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Site-Specific Post-translational Surface Modification of Adeno-Associated Virus Vectors Using Leucine Zippers

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Abstract

Adeno-associated virus (AAV) is widely favored as a gene therapy vector, tested in over 200 clinical trials internationally. To improve targeted delivery a variety of genetic capsid modifications, such as insertion of targeting proteins/peptides into the capsid shell, have been explored with some success but larger insertions often have unpredictable deleterious impacts on capsid formation and gene delivery. Here, we demonstrate a modular platform for the integration of exogenous peptides and proteins onto the AAV capsid post-translationally while preserving vector functionality. We decorated the AAV capsid with leucine-zipper coiled-coil binding motifs that exhibit specific noncovalent heterodimerization. AAV capsids successfully display

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Author Contributions

N.N.T., C.L., G.B., and J.S. conceived of the study. N.N.T., J.Y., B.M., and M.Y.C. planned and conducted experiments and collected and analyzed data. G.B. and J.S. supervised the project. N.N.T. wrote the manuscript and all authors reviewed and edited the manuscript.

Supporting Information

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Quantification of zipper incorporation in Velcro-AAV; E3 Velcro-AAV transduction in HUVECs; Velcro-AAV nickel affinity column chromatography elution fraction quantification ([PDF](#))

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hexahistidine tagged-peptides using this approach, as demonstrated through nickel column affinity. This protein display platform may facilitate the incorporation of biological moieties on the AAV surface, expanding possibilities for vector enhancement and engineering.

Graphical Abstract

Keywords

adeno-associated virus; protein engineering; gene delivery; synthetic virology

The adeno-associated virus (AAV) has demonstrated strong potential as a gene therapy vector in clinical trials due to its high gene delivery efficiency, nonpathogenicity, and low immunogenicity.¹ While AAV has shown great promise, improvements to the vector are still needed to optimize its tropism and therapeutic efficacy. To improve gene delivery, efforts have focused on altering AAV's interactions with extracellular proteins, cellular receptors, and intracellular trafficking machinery using both rational and combinatorial design strategies.^{2–5}

Attachment of moieties to the assembled AAV capsid has previously been investigated as a strategy for vector engineering.⁶ One approach used a bispecific antibody to bind both the AAV capsid and a cell-specific receptor found on human megakaryocytes.⁷ Alternatively, both nonspecific biotinylation and specific metabolic biotinylation of small peptide insertions have been used to attach a variety of targeting ligands and probes to the AAV capsid.^{8,9} Use of biotinylation allows for a modular capsid binding site capable of picomolar binding affinities, but the proteins to be attached must be conjugated to streptavidin or avidin—large domains that may interfere with protein folding or function. Chemical conjugation approaches have also been applied to the AAV capsid, both nonspecifically on surface-exposed lysines and at specific sites introduced using unnatural amino acids.^{10,11} These approaches have been used to attach targeting moieties and probes as well as chemotherapy drugs and nucleic acids. However, chemical conjugation reactions must be optimized to achieve high yield and frequently utilize catalysts that are not biocompatible, requiring additional purification steps.^{12,13}

As an alternative to these previous approaches, we developed a coiled-coil based platform for noncovalent attachment of proteins/peptides to the surface of the AAV capsid (named Velcro-AAV). The prototypical coiled-coil consists of repeating heptads with hydrophobic domains stabilizing the periodic superhelical structure and charged residues facilitating electrostatic interactions between α -helices.¹⁴ It has been well established that leucine zipper (LZ) peptides engineered using principles gleaned from the study of naturally occurring LZs form coiled-coil pairs with high affinity and specificity.¹⁵ Because of these

properties and the reduced potential for cross-talk between *de novo* LZ designs and native signaling cascades, LZs have been widely adopted by protein engineers.¹⁶ LZs have served as the linker between components of gold, protein, and polymer delivery vectors.^{17–20} LZs have previously been used to promote receptor cross-linking as a drug-free macromolecular therapy *in vivo*, with no observed toxicity in tissues evaluated through histology after intravenous administration to SCID mice. Minimal cytokine release was observed in macrophages exposed to these LZs.²¹ Through these studies, researchers have demonstrated the modularity and biocompatibility of LZs as adaptors in delivery vector designs.

Our platform uses the engineered E3 (EIAALEKEIAALEKEIAALEK) and K3 (KIAALKEKIAALKEKIAALKE) heterodimerizing pair, optimized to maintain binding affinity with shorter peptide lengths.²² These zippers are incorporated in a surface-loop of the AAV9 capsid along with an enterokinase digestion site to facilitate zipper linearization. After enterokinase digestion, zippers are then available to bind their pair and display an attached peptide or protein. This Velcro AAV platform uses a relatively small genetic insertion to facilitate attachment of potentially large proteins to the assembled virus capsid. This approach may be suitable for the modular attachment of large proteins that may disrupt capsid formation if inserted genetically, toxic proteins that may damage producer cells, or delicate proteins that are unlikely to retain activity when purified along with the AAV capsid. For example, antibodies and other large targeting moieties may be attached to the capsid for modular retargeting to cell types. The use of a small genetically inserted adaptor that mediates postassembly attachment preserves vector production and stability while permitting site-specific surface display.

RESULTS AND DISCUSSION

We developed a method to attach proteins/peptides onto the AAV capsid surface using LZ coiled-coil heterodimerization, allowing for modular attachment of protein components to the capsid postassembly. We designed genetic modifications to the AAV9 *cap* gene to incorporate a LZ peptide motif after residue G453, a site previously demonstrated to tolerate insertion of small peptides and to result in surface-display.²³ We selected an engineered LZ motif with a short three-heptad (21 residues) sequence optimized for maximum stability and designed peptide inserts for the K3 and E3 components of the heterodimer. As G453 occurs in the middle of the capsid protein sequence, insertions at this point take on a loop conformation with both N- and C-termini of the protein/peptide anchored to the capsid. To liberate the LZ on one end, we incorporated an enterokinase cleavage motif at the C-terminus of the LZ sequence.²⁴ We also included GGS linkers to increase flexibility of the insertion, facilitating VP folding and virus assembly.²⁵ As capsid insertions have the potential to interfere with viral assembly and function, we used a mosaic capsid design to incorporate LZ inserts on a fraction of capsid subunits. AAV typically assembles with the capsid subunits VP1, VP2, and VP3 incorporated in a 1:1:10 ratio.²⁶ In our design, inserts were incorporated in a modified AAV9 VP2-only plasmid. We then assembled Velcro-AAV vectors through cotransfection of a plasmid expressing VP1 and VP3 only (VP1/3) and a plasmid expressing VP2 only with E3 (VP2-E3) or K3 insertion (VP2-K3), with the aim of incorporating zippers on approximately 1/12th of capsid subunits (Figure 1).

Characterization of Velcro-AAV Assembly, Zipper Incorporation, Genomic Protection, and Transduction.

Velcro-AAV capsids were assembled using 4-plasmid transfection, purified, and quantified. AAV9-E3 and AAV9-K3 vectors form at ~25% of unmodified AAV9 titers (Figure 2A). These reduced titers are comparable to the titers of other AAV serotypes with lower production yields than AAV9.²⁷ Other inserts at the same site have not impacted capsid assembly and genome packaging.²³ Only one of the inserts previously tested at this site formed at reduced titers, approximately 25% of AAV9.²⁸ Tolerance of the AAV capsid to insertion has been demonstrated to vary with the size and sequence of insertion.^{29,30}

Assembled capsids were digested with enterokinase or sham, resultant capsids were denatured, and constituent capsid proteins were visualized on a Western blot (Figure 2B). AAV9-E3 and AAV9-K3 both incorporate modified VP2-LZ protein subunits, as designed. Interestingly, these viruses contain some modified VP3-LZ proteins, as indicated by the faint band directly above the VP3 band in sham-treated lanes. This may be due to leaky translation from the CTG codon used to replace VP3 start codons in the VP2-LZ plasmids, as previously observed with other genes in mammalian cells.³¹ In total, the AAV9 E3 viruses appear to incorporate ~12.2% zipper-containing subunits, while the AAV9 K3 viruses incorporate ~7.5% zipper-containing subunits (Supplemental Figure 1). After digestion with enterokinase, the VP2-LZ and VP3-LZ bands disappear and the C-terminal cleavage product appears (predicted mass is 32 kDa), indicating complete digestion.

Enterokinase-treated capsids were then assayed for viral genome protection against nuclease digestion to determine if the proteolytic cleavage reduced capsid structural integrity (Figure 3A). AAV-E3 and AAV-K3 exhibit approximately 75–100% genomic protection, similar to AAV9, regardless of enterokinase treatment. This indicates that the enterokinase digestion step required for zipper linearization does not appear to impact capsid integrity. As this platform is further developed for *in vivo* applications, it will be useful to determine if this enterokinase cleavage step has any impact on long-term capsid stability that may result in challenges for Velcro-AAV production pipelines or storage.³²

Vectors were also screened for their ability to transduce cells (Figure 3B). CHO-Lec2 cells were transduced with AAV-E3 and AAV-K3 treated with either enterokinase or sham buffer. AAV-E3 exhibits a 50% drop in transduction index, while AAV-K3's transduction index is not reduced as compared to AAV9. These results appear to be variable depending on the cell type or multiplicity of infection (MOI) tested, as AAV-E3 postenterokinase digestion exhibits comparable transduction to AAV9 in human umbilical vein embryonic cells (HUVECs) transfected at an MOI of 100 000 (Supplemental Figure 2). Differences between AAV-E3 and AAV-K3 transduction abilities in CHO-Lec2 cells may be a charge-based effect, as the AAV-E3 insert bears an overall negative charge (−6) while the AAV-K3 insert bears a neutral charge. Negatively charged domains at this insertion site have previously been shown to interfere with AAV9 galactose receptor binding and to inhibit CHO-Lec2 transduction.^{23,28} If negative charge at the insertion site is resulting in reduced AAV-E3 transduction, future designs of Velcro-AAV could mitigate this effect by incorporating positive charges on the capsid in between the zipper sequence and the enterokinase cleavage motif. While AAV-E3's transduction is not impacted by enterokinase digestion, AAV-K3

shows a slight reduction in transduction postenterokinase digestion. The results thus far demonstrate that Velcro-AAV assemble at reduced titers compared to AAV9, retain full genomic protection after zippers are liberated, and transduce cells although transduction levels relative to AAV9 are dependent on zipper sequence and cell line.

Attachment of Peptide to Surface of Velcro-AAV.

To determine if Velcro-AAVs exhibit binding to their complementary zippers, we modified a previously developed assay based on nickel affinity chromatography.³³ We designed LZ peptides with C-terminal His₆ tags. AAV-E3 was incubated with the K3-His₆ peptide and AAV-K3 was incubated with the E3-His₆ peptide. Virus binding to a nickel affinity column was then quantified using qPCR.

We hypothesized the Velcro-AAV would bind to its complementary LZ peptide, which would in turn bind to the affinity column through its His₆ tag. AAV-E3, when incubated with 300 000× (300 000:1 molar ratio) of K3-His₆ peptide, results in ~80% of capsids binding the column (Figure 4A). The 30 000× molar ratio of peptide results in ~20% of capsids binding the column. However, lower molar ratios tested are not sufficient to induce virus column binding. At all peptide concentrations, AAV9 does not exhibit column binding as desired, suggesting that this behavior is due to the interaction of the LZ heterodimerizing pair. Notably, AAV-K3 also does not exhibit column binding—almost all of the virus is recovered in the flow through and wash fractions (Figure 4B).

The results demonstrate the AAV-E3 vector is able to bind K3-His₆ peptides successfully, but the AAV-K3 vector does not show evidence of binding to E3-His₆ peptides. The AAV-K3 insertion may interact with the surrounding capsid, limiting its capacity for interaction with the E3 peptide. Although we observed AAV-E3 binding to the K3-His₆ peptide via nickel affinity assay, 300 000× more peptide than virus was required to observe statistically significant column binding. These results may be due to the tendency of K3 peptide and K3-fusion protein constructs to homo-oligomerize at high concentrations.^{34,35} Future optimization of binding conditions such as peptide concentrations, temperature, and buffer may reduce the amount of peptide required.

Velcro-AAV vectors were also screened for changes in transduction after incubation with complementary His₆-tagged peptides. For the peptides tested, no impact on Velcro-AAV transduction was observed (Figure 4C). This lack of impact on transduction may be specific to the tested peptides—applications of the Velcro-AAV platform will require screening attachments of various sizes and amino acid composition to determine potential impacts on transduction. Additionally, further experiments are required to determine what fraction of capsid zipper sites are bound to peptides postincubation, as it is possible that peptide incubation does not appear to affect transduction because the majority of zipper sites on each capsid remain unbound.

CONCLUSION

To facilitate modular attachment of peptides to AAV, we integrated LZ adaptors into the AAV9 capsid surface and demonstrated the modified capsid's ability to bind peptides with

complementary adaptors. Velcro-AAV formed structurally intact capsids and retained transduction ability with variable levels depending on insertion and cell type. The E3 Velcro-AAV variant exhibits binding to complementary K3 peptides, although a large molar ratio of K3 peptides is required to observe binding in the nickel column assay used in this study. Viral transduction appears to be unimpaired by peptide binding, although this may vary with composition and size of the attached peptides.

While this prototype of Velcro-AAV shows promise in facilitating AAV peptide display, future iterations may require application-specific optimization of the number of zippers incorporated in the capsid—the incorporation of more zipper-containing subunits may result in higher levels of peptide/protein display, but also reduced vector production yields and potential impacts on transduction depending on the targeted cell type. This optimization may be performed using strategies such as adjusting the plasmid ratio of VP2-LZ and VP1/3 used in vector production or incorporating a strong start codon at VP2 in the VP2-LZ plasmid to reduce VP3-LZ expression and thus reduce the number of zipper-containing subunits incorporated.²⁹

In addition to functionalization with bioactive peptides, this Velcro-AAV platform may potentially be functionalized with large motifs such as antibody fragments or enzymes. Velcro-AAV may also be expanded using orthogonal zipper pairs to display multiple ligands for combinatorial targeting and delivery. Future work deploying this platform technology *in vivo* may draw on prior research developing stimulus-responsive coiled-coil motifs to promote the release of zipper-attached cargo at target sites,³⁶ or using cross-linking to strengthen coiled-coil linkers for systemic delivery.³⁷ The development of a modular display platform for the AAV capsid surface may expand the available toolkit for retargeting gene therapy vectors.

METHODS

Cloning of Plasmid Constructs.

AAV9 VP2 LZ constructs were created through modification of the pAAV2/9 plasmid. Restriction digest sites for NgoMIV and KasI were inserted after residue G453 in the AAV9 *cap* gene, and extraneous restriction sites and start codons for VP1 and VP3 were removed from the plasmid backbone through site directed mutagenesis (SDM) using the QuikChange protocol with Pfu Ultra polymerase (Agilent Technologies). Similarly, AAV9 VP1/3 was created through SDM to remove the weak start codon for VP2. As previously described for the generation of AAV2 capsid protein knockdowns, VP1 and VP3 start codons were modified to CTG, and VP2 weak start codon was modified to GCG.²⁹

K3 and E3 zipper inserts with enterokinase cleavage sites, spacers, and restriction digest sites were designed and purchased as gBlocks (Integrated DNA Technologies). Backbone plasmid and gBlocks were double-digested using NgoMIV and KasI (New England Biolabs) according to manufacturer protocol. Reactions were heat-inactivated. The backbone plasmid was dephosphorylated using Antarctic phosphatase (New England Biolabs) and gel purified using a Zymoclean gel DNA recovery kit (Zymo Research). Inserts were purified using a DNA clean and concentrator kit (Zymo Research). Backbone and insert fragments were

ligated using T4 DNA ligase (New England Biolabs) according to manufacturer protocol. Plasmid products were sequence-verified through an external vendor (Genewiz).

Virus Production and Purification.

Velcro-AAV vectors were produced by a quadruple-plasmid polyethylenimine transfection in HEK 293T cells consisting of (1) pXX6-80 helper plasmid encoding adenoviral helper genes, (2) pITR-GFP encoding a GFP transgene flanked by inverted terminal repeats, (3) VP1/3 (pAAV2/9 with VP2 start codon knocked out) and (4) VP2 LZ construct. After 48 h, cells were lysed through 3 freeze–thaws, then treated with 50 units/mL benzonase nuclease (Sigma) and centrifuged to remove excess nucleic acids. The supernatant was layered atop a 15%–54% iodixanol step gradient, and the tubes were centrifuged at 48 000 rpm in a Beckman Type 70Ti rotor for 105 min at 18 °C. Viruses were then extracted from the 40% iodixanol layer and concentrated into a gradient buffer (10 mM Tris, pH 7.6, 10 mM MgCl₂, 150 mM NaCl) + 0.001% Pluronic F-68 (GB-PF68, Thermo Fisher) using Amicon Ultra 100 kDa centrifugal filters (EMD Millipore).

Virus Quantification.

Viral titers were quantified using quantitative polymerase chain reaction (qPCR). Viral capsids were denatured in 2 M NaOH at 56 °C for 30 min to release genomes. The mixture was then neutralized using 2 M HCl. Samples were diluted in 10 ng/μL salmon sperm in ultrapure water and reactions prepared using SYBR Green PCR Master Mix (Life Technologies) and primers targeting the CMV promoter. Serially diluted recombinant AAV transgene was used to generate a standard curve. Samples were analyzed using a BioRad CFX96 qPCR machine.

Enterokinase Digestion.

LZ motifs were liberated on one end from the capsid through digestion with enterokinase. Virus was incubated with either sham (20 mM Tris-HCl, 200 mM NaCl, 2 mM CaCl₂, 50% glycerol, pH 7.2) or enterokinase enzyme (Light Chain, NEB) in 2 mM CaCl₂ at 23 °C for 24 h. To remove enterokinase from the virus digest, Pierce microcentrifuge spin columns (Thermo Fisher) were prepared with Trypsin-inhibitor agarose beads (Sigma). Beads were prepared by flowing stripping buffer (0.1 M NaCl, 0.1 M formic acid) through the columns three times, followed by 1 × GB-PF68. The virus-enterokinase reaction was incubated with the Trypsin-inhibitor agarose beads on the column for 15 min with agitation, before spinning at 2000g for 2 min to elute the virus.

Western Blot of Capsid Proteins.

Viral capsid proteins were characterized using Western blot to determine capsid composition and monitor enterokinase cleavage. Viruses were denatured with LDS sample buffer (NuPAGE, Life Technologies). Samples were electrophoresed in 4–12% Bis-Tris gels (NuPAGE, Life Technologies) and bands were wet-transferred to a nitrocellulose membrane. Membranes were then blocked in 5% milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.2% Tween) and washed in PBS-T. Membranes were then incubated in monoclonal mouse B1 primary antibody (1:50 dilution, American Research

Products). Membranes were washed, then incubated in HRP-conjugated goat antimouse IgG secondary antibody (1:2000 dilution, Santa Cruz Biotechnology). Blots were then PBS-T washed and treated with LumiLight Western blotting substrate (Roche Applied Science). Blots were imaged using a GE Healthcare Image-Quant LAS 4000 imager.

Viral Genome Protection Assay.

Virus samples treated with sham or enterokinase were diluted 1/10 in endo buffer (1.5 mM MgCl₂, 0.5 mg/mL BSA, 50 mM Tris, pH 8.0). Samples were divided into two 20 μ L reactions and either 0.5 μ L benzonase nuclease (Sigma) or 0.5 μ L sham buffer (50% glycerol, 50 mM Tris-HCl, 20 mM NaCl, 2 mM MgCl₂, pH 8.0) was added. Reactions were incubated at 37 °C for 30 min, and then nuclease activity was terminated through the addition of 0.0125 μ M EDTA. Remaining genomes were quantified through qPCR, and genomic protection was calculated as the ratio of genomes in benzonase-treated samples to genomes in sham-treated samples.

Quantification of Viral Transduction.

Viruses pre- and postenterokinase cleavage were evaluated for their transduction abilities. HUVEC or CHO-Lec2 cells were seeded on tissue-culture treated poly-L-lysine coated 48-well plates. At 70% confluency, cells were transduced with the virus in serum-free media at the indicated multiplicity of infection. Media was changed to media with serum at 24 h after transduction. Cells were harvested at 48 h post-transduction and analyzed through flow cytometry (BD FACS Canto II). The transduction index (TI), a linear indicator of transduction efficiency, was computed as the product of %GFP⁺ cells and the geometric mean fluorescence intensity (gMFI).³⁸

Nickel Column Binding Assay.

Samples of 3×10^9 vg of amicon-purified Velcro-AAV were diluted in 120 μ L of binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) along with his-tagged complementary zipper peptide (purchased from GenScript). The samples were incubated at RT for 30 min. His SpinTrap columns (GE Life Sciences) were equilibrated with binding buffer. All spins were conducted at 100g for 30 s. Samples were loaded onto the columns, and then the columns were washed with 600 μ L of binding buffer. Samples were eluted with 200 μ L of four elution buffers with increasing concentrations of imidazole (20 mM sodium phosphate, 500 mM NaCl, [100, 250, 500, 1000] mM imidazole, pH 7.4). Each flow through was collected, and viral titers were determined using qPCR. Titers were normalized to the total amount of virus recovered from the load, wash, and elution fractions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AAV	Adeno-associated virus
BSA	bovine serum albumin
CMV	cytomegalovirus
HUVEC	human umbilical vein embryonic cell
gMFI	geometric mean fluorescence intensity
LZ	leucine zipper
MOI	multiplicity of infection
qPCR	quantitative polymerase chain reaction
TI	transduction index

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Figure 1.

Plasmids used to assemble Velcro-AAV. LZ sequences were inserted after residue G453 in the AAV9 *cap* gene, along with spacers (red) and an enterokinase cleavage sequence (orange). For VP2-E3 and VP2-K3 constructs, the start codons for VP1 and VP3 were replaced with CTG to ablate their translation. Modified VP2-E3 and VP2-K3 plasmids were cotransfected with VP1/3 plasmid to generate mosaic capsids.

Figure 2.

Characterization of Velcro-AAV vector formation and enterokinase digestion. (A) Genomic titers of 1-plate virus preps of E3 and K3 Velcro-AAV. Titters are expressed in terms of viral genomes per milliliter and were determined using qPCR. AAV9 is included as a control. $N=3$ independent virus preps; error bars are SEM. One-way ANOVA was performed with Dunnett's *posthoc* multiple comparison test to compare Velcro-AAV to AAV9. ** $p < 0.01$. (B) Western blot of enterokinase (Ek)- or sham (S)-treated samples. Purified viruses were

treated with enterokinase or sham, denatured, and VPs were analyzed on a Western blot using B1 antibody. B1 antibody recognizes an epitope present in the C-terminus of all VPs.

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Figure 3.

Velcro-AAV genomic protection and transduction index after enterokinase digestion. (A) Benzonase genomic protection assay. Velcro-AAV vectors pre- and postenterokinase digestion were treated with either benzonase or sham buffer and the fraction of genomes remaining in benzonase-treated samples as compared to sham was quantified using qPCR. (B) CHO-Lec2 cells were transduced at an MOI of 5000 with Velcro-AAV. Transgene expression was measured by flow cytometry at 48 h post-transduction. Transduction index (the product of %+GFP cells and geometric mean fluorescence intensity) is reported relative

to AAV9. In both panels, $N=3$ experiments were conducted and error bars are SEM. One-way ANOVA was performed with Sidak's *posthoc* multiple comparison test to compare Velcro-AAV to AAV9 and to compare pre- and postenterokinase digested Velcro-AAV. ** $p < 0.01$, *** $p < 0.001$.

Figure 4.

Characterization of Velcro-AAV interaction with complementary LZ peptides. Velcro-AAV E3 (A) and K3 (B) vectors were incubated with complementary His₆ tagged peptides (300 000×, 30 000×, 3000×, or 300×), then the mixture was loaded onto nickel columns, and the fraction of total virus eluted in the flow through (S), wash (W), and elution fractions (E1, E2, E3, E4) was quantified via qPCR. Total virus in elution fractions was summed; the virus in individual fractions is plotted in Figure S3. *N* = 3 independent experiments, error bars are SEM. Two-way ANOVA was performed with Dunnett's *posthoc* multiple comparison test to

compare mutants to AAV9 with 300 000× peptide in each wash fraction. ** $p < 0.01$, *** $p < 0.001$. C) Velcro-AAV were incubated with complementary His₆ tagged peptides at 300 000× and then used to transduce CHO-Lec2 cells at an MOI of 5000. Transgene expression was measured by flow cytometry at 48 h post-transduction. Transduction index (the product of %+GFP cells and geometric mean fluorescence intensity) is reported relative to AAV9 with no peptide. $N = 3$ independent experiments; error bars are SEM. One-way ANOVA was performed with Sidak's *posthoc* multiple comparison test to compare all experimental conditions to AAV9 with no added peptide and to compare each capsid–peptide transduction to the corresponding capsid–no peptide control. *** $p < 0.001$.