAV2, by cryo-electron microscopy (cryo-EM) and image reconstruction. The structures of rAAV2.7m8 and AAV2 were determined to 2.91 and 3.02 Å resolution, respectively. The rAAV2.7m8 amino acid side-chains for residues 219-745 (the last C-terminal residue) were interpretable in the density map with the exception of the 10 inserted amino acids. While observable in a low sigma threshold density, side-chains were only resolved at the base of the insertion, likely due to flexibility at the top of the loop. A comparison to parental AAV2 (ordered from residues 217-735) showed the structures to be similar, except at some side-chains that had different orientations and, in VR-VIII containing the 10 amino acid insertion. VR-VIII is part of an AAV2 antigenic epitope, and the

*Co-corresponding authors: M. Gasmi, mehdi@adverum.com; M. Agbandje-McKenna, mckenna@ufl.edu.

†These authors contributed equally to this work.

Author contributions

M. Agbandje-McKenna, M. Gasmi and A. Keravala conceived the project. R. Aeraan and Y.S. Tseng conducted molecular cloning. Duncan Sousa and John Spear collected the AAV2 cryo-EM data, T. Makal prepared the AAV2.7m8 sample for data collection and ran the Native immunodot blot assays, M. Agbandje-McKenna determined the AAV2.7m8 and AAV2 structures by cryo-EM and image reconstruction, Justin Kurian refined the AAV2 structure, and Antonette Bennett refined the AAV2.7m8 structure. A. Bennett, B. Belbellaa, M. Gasmi, and M. Agbandje-McKenna wrote the manuscript.

Conflict of interest statement

M. Agbandje-McKenna (MAM) is a SAB member for Voyager Therapeutics, Inc., and AGTC, has a sponsored research agreement with AGTC and Voyager Therapeutics, and is a consultant for Intima Biosciences, Inc. MAM is a co-founder of StrideBio, Inc. MAM and Antonette Bennett have IP on AAV vectors. A. Keravala (AK), R. Aeraan (RA) and YS. Tseng (YST) were employees of Adverum Biotechnology Inc, at the time of this study. Currently, AK is employed by Rocket Pharmaceuticals, RA by Casebia Therapeutics and YST by Biomarin. M. Gasmi is CSO and president and Brahim Belbellaa employee of Adverum Biotechnologies and both hold stock grants. Adverum Biotechnologies Inc has been licensed the exclusive exploitation rights to the rAAV2.7m8 vector.

difference is consistent with rAAV2.7m8's escape from a known AAV2 monoclonal antibody, C37-B. The observations provide valuable insight into the configuration of inserted surface peptides on the AAV capsid and structural differences to be leveraged for future AAV vector rational design, especially for retargeted tropism and antibody escape.

Keywords

Adeno-associated virus; AAV; cryo-EM; AAV2.7m8; gene delivery

INTRODUCTION

Adeno-Associated Viruses (AAVs) are non-enveloped, single-stranded DNA viruses that belong to the *Dependoparvovirus* genus of the *Parvoviridae*. Thus, they require coinfection with a helper virus for replication (Cotmore et al., 2014; Cotmore et al., 2019). They infect a diverse range of vertebrate hosts, including humans, and are not associated with any known medical disorder in the human population (Mingozzi and High, 2013). More than 130 different naturally occurring serotypes and genotypes have been identified, which vary in capsid protein sequence and structure (Gao et al., 2004; Mietzsch et al., 2019; Zinn and Vandenberghe, 2014). This capsid diversity results in a large variety of cell tropisms, which makes the AAV vector a very attractive gene delivery vehicle, as most organs and cell-types can be targeted with one or several of these naturally occurring AAV serotypes (Wang et al., 2019). In the past few decades, recombinant AAV (rAAV) vectors have been successful in numerous clinical trials addressing rare genetic diseases. This has led to the approval of two biologics by the FDA, Luxturna (FDA STN#125610; EMEA/H/C/004451) and Zolgensma (FDA STN #125694), and Glybera by the EMA (Wang et al., 2019), with several other potential FDA approvals in the pipeline (<https://clinicaltrials.gov/>).

In addition to naturally occurring AAV serotypes, several engineered capsids have been generated using either rational design based on 3D structures (reviewed in (Mietzsch et al., 2019)) or directed evolution (e.g. (Asokan et al., 2012; Asokan et al., 2010; Kotterman and Schaffer, 2014; Shen et al., 2013; Tse et al., 2017)) towards enhancing transduction efficiency or immunological profile *in vivo* (Buning and Srivastava, 2019). This includes the recombinant AAV vector rAAV2.7m8 (Dalkara et al., 2013), which is derived from the natural serotype AAV2 and was isolated by directed evolution when screening for increased transduction of the mouse retina following intravitreal (IVT) administration. In this vector, peptide LALGETTRPA is inserted after amino position R588 of AAV2, located in a capsid surface loop designated variable region VIII (VR-VIII) based on 3D structure comparison (Govindasamy et al., 2006). This insertion was predicted and shown to disrupt the binding of AAV2 to its primary receptor, heparan sulfate proteoglycan (HSPG) (Dalkara et al., 2013). The reduction in HS binding was predicted to facilitate spread of the vector in the neuroretina. Furthermore, rAAV2.7m8 was shown to cross the inner limiting membrane in the retina, where other naturally occurring AAV serotypes, including its parental AAV2, have poor retina penetration (Aartsen et al., 2010; Dalkara et al., 2009; Dalkara et al., 2013; Giove et al., 2010; Hellstrom et al., 2009). This vector has shown widespread transduction of the neuroretina and strong transgene expression in small and large animal models following

intravitreal delivery (Dalkara et al., 2013; Grishanin et al., 2019; Khabou et al., 2016; Kleine Holthaus et al., 2018). This route of administration is less invasive than the traditional sub-retinal route, which can be harmful to retinal structure and function, especially when the patient retina is already compromised and/or ongoing degeneration. Recently, rAAV2.7m8 encoding aflibercept (ADVM-022) showed safety and efficacy in the laser-induced choroid neovascularization non-human primate model of wet macular degeneration (wet-AMD) (Grishanin et al., 2019). This led to a phase 1 dose-escalating clinical trial for wet AMD with IVT administration of ADVM-022 (NCT03748784). The ability of rAAV2.7m8 to cross biologic barriers also been demonstrated for inner ear gene transfer in mice, suggesting potential use as a vector for the treatment of inherited or acquired auditory disorders (Isgrig et al., 2019).

The AAV capsid is composed of 3 overlapping capsid viral proteins (VPs), VP1 (~87kDa, 735aa), VP2 (~73kDa, 598aa), and VP3 (~61kDa, 532aa), assembled in a predicted ratio of 1:1:10, respectively (Johnson et al., 1971; Snijder et al., 2014). All the sequence of VP3 is within VP2 and all the sequence of VP2 is within VP1, as they all share a common C-terminus. The minor VP1 contains a unique region, VP1u, encoding a phospholipase A₂ (PLA₂) enzyme essential for infection. To date, the high resolution structures of several AAV serotypes, AAV1-3, 6-9, clonal isolates AAV4 and AAV5, and rhesus isolates AAVrh8 and AAVrh32, have been determined by cryo-EM and/or X-ray crystallography (DiMattia et al., 2012; Drouin et al., 2016; Govindasamy et al., 2006; Govindasamy et al., 2013; Halder et al., 2015; Kronenberg et al., 2001; Lerch et al., 2010; Mikals et al., 2014; Nam et al., 2007; Ng et al., 2010; Padron et al., 2005; Tan et al., 2018; Xie et al., 2002). In all the AAV structures determined to date, only the overlapping VP3 region has been resolved. This is likely due to the low copy number of VP1 and VP2 that is incompatible with the icosahedral symmetry applied during structure determination, and the disorder predicted for the overlapping VP1/2 common region. The structure of the overlapping VP3 common region is composed of an eight stranded β barrel motif (β BIDG, and β CHEF), which forms the interior and body of the capsid, as well as a conserved α helix, α A. There are interconnecting loops between the β strands that form the exterior of the capsid that vary in sequence and length between the different serotypes. These interconnecting loops interact at the 2-, 3- and 5-fold axes and are responsible for assembling characteristic features of the capsid, including a depression, protrusions, and a channel, respectively (reviewed in (Mietzsch et al., 2019)). Differences on the capsid surface, due to variation in the interconnecting loops, dictate the functional variation observed among the serotypes.

Structural characterization of engineered capsids, for example, AAV2.5 and AAV9_L001, provide valuable information about the regions of the capsid that contributed to altered tropism compared to their parental serotypes (Burg et al., 2018; Guenther et al., 2019). The recombinant vector AAV2.5, created by rational design, combined the muscle tropism of AAV1 with the HSPG receptor attachment properties of AAV2, and was capable of escaping AAV2 antibodies. The design, confirmed by the high-resolution capsid structure determined by cryo-EM, showed AAV2.5 and parental AAV2 to be the similar except at the surface regions where the sequence was changed to that of AAV1 (Bowles et al., 2012; Burg et al., 2018). At these altered positions, AAV2.5 resembled AAV1 indicating that the sequence dictated the structure topology, and that the specific structures enabled function. For the

AAV9_L001 vector, a matrix Metalloproteinase (MMP) activatable peptide was inserted into VR-VIII for targeting cardiac tissue. The structures of the variant and parental AAV9 vectors also shared similar overall features except at the site of peptide insertion consistent with the maintenance of the integrity and function of the capsid with the additional benefit of tissue specificity (Guenther et al., 2019).

In the present study, we determined the structures of rAAV2.7m8 and parental AAV2 capsids by cryo-EM to 2.91 Å and 3.02 Å resolution, respectively, to help identify the effect of the 7m8 peptide insertion on the capsid structure and correlate it to its function when the two viruses are compared. Consistent with the structure of other AAVs, including previously determined AAV2 structures, only the VP3 common region is resolved for rAAV2.7m8 and AAV2. Electron density was observed for the entire loop formed by the 7m8 insertion peptide; however, the side-chain density could only be resolved for six out of the ten amino acids and the remaining residues were only observed at a sigma threshold of 1. This observation is consistent with flexibility of the loop resulting in different conformations. The HS binding residues R588 and R598/589 are structurally similar in rAAV2.7m8 and AAV2, consistent with the ability of rAAV2.7m8 to still bind HS. The reduced affinity reported (Dalkara et al., 2013) is thus likely due to steric hindrance of the critical R585 and R598/588 residues by the inserted peptide localized radially above them in the rAAV2.7m8 structure. Several identical amino acids differed in side-chain conformation between rAAV2.7m8 and AAV2. This suggests conformational freedom within the capsid and beyond the site of the loop insertion. The rAAV2.7m8 insertion modified the C37-B antibody binding site (Gurda et al., 2013) creating an escape variant. Altogether, these results provide valuable insights into the structural juxtaposition of surface loop insertions and their functional conformation. It can also inform future rational AAV vector design to target specific tissues while evading a host immune response, a necessity in the AAV gene delivery field.

MATERIALS AND METHODS

rAAV2.7m8 vector production and purification

The AAV2.7m8.CMV-eGFP (rAAV2.7m8) capsid was produced in the *baculovirus* expression vector system (BEVS), using the *Spodoptera frugiperda* 9 (Sf9) cell line (Vaughn et al., 1977), as previously described (Chen, 2008). Briefly, two baculoviruses were generated, one encoding both the AAV2 Rep and the AAV2.7m8 Cap gene (rBV-R2C.7m8) and another one encoding the expression cassette composed of the eGFP cDNA under the control of the human CMV IE1 promoter (rBV-eGFP). Sf9 cells were cultured in shaker flasks and infected with rBV-R2C.7m8 and rBV-eGFP. To prevent proteolytic cleavage of rAAV particles, a protease inhibitor was added to the culture post-infection. After approximately three days, Sf9 cells were lysed and the lysate was processed through a series of filtration membranes to generate clarified supernatant or cell lysate. In the subsequent steps, rAAV particles were purified by successive chromatography steps. Following sterile filtration, the rAAV2.7m8 vector was formulated in a phosphate buffer. The purity of the vector stock was assayed using silver stained SDS-PAGE with the SilverQuest™ Silver Staining Kit (ThermoFisher Scientific, ref LC6070). The sample was titrated by Taqman qPCR. The capsid integrity was visualized using negative-stain electron microscopy (EM).

Five microliters of purified sample were loaded on to a glow discharged carbon coated copper grids for 30 sec, blotted, stained with 2% uranyl acetate, and examined on an FEI Spirit Transmission electron microscope.

AAV2 VLP production and purification

A baculovirus expressing AAV2 virus-like particles (VLPs) was generously provided by Sergei Zolotukhin (University of Florida). The virus was used to generate AAV2 VLPs in Sf9 cells according to the Bac-to-Bac expression system (Invitrogen) as previously reported for AAV9 VLP (DiMattia et al., 2005). The expressed VLPs were purified by a discontinuous step iodixanol gradient and ion exchange chromatography, according to previous established protocols (Zolotukhin et al., 2002). The purity was confirmed by Coomassie blue stained SDS PAGE and capsid integrity by negative stain EM as described above.

Native immunodot blot

AAV2 VLPs at 6×10^{12} particles/ml and rAAV2.7m8 vectors at 5×10^{12} vg/ml, in 50 μ l of PBS, were loaded onto duplicate nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk (Lab Scientific M-0842) in 0.05% Tween-PBS (T-PBS) for 1 hr at RT. Primary antibodies A20 (1:500) or C37-B (1:3000) diluted with 1% non-fat dry milk in T-PBS were added to the blots and incubated with rocking for 1 hr at RT. The blots were washed three times for 10 min each in T-PBS prior to incubation in secondary antibody, anti-mouse IgG HRP-conjugated antibody (GE Healthcare, cat. # NA931), diluted 1:10,000 with 1% non-fat dry milk in T-PBS, for 1 hr at RT. The blots were washed three times for 10 min and visualization with Immobilon chemiluminescent substrate (Millipore, cat. # WBKLS0500).

Cryo-EM and data collection

Three microliters of rAAV2.7m8 and AAV2, at ~ 1.0 mg/mL, were applied to C-flat holey carbon grids (Protochips, Inc.), following glow discharging to increase hydrophilicity, and vitrified with a Vitrobot Mark IV (FEI Co.). The grids were screened with a Tecnai G2 F20-TWIN transmission electron microscope operated at 200 kV under low-dose conditions (~ 20 e-/Å²) for suitable particle distribution and ice thickness prior to high resolution data collection. Cryo-electron micrograph movie frames were collected on a Titan Krios electron microscope (FEI Co.) operated at 300 kV with a K2 detector (Gatan) as part of the West/Midwest Consortium for High-Resolution Cryo Electron Microscopy (for rAAV2.7m8) or the Southeastern Center for Microscopy of MacroMolecular Machines (SECM4) (for AAV2). Micrograph frame alignment was performed using the MotionCor2 application with dose weighting (Zheng et al., 2017). The data collection parameters are summarized in Table 1.

Structure determination by 3D image reconstruction, model building, and structure refinement

Particles were selected from the aligned micrographs using the automated particle picking option AUTOPP (M), a subroutine in the program AUTO3DEM (Yan et al., 2007a).

AUTOPP subroutines (F and O) were used for the normalization and apodization of the extracted particles (Yan et al., 2007a). Microscope related contrast transfer functions were corrected based on the estimated defocus values for each micrograph, using the CTFFIND4 program (Rohou and Grigorieff, 2015). The Initial model was generated from a 100 particle images using the ab initio model subroutine in AUTO3DEM and by applying icosahedral symmetry (Yan et al., 2007b). The random model was used to search particle origin and orientation for the data sets for 10 cycles. This was followed by cycles of solvent flattening and CTF refinement using AUTO3DEM (Yan et al., 2007a). AUTO3DEM option “score fractions” was used to remove outlier particles based on their deviation from refined origins and orientations (Yan et al., 2007a). The gold standard protocol was followed throughout the structure determination process. The resolution for the reconstructed maps were estimated at FSC = 0.143. Table 1 contains the data collection and refinement parameters. To sharpen the high-resolution features of the map, B-factors 1/50, 1/100, 1/150, and 1/200 Å², were applied to the maps followed by visual inspection in the Coot program (Emsley et al., 2010). All the maps were used for model building of the main- and side-chains in Coot (Emsley et al., 2010), but the 1/100 and 1/50 map was selected for refinement of the structures of rAAV2.7m8 and AAV2, respectively, in the Phenix program (Afonine et al., 2013).

The rAAV2.7m8 and AAV2 density maps were initially interpreted using the coordinates from the AAV2 crystal structure (RCSB PDB ID # 1LP3). To build rAAV2.7m8, 1LP3 was supplied as a template in the SWISS-MODEL online modeling program to generate a VP monomer model (Biasini et al., 2014). A 60mer was generated for both rAAV2.7m8 and AAV2 from a VP monomer using the Oligomer generator subroutine within the ViperDB online server (Carrillo-Tripp et al., 2009). The 60mer models were docked into the cryo-reconstructed maps using the “fit in map” subroutine in the UCSF-Chimera program (Pettersen et al., 2004; Yang et al., 2012). A pixel size screen was used to maximize correlation coefficient (CC) between the maps and models. The maps were then converted to Xplor map format using both the “e2proc3D.py” subroutine in EMAN2 (Tang et al., 2007) and the optimized voxel size. Each Xplor map was converted to the CCP4 format using the program MAPMAN (Kleywegt and Jones, 1996) for further model building and refinement.

The rAAV2.7m8 density map, at 2.9 Å resolution (FSC 0.143), enabled the interpretation of individual VP3 amino acid positions from N-terminal residue 219–736 (C-terminus) using interactive model building and the real-space-refine option in Coot (Emsley et al., 2010), followed by the refinement of the 60mer model against the cryo-reconstructed density map in the Phenix program (Afonine et al., 2013). This involved rigid body, real-space, and B-factor refinement, with the default settings. The model was inspected with Coot (Emsley et al., 2010) between refinement steps, and side- and main-chain modifications were made, when necessary. The final model statistics are listed in Table 1. The structure determination and refinement of the parental AAV2 followed the same protocol as used for rAAV2.7m8 with the exception that a new model was not built. The coordinates from the crystal structure, RCSB PDB # 1LP3, served as the starting model. The Ca atoms of final refined VP3 structure of the for rAAV2.7m8 and AAV2 were superposed using the sequence and secondary structure matching (SSM) option in the Coot program (Emsley et al., 2010) followed by comparison of the main- and side-chains. The cryo-EM structure of AAV2 VP3 was also compared with 1LP3 using SSM. The side-chain density images and cartoon

representations were generated with the PyMOL program (DeLano, 2002). Surface representations were generated with the UCSF-Chimera program (Yang et al., 2012). Road maps were generated with the RIVEM program (Xiao and Rossmann, 2007).

Structure accession numbers

The cryo-EM reconstructed density maps and atomic models built for rAAV2.7m8 and AAV2 were deposited with accession numbers EMD-20609, PDB 6U0R and EMD-20610, PDB 6U0V, respectively, in the Electron Microscopy Data Bank (EMDB) and Protein Data Bank (PDB).

RESULTS AND DISCUSSION

The purity of rAAV2.7m8 and AAV2 were confirmed by gel electrophoresis, and the integrity of the capsids was confirmed by EM (Fig. 1A). The capsid structure of rAAV2.7m8 was determined to a resolution of 2.91 Å (FSC 0.143), from 421 aligned micrographs and 27,364 extracted particles (Fig. 1B and Table 1). For AAV2, the structure was determined from 993 aligned micrographs and 6,778 extracted particles to a resolution of 3.02 Å (FSC 0.143) (Fig. 1C and Table1). The rAAV2.7m8 capsid maintains the capsid morphology of the parvoviruses that includes a depression at the icosahedral 2-fold axes and surrounding the 5-folds axes, protrusions surround the 3-fold axes, and a channel at the 5-fold axes (Fig. 2A). It also contains a raised capsid region between the 2- and 5-fold axes termed the 2/5-fold wall. The rAAV2.7m8 and AAV2 structures are similar with the exception of the 3-fold protrusions containing the 7m8 insertion (Fig. 2). The 3-fold protrusions are more pronounced in rAAV2.7m8 due to the 10 amino acid insertion that projects radially outwards from the capsid surface (Fig. 2A and C). The 3-fold region, including the protrusions, contain receptor binding, infectivity, and antigenicity controlling residues (reviewed in (Mietzsch et al., 2019)). The 2-fold and 2/5-fold regions serve as antigenic regions and contribute to receptor attachment (Gurda et al., 2012; Gurda et al., 2013; Huang et al., 2014; Huang et al., 2016; McCraw et al., 2012; Tseng and Agbandje-McKenna, 2014; Tseng et al., 2016; Tseng et al., 2015). The pore at the 5-fold axes is postulated to be the site for externalization of the PLA₂ enzyme within VP1u required for endosomal escape during infection and for genome packaging and release during infection (Bleker et al., 2005; Grieger et al., 2007; Kronenberg et al., 2005; Sonntag et al., 2006).

The high-resolution of the rAAV2.7m8 and AAV2 maps allowed for accurate fitting of the VP sequence side-chains (example given in Fig. 3A for rAAV2.7m8). The rAAV2.7m8 was ordered from residue 219 to 745 (last C-terminal residue, VP1 numbering) while AAV2 was ordered from residue 217 to 735 (last C-terminal residue). The lack of VP1u (residues 1-137), VP1/2 (residues 138-202), and VP3 N-terminus (residues 203-216/218) ordering is consistent with the previous report for the AAV2 crystal structure as well as reports for other AAV capsid structures (reviewed in (Mietzsch et al., 2019)). While structure prediction indicates that the VP1u is mostly helical in nature (not shown) as reported for the structures of most PLA₂ domains, the VP1/2 common region and N-terminal residues of VP3 are predicted to be highly disordered (Mietzsch et al., 2019; Venkatakrishnan et al., 2013). This flexibility and the low copy number of VP1 and VP2 incorporated into the capsids, is

incompatible with the icosahedral symmetry applied during structure determination of these capsids resulting in lack of resolution of the density for these regions in the reconstructed maps. As noted above, the most significant difference between rAAV2.7m8 and parental AAV2 resides at the protrusions surrounding the icosahedral 3-fold axes containing the 10 residues inserted into AAV VR-VIII (Fig. 2, 3C, and D). The main- and side-chains of AAV2 residues T581-Q589 were readily fitted into the reconstructed density map without any ambiguity at a sigma (σ) threshold of 2 (Fig. 3C). While the density for residues T581-Q599 was evident at 1σ in rAAV2.7m8, the three residues at the top of this loop, L590-E592, were not ordered at 2σ (Fig. 3D and 4). At 3.0σ , side-chain density was observable for up to residue N587 on the ascending arm and after R595 on the descending arm of the inserted loop (Fig. 4A). Thus, the base of the inserted peptide was the most ordered. Despite flexibility at the top of the peptide insertion (Fig. 4), it did not fold down to interact with residues in adjacent VR-V.

Several AAV structures have been reported to contain nucleotide density in the capsid interior, referred to as the DNA binding pocket (Govindasamy et al., 2006; Halder et al., 2015; Lerch et al., 2010; Mikals et al., 2014; Nam et al., 2007; Ng et al., 2010). This density was ordered in VLP structures as well as virions packaging wild-type and reporter genes (Govindasamy et al., 2006; Halder et al., 2015; Lerch et al., 2010; Mikals et al., 2014; Nam et al., 2007; Ng et al., 2010). The residues forming the nucleotide binding pocket are V418, P419, H639, P690, S641, P642, G648, L649, and F647, and they are conserved in all AAVs. Despite this conservation, no nucleotide was observed in the AAV2, AAV5, and AAV9 structures (DiMattia et al., 2012; Govindasamy et al., 2013; Guenther et al., 2019; Xie et al., 2002). It has been proposed that nucleotide binding may play a role in capsid/DNA stabilization or genome packaging because of conservation of the binding site amino acid sequences and not the sequence of packaged genome (Ng et al., 2010). This proposal is yet to be tested. As reported for other AAV structures, density for a single purine nucleotide (dAMP) was observed in the rAAV2.7m8 capsid interior the DNA binding pocket (Fig. 3B). Interestingly and similar to the AAV2 crystal structure, the high-resolution AAV2 cryo-EM density map reported here also lacks the nucleotide density, while the low-resolution AAV2-HS complex structure had ordered density interpreted nucleotides (Levy et al., 2009). These observations suggest that despite the conservation of the binding pocket, the affinity for the packaged genome is likely reduced in AAV2 resulting in disorder as resolution increases. The observation of a nucleotide in the rAAV2.7m8, which like the AAV2-HS structure was modified at the icosahedral 3-fold axis, suggests that this is a stabilizing interaction for AAV2.

The rAAV2.7m8 VP structure conserved the secondary structure elements reported for parvoviruses. This includes an eight-stranded anti-parallel β -barrel core, with the BIDG sheet facing the virus interior, and the α A helix forming the wall of the 2-fold axes (Fig. 5A). Large loops interconnect the secondary structure elements with regions of high variability between different serotypes defined as VR-I to VR-IX based on two diverse serotypes, AAV2 and AAV4 (Govindasamy et al., 2006). These VRs form features observed at the capsid surface (Fig. 2 and 5). The wall of the 2-fold depression is formed by VR-VI, the 2/5-fold wall is formed by VR-I, VR-III, and VR-IX, the top of the 5-fold channel is formed by VR-II, and the protrusions surrounding the 3-fold axis is composed of VR-IV,

VR-V, and VR-VIII (Fig. 2, 5B, and C). Residues within these VRs are important for various lifecycle functions. For example, for AAV2, two critical residues required for binding HS, R585 and R588, reside within VR-VIII, and the 511-NGR-513 proposed to play a role in $\alpha 5\beta 1$ co-receptor binding is flanked by VR-I and VR-IV (Asokan et al., 2006; Kern et al., 2003; Levy et al., 2009; Ng et al., 2010; Opie et al., 2003; Shen et al., 2015; Summerford and Samulski, 1998). Furthermore, residues forming antigenic epitopes on the AAV2 capsid surface are localized to VR-I, VR-III, VR-IV, VR-V, VR-VI, VR-VII, VR-VIII, and VR-IX (Bennett et al., 2018; Giles et al., 2018; Gurda et al., 2012; Gurda et al., 2013; McCraw et al., 2012; Tseng and Agbandje-McKenna, 2014; Tseng et al., 2016; Tseng et al., 2015). For several other AAVs, receptor attachment sites, and residues dictating transduction efficiency are also located within the VRs (e.g. (Adachi et al., 2014; Aslanidi et al., 2013; Bell et al., 2012; Bowles et al., 2012; Girod et al., 1999; Huang et al., 2016; Kern et al., 2003; Ling et al., 2011; Lochrie et al., 2006; Maheshri et al., 2006; Nam et al., 2011; Raupp et al., 2012; Salganik et al., 2014; Shen et al., 2013; Summerford and Samulski, 1998; Summerford et al., 2016; Tenney et al., 2014; Wobus et al., 2000; Wu et al., 2000; Wu et al., 2006).

The 10 amino acid peptide insertion in rAAV2.7m8 is located at the top of VR-VIII (Fig. 5A and B) that forms the side of the 3-fold protrusion facing the icosahedral 3-fold axis that now extends further out than VR-IV, unlike in the parental AAV2 (Fig. 5A, B, 6A, and B). As mentioned above, this insertion was reported to reduce HS binding affinity compared to parental AAV2 (Dalkara et al., 2013). The reported AAV2 HS binding residues are R484, R487, K532, R585, and R588 (Fig. 6C) (Kern et al., 2003; Opie et al., 2003) corresponding to residues R484, R487, K532, R585, and R598 in rAAV2.7m8. Critical residues R585 and R588/598, are positioned adjacent to each other from the ascending and descending arms, respectively, of VR-VIII, and together with the other residues, located at the base of the 3-fold protrusions, form a basic patch on the capsid surface (Fig. 6). This patch forms the HS binding footprint. The rAAV2.7m8 peptide insertion does not alter the position or structure of the R585 and R598 (Fig. 6A and B) nor that of the other HS binding residues (not shown). However, a surface view of rAAV2.7m8 and AAV2 structures indicates that the insertion in the latter obscures access to some of the residues, although not R585 or R598 (Fig. 6C and D). This insertion occurs on all 60 VP subunits assembling the capsid (Fig. 7), and as such the HS binding sites could experience a block from a receptor molecule approaching the basic patch from above the capsid. The 10 amino acid peptide insertion may be the cause of the reduced HS binding reported for AAV2.7m8.

AAV2 is the most extensively studied AAV serotype, including the functional characterization of all charged residues in the VP (Choi et al., 2005; Grieger et al., 2006; Lochrie et al., 2006; Mietzsch et al., 2019; Wang et al., 2019; Wu et al., 2000). To date, the receptor binding phenotype of rAAV2.7m8 is the only function analyzed, as described above. The main-chain and secondary structure elements of rAAV2.7m8 and AAV2 are structurally conserved (Fig. 5A and 5B), except for the 10 residue insertion in AAV2.7m8 as described above. In addition to this difference, several side-chains of rAAV2.7m8 have either dual or alternate conformations when compared to AAV2 (Table 2). Previous mutagenesis and biophysical studies have reported the importance of some of these residues in virus assembly, production (Grieger et al., 2006; Wu et al., 2000), infectivity (Zhong et al., 2008), and pH-induced changes during trafficking (Nam et al., 2011; Salganik et al., 2012). Dual

and alternate side-chain conformations were observed for five (R238, R298, R310, K314, and R389, Table 2) of the fifty-eight basic residues found in rAAV2.7m8 VP3 when compared to AAV2 VP3. These basic residues are located in the 2-fold symmetry related interface, the conserved secondary structures, and facing the luminal surface of the capsid (Table 2). R238 of rAAV2.7m8 is involved in one additional interaction at the 2-fold axes possibly resulting in a more stable capsid compared to AAV2 (not shown). R389 has a dual conformation in rAAV2.7m8 but not AAV2, and has been previously characterized as one of four residues (R389, H526, E563 and Y704 - pH quartet) that undergo pH-induced conformational change, an essential step in viral infectivity (Bartlett et al., 2000; Douar et al., 2001; Hansen et al., 2001; Nam et al., 2011; Nam et al., 2007). In AAV2, all the pH quartet residues have the same conformational as in AAV8 at pH 7.4 (Nam et al., 2011), however, acidic residue E563 was less ordered. Two of the residues in the pH quartet, Y714 (Y704 in AAV2) and E563, are in the reported AAV8 pH4 conformation (Nam et al., 2011) in rAAV2.7m8, implying that rAAV2.7m8 is already primed for endosomal escape or that this capsid region samples at least two conformations. Residue C361 (Table 2) has an alternate conformation in rAAV2.7m8 compared to AAV2, and is involved in an interaction with H288 through additional density present between the two residues. This additional interaction would potentially contribute to a more stable rAAV2.7m8 when compared to the AAV2. Residue E416 (Table 2), proximal to the DNA binding pocket, varies in its interactions in the two structures. rAAV2.7m8 E416 has additional density extending to the luminal surface towards the base of the dAMP, while AAV2 E416 interacts with K640, which has a dual conformation in AAV2 not rAAV2.7m8. Overall rAAV2.7m8 appears to have more conformational freedom than AAV2, despite their sequence identity. A role for this difference is yet to be determined.

Native dot immunoblot analysis of AAV2.7m8 capsid and AAV2 VLP in the presence of monoclonal antibodies A20 and C37-B confirmed escape from the latter by AAV2.7m8 capsid (Fig. 8A). This observation is consistent with the positioning of the peptide insert above at the epitope mapped for C37-B to VR-VIII using cryo-EM of the AAV2-C37-B complex (Fig. 8B) (Gurda et al., 2013). While the A20 epitope is positioned from the 2/5-fold wall towards the 5-fold axes and shares no residues with the peptide (McCraw et al., 2012), the C37-B epitope is significantly overlapped by the position of the inserted peptide (Fig. 8B). This observation supports a two-pronged approach involving peptide insertion for altering tissue tropism as well as ablating antigenic reactivity.

SUMMARY

This study utilized cryo-EM to determine the atomic structure of AAV2.7m8, a vector identified from a directed evolution approach to isolate viruses with altered tropism relative to the parental virus. In this case, the new vector's structure retained the overall VP topology and capsid morphology of the parental AAV2 except at the 3-fold protrusion important for receptor binding and antigenicity. For AAV2.7m8, this region is also important for cellular trafficking, given its ability to reach the retina following intravitreal administration, a capability not shared by parental AAV2. Residues exhibiting altered or dual conformations, despite identity between the two viruses, were in functional regions and localized to areas with conformational freedom. The data presented provides information for engineering AAV

capsid locations that can tolerate insertions for the development of a repertoire of AAV variants with improved desired functions.

Acknowledgment

This work and its publication were supported by Adverum Biotechnologies Inc., through a Sponsored Research Agreement and NIH R01 GM109524. We thank Mrs. Christopher Cruz, Kyle Hu and Jason Chan for the production and QC of the vector. We thank Drs. Diana Cepeda, Pallavi Sharma, Baljit Singh and Dr. Claire Gelfman for scientific discussions. Data collection at Florida State University (AAV2) was made possible by NIH grants S10 OD018142-01 Purchase of a direct electron camera for the Titan-Krios at FSU (PI Taylor), S10 RR025080-01 Purchase of a FEI Titan Krios for 3-D EM (PI Taylor), and U24 GM116788 The Southeastern Consortium for Microscopy of MacroMolecular Machines (PI Taylor). Data collection on AAV2.7m8 was made possible by NIH U24GM116792, The West/Midwest Consortium for High-Resolution Cryo Electron Microscopy (MPI, contact PI, Zhou).

REFERENCES

- Aartsen WM, van Cleef KW, Pellissier LP, Hoek RM, Vos RM, Blits B, Ehlert EM, Balaggan KS, Ali RR, Verhaagen J, Wijnholds J, 2010 GFAP-driven GFP expression in activated mouse Muller glial cells aligning retinal blood vessels following intravitreal injection of AAV2/6 vectors. *PLoS One* 5, e12387. [PubMed: 20808778]
- Adachi K, Enoki T, Kawano Y, Veraz M, Nakai H, 2014 Drawing a high-resolution functional map of adeno-associated virus capsid by massively parallel sequencing. *Nat Commun* 5, 3075. [PubMed: 24435020]
- Afonine PV, Headd JJ, Terwilliger TC, Adams PD, 2013 New tool: phenix.real_space_refine. *Computational Crystallography Newsletter* 4, 43–44.
- Aslanidi GV, Rivers AE, Ortiz L, Song L, Ling C, Govindasamy L, Van Vliet K, Tan M, Agbandje-McKenna M, Srivastava A, 2013 Optimization of the capsid of recombinant adeno-associated virus 2 (AAV2) vectors: the final threshold? *PLoS One* 8, e59142. [PubMed: 23527116]
- Asokan A, Schaffer DV, Samulski RJ, 2012 The AAV vector toolkit: poised at the clinical crossroads. *Molecular therapy : the journal of the American Society of Gene Therapy* 20, 699–708. [PubMed: 22273577]
- Asokan A, Hamra JB, Govindasamy L, Agbandje-McKenna M, Samulski RJ, 2006 Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. *J Virol* 80, 8961–8969. [PubMed: 16940508]
- Asokan A, Conway JC, Phillips JL, Li C, Hegge J, Sinnott R, Yadav S, DiPrimio N, Nam HJ, Agbandje-McKenna M, McPhee S, Wolff J, Samulski RJ, 2010 Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. *Nat Biotechnol* 28, 79–82. [PubMed: 20037580]
- Bartlett JS, Wilcher R, Samulski RJ, 2000 Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 74, 2777–2785. [PubMed: 10684294]
- Bell CL, Gurda BL, Van Vliet K, Agbandje-McKenna M, Wilson JM, 2012 Identification of the galactose binding domain of the adeno-associated virus serotype 9 capsid. *J Virol* 86, 7326–7333. [PubMed: 22514350]
- Bennett AD, Wong K, Lewis J, Tseng YS, Smith JK, Chipman P, McKenna R, Samulski RJ, Kleinschmidt J, Agbandje-McKenna M, 2018 AAV6 K531 serves a dual function in selective receptor and antibody ADK6 recognition. *Virology* 518, 369–376. [PubMed: 29604478]
- Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L, Schwede T, 2014 SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 42, W252–258. [PubMed: 24782522]
- Bleker S, Sonntag F, Kleinschmidt JA, 2005 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. [PubMed: 15681453]

- Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, Li J, Wang B, Monahan PE, Rabinowitz JE, Grieger JC, Govindasamy L, Agbandje-McKenna M, Xiao X, Samulski RJ, 2012 Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Mol Ther* 20, 443–455. [PubMed: 22068425]
- Buning H, Srivastava A, 2019 Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors. *Mol Ther Methods Clin Dev* 12, 248–265. [PubMed: 30815511]
- Burg M, Rosebrough C, Drouin LM, Bennett A, Mietzsch M, Chipman P, McKenna R, Sousa D, Potter M, Byrne B, Jude Samulski R, Agbandje-McKenna M, 2018 Atomic structure of a rationally engineered gene delivery vector, AAV2.5. *J Struct Biol*.
- Carrillo-Tripp M, Shepherd CM, Borelli IA, Venkataraman S, Lander G, Natarajan P, Johnson JE, Brooks CL 3rd, Reddy VS, 2009 VIPERdb2: an enhanced and web API enabled relational database for structural virology. *Nucleic Acids Res* 37, D436–442. [PubMed: 18981051]
- Chen H, 2008 Intron splicing-mediated expression of AAV Rep and Cap genes and production of AAV vectors in insect cells. *Molecular therapy : the journal of the American Society of Gene Therapy* 16, 924–930. [PubMed: 18388928]
- Choi VW, McCarty DM, Samulski RJ, 2005 AAV hybrid serotypes: improved vectors for gene delivery. *Curr Gene Ther* 5, 299–310. [PubMed: 15975007]
- Cotmore SF, Agbandje-McKenna M, Chiorini JA, Mukha DV, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen P, Gatherer D, Davison AJ, 2014 The family Parvoviridae. *Arch Virol* 159, 1239–1247. [PubMed: 24212889]
- Cotmore SF, Agbandje-McKenna M, Canuti M, Chiorini JA, Eis-Hubinger AM, Hughes J, Mietzsch M, Modha S, Ogliastro M, Penzes JJ, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen P, Ictv Report C, 2019 ICTV Virus Taxonomy Profile: Parvoviridae. *J Gen Virol* 100, 367–368. [PubMed: 30672729]
- Dalkara D, Kolstad KD, Caporale N, Visel M, Klimczak RR, Schaffer DV, Flannery JG, 2009 Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. *Mol Ther* 17, 2096–2102. [PubMed: 19672248]
- Dalkara D, Byrne LC, Klimczak RR, Visel M, Yin L, Merigan WH, Flannery JG, Schaffer DV, 2013 In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. *Sci Transl Med* 5, 189ra176.
- DeLano WL, 2002 The PyMOL Molecular Graphics System.
- DiMattia M, Govindasamy L, Levy HC, Gurda-Whitaker B, Kalina A, Kohlbrenner E, Chiorini JA, McKenna R, Muzyczka N, Zolotukhin S, Agbandje-McKenna M, 2005 Production, purification, crystallization and preliminary X-ray structural studies of adeno-associated virus serotype 5. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 61, 917–921.
- DiMattia MA, Nam HJ, Van Vliet K, Mitchell M, Bennett A, Gurda BL, McKenna R, Olson NH, Sinkovits RS, Potter M, Byrne BJ, Aslanidi G, Zolotukhin S, Muzyczka N, Baker TS, Agbandje-McKenna M, 2012 Structural insight into the unique properties of adeno-associated virus serotype 9. *J Virol* 86, 6947–6958. [PubMed: 22496238]
- Douar AM, Poulard K, Stockholm D, Danos O, 2001 Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol* 75, 1824–1833. [PubMed: 11160681]
- Drouin LM, Lins B, Janssen M, Bennett A, Chipman P, McKenna R, Chen W, Muzyczka N, Cardone G, Baker TS, Agbandje-McKenna M, 2016 Cryo-electron Microscopy Reconstruction and Stability Studies of the Wild Type and the R432A Variant of Adeno-associated Virus Type 2 Reveal that Capsid Structural Stability Is a Major Factor in Genome Packaging. *J Virol* 90, 8542–8551. [PubMed: 27440903]
- Emsley P, Lohkamp B, Scott WG, Cowtan K, 2010 Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66, 486–501. [PubMed: 20383002]
- Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM, 2004 Clades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol* 78, 6381–6388. [PubMed: 15163731]
- Giles AR, Govindasamy L, Somanathan S, Wilson JM, 2018 Mapping an Adeno-associated Virus 9-Specific Neutralizing Epitope To Develop Next-Generation Gene Delivery Vectors. *J Virol* 92.

- Giove TJ, Sena-Esteves M, Eldred WD, 2010 Transduction of the inner mouse retina using AAVrh8 and AAVrh10 via intravitreal injection. *Exp Eye Res* 91, 652–659. [PubMed: 20723541]
- Girod A, Ried M, Wobus C, Lahm H, Leike K, Kleinschmidt J, Deleage G, Hallek M, 1999 Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* 5, 1052–1056. [PubMed: 10470084]
- Govindasamy L, Padron E, McKenna R, Muzyczka N, Kaludov N, Chiorini JA, Agbandje-McKenna M, 2006 Structurally mapping the diverse phenotype of adeno-associated virus serotype 4. *J Virol* 80, 11556–11570. [PubMed: 16971437]
- Govindasamy L, DiMattia MA, Gurda BL, Halder S, McKenna R, Chiorini JA, Muzyczka N, Zolotukhin S, Agbandje-McKenna M, 2013 Structural insights into adeno-associated virus serotype 5. *J Virol* 87, 11187–11199. [PubMed: 23926356]
- Grieger JC, Snowdy S, Samulski RJ, 2006 Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly. *J Virol* 80, 5199–5210. [PubMed: 16699000]
- Grieger JC, Johnson JS, Gurda-Whitaker B, Agbandje-McKenna M, Samulski RJ, 2007 Surface-exposed adeno-associated virus Vp1-NLS capsid fusion protein rescues infectivity of noninfectious wild-type Vp2/Vp3 and Vp3-only capsids but not that of fivefold pore mutant virions. *J Virol* 81, 7833–7843. [PubMed: 17507473]
- Grishanin R, Vuilleminot B, Sharma P, Keravala A, Greengard J, Gelfman C, Blumenkrantz M, Lawrence M, Hu W, Kiss S, Gasmi M, 2019 Preclinical Evaluation of ADV-M-022, a Novel Gene Therapy Approach to Treating Wet Age-Related Macular Degeneration. *Molecular therapy : the journal of the American Society of Gene Therapy* 27, 118–129. [PubMed: 30528929]
- Guenther CM, Brun MJ, Bennett AD, Ho ML, Chen W, Zhu B, Lam M, Yamagami M, Kwon S, Bhattacharya N, Sousa D, Evans AC, Voss J, Seveck-Muraca EM, Agbandje-McKenna M, Suh J, 2019 Protease-Activatable Adeno-Associated Virus Vector for Gene Delivery to Damaged Heart Tissue. *Mol Ther* 27, 611–622. [PubMed: 30772143]
- Gurda BL, Raupp C, Popa-Wagner R, Naumer M, Olson NH, Ng R, McKenna R, Baker TS, Kleinschmidt JA, Agbandje-McKenna M, 2012 Mapping a neutralizing epitope onto the capsid of adeno-associated virus serotype 8. *J Virol* 86, 7739–7751. [PubMed: 22593150]
- Gurda BL, DiMattia MA, Miller EB, Bennett A, McKenna R, Weichert WS, Nelson CD, Chen WJ, Muzyczka N, Olson NH, Sinkovits RS, Chiorini JA, Zolotukhin S, Kozyreva OG, Samulski RJ, Baker TS, Parrish CR, Agbandje-McKenna M, 2013 Capsid antibodies to different adeno-associated virus serotypes bind common regions. *J Virol* 87, 9111–9124. [PubMed: 23760240]
- Halder S, Van Vliet K, Smith JK, Duong TT, McKenna R, Wilson JM, Agbandje-McKenna M, 2015 Structure of neurotropic adeno-associated virus AAVrh.8. *J Struct Biol* 192, 21–36. [PubMed: 26334681]
- Hansen J, Qing K, Srivastava A, 2001 Adeno-associated virus type 2-mediated gene transfer: altered endocytic processing enhances transduction efficiency in murine fibroblasts. *J Virol* 75, 4080–4090. [PubMed: 11287557]
- Hellstrom M, Ruitenber MJ, Pollett MA, Ehlert EM, Twisk J, Verhaagen J, Harvey AR, 2009 Cellular tropism and transduction properties of seven adeno-associated viral vector serotypes in adult retina after intravitreal injection. *Gene Ther* 16, 521–532. [PubMed: 19092858]
- Huang LY, Halder S, Agbandje-McKenna M, 2014 Parvovirus glycan interactions. *Curr Opin Virol* 7, 108–118. [PubMed: 25047752]
- Huang LY, Patel A, Ng R, Miller EB, Halder S, McKenna R, Asokan A, Agbandje-McKenna M, 2016 Characterization of the Adeno-Associated Virus 1 and 6 Sialic Acid Binding Site. *J Virol* 90, 5219–5230. [PubMed: 26962225]
- Ilyas M, Mietzsch M, Kailasan S, Vaisanen E, Luo M, Chipman P, Smith JK, Kurian J, Sousa D, McKenna R, Soderlund-Venermo M, Agbandje-McKenna M, 2018 Atomic Resolution Structures of Human Borna Disease Virus Determined by Cryo-Electron Microscopy. *Viruses* 10.
- Isgrig K, McDougald DS, Zhu J, Wang HJ, Bennett J, Chien WW, 2019 AAV2.7m8 is a powerful viral vector for inner ear gene therapy. *Nat Commun* 10, 427. [PubMed: 30683875]
- Johnson FB, Ozer HL, Hoggan MD, 1971 Structural proteins of adenovirus-associated virus type 3. *J Virol* 8, 860–863. [PubMed: 5172922]

- Kern A, Schmidt K, Leder C, Muller OJ, Wobus CE, Bettinger K, Von der Lieth CW, King JA, Kleinschmidt JA, 2003 Identification of a heparin-binding motif on adeno-associated virus type 2 capsids. *J Virol* 77, 11072–11081. [PubMed: 14512555]
- Khabou H, Desrosiers M, Winckler C, Fouquet S, Auregan G, Bemelmans AP, Sahel JA, Dalkara D, 2016 Insight into the mechanisms of enhanced retinal transduction by the engineered AAV2 capsid variant –7m8. *Biotechnol Bioeng* 113, 2712–2724. [PubMed: 27259396]
- Kleine Holthaus SM, Ribeiro J, Abelleira-Hervas L, Pearson RA, Duran Y, Georgiadis A, Sampson RD, Rizzi M, Hoke J, Maswood R, Azam S, Luhmann UFO, Smith AJ, Mole SE, Ali RR, 2018 Prevention of Photoreceptor Cell Loss in a Cln6(nclf) Mouse Model of Batten Disease Requires CLN6 Gene Transfer to Bipolar Cells. *Molecular therapy : the journal of the American Society of Gene Therapy* 26, 1343–1353. [PubMed: 29606505]
- Kleywegt GJ, Jones TA, 1996 xdlMAPMAN and xdlDATAMAN - programs for reformatting, analysis and manipulation of biomacromolecular electron-density maps and reflection data sets. *Acta Crystallogr D Biol Crystallogr* 52, 826–828. [PubMed: 15299647]
- Kotterman MA, Schaffer DV, 2014 Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet* 15, 445–451. [PubMed: 24840552]
- Kronenberg S, Kleinschmidt JA, Bottcher B, 2001 Electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 empty capsids. *EMBO Rep* 2, 997–1002. [PubMed: 11713191]
- Kronenberg S, Böttcher B, von der Lieth CW, Bleker S, Kleinschmidt JA, 2005 A conformational change in the adeno-associated virus type 2 capsid leads to the exposure of hidden VP1 N termini. *Journal of virology* 79, 5296–5303. [PubMed: 15827144]
- Leach TF, Xie Q, Chapman MS, 2010 The structure of adeno-associated virus serotype 3B (AAV-3B): insights into receptor binding and immune evasion. *Virology* 403, 26–36. [PubMed: 20444480]
- Levy HC, Bowman VD, Govindasamy L, McKenna R, Nash K, Warrington K, Chen W, Muzyczka N, Yan X, Baker TS, Agbandje-McKenna M, 2009 Heparin binding induces conformational changes in Adeno-associated virus serotype 2. *J Struct Biol* 165, 146–156. [PubMed: 19121398]
- Ling C, Lu Y, Cheng B, McGoogan KE, Gee SW, Ma W, Li B, Aslanidi GV, Srivastava A, 2011 High-efficiency transduction of liver cancer cells by recombinant adeno-associated virus serotype 3 vectors. *J Vis Exp*.
- Lochrie MA, Tatsuno GP, Christie B, McDonnell JW, Zhou S, Surosky R, Pierce GF, Colosi P, 2006 Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. *J Virol* 80, 821–834. [PubMed: 16378984]
- Maheshri N, Koerber JT, Kaspar BK, Schaffer DV, 2006 Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat Biotechnol* 24, 198–204. [PubMed: 16429148]
- McCraw DM, O'Donnell JK, Taylor KA, Stagg SM, Chapman MS, 2012 Structure of adeno-associated virus-2 in complex with neutralizing monoclonal antibody A20. *Virology* 431, 40–49. [PubMed: 22682774]
- Mietzsch M, Penzes JJ, Agbandje-McKenna M, 2019 Twenty-Five Years of Structural Parvovirology. *Viruses* 11.
- Mietzsch M, Kailasan S, Garrison J, Ilyas M, Chipman P, Kantola K, Janssen ME, Spear J, Sousa D, McKenna R, Brown K, Soderlund-Venermo M, Baker T, Agbandje-McKenna M, 2017 Structural Insights into Human Bocaparvoviruses. *Journal of virology* 91.
- Mikals K, Nam HJ, Van Vliet K, Vandenberghe LH, Mays LE, McKenna R, Wilson JM, Agbandje-McKenna M, 2014 The structure of AAVrh32.33, a novel gene delivery vector. *J Struct Biol* 186, 308–317. [PubMed: 24704217]
- Mingozzi F, High KA, 2013 Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* 122, 23–36. [PubMed: 23596044]
- Nam HJ, Gurda BL, McKenna R, Potter M, Byrne B, Salganik M, Muzyczka N, Agbandje-McKenna M, 2011 Structural studies of adeno-associated virus serotype 8 capsid transitions associated with endosomal trafficking. *J Virol* 85, 11791–11799. [PubMed: 21900159]
- Nam HJ, Lane MD, Padron E, Gurda B, McKenna R, Kohlbrenner E, Aslanidi G, Byrne B, Muzyczka N, Zolotukhin S, Agbandje-McKenna M, 2007 Structure of adeno-associated virus serotype 8, a gene therapy vector. *J Virol* 81, 12260–12271. [PubMed: 17728238]

- Ng R, Govindasamy L, Gurda BL, McKenna R, Kozyreva OG, Samulski RJ, Parent KN, Baker TS, Agbandje-McKenna M, 2010 Structural characterization of the dual glycan binding adeno-associated virus serotype 6. *J Virol* 84, 12945–12957. [PubMed: 20861247]
- Opie SR, Warrington KH, Agbandje-McKenna M, Zolotukhin S, Muzyczka N, 2003 Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. *J Virol* 77, 6995–7006. [PubMed: 12768018]
- Padron E, Bowman V, Kaludov N, Govindasamy L, Levy H, Nick P, McKenna R, Muzyczka N, Chiorini JA, Baker TS, Agbandje-McKenna M, 2005 Structure of adeno-associated virus type 4. *J Virol* 79, 5047–5058. [PubMed: 15795290]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE, 2004 UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25, 1605–1612. [PubMed: 15264254]
- Raup C, Naumer M, Muller OJ, Gurda BL, Agbandje-McKenna M, Kleinschmidt JA, 2012 The threefold protrusions of adeno-associated virus type 8 are involved in cell surface targeting as well as postattachment processing. *J Virol* 86, 9396–9408. [PubMed: 22718833]
- Rohou A, Grigorieff N, 2015 CTFFIND4: Fast and accurate defocus estimation from electron micrographs. *J Struct Biol* 192, 216–221. [PubMed: 26278980]
- Salganik M, Aydemir F, Nam HJ, McKenna R, Agbandje-McKenna M, Muzyczka N, 2014 Adeno-associated virus capsid proteins may play a role in transcription and second-strand synthesis of recombinant genomes. *J Virol* 88, 1071–1079. [PubMed: 24198419]
- Salganik M, Venkatakrishnan B, Bennett A, Lins B, Yarbrough J, Muzyczka N, Agbandje-McKenna M, McKenna R, 2012 Evidence for pH-dependent protease activity in the adeno-associated virus capsid. *J Virol* 86, 11877–11885. [PubMed: 22915820]
- Shen S, Horowitz ED, Troupes AN, Brown SM, Pulicherla N, Samulski RJ, Agbandje-McKenna M, Asokan A, 2013 Engraftment of a galactose receptor footprint onto adeno-associated viral capsids improves transduction efficiency. *J Biol Chem* 288, 28814–28823. [PubMed: 23940044]
- Shen S, Berry GE, Castellanos Rivera RM, Cheung RY, Troupes AN, Brown SM, Kafri T, Asokan A, 2015 Functional analysis of the putative integrin recognition motif on adeno-associated virus 9. *J Biol Chem* 290, 1496–1504. [PubMed: 25404742]
- Snijder J, van de Waterbeemd M, Damoc E, Denisov E, Grinfeld D, Bennett A, Agbandje-McKenna M, Makarov A, Heck AJ, 2014 Defining the stoichiometry and cargo load of viral and bacterial nanoparticles by Orbitrap mass spectrometry. *J Am Chem Soc* 136, 7295–7299. [PubMed: 24787140]
- Sonntag F, Bleker S, Leuchs B, Fischer R, Kleinschmidt JA, 2006 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. *J Virol* 80, 11040–11054. [PubMed: 16956943]
- Summerford C, Samulski RJ, 1998 Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 72, 1438–1445. [PubMed: 9445046]
- Summerford C, Johnson JS, Samulski RJ, 2016 AAVR: A Multi-Serotype Receptor for AAV. *Mol Ther* 24, 663–666. [PubMed: 27081719]
- Tan YZ, Aiyer S, Mietzsch M, Hull JA, McKenna R, Grieger J, Samulski RJ, Baker TS, Agbandje-McKenna M, Lyumkis D, 2018 Sub-2 A Ewald curvature corrected structure of an AAV2 capsid variant. *Nat Commun* 9, 3628. [PubMed: 30194371]
- Tang G, Peng L, Baldwin PR, Mann DS, Jiang W, Rees I, Ludtke SJ, 2007 EMAN2: an extensible image processing suite for electron microscopy. *J Struct Biol* 157, 38–46. [PubMed: 16859925]
- Tenney RM, Bell CL, Wilson JM, 2014 AAV8 capsid variable regions at the two-fold symmetry axis contribute to high liver transduction by mediating nuclear entry and capsid uncoating. *Virology* 454–455, 227–236. [PubMed: 24725949]
- Tse LV, Klinc KA, Madigan VJ, Castellanos Rivera RM, Wells LF, Havlik LP, Smith JK, Agbandje-McKenna M, Asokan A, 2017 Structure-guided evolution of antigenically distinct adeno-associated virus variants for immune evasion. *Proc Natl Acad Sci U S A* 114, E4812–e4821. [PubMed: 28559317]

- Tseng YS, Agbandje-McKenna M, 2014 Mapping the AAV Capsid Host Antibody Response toward the Development of Second Generation Gene Delivery Vectors. *Front Immunol* 5, 9. [PubMed: 24523720]
- Tseng YS, Vliet KV, Rao L, McKenna R, Byrne BJ, Asokan A, Agbandje-McKenna M, 2016 Generation and characterization of anti-Adeno-associated virus serotype 8 (AAV8) and anti-AAV9 monoclonal antibodies. *J Virol Methods* 236, 105–110. [PubMed: 27424005]
- Tseng YS, Gurda BL, Chipman P, McKenna R, Afione S, Chiorini JA, Muzyczka N, Olson NH, Baker TS, Kleinschmidt J, Agbandje-McKenna M, 2015 Adeno-associated virus serotype 1 (AAV1)- and AAV5-antibody complex structures reveal evolutionary commonalities in parvovirus antigenic reactivity. *J Virol* 89, 1794–1808. [PubMed: 25410874]
- Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P, 1977 The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In Vitro* 13, 213–217.
- Venkatakrishnan B, Yarbrough J, Domsic J, Bennett A, Bothner B, Kozyreva OG, Samulski RJ, Muzyczka N, McKenna R, Agbandje-McKenna M, 2013 Structure and dynamics of adeno-associated virus serotype 1 VP1-unique N-terminal domain and its role in capsid trafficking. *Journal of virology* 87, 4974–4984. [PubMed: 23427155]
- Wang D, Tai PWL, Gao G, 2019 Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov* 18, 358–378. [PubMed: 30710128]
- Wobus CE, Hügler-Dorr B, Girod A, Petersen G, Hallek M, Kleinschmidt JA, 2000 Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection. *J Virol* 74, 9281–9293. [PubMed: 10982375]
- Wu P, Xiao W, Conlon T, Hughes J, Agbandje-McKenna M, Ferkol T, Flotte T, Muzyczka N, 2000 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. [PubMed: 10954565]
- Wu Z, Asokan A, Grieger JC, Govindasamy L, Agbandje-McKenna M, Samulski RJ, 2006 Single amino acid changes can influence titer, heparin binding, and tissue tropism in different adeno-associated virus serotypes. *J Virol* 80, 11393–11397. [PubMed: 16943302]
- Xiao C, Rossmann MG, 2007 Interpretation of electron density with stereographic roadmap projections. *J Struct Biol* 158, 182–187. [PubMed: 17116403]
- Xie Q, Bu W, Bhatia S, Hare J, Somasundaram T, Azzi A, Chapman MS, 2002 The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc Natl Acad Sci U S A* 99, 10405–10410. [PubMed: 12136130]
- Yan X, Sinkovits RS, Baker TS, 2007a AUTO3DEM--an automated and high throughput program for image reconstruction of icosahedral particles. *J Struct Biol* 157, 73–82. [PubMed: 17029842]
- Yan X, Dryden KA, Tang J, Baker TS, 2007b Ab initio random model method facilitates 3D reconstruction of icosahedral particles. *J Struct Biol* 157, 211–225. [PubMed: 16979906]
- Yang Z, Lasker K, Schneidman-Duhovny D, Webb B, Huang CC, Pettersen EF, Goddard TD, Meng EC, Sali A, Ferrin TE, 2012 UCSF Chimera, MODELLER, and IMP: an integrated modeling system. *J Struct Biol* 179, 269–278. [PubMed: 21963794]
- Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng Y, Agard DA, 2017 MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* 14, 331–332. [PubMed: 28250466]
- Zhong L, Li B, Mah CS, Govindasamy L, Agbandje-McKenna M, Cooper M, Herzog RW, Zolotukhin I, Warrington KH Jr., Weigel-Van Aken KA, Hobbs JA, Zolotukhin S, Muzyczka N, Srivastava A, 2008 Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci U S A* 105, 7827–7832. [PubMed: 18511559]
- Zinn E, Vandenberghe LH, 2014 Adeno-associated virus: fit to serve. *Curr Opin Virol* 8, 90–97. [PubMed: 25128609]
- Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO, 2002 Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* 28, 158–167. [PubMed: 12413414]

Figure 1.

Purity, capsid integrity, and reconstructed resolution of rAAV2.7m8 and AAV2. (A) SDS-PAGE and aligned cryo-micrographs of rAAV2.7m8 (left) and AAV2 (right). VP1, VP2, and VP3 are observed for both samples. (B) Fourier Shell Correlation (FSC) plotted against inverse resolution (FSC plot) for the rAAV2.7m8 structure. The structure was determined to 3.03 Å (FSC 0.5) and 2.90 Å (FSC 0.143) resolution. (C) FSC plot for AAV2, the structure is at 3.3 Å (FSC 0.5) and 3.03 Å (FSC 0.143) resolution.

Figure 2.

Comparison of the rAAV2.7m8 and AAV2 structures. (A) The reconstructed density map for rAAV2.7m8 radially colored from the capsid center to the surface according to the color key. (B) The reconstructed density map for AAV2 radially colored as shown in the color key. (C) Superposition of the rAAV2.7m8 and AAV2 reconstructed density maps highlighting the peptide loop insertion in rAAV2.7m8 (colored red). The icosahedral 2-fold, 3-fold, and 5-fold axes are represented as an oval, triangle, and pentagon, respectively. The figures were generated using the program UCSF-Chimera (Pettersen et al., 2004; Yang et al., 2012).

Figure 3.

The atomic structures of rAAV2.7m8 and AAV2. (A) Electron density map of rAAV2.7m8 fitted with model residues C230 – W234 (part of the β A strand) contoured to 2σ . (B) Electron density map and model of a dAMP in rAAV2.7m8 colored gray and electron density map of AAV2 and model colored blue and surrounding residues. Carbon atoms are colored gray (rAAV2.7m8) and blue (AAV2), nitrogen in blue, oxygen in red, phosphate in orange. The maps are contoured at a threshold of 2σ . (C) The electron density map and model of AAV2 residues T581-Q589. The carbon and nitrogen are colored in different

shades of blue and the oxygen is in red. The map is contoured at 2σ . (D) The electron density map and model of rAAV2.7m8 residues T581-Q599 that includes the point of peptide insertion (L588 is the first inserted residue). The map is contoured at 2σ . The carbon is colored gray, nitrogen is in blue, and oxygen is in red. The figures were generated using the program UCSF-Chimera (Pettersen et al., 2004; Yang et al., 2012).

Figure 4.

The structure of VR-VIII. The density map and model of the rAAV2.7m8 VR-VIII superposed onto the backbone of the same region of AAV2, with the map contoured at (A) 3σ, (B) 2σ, and (C) 1σ. The AAV2 loop is colored blue, and the model of rAAV2.7m8 is colored as in figure 3D. The figures were generated using the program UCSF-Chimera (Pettersen et al., 2004; Yang et al., 2012)

Figure 5.

Model and sequence of rAAV2.7m8. (A) Cartoon ribbon diagram of rAAV2.7m8 colored gray with loop insertion colored red within a transparent surface. The core β barrel motif (β BIDG and β CHEF), the α A helix, the N- and C-terminal, the DE loop, the HI loop, the interior and exterior (of the capsid) are labelled. The approximate 2-, 3-, and 5-fold axes are depicted as an oval, triangle, and pentagon, respectively. (B) Cartoon loop diagram of rAAV2.7m8 colored gray with peptide insertion colored red. Variable regions (I-IX) colored: I (purple), II (blue), III (yellow), IV (red), V (dark gray), VI (pink), VII (cyan), VIII (green), IX (brown).

and IX (brown). (C) Sequence and secondary structure depiction with a white arrow for β -strands, and black barrel for α A helix. Start sites for VP1, VP2, and VP3 are marked with a small red arrow, the first residue fitted into the cryo-EM map is marked with a blue arrow, and the sequences defined as VRs are colored as in (B). This figure was generated using the program PyMol (DeLano, 2002).

Figure 6.

Structure and location of rAAV2.7m8 and AAV2 HS binding residues. (A) VR-VIII density map and model for AAV2 with side-chain for residues R585 and R588 shown in stick representation. The loop is in blue and density map is contoured at 2σ . (B) VR-VIII density map and model for rAAV2.7m8 with side-chain for R585 and R598 shown in stick representation. The peptide insertion is shown in red and the remainder of the VR in gray. The electron density map is contoured at 2σ . (C) Stereographic roadmap projection of AAV2 viewed down the icosahedral 2-fold axis. Residues are labeled as 3 letter codes and with AAV2 VLP sequence number. The boundary of each residue is shown in black, and the exterior amino acids that are visible are shown in the image. HS binding residues R484, R487, K532, R585, and R588 are colored blue. (D) Stereographic roadmap projection of rAAV2.7m8 viewed down the icosahedral 2-fold axis. HS binding residues R484, R487,

K532, R585, and R598 are colored blue. The residues of the peptide insertion are colored red. The viral asymmetric unit is shown as a black triangle and bounded by 2-, 3-, and 5-fold axes, depicted by an oval, triangle, and pentagon, respectively. The roadmaps were generated by the program RIVEM (Xiao and Rossmann, 2007).

Figure 7.

The rAAV2.7m8 multimer. (A) A monomer VP of rAAV2.7m8 colored gray with the peptide insert colored red, superposed onto an AAV2 monomer colored blue. A zoom of the insertion is shown below with a 90° rotation shown on its right. (B) A 60mer of rAAV2.7m8 colored gray with reference monomer colored dark gray and a different color for the insert on each monomer. The viral asymmetric unit is depicted with a white triangle. The approximate 2-, 3-, and 5-fold axes are shown as an oval, triangle, and pentagon, respectively. This figure was generated by the program PyMol (DeLano, 2002).

Figure 8.

Comparative antigenic profile of rAAV2.7m8 and AAV2. A) Native immunoblot probed with primary monoclonal antibodies A20 and C37-B with epitopes at the 2/5-fold wall and 3-fold protrusion, respectively. The C37-B epitope is located at the peptide insert site. (B) Stereographic roadmap projection of rAAV2.7m8 viewed down the icosahedral 2-fold axis. Equivalent AAV2 VLP residues identified as important for A20 binding are colored blue and for C37-B binding are colored yellow. The peptide insert is colored red. The residues that overlap between the peptide insert and the C37-B contact residues are colored orange. The

viral asymmetric unit is shown as a black triangle and bounded by a 2-, 3-, and 5-fold axes, depicted by an oval, triangle, and pentagon, respectively. The roadmap was generated by the program RIVEM (Xiao and Rossmann, 2007).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1.

Data collection parameters and statistics of final model.

	rAAV2.7m8	AAV2
Total number of aligned micrographs	410	993
Defocus range (μm)	1.6 – 3.28	0.02 – 3.59
Electron dose ($\text{e}^-/\text{\AA}^2$)	75	36
No. of frames/micrograph	50	50
Pixel size ($\text{\AA}/\text{pixel}$)	1.06	0.91
Starting no. of particles	27,365	13,557
No. of particles used for final map	27,364	6,778
Inverse B factor used for final map ($\text{\AA}^2$)	100	50
Resolution of final map ($\text{\AA}$)	2.91	3.02
PHENIX model refinement statistics		
Residue range	219-745	217-735
Map CC	0.88	0.82
RMSD ($\text{\AA}$)		
Bonds	0.01	0.01
Angles	0.9	0.8
All-atom clash score	11.24	7.66
Ramachandran plot (%)		
Favored	92.95	97.1
Allowed	6.1	2.9
Outliers	0.95	0
Rotamers outliers	0	0.22
No. of C β deviations	0	0

Table 2.

Conformational Variation Between the rAAV2.7m8 and AAV2 Structures

Residue position rAAV2.7m8	Interacting Residue	Residue position on AAV2	Interacting residue	Location on capsid
R238 ^{D1}	D237 E693 E695	R238 ^{D1}	E683 E685	Interior surface, βB
R298 ^{D2}	D295 E699 S711	R298 ^{D2}	D295 E689 S701	2-fold axis, αA
R310 ^{D1}	T414	R310 ^{D1}	E683	Interior surface, βB
K314 ^{D2}	NI	K314 ^{D2}	NI	Interior surface, βB
C361 [*]	H288 through additional density	C361 [*]	NI	Core of the capsid
<u>R389</u> ^D	NI	<u>R389</u>	NI	Exterior surface, VR-III
E416	dAMP through extra density	E416	K640 [*]	Interior surface
<u>Y714</u> [*]	NI	<u>Y704</u> [*]	NI	Exterior surface at the 2-fold axis

^D Dual conformation of the same residue in either AAV2 or rAAV2.7m8.

^{D1} Dual conformation: one in the same orientation and the other different.

^{D2} Dual conformation in both AAV2 and AAV2.7m8

^{*} Side chain with different orientation.

Underlined residues are a part of the pH quartet.

NI No interaction.