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(54) Title: METHODS AND COMPOSITIONS FOR MODIFYING ASSEMBLY-ACTIVATING PROTEIN (AAP)-DEPENDENCE OF VIRUSES

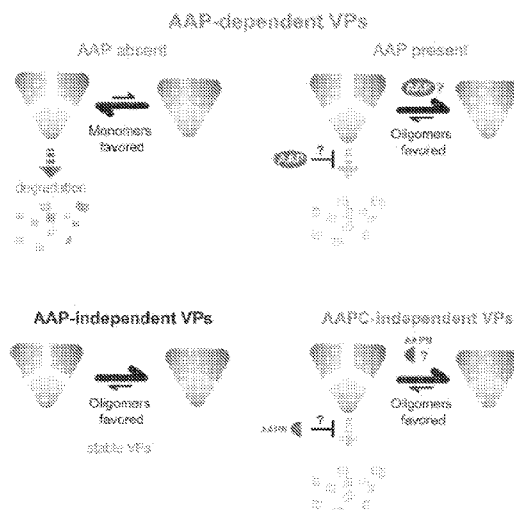


FIG. 7

(57) Abstract: This disclosure describes and demonstrates the utility of a particular sequence motif within an Adeno-associated virus (AAV) capsid protein that enables the assembly-activating protein (AAP)-dependence of the AAV to be modified. Thus, this sequence motif can be used to address and alleviate at least one of the bottlenecks encountered in the production of virus vectors. In particular, this disclosure describes a minimal motif defined through a novel phenotype-to-phylogeny mapping method that can be used to modify the AAP dependence of a virus.



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## METHODS AND COMPOSITIONS FOR MODIFYING ASSEMBLY- ACTIVATING PROTEIN (AAP)-DEPENDENCE OF VIRUSES

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. Application No. 62/504,318, filed on May 10, 2017 and U.S. Application No. 62/669,901 filed May 10, 2018, both of which are incorporated herein by reference in their entirety.

### TECHNICAL FIELD

This disclosure generally relates to viral vector systems.

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### BACKGROUND

Adeno-associated virus (AAV) is a leading platform in therapeutic gene transfer, primarily for *in vivo* gene therapy approaches. While preclinical and clinical studies continue to demonstrate AAV's potential as a reagent for safe and efficient gene delivery to alleviate a number of diseases, a bottleneck to its broader application is the production of sufficient vector quantities to treat these patient populations.

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### SUMMARY

In general, this disclosure describes and demonstrates the utility of a particular sequence motif within an AAV capsid protein that enables the assembly-activating protein (AAP)-dependence of the AAV to be modified. Thus, this sequence motif can be used to address and alleviate at least one of the bottlenecks encountered in the production of virus vectors. In particular, this disclosure describes a minimal motif defined through a novel phenotype-to-phylogeny mapping method that can be used to modify the AAP-dependence of a virus. Briefly, a number of ancestral AAVs that have been developed (see, for example, WO 2015/054653 and WO 2017/019994, which are incorporated herein by reference in their entirety) were used to examine AAP dependence across a wide structural differential. This analysis allowed for the identification of a minimal motif that determines AAP dependency.

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In one aspect, the disclosure features adeno-associated virus (AAV) capsid polypeptides including an amino acid sequence having at least 95% sequence identity (e.g., at least 99% sequence identity) to the amino acid sequence of SEQ ID NO: 3. In

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some embodiments, the AAV capsid polypeptide has the amino acid sequence of SEQ ID NO:3. In some embodiments, the AAV capsid polypeptides are encoded by the nucleic acid sequence of SEQ ID NO: 4. In some embodiments, the AAV capsid polypeptide has the amino acid sequence of SEQ ID NO:1, but contains the amino acid residues at the indicated positions shown in Table 6 for “independence” or “dependence” with respect to AAP.

This disclosure also features virus particles including any of the adeno-associated virus (AAV) capsid polypeptides described herein. Such virus particles can further include a transgene.

In another aspect, the disclosure features nucleic acid molecules including a nucleic acid sequence having at least 95% sequence identity (e.g., at least 99% sequence identity) to the nucleic acid sequence of SEQ ID NO: 4 and encoding an adeno-associated virus (AAV) capsid polypeptide. In some embodiments, the nucleic acid molecule has the nucleic acid sequence of SEQ ID NO:4. In some embodiments, the nucleic acid molecule encodes the amino acid sequence of SEQ ID NO: 3.

The disclosure also provides vectors including any of the nucleic acid molecules described herein, as well as host cells including any of the nucleic acid molecules and/or vectors described herein. In some embodiments, the host cell is a packaging cell.

In another aspect, the disclosure features packaging cells including a nucleic acid molecule encoding an adeno-associated virus (AAV) capsid polypeptide, wherein the AAV capsid polypeptide has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3. In some embodiments, the packaging cell lacks the assembly activating protein (AAP).

In another aspect, the disclosure includes methods of reducing the assembly activating protein (AAP)-dependence of an adeno-associated virus (AAV). Such methods include providing an AAV having a capsid polypeptide that has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3.

In yet another aspect, the disclosure features methods of relieving, at least partially, the assembly activating protein (AAP)-dependence of an adeno-associated virus (AAV), the method including: incorporating a capsid polypeptide into the AAV that has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3.

In still another aspect, the disclosure provides methods of engineering an adeno-associated virus (AAV) to reduce its dependence on assembly activating protein (AAP),

including: engineering an AAV that comprises a capsid polypeptide that has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3.

Any of the methods described herein can further include culturing the adeno-associated virus (AAV) in the absence of the assembly activating protein (AAP). Any of the methods described herein further can include sequencing the engineered adeno-associated virus (AAV). Any of the methods described herein further can include comparing the assembly activating protein (AAP)-dependence of the engineered adeno-associated virus (AAV) relative to a non-engineered or wild type AAV. Any of the methods described herein further can include aligning the engineered adeno-associated virus (AAV) with the non-engineered or wild type AAV.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

### BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a series of schematic diagrams, gels, and bar graphs of data demonstrating that the requirement for AAP ranges broadly across all clades of AAV. Panel A are schematics of AAPstop60 and AAP-HA construct maps. Black arrows: transcription start sites at p5, p19, and p40 viral promoters. Grey arrows: *cap* gene product translation start codons. Early stop codon (red) introduced by site directed mutagenesis ~60 aa into the AAP ORF. HA tag (orange) inserted at a conserved BsiWI site near AAP C-terminus. Panel B is a photograph of a gel demonstrating that AAV1 and AAV3 AAP-HA constructs were generated in both WT AAP and AAPstop60 context. Lysates from transfected HEK293 cells were harvested after 36 h, clarified by centrifugation, and interrogated for AAP by Western Blot with anti-HA antibody. Panel C is a graph demonstrating the vector produced from WT or AAPstop60 *rep-cap* constructs was titrated by qPCR to quantify DNase resistant particles. AAPstop60 titers are reported as a percentage of each WT serotype titer and represent the average of at least 3 independent experiments  $\pm$  SEM. Bar color corresponds to heatmap color on y-

axis. † AAPstop60 titer below background level for at least one trial (no *cap* gene control). See Table 2 for statistics. Panel D is a graph of vector produced from WT and AAPstop60 constructs of AAV2 and AAV3 were titrated by A20 capsid ELISA, reported as a percentage of WT titer (average of two experiments; see Table 1 for individual data of the titration by ELISA and qPCR). See also FIG. 11.

FIG 2 is a series of gels and graphs of experimental data showing VP protein levels in natural serotypes. HEK 293 cells were transfected with helper and *rep-cap* plasmids as denoted above the lane: (wt) WT AAP; (s) AAPstop60; (r) AAPstop60 plus a CMV-driven AAP2. Whole cell lysates were harvested after 36 hours, clarified by centrifugation, and VP levels interrogated with B1 antibody (VP1/2/3). Actin was used as a loading control. Serotypes of *rep-cap* plasmids indicated above each blot, with (Panel A) AAPstop60 titers  $\geq 10\%$  WT titer and (Panel B) AAPstop60 titers  $< 10\%$  WT titer. Panel C is a blot showing RNA quantified from AAV2 transfections as above, normalized to GAPDH, reported relative to AAV2 WT. Minor and major splice isoforms, as well as unspliced transcript levels, were examined as denoted on x-axis and diagrammed at right; primers indicated by arrows. Graph represents the mean of three independent experiments  $\pm$ SEM; there is no statistically significant difference between groups (see Table 2 for statistics). Panel D are blots of HEK 293 cells transfected with helper and AAV8 WT or AAPstop60 *rep-cap* plasmids as indicated. At 24h, AAPstop60 transfected cells were treated with concentrations of Bortezomib, MLN7243, or Bafilomycin as indicated above lanes, and incubated an additional 8 h before whole cell lysates were harvested as in (Panel A) and (Panel B). VP levels were interrogated by Western Blot with B1 antibody (top). Blot was stripped and reprobed for Ubiquitin (bottom). Actin was used as a loading control (middle). Panel E are dot blots using lysates from Panel D treated with DMSO or 1  $\mu$ M Bortezomib, MLN7243, or Bafilomycin as listed below were assayed for the presence of assembled capsids by dot blot with the ADK8 antibody (recognizes a conformational epitope only present in assembled AAV8 capsids). The experiment was repeated in the presence of AAP to control for any effects of the drugs on capsid assembly (right panel). See also FIG. 8.

FIG. 3 is a series of gels, charts, and schematics representing experimental data demonstrating that the requirement for AAP exhibits branch specificity in the context of a putative AAV phylogeny. Panel A is a graph showing that AAPstop60s were generated for the nine putative ancestral AAVs. Vector produced from WT or AAPstop60 *rep-cap*

constructs was titrated by qPCR to quantify DNase resistant particles. AAPstop60 titers are reported as a percentage of each serotype's WT titer and represent the mean of at least 3 independent experiments  $\pm$  SEM. Bar color corresponds to heatmap color on y-axis and is used also in (Panel D) and (Panel E). † AAPstop60 titer below background (no *cap* gene control) for at least one trial. See Table 2 for statistics. \* Anc126 produces at consistently low titers (below  $1e^9$  GC/mL) for WT and AAPstop60. Panel B is a graph showing that AAPstop20s were generated for AAV4 and all AAV variants with AAPstop60 titers  $\geq 10\%$  by introducing early stop codons at  $\sim 20$  aa into the AAP ORF. Vector was produced and titrated as in Panel A, adding the AAPstop20 condition (light grey bars) and AAPstop20 plus a CMV-driven construct expressing a homologous AAP (dark grey bars) (mean of two experiments; see Table 3 for individual AAPstop60, stop20, and AAP rescue vector titration data). † Titer below background (no *cap* gene control) for at least one trial. ‡ Rescue performed with AAP2. Panel C are gels of HEK 293 cells transfected with helper and *rep-cap* plasmids as denoted above lane: (wt) WT AAP; (s60) AAPstop60; (s20) AAPstop20; (r) AAPstop20 plus CMV-driven homologous AAP. Whole cell lysates were harvested after 36 hours and VP levels interrogated by Western Blot. Tubulin was used as a loading control. Panel D is the categorization of AAP phenotypes. Boxes below each serotype indicate AAPstop60 percentage of WT titer. Black boxes indicate AAP-independent (AAPstop20 titer is  $\gg 1\%$ ). Serotypes with AAPstop60 titers  $> 10\%$  (green) indicate assembly in absence of C-terminal two-thirds of AAP (AAPC-independent). Panel E is a reconstructed AAV phylogeny, branches colored as in Panel D. Grey numbers on branches indicate number of divergent amino acids between the two serotypes flanking the branch segment. See also FIGs. 8, 9, 10, and 12.

FIG. 4 is a series of schematics, graphs, and gels experimental data showing the characterization of 82DI, an AAPC-independent gain-of-function mutant. Panel A are schematics of 82DI generated by introducing Branch I residue identities into an Anc82 *rep-cap* plasmid by site directed mutagenesis. Panel B is a graph of vector produced from Anc82, 82DI, and their AAPstop60s was titrated by quantifying DNase resistant particles (DRP), and is reported as a percentage of Anc82 WT titer. Graph represents the mean of four independent experiments,  $\pm$  SEM. See Table 2 for statistics. Panel C is a graph, where each site in 82DI and 82DIAAPstop60 was reverted to its Anc82 identity individually by site directed mutagenesis. Vector titers quantifying DRPs are reported as a percentage of 82DI WT titer and represent the mean of 2 trials (see Table 4 for

individual 82DI single revertant vector titration data). Panel D is a photograph of a gel of HEK 293 cells transfected with helper and rep-cap plasmids as denoted above lane: (WT) WT AAP; (s60) AAPstop60; (s20) AAPstop20; (r) AAPstop20 plus CMV-driven AAP2. Whole cell lysates were harvested after 36 hours, and VP levels interrogated by Western Blot. Tubulin was used as a loading control. Panel E is normalized SYPRO® Orange fluorescence signals obtained for Anc82 and 82DI. Panel F are photographs of GFP fluorescence detected in murine livers 30 d after systemic injection with  $1 \times 10^{11}$  vg/mouse of Anc82, 82DI, or AAV8. Each image is representative of an individual animal. See also FIGs. 12 and 13.

10 FIG. 5 is a series of molecular-level and atomic-level schematics demonstrating that sites of interest map to the trimer interface, suggesting stronger inter-monomeric interactions in AAPC-independent serotypes. Panel A is a summary of the ten sites (twelve residues) identified by Branch D/Branch I multiple sequence alignment, numbered from VP1 start codon. Branch D residues include Anc80, Anc81, Anc82, Anc83, Anc84, AAV8, and rh10; Branch I residues include Anc110, rh8, and AAV9. Variations in identity for AAV8 and AAV9 are indicated in parentheses and are exclusive to these members of their respective branches. Panel B is a side view of an AAV9 trimer, showing planes of view in Panel C. Each monomer is represented as one color, and each site of interest in a darker shade of that color. Numbered arrows indicate each site within the red monomer. Panels D-F are atomic-level views of select sites in AAV8 and AAV9 trimers.

25 FIG. 6 is a series of schematics and gels of experimental data demonstrating that AAP promotes VP-VP interactions. Panel A is a schematic of expression constructs for AAP2 and VP1 and VP3 of AAV2, AAV3, Anc82, and 82DI. In CMV-HA-VP1, the VP2 and VP3 start codons were modified to silence their expression, and the AAPstop60 mutation (red rectangle) was included. Panel B are photographs of HEK293 cells transfected with CMV-HA-VP1 and CMV-VP3 of serotype indicated above each lane, +/- CMV-AAP2, and lysates harvested after 48 h. Immunoprecipitation was performed using anti-HA antibody; VPs detected by Western Blot using the B1 antibody. Panel C are photographs of lysates from CMV-HA-VP1, CMV-VP3, +/- CMV-AAP2 (all AAV2 proteins) transfected HEK293 cells treated with DMSO, 5 mM disuccinimidyl glutarate (DSG), or 5 mM disuccinimidyl suberate (DSS) as indicated above columns. VPs were

detected by Western Blot with B1 antibody. Approximate molecular weights are shown to the right of each row. See also FIG. 13.

FIG. 7 is a schematic model for early steps of capsid assembly across the AAP phenotypes. Whether a serotype is AAP-dependent, AAP-independent, or AAPC-  
5 independent, nucleating capsid assembly is likely dependent on both the stability and oligomerization of VP proteins. The findings herein demonstrate AAP is active in both functions. Whether or not these functions are separate is unclear, and indicated by question marks in the model.

FIG. 8 is a series of representations of gels showing AAV8 and AAV3 VP  
10 degradation. Related to FIG. 2 and FIG. 3. Panel A are photographs of HEK 293 cells transfected with helper and AAV8 wt or AAPstop60 *rep-cap* plasmids as indicated. At 24 h, AAPstop60 transfected cells were treated with 50  $\mu$ M cycloheximide (CHX) and lysates harvested at progressive time points. VP levels were interrogated by Western Blot with B1 antibody, and p62 was blotted for as a positive control for CHX effectiveness.  
15 The exposure shown for AAV8AAPstop60 transfected cells is a long exposure with a higher sensitivity detection reagent, to demonstrate that AAV8 VPs could not be detected in the absence of AAP. Panel B are photographs of HEK 293 cells transfected with helper and AAV3 wt or AAPstop20 *rep-cap* plasmids as indicated. At 24 h, AAPstop20 transfected cells were treated with concentrations of Bortezomib, MLN7243, or  
20 Bafilomycin as indicated above lanes, and incubated an additional 8 h before whole cell lysates were harvested. VP levels were interrogated by Western Blot with B1 antibody (top). Blot was stripped and reprobbed for Ubiquitin (bottom). Actin was used as a loading control (middle).

FIG. 9 is a schematic showing the conservation of AAP across 21 serotypes.  
25 Related to FIGs. 1, 2, 3, 6, and 7. Multiple protein sequence alignment (ClustalW) for AAPs of all 21 serotypes examined in this study. Ancestral sequence reconstruction to generate the Anc-AAVs (and by extension, Anc-AAPs) is detailed elsewhere (Zinn et al., 2015, Cell Rep., 12:1056-68). In brief, VP coding sequence for AncAAVs was determined first on a protein level, and then reverse translated to DNA for subsequent  
30 synthesis using a codon table from the most similar extant AAV sequence available. The conserved core (black bar) identified previously (Naumer et al., 2012, J. Virol., 86:13038-48) retains high conservation across the Anc-AAPs, and may confer the chaperone function suggested by the data for AAPN (purple bar) in the present work. The work

described herein additionally points to a scaffolding function for AAP that may be largely contained in the C-terminal two-thirds of AAP (AAPC, grey bar).

FIG. 10 is a series of schematics and graphs of experimental data demonstrating that AAV3 AAPN does not rescue AAP-dependent viral production. Related to FIG. 3. Panel A are constructs expressing only AAP generated by adding early stop codons in the VP1, VP2, and VP3 ORFs of an AAV3 genome, and the AAPstop60 mutation was included to generate a construct expressing only AAPN. The VP3 early stop codon is a silent mutation in the AAP ORF. Panel B is a graph from constructs in (A) used to trans-complement AAPstop20 viral production in AAV2 and AAV3 (green and red bars) and viral titers reported as a percentage of their WT titer. Graph represents average of two trials. † Titer below background (no *cap* gene control) in at least one trial. Panel C shows the individual data for both trials.

FIG. 11 is a series of representations of microscope images and graphs that show experimental data demonstrating that AAPstop60 virus is indistinguishable from wt AAP virus. Related to FIG. 1. Panel A are photographs of AAV3 and AAV3AAPstop60.CMV.EGFP.T2A.Luciferase vector stained with uranyl acetate and imaged by TEM. Panels B and C are results from HEK293 cells incubated with hAd5 (MOI=20) overnight, then AAV3, AAV3AAPstop60, AAV9 or AAV9AAPstop60.CMV.EGFP.T2A.Luciferase was added at GC/well indicated on x-axis in Panel C. GFP fluorescence was imaged at 48 h (Panel B; images represent the highest titer for each vector). Luciferase activity was quantified at 48 h (Panel C). Panel D is normalized SYPRO® Orange fluorescence signals obtained for AAV3 and AAV9 WT and AAPstop60 vectors.

FIG. 12 is a series of graphs that show experimental data of Anc82 vs Anc82DI *in vitro* and *in vivo* as follows. Related to FIG. 4. Panel A is a graph of HEK293 cells incubated with hAd5 (MOI=20) overnight, then Anc82 or Anc82DI.CMV.EGFP.T2A.Luciferase vector added at  $1 \times 10^9$  or  $1 \times 10^8$  GC/well as indicated. Luciferase activity was measured after 48 h. Panel B is a graph of mice injected systemically with  $1 \times 10^{11}$  vg/mouse of Anc82, 82DI, or AAV8.CB7.CI.EGFP.FF2A.hA1AT.RBG. Human  $\alpha$ -1 antitrypsin (hA1AT) levels were measured by ELISA in serum sampled on time points indicated on x axis.

FIG. 13 is a pair of bar graphs of experimental data showing that the IP fraction does not contain fully assembled capsids. Related to FIG. 6. Rep, helper, and ITR-CMV-

EGFP-T2A-Luc\_ITR reporter genome plasmids were transfected with the AAV2 protein expression constructs indicated on x-axis. Fully assembled vectors in the input, IP, and supernatant fractions were quantified by (Panel A) qPCR on DNase resistant genomes or (Panel B) A20 capsid ELISA. Graphs are representative of two independent experiments.

5 † At least one measurement falls below the limit of detection.

### DETAILED DESCRIPTION

Gene transfer, either for experimental or therapeutic purposes, relies upon a vector or vector system to shuttle genetic information into target cells. The vector or vector system is considered the major determinant of efficiency, specificity, host response, pharmacology, and longevity of the gene transfer reaction. Currently, the most efficient and effective way to accomplish gene transfer is through the use of vectors or vector systems based on viruses that have been made replication-defective. One of the most common viruses to be made replication-defective and used in gene transfer is adeno-associated virus (AAV).

15 The AAV capsid is a non-enveloped, icosahedral 60-mer of three repeating protein monomer subunits called viral protein 1 (VP1), VP2, and VP3. A single transcript expressed from the AAV cap gene containing nested open reading frames (ORFs) is alternately spliced, resulting in three distinct protein products that share C-terminal identity the length of VP3. A 1:1:10 stoichiometry of VP1:VP2:VP3 in the assembled capsid is thought to be a consequence of the relative abundance of each protein, which is, in turn, regulated by splice product abundance and a non-canonical ACG translation start codon for VP2.

25 The Assembly-Activating Protein (AAP) is a non-structural protein expressed from a non-canonical CTG start codon of an overlapping reading frame embedded within the capsid (*cap*) gene of AAV. AAV serotypes have different requirements for AAP, with some AAV serotypes exhibiting AAP-dependence (e.g., AAV8, rh10, Anc80, Anc81, Anc82, Anc83, and Anc84) and other AAV serotypes exhibiting AAP-independence (e.g., AAV9, rh8, and Anc110).

30 As used herein, an ancestral scaffold sequence refers to a sequence that is constructed using evolutionary probabilities and evolutionary modeling and is not known to have ever existed or to presently exist in nature. These scaffold sequences were leveraged herein to interrogate AAP function and delineate structural determinants within the capsid relevant to the virus' requirement for AAP.

This disclosure provides methods of modifying the AAP-dependence of an AAV. For example, an AAV capsid sequence can be engineered to include the motif identified herein, which reduces the AAP-dependence (or, conversely, increases the AAP-independence) during packaging of the AAV. This provides a number of benefits during manufacturing including, without limitation, the ability to reduce the number of components needed for productive particle assembly in any AAV production system (e.g. mammalian, yeast, insect cell), the ability to optimize AAV capsid structure with reduced constraints imposed by AAP, the potential of AAV capsid self assembly from minimal components, and the reduction of AAP contamination concerns in the final vector preparations.

***Adeno-Associated Virus (AAV) Nucleic Acid and Polypeptide Sequences Imparting Modified AAP-Dependency***

An AAV capsid sequence, based originally on the Anc82 sequence (SEQ ID NO:1, encoded by SEQ ID NO:2), which exhibits AAP-dependence during packaging, has been modified as described herein to produce Anc82DI (SEQ ID NO:3, encoded by SEQ ID NO:4). Anc82DI exhibits AAP-independence during packaging, but appears to retain functionality as a potent gene transfer vector. The sequence motif that imparts AAP-independence on AAP-dependent sequences is provided in Table 6.

**Table 6. Motif for Modifying AAP-Dependence**

Site	Residue identity and position in Anc80, Anc81, Anc82, Anc84, rh10 and AAV8 “Independence”	Aligns to this residue in Anc110, rh8, and AAV9 “Dependence”
1	K163	T162 (S in AAV9)
2	A206	S205
3	K478	R476
4	L481	V479 (I in AAV9)
5	V520 (A in AAV8)	M518
6	T528	S526
7	L586	N584 (H in AAV9)
8	A592 – P593	Q590 – A591
9	N599 – S600	H597 – N598 (QN in AAV9)
10	A603	V601 (I in AAV9)

Anc82 protein (SEQ ID NO:1)  
 MAADGYLPDWLEDNLSEGI REWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPENGLDKGEPVNAADAAA  
 LEHDKAYDQQLKAGDNPYLRYNHADAQEFQERLQEDTSFGGNLGRAVFAQKRVLEPLGLVEEGAKTAPGKKR

PVEQSPQREPDSSTGIGKKGQQPAKKRLNFGQTGDSESVDPDQPLGEP PAAPSGVGSNTMAAGGGAPMADNN  
 EGADGVGNS SGNWHDSTWLGDRVITTTSTRTWALPTYNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDNF  
 RFHCHFS PRDWQRLINNNWGFPRKRLNFKLFNIQVKEVTTNEGKTIANNLTSTVQVFTDSEYQLPYVLGSA  
 HQGCLPPFPADV FMI PQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYTFEDVFPFHSSYAHSQS  
 5 LDRLMNPLIDQYLYLSRTQTTGGTAGTQTLQFSQAGPSSMANQAKNWLPGPCYRQQRVSTTTNQNNSNFA  
 WTGATKYHLNGRDSLNVPGVAMATHKDDERDFPSSGVLI FGKQGAGNDNDVDYSNVMITSEEEIKTTNPVAT  
 EEYGVVATNLQ SANTAPQTGTVNSQ GALPGMVWQNRDVYLOGPIWAKI PHTDGNFHP SPLMGGFGLKHP PPQ  
 ILIKNTPVPADPPTTFNQAKLNSFITQYSTGQVSVEIEWELQKENS KRWNPEIQYTSNYKSTNVDFAVNTE  
 GVYSEPRPIGTRYLTRNL

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Anc82 DNA (SEQ ID NO:2)

ATGGCTGCCGATGGTATCTTCCAGATTGGCTCGAGGACAACCTCTCTGAGGGCATT CGCGAGTGGTGGGAC  
 CTGAAACCTGGAGCCCCGAAACCCAAAGCCAACCAGCAAAAAGCAGGACGACGGCCGGGGTCTGGTGCCTTCCCT  
 GGCTACAAGTACCTCGGACCCTTCAACGGACTCGACAAGGGGGAGCCCGTCAACGCGGCGGACGCAGCGGCC  
 15 CTCGAGCAGCACAAGGCTACGACCAGCAGCTCAAAGCGGGTGACAATCCGTACCTGCGGTATAATCACGCC  
 GACGCCGAGTTTCAGGAGCGTCTGCAAGAAGATACGTCTTTTGGGGCAACCTCGGGCGAGCAGTCTTCCAG  
 GCCAAGAAGCGGGTCTCGAACCTCTCGGTCTGGTTGAGGAAGGCGCTAAGACGGCTCCTGGAAAGAAGAGG  
 CCGGTAGAGCAGTCAACACAGCGTGAGCCCGACTCCTCCACGGGCATCGGCAAGAAAGGCCAGCAGCCCGCC  
 AAAAAGAGACTCAATTTCCGTGACTGGGACTCAGAGTCAAGTCCCCGACCCCTCAACCTCTCGGAGAACCT  
 20 CCAGCAGCGCCCTCTGGTGTGGGATCTAATAACAATGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC  
 GAAGGTGCCGAGTGGTAATCTCGGGAATGGCATTGCGATTCCACATGGCTGGCGACAGAGTC  
 ATCACCACCAGCACCGAACCTGGGCCCTGCCCACTACAACAACCACCTCTACAAGCAAATCTCCAACGGG  
 ACCTCGGGAGGCAGCACCAACGACAACACCTACTTTGGTACAGCACCCCTGGGGGTATTTTACTTTAAC  
 AGATTCCACTGCCACTTCTCACCACGTGACTGGCAGCGACTCATCAACAACAACCTGGGGATTCCGGCCCAAG  
 25 AGACTCAACTTCAAGCTCTTCAACATCCAGGTCAAAGAGGTCACGACGAATGAAGGCACCAAGACCATCGCC  
 AATAACCTCACCAGCACCGTCCAGGTGTTTACGGACTCGGAATACCAGCTGCCGTACGTCTCTCGGCTCTGCC  
 CACCAGGGCTGCCCTGCCCTCCGTTCCCGGGCGGACGTCTTCATGATTCCCTCAGTACGGCTACCTGACTCTCAAC  
 AACGGTAGTCAGGCCGTGGGACGTTCTCTCTACTGCCTGGAGTACTTCCCTCTCAGATGCTGAGAACG  
 GGCACAACAATTTCAATT CAGCTACACTTTCCGAGGACGTGCCTTTCCACAGCAGCTACGCGCAGCAGCCAGAGT  
 30 TTGGACAGGCTGATGAATCCTCTCATCGACTACCTGTACTACCTGTCAAGAACCCAGACTACGGGAGGC  
 ACAGCGGGAACCCAGACGTTGCAGTTTTCTCAGCCGGGCTAGCAGCATGGCGAATCAGGCCAAAACTGG  
 CTGCCTGGACCCTGCTACAGACAGCAGCGCGTCTCCACGACAACGAATCAAAAACAACAACAGCAACTTTGCC  
 TGGACTGGTGCCACCAAGTATCATCTGAACGGCAGAGACTCTCTGGTGAATCCGGGCGTGCCTATGGCAACC  
 CACAAGGACGACGAGGACCGCTTCTTCCCATCCAGCGCGTCTCATATTTGGCAAGCAGGGAGCTGGAAAT  
 35 GACAACGTGGACTATAGCAACGTGATGATAACGCGAGGAAGAAATCAAGACCACCAACCCCGTGGCCACA  
 GAAGAGTATGGCGTGGTGGTACTAACCCTACAGTCCGCAAAACACCGCTCCTCAAACGGGGACCGTCAACAGC  
 CAGGGAGCCTTACCTGGCATGGTCTGGCAGAACCAGGGACGTGTACCTGCAGGGTCTATTTGGGCCAAGATT  
 CCTCACACAGATGGCAACTTTCAACCGTCTCCTTTAATGGGCGGCTTTGGACTTAAACATCCCGCTCCTCAG  
 40 ATCCTCATCAAAAACTCCTGTTCTGTTCCGGATCCTCCAACAACGTTCAACCAGGCCAAGCTGAATTTCTTTC  
 ATCAGCAGTACAGCACCGGACAAGTCAGCGTGGAGATCGAGTGGGAGCTGCAGAAGGAGAACAGCAAGCGC  
 TGGAACCCAGAGATT CAGTATACTTCCAAC TACTACAAATCTACAAATGTGGACTTTGCTGTTAATACTGAG  
 GGTGTTTACTCTGAGCCTCGCCCCATTGGCACTCGTTACCTCACCCGTAATCTGTAA

Anc82DI protein (SEQ ID NO:3):

MAADGYLPDWLEDNLSEGIREWWDLKP GAKPKANQQKQDDGRGLVLPGYKYLGPFNGLDKGEPVNAADAAA  
 LEHDKAYDQQLKAGDNPYLRYNHADA E FQERLQEDT SFGGNLGRAVFQAKKRVL EPLGLVEEGAKTAPGKKR  
 PVEQSPQREPDSSTGIGKSGQQPAKKRLNFGQTGDSESVDPDQPLGEP PAAPSGVGSNTMASGGGAPMADNN  
 EGADGVGNS SGNWHDSTWLGDRVITTTSTRTWALPTYNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDNF  
 5 RFHCHFS PRDWQRLINNNWGFPRKRLNFKLFNIQVKEVTTNEGKTIANNLTSTVQVFTDSEYQLPYVLGSA  
 HQGCLPPFPADV FMI PQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYTFEDVFPFHSSYAHSQS  
 LDRLMNPLIDQYLYLSRTQTTGGTAGTQTLQFSQAGPSSMANQARNWVPGPCYRQQRVSTTTNQNNSNFA  
 WTGATKYHLNGRDSL MNPGVAMASHKDDERDFPSSGVLI FGKQGAGNDNDVDYSNVMITSEEEIKTTNPVAT  
 EEYGVVATNHQSANTQAQTGTVQNQGI LPGMVWQNRDVYLOGPIWAKI PHTDGNFHP SPLMGGFGLKHP PPQ  
 ILIKNTPVPADPPTTFNQAKLNSFITQYSTGQVSVEIEWELQKENS KRWNPEIQYTSNYKSTNVDFAVNTE  
 55 GVYSEPRPIGTRYLTRNL

Anc82DI DNA (SEQ ID NO:4):

ATGGCTGCCGATGGTATCTTCCAGATTGGCTCGAGGACAACCTCTCTGAGGGCATT CGCGAGTGGTGGGAC  
 CTGAAACCTGGAGCCCCGAAACCCAAAGCCAACCAGCAAAAAGCAGGACGACGGCCGGGGTCTGGTGCCTTCCCT  
 60 GGCTACAAGTACCTCGGACCCTTCAACGGACTCGACAAGGGGGAGCCCGTCAACGCGGCGGACGCAGCGGCC  
 CTCGAGCAGCACAAGGCTACGACCAGCAGCTCAAAGCGGGTGACAATCCGTACCTGCGGTATAATCACGCC

GACGCCGAGTTTCAGGAGCGTCTGCAAGAAGATACGTCTTTTGGGGGCAACCTCGGGCGAGCAGTCTCCAG  
 GCCAAGAAGCGGGTTCTCGAACCTCTCGGTCTGGTTGAGGAAGGCGCTAAGACGGCTCCTGGAAAAGAAGAGG  
 CCGGTAGAGCAGTACCACAGCGTGAGCCCGACTCCTCCACGGGCATCGGCAAGAGCGGCCAGCCCCGCC  
 AAAAAAGAGACTCAATTTCCGGTCAGACTGGCGACTCAGAGTCAGTCCCCGACCCCTCAACCTCTCGGAGAACCT  
 5 CCAGCAGCGCCCTCTGGTGTGGGATCTAATACAATGGCTTCAGGCGGTGGCGCACCAATGGCAGACAATAAC  
 GAAGGTGCCGACGGAGTGGGTAATTCCTCGGGAAATTTGGCATTGCGATTCCACATGGCTAGGCGACAGAGTC  
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 ACCTCGGGAGGCAGCACCAACGACAACACCTACTTTGGCTACAGCACCCCTGGGGGTATTTTACTTTAAC  
 10 AGATTCCACTGCCACTTCTCACCACGTGACTGGCAGCGACTCATCAACAACAACCTGGGGATTCCGGCCCAAG  
 AGACTCAACTTCAAGCTCTTCAACATCCAGGTCAAAGAGGTCACGACGAATGAAGGCACCAAGACCATCGCC  
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 CACCAGGGTGCCCTGCCTCCGTTCCCGGGCGGACGTCTTTCATGATTCCCTCAGTACGGCTACCTGACTCTCAAC  
 AACGGTAGTCAGGCCGTGGGACGTTCCCTCTTCTACTGCCTGGAGTACTTCCCCTCTCAGATGCTGAGAACG  
 15 GGCAACAACTTTCAATTCAAGCTACTTTTCAGGACGTCGCTTTCCACAGCAGCTACGCGCACAGCCAGAGT  
 TTGGACAGGCTGATGAATCCTCTCATCGACCAGTACTGTACTACCTGTCAAGAACCAGACTACGGGAGGC  
 ACAGCGGGAACCCAGACGTTGCAGTTTTCTCAGGCCGGCCCTAGCAGCATGGCGAATCAGGCCAGAACTGG  
 GTGCCTGGACCTGCTACAGACAGCAGCGCGTCTCCACGACAACGAATCAAAACAACAACAGCAACTTTGCC  
 TGGACTGGTGCCACCAAGTATCATCTGAACGGCAGAGACTCTCTGATGAATCCGGGCGTCGCCATGGCAAGC  
 20 CACAAGGACGACGAGGACCGCTTCTCCCATCCAGCGGCGTCTCATATTTGGCAAGCAGGGAGCTGGAAAT  
 GACAACGTGGACTATAGCAACGTGATGATAACCAGCGAGGAAGAAATCAAGACCACCAACCCCGTGGCCACA  
 GAAGAGTATGGCGTGGTGGCTACTAACCACCACTCGGCAACACCCAGGCTCAAACGGGGACCGTCCAAAAAC  
 CAGGGAATCTTACCTGGCATGGTCTGGCAGAACCAGGACGTGTACCTGCAGGGTCTATTTGGGCCAAGATT  
 CCTCACACAGATGGCAACTTTACCCGTCCTCTTAAATGGGCGGCTTTGGACTTAAACATCCGCCTCCTCAG  
 25 ATCCTCATCAAAAACACTCCTGTTCTGCGGATCCTCCAACAACGTTCAACCAGGCCAAGCTGAATTTCTTTC  
 ATCAGCAGTACAGCACCGGACAAGTCAGCGTGGAGATCGAGTGGGAGCTGCAGAAGGAGAACAGCAAGCGC  
 TGGAACCCAGAGATTCAGTATACTTCCAACCTACTACAAATCTACAAATGTGGACTTTGCTGTTAATACTGAG  
 GGTGTTTACTCTGAGCCTCGCCCCATTGGCACTCGTTACCTCACCCGTAATCTGTAA

30 In addition to the polypeptides having the amino acid sequences shown in SEQ ID  
 NOs: 1 and 3, polypeptides are provided that have at least 95% sequence identity (e.g., at  
 least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity) to the  
 polypeptides having the amino acid sequences shown in SEQ ID NOs: 1 and 3.  
 Similarly, nucleic acid molecules are provided that have at least 95% sequence identity  
 35 (e.g., at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity) to  
 the nucleic acid molecules shown in SEQ ID NOs: 2 and 4.

In calculating percent sequence identity, two sequences are aligned and the  
 number of identical matches of nucleotides or amino acid residues between the two  
 sequences is determined. The number of identical matches is divided by the length of the  
 40 aligned region (i.e., the number of aligned nucleotides or amino acid residues) and  
 multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated  
 that the length of the aligned region can be a portion of one or both sequences up to the  
 full-length size of the shortest sequence. It also will be appreciated that a single sequence  
 can align with more than one other sequence and hence, can have different percent  
 45 sequence identity values over each aligned region.

The alignment of two or more sequences to determine percent sequence identity  
 can be performed using the algorithm described by Altschul et al. (1997, Nucleic Acids

Res., 25:3389 3402) as incorporated into BLAST (basic local alignment search tool) programs, available at ncbi.nlm.nih.gov on the World Wide Web. BLAST searches can be performed to determine percent sequence identity between a sequence (nucleic acid or amino acid) and any other sequence or portion thereof aligned using the Altschul et al. algorithm. BLASTN is the program used to align and compare the identity between nucleic acid sequences, while BLASTP is the program used to align and compare the identity between amino acid sequences. When utilizing BLAST programs to calculate the percent identity between a sequence and another sequence, the default parameters of the respective programs generally are used.

This disclosure also provides vectors containing nucleic acid molecules that encode polypeptides. Vectors, including expression vectors, are commercially available or can be produced by recombinant technology. A vector containing a nucleic acid molecule can have one or more elements for expression operably linked to such a nucleic acid molecule, and further can include sequences such as those encoding a selectable marker (e.g., an antibiotic resistance gene), and/or those that can be used in purification of a polypeptide (e.g., 6xHis tag). Elements for expression include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences. One example of an expression element is a promoter sequence. Expression elements also can include one or more of introns, enhancer sequences, response elements, or inducible elements that modulate expression of a nucleic acid molecule. Expression elements can be of bacterial, yeast, insect, mammalian, or viral origin and vectors can contain a combination of expression elements from different origins. As used herein, operably linked means that elements for expression are positioned in a vector relative to a coding sequence in such a way as to direct or regulate expression of the coding sequence.

A nucleic acid molecule, e.g., a nucleic acid molecule in a vector (e.g., an expression vector, such as a viral vector) can be introduced into a host cell. The term "host cell" refers not only to the particular cell(s) into which the nucleic acid molecule has been introduced, but also to the progeny or potential progeny of such a cell. Many suitable host cells are known to those skilled in the art; host cells can be prokaryotic cells (e.g., *E. coli*) or eukaryotic cells (e.g., yeast cells, insect cells, plant cells, mammalian cells). Representative host cells can include, without limitation, A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, 293 cells, Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and

myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster. Methods for introducing nucleic acid molecules into host cells are well known in the art and include, without limitation, calcium phosphate precipitation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer (e.g., transduction).

With respect to polypeptides, “purified” refers to a polypeptide (i.e., a peptide or a polypeptide) that has been separated or purified from cellular components that naturally accompany it. Typically, the polypeptide is considered “purified” when it is at least 70% (e.g., at least 75%, 80%, 85%, 90%, 95%, or 99%) by dry weight, free from the polypeptides and naturally occurring molecules with which it is naturally associated. Since a polypeptide that is chemically synthesized is, by nature, separated from the components that naturally accompany it, a synthetic polypeptide is considered “purified,” but further can be removed from the components used to synthesize the polypeptide (e.g., amino acid residues). With respect to nucleic acid molecules, “isolated” refers to a nucleic acid molecule that is separated from other nucleic acid molecules that are usually associated with it in the genome. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule.

Polypeptides can be obtained (e.g., purified) from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and/or hydroxyapatite chromatography. A purified polypeptide also can be obtained, for example, by expressing a nucleic acid molecule in an expression vector or by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. Similarly, nucleic acid molecules can be obtained (e.g., isolated) using routine methods such as, without limitation, recombinant nucleic acid technology (e.g., restriction enzyme digestion and ligation) or the polymerase chain reaction (PCR; see, for example, PCR Primer: A Laboratory Manual, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, 1995). In addition, isolated nucleic acid molecules can be chemically synthesized.

### ***Methods of Making Virus Particles Having Modified AAP-Dependence***

After the desired sequence of a virus or portion thereof has been determined (e.g., having a modified AAP-dependency), the actual nucleic acid molecule and/or polypeptide(s) can be generated, e.g., synthesized. Methods of generating an artificial  
5 nucleic acid molecule or polypeptide based on a sequence obtained, for example, *in silico*, are known in the art and include, for example, chemical synthesis or recombinant cloning. Additional methods for generating nucleic acid molecules or polypeptides are known in the art and are discussed in more detail below.

Once a polypeptide has been produced, or once a nucleic acid molecule has been  
10 generated and expressed to produce a polypeptide, the polypeptide can be assembled into a virus particle using, for example, a packaging host cell. The components of a virus particle (e.g., rep sequences, cap sequences, inverted terminal repeat (ITR) sequences) can be introduced, transiently or stably, into a packaging host cell using one or more vectors as described herein.

15 Virus particles can be purified using routine methods. As used herein, “purified” virus particles refer to virus particles that are removed from components in the mixture in which they were made such as, but not limited to, viral components (e.g., rep sequences, cap sequences), packaging host cells, and partially- or incompletely-assembled virus particles.

20 Once assembled, the virus particles can be screened for, e.g., the ability to replicate; gene transfer properties; receptor binding ability; and/or seroprevalence in a population (e.g., a human population). Determining whether a virus particle can replicate is routine in the art and typically includes infecting a host cell with an amount of virus particles and determining if the virus particles increase in number over time. Determining  
25 whether a virus particle is capable of performing gene transfer also is routine in the art and typically includes infecting host cells with virus particles containing a transgene (e.g., a detectable transgene such as a reporter gene, discussed in more detail below). Following infection and clearance of the virus, the host cells can be evaluated for the presence or absence of the transgene. Determining whether a virus particle binds to its  
30 receptor is routine in the art, and such methods can be performed *in vitro* or *in vivo*.

Determining the seroprevalence of a virus particle is routinely performed in the art and typically includes using an immunoassay to determine the prevalence of one or more antibodies in samples (e.g., blood samples) from a particular population of individuals.

Seroprevalence is understood in the art to refer to the proportion of subjects in a population that is seropositive (i.e., has been exposed to a particular pathogen or immunogen), and is calculated as the number of subjects in a population who produce an antibody against a particular pathogen or immunogen divided by the total number of individuals in the population examined. Immunoassays are well known in the art and include, without limitation, an immunodot, Western blot, enzyme immunoassays (EIA), enzyme-linked immunosorbent assay (ELISA), or radioimmunoassay (RIA). Simply by way of example, see Xu et al. (2007, *Am. J. Obstet. Gynecol.*, 196:43.e1-6); Paul et al. (1994, *J. Infect. Dis.*, 169:801-6); Sauerbrei et al. (2011, *Eurosurv.*, 16(44):3); Boutin et al. (2010, *Hum. Gene Ther.*, 21:704-12); Calcedo et al. (2009, *J. Infect. Dis.*, 199:381-90); and Sakhria et al. (2013, *PLoS Negl. Trop. Dis.*, 7:e2429), each of which determined seroprevalence for a particular antibody in a given population.

As described herein, virus particles can be neutralized by a person's, e.g., patient's, immune system. Several methods to determine the extent of neutralizing antibodies in a serum sample are available. For example, a neutralizing antibody assay measures the titer at which an experimental sample contains an antibody concentration that neutralizes infection by 50% or more as compared to a control sample without antibody. See, also, Fisher et al. (1997, *Nature Med.*, 3:306-12) and Manning et al. (1998, *Human Gene Ther.*, 9:477-85).

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#### ***Methods of Using Viruses or Portions Thereof Having Modified AAP-Dependence***

A virus or portion thereof that has a modified AAP-dependence as described herein can be used in a number of research and/or therapeutic applications. For example, a virus or portion thereof that has a modified AAP-dependence as described herein can be used in human or animal medicine for gene therapy (e.g., in a vector or vector system for gene transfer) or for vaccination (e.g., for antigen presentation). More specifically, a virus or portion thereof that has a modified AAP-dependence as described herein can be used for gene addition, gene augmentation, genetic delivery of a polypeptide therapeutic, genetic vaccination, gene silencing, genome editing, gene therapy, RNAi delivery, cDNA delivery, mRNA delivery, miRNA delivery, miRNA sponging, genetic immunization, optogenetic gene therapy, transgenesis, DNA vaccination, or DNA immunization.

30

A host cell can be transduced or infected with a virus or portion thereof having a modified AAP-dependence *in vitro* (e.g., growing in culture) or *in vivo* (e.g., in a subject).

Host cells that can be transduced or infected with a virus or portion thereof having a modified AAP-dependence *in vitro* are described herein; host cells that can be transduced or infected with an ancestral virus or portion thereof *in vivo* include, without limitation, brain, liver, muscle, lung, eye (e.g., retina, retinal pigment epithelium), kidney, heart, gonads (e.g., testes, uterus, ovaries), skin, nasal passages, digestive system, pancreas, islet cells, neurons, lymphocytes, ear (e.g., inner ear), hair follicles, and/or glands (e.g., thyroid).

A virus or portion thereof having a modified AAP-dependence as described herein can be modified to include a transgene (in *cis* or *trans* with other viral sequences). A transgene can be, for example, a reporter gene (e.g., beta-lactamase, beta-galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent polypeptide (GFP), chloramphenicol acetyltransferase (CAT), or luciferase, or fusion polypeptides that include an antigen tag domain such as hemagglutinin or Myc) or a therapeutic gene (e.g., genes encoding hormones or receptors thereof, growth factors or receptors thereof, differentiation factors or receptors thereof, immune system regulators (e.g., cytokines and interleukins) or receptors thereof, enzymes, RNAs (e.g., inhibitory RNAs or catalytic RNAs), or target antigens (e.g., oncogenic antigens, autoimmune antigens)).

The particular transgene will depend, at least in part, on the particular disease or deficiency being treated. Simply by way of example, gene transfer or gene therapy can be applied to the treatment of hemophilia, retinitis pigmentosa, cystic fibrosis, leber congenital amaurosis, lysosomal storage disorders, inborn errors of metabolism (e.g., inborn errors of amino acid metabolism including phenylketonuria, inborn errors of organic acid metabolism including propionic academia, inborn errors of fatty acid metabolism including medium-chain acyl-CoA dehydrogenase deficiency (MCAD)), cancer, achromatopsia, cone-rod dystrophies, macular degenerations (e.g., age-related macular degeneration), lipopoly peptide lipase deficiency, familial hypercholesterolemia, spinal muscular atrophy, Duchenne's muscular dystrophy, Alzheimer's disease, Parkinson's disease, obesity, inflammatory bowel disorder, diabetes, congestive heart failure, hypercholesterolemia, hearing loss, coronary heart disease, familial renal amyloidosis, Marfan's syndrome, fatal familial insomnia, Creutzfeldt-Jakob disease, sickle-cell disease, Huntington's disease, fronto-temporal lobar degeneration, Usher syndrome, lactose intolerance, lipid storage disorders (e.g., Niemann-Pick disease, type C), Batten disease, choroideremia, glycogen storage disease type II (Pompe disease),

ataxia telangiectasia (Louis-Bar syndrome), congenital hypothyroidism, severe combined immunodeficiency (SCID), and/or amyotrophic lateral sclerosis (ALS).

A transgene also can be, for example, an immunogen that is useful for immunizing a subject (e.g., a human, an animal (e.g., a companion animal, a farm animal, an endangered animal). For example, immunogens can be obtained from an organism (e.g., a pathogenic organism) or an immunogenic portion or component thereof (e.g., a toxin polypeptide or a by-product thereof). By way of example, pathogenic organisms from which immunogenic polypeptides can be obtained include viruses (e.g., picornavirus, enteroviruses, orthomyxovirus, reovirus, retrovirus), prokaryotes (e.g., *Pneumococci*, *Staphylococci*, *Listeria*, *Pseudomonas*), and eukaryotes (e.g., amebiasis, malaria, leishmaniasis, nematodes). It would be understood that the methods described herein and compositions produced by such methods are not to be limited by any particular transgene.

A virus or portion thereof having a modified AAP-dependence, usually suspended in a physiologically compatible carrier, can be administered to a subject (e.g., a human or non-human mammal). Suitable carriers include saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline), lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, and water. The virus or portion thereof having a modified AAP-dependence is administered in sufficient amounts to transduce or infect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to an organ such as, for example, the liver or lung, orally, intranasally, intratracheally, by inhalation, intravenously, intramuscularly, intraocularly, subcutaneously, intradermally, transmucosally, or by other routes of administration. Routes of administration can be combined, if desired.

The dose of the virus or portion thereof having a modified AAP-dependence that is administered to a subject will depend primarily on factors such as the condition being treated, and the age, weight, and health of the subject. For example, a therapeutically effective dosage of a virus or portion thereof having a modified AAP-dependence that is to be administered to a human subject generally is in the range of from about 0.1 ml to about 10 ml of a solution containing concentrations of from about  $1 \times 10^1$  to  $1 \times 10^{12}$  genome copies (GCs) of viruses (e.g., about  $1 \times 10^3$  to  $1 \times 10^9$  GCs). Transduction and/or

expression of a transgene can be monitored at various time points following administration by DNA, RNA, or protein assays. In some instances, the levels of expression of the transgene can be monitored to determine the frequency and/or amount of dosage. Dosage regimens similar to those described for therapeutic purposes also may be utilized for immunization.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.

## EXAMPLES

### Example 1—Vectors and Sequences

Adeno-associated viral vectors were pseudotyped with either extant or ancestral viral capsids. Extant capsids include AAV1 (Genbank [GB] AAD27757.1), AAV2 (GB AAC03780.1), AAV3 (GB U48704.1), AAV4 (GB U89790.1), AAV5 (GB AAD13756.1), AAV6 (GB AF028704.1), AAV7 (NC\_006260.1) Rh.10 (gb AAO88201.1), AAV8 (GB AAN03857.1), AAV9 (GB AAS99264.1), and Rh32.33 (GB EU368926). Ancestral AAV capsids include Anc80L65, Anc81, Anc82, Anc83, Anc84, Anc110, Anc113, Anc126, and Anc127 (KT235804-KT235812). In this study, Anc83 has the following mutation in the presumed AAP ORF: Q1L (83AAP-KI).

### Example 2—Site Directed Mutagenesis

AAPstop60, AAPstop20, and 82DI single revertant mutations were generated using the QuikChange® II Site-Directed Mutagenesis Kit according to the manufacturer's instructions. To generate 82DI, the QuikChange® Lightning Multi Site-Directed Mutagenesis Kit was used according to the manufacturer's instructions, in two phases: first, five sites were mutated on an Anc82 backbone, then the remaining five mutations were introduced into this quintuple mutant backbone.

### Example 3—Crude Virus Preparations / Titration

Virus preparations to assay production in all serotypes and mutants were prepared as follows: Polyethylenimine transfections of AAV *cis* ITR-CMV-EGFP-T2A-Luc-ITR

(2 $\mu$ g), AAV *trans rep-cap* (2 $\mu$ g), and adenovirus helper plasmid (4 $\mu$ g) were performed on HEK 293 cells at 90% confluency in 6-well dishes. PEI Max (Polysciences)/DNA ratio was maintained at 1.375:1 (w/w) in serum-free media. Virus was harvested after 72 h by three freeze/thaw cycles followed by centrifugation at 15000 x g.

5 For DRP titers, crude preps were DNaseI treated, and resistant (packaged) vector genome copies were used to titrate preps by TaqMan qPCR amplification (Applied Biosystems 7500, Life Technologies) with primers and probes detecting CMV promoter regions of the transgene cassette.

#### 10 Example 4—Thermostability Assay (AAV-ID)

Thermostability of purified vector was assayed by AAV-ID (Pacouret et al., 2017, Mol. Ther., 25:1375-86). Briefly, A 500 uL sample of SYPRO® Orange 50X was prepared using PBS<sup>2+</sup> (21-030-CV, Corning Inc., Corning, NY) as a solvent. 96-well plates were loaded with 45 uL samples, supplemented with 5 uL Sypro Orange 50X.  
15 PBS<sup>2+</sup> and 0.25 mg/mL Lysozyme (L6876, SIGMA-ALDRICH, St. Louis, MO, USA) solutions were used as negative and positive controls, respectively. Plates were sealed and centrifuged at 3000 rpm for 2 min, and subsequently loaded into a 7500 Real-Time PCR System (ThermoFisher SCIENTIFIC). Samples were incubated at 25°C for 2 min prior to undergo a temperature gradient (25 to 99°C, ~2°C/10min, step and  
20 hold mode with 0.4°C temperature increments), while monitoring the fluorescence of the SYPRO® Orange dye using the ROX filter cube available on both qPCR systems. Fluorescence signals F were normalized between 0 and 100% and melting temperatures were defined as the temperature for which the numerical derivative dF/dT reached its maximum.

25

#### Example 5—Enzyme-Linked Immunosorbent Assays

A20 capsid ELISAs were performed on crude virus preparations with the PROGEN AAV 2 Titration ELISA kit (ref# PRATV), according to the manufacturer's instructions.

30 hA1AT ELISAs were performed on 1:1250-1:10000 serial dilutions of mouse serum with the Cloud-Clone ELISA kit for  $\alpha$ -1 antitrypsin (SEB697Hu, 96 tests) according to the manufacturer's instructions.

### Example 6—Animal Studies

C57BL/6 male mice (6-8 weeks) were purchased from Jackson Laboratories. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Schepens Eye Research  
5 Institute.

Mice were anesthetized with Ketamine/Xylazine intraperitoneally. Each animal was injected retro-orbitally (100  $\mu$ l) with 1.00E+11 VG/mouse of the following vectors: Anc82.CB7.CI.EGFP.FF2A.hA1AT.RBG and  
10 Anc82DI.CB7.CI.EGFP.FF2A.hA1AT.RBG. Blood was collected via submandibular bleeds using GoldenRod animal lancets (MEDipoint, Inc.) prior injection, and 3, 7, 15 and 28 days post injection. Samples were centrifuged at 8,000 rpm for 7.5 minutes and the serum was collected.

Animals were euthanized, and livers were collected and submerged in 4% paraformaldehyde solution (Electron Microscopy Sciences) for 30 minutes, then placed in  
15 30% sucrose overnight. The next day the liver was mounted in Tissue-Tek O.C.T. Compound (Sakura Finetek) and flash frozen in cool isopentane.

### Example 7—Tissue Histology

To visualize eGFP expression in liver, 15  $\mu$ m sections were mounted with  
20 VECTASHIELD® Hard Set™ mounting medium with DAPI (H-1500) and imaged with a Zeiss Axio Imager M2, at same gain and intensity across all sections.

### Example 8—Molecular Representations

All molecular representations in this study were generated using PyMOL and  
25 Protein Data Bank files 2QA0 (AAV8) and 3UX1 (AAV9).

### Example 9—Production and Purification of AAV3, AAV3s, AAV9 and AAV9s

Vectors were purified by affinity chromatography using either AVB Sepharose HP (25-4112-11, GE Healthcare) (AAV3 and AAV3s) or POROS CaptureSelect AAV9  
30 affinity resin (Thermo Fisher) per the manufacturer instructions (AAV9 and AAV9s).

Large scale crude preps were treated with benzonase (250 U/mL, 1 h, 37°C) before the centrifugation step (1 h, 10,000 rpm, 20°C), then filtered using a 0.2  $\mu$ m Nalgene Rapid-Flow filter. Vectors were purified by affinity chromatography using

either HiTrap columns prepacked with 1 mL AVB Sepharose HP (25-4112-11, GE Healthcare) (AAV3 and AAV3s) or a 5 x 125 mm Econoline column (TAC05/125PE0-AB-3, essentialLife Solutions) packed with 1 mL POROS CaptureSelect AAV9 affinity resin (Thermo Fisher) per the manufacturer instructions (AAV9 and AAV9s). Columns were sanitized with 5 column volumes (CV) 0.1 M H<sub>3</sub>PO<sub>4</sub>, 1 M NaCl, pH 2 (1 mL/min) and equilibrated with 5 CV PBS (21-030-CV, Corning) (1 mL/min). Clarified lysates were injected at 1 mL/min. Columns were further washed with 10 CV PBS (1 mL/min). Vector particles were eluted in 3 mL 0.1 M NaOAc, 0.5 M NaCl, pH 2.5 (1 mL/min) and immediately neutralized with 400 µL of 1 M Tris-HCl, pH 10. Samples were further buffer-exchanged in PBS and concentrated by Amicon filtration (UFC910024, EMD Millipore) per the manufacturer instructions. Sample purity was assessed by SDS-PAGE, whereas DNase I-resistant vector genomes were quantified by quantitative polymerase chain reaction (qPCR) using the TaqMan (Life Technologies) system with primers and probes targeting SV40 or eGFP.

15

#### Example 10—Statistical Methods

All data were analyzed using R prior to normalization for reporting in the figures (unless otherwise indicated). P-values are reported in Table 2. Viral titers were compared using a paired, one-tailed Student's t test and RNA levels were compared using a paired, two-tailed Student's t test.

20

Table 2 shows the statistical analysis related to FIGs. 1, 2, 3, and 4. All statistical analysis was performed in R, on data prior to normalization for reporting in the main figures indicated at left of table. Panel A compares WT and AAPstop60 viral titers measured by qPCR after background subtraction (no cap gene control). P-values resulting from a paired, one-tailed t-test. ND="not determined"; a t-test could not be performed on serotypes with one or more trials within 3 standard deviations of measured background. Panel B compares AAPstop60 or rescue to WT levels of RNA (all normalized to GAPDH). P-values resulting from a paired, two-tailed t-test.

25

#### Example 11—Expression Constructs

30

AAP-HA: Complimentary oligonucleotides encoding the Hemagglutinin (HA) tag with BsiWI overhangs (5' GTAC, 3' CATG) were annealed in T4 Ligase Buffer ramping

from 95°C to 25°C at 5°/min, PNK treated, and ligated into BsiWI digested and CIP treated AAV1 and AAV3 wt and AAPstop60 *rep-cap* plasmids.

CMV-HA-VP1 and CMV-VP3: gBlocks® Gene Fragments (IDT) of bp #4 - 696 of VP1 for AAV2, AAV3, Anc82, and 82DI were obtained, with the following  
5 modifications: an EcoRI site, start codon, and HA sequence added to 5' end, ACG to ACC mutation of VP2 start codon, and ATG to CTG mutation of VP3 start codon. The gBlocks® include a BsrDI restriction site conserved in *cap*; gBlocks were digested with EcoRI and BsrDI. VP3 sequences were PCR amplified from the appropriate AAPstop60  
10 *rep-cap* plasmids with primers incorporating 5' EcoRI and 3' HindIII restriction sites, then digested with either EcoRI and HindIII (for CMV-VP3) or BsrDI and HindIII (For CMV-HA-VP1). Fragments were ligated into pCDNA3.1(-) in the appropriate combinations. For CMV-AAP2, AAP was amplified from AAV2 *rep-cap* plasmid and ligated into pCDNA3.1(-).

#### 15 Example 12—Protein Lysate Preparation and Degradation / Turnover Studies

Transfections were performed as in Crude Virus Preparation. At 36 h, supernatant was aspirated and cells lysed on plate with 100 µL (FIG. 2) or 150 µL (FIGs. 3 and 4) lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH8, plus cOmplete Mini™ protease inhibitor). Lysate was clarified by centrifugation at 15,000 x g, diluted 1:50 in  
20 lysis buffer for actin or 1:100 for tubulin loading control blots, and denatured in 4x NuPAGE® LDS sample buffer +0.5% βME at 90°C. 100 µg (FIG. 2) or 50 µg (FIGs. 3 and 4) total protein (or dilution thereof for loading control blot) per well were loaded and electrophoresed on NuPAGE® 4-12% Bis-Tris gels. For proteasome and lysosome inhibition experiments, media was removed 24 h after transfection and replaced with  
25 media containing the appropriate concentration of Bortezomib (Selleckchem PS-341), MLN7243 (Chemgood C-1233), Bafilomycin (Enzo BML-CM110-0100), or DMSO (for wt and AAPstop untreated samples) and incubated an additional 8 h. 25 µg total protein were loaded per well (diluted 1:10 for loading control). For protein turnover experiments by blocking protein synthesis, media was removed 24 h after transfection and replaced  
30 with media containing 50 µg/mL CHX (Sigma C7698) and lysates were harvested as described above at 1, 2, 4, 6, and 8 h time points, and for the 0 h time point media was not replaced but lysates were harvested. 10% FBS was maintained throughout all transfections and drug incubations described here.

#### Example 13—Western / Dot Blotting

Electrophoresed proteins were transferred to PVDF membranes, incubated with primary antibody (B1, 1:250, ARP#03-65158; Actin, 1:20000, Abcam 8227; Tubulin, 5 1:20000, Abcam 7291; HA, 1:5000, Abcam 9110; p62, 1:1000, Cell Signalling 5114) overnight, and detected with Anti-mouse (GE Healthcare LNXA931/AE) or Anti-rabbit (Sigma A0545) HRP conjugated secondary antibody and Thermo Super Signal® West Pico or Femto.

For dot blots, protein lysates were diluted 1:100 and 2 µL was spotted onto 10 nitrocellulose membranes, allowed to dry, blocked in 5% milk, and incubated with ADK8 (ARP#03-651160) overnight.

#### Example 14—Immunoprecipitations

PEI transfections were performed with 10 µg each of CMV-HA-VP1, CMV-VP3, 15 and CMV-AAP2 plasmid of the appropriate serotype (CMV-AAP2 added only where indicated) on 10 cm dishes of HEK 293 cells at ~80% confluency. PEI Max (Polysciences)/DNA ratio was maintained at 1.375:1 (w/w) in serum-free media. At 24h post transfection, cells were pelleted and resuspended in 1 mL lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH8, plus cOmplete Mini™ protease inhibitor). 20 Immunoprecipitation was performed with rb Anti-HA antibody (Abcam 9110) and Pierce Protein A/G Plus Agarose beads. Precipitated proteins were eluted in 4x NuPAGE® LDS sample buffer +0.5% βME at 90°C for 10 minutes. 10 µL (IP) or 30 µL (input) were loaded and electrophoresed on NuPAGE® 4-12% Bis-Tris gels and detected in Western Blotting above. For detection of full virions (FIG. 6C), ITR-CMV-EGFP-T2A-Luc-ITR 25 and pRep plasmids were added to the above transfection with AAV2. To avoid heat-denaturation of AAV2 capsids, complexes were instead eluted with 0.2 M glycine, pH 2.8, and eluates neutralized with equal volume Tris pH 8.5. Purified AAV2 preps were treated in parallel with elution and neutralization buffers to ensure these conditions did not denature the capsids. Control, IP, input, and supernatant fractions were DNaseI 30 treated, and resistant (packaged) vector genome copies were quantified by TaqMan qPCR amplification (Applied Biosystems 7500, Life Technologies) with primers and probes detecting CMV promoter regions of the transgene cassette.

#### Example 15—Crosslinking

PEI transfections were performed with 2 µg each of CMV-HA-VP1, CMV-VP3, and CMV-AAP2 plasmid of the appropriate serotype (CMV-AAP2 added only where indicated) on 6-well plates of HEK 293 cells at ~80% confluency. PEI Max (Polysciences)/DNA ratio was maintained at 1.375:1 (w/w) in serum-free media. After 36 h, on-plate lysis was performed with M-PER™ (Thermo) buffer supplemented with cComplete™ mini protease inhibitor. Lysate was divided into three and treated with 5 mM final concentration of disuccinimidyl glutarate (DSG, Thermo), disuccinimidyl suberate (DSS, Thermo), or an equal volume of DMSO as a mock treatment. Reactions were incubated on ice for 1 h, mixing periodically, then quenched with 1 M Tris. NuPAGE® LDS Sample Buffer + 2-Mercaptoethanol were added and samples boiled for 10 minutes, loaded onto SDS-PAGE gel, and interrogated by Western Blot with B1 antibody.

#### Example 16—RNA Quantification

Transfections were performed as described in Crude Virus Preparations / Titration. RNA was harvested after 36 h with Qiagen RNeasy mini ® kit according to the manufacturer's instructions. TURBO DNA-free™ kit (Invitrogen) was used according to manufacturer's instructions (Rigorous DNase treatment protocol) to eliminate contaminating DNA from samples. cDNA synthesis was performed with iScript™ kit (BioRad) using 250 ng total RNA from each sample. Reactions were then diluted 10-fold, and 5 µL of diluted cDNA used in each qPCR reaction, prepared with PowerUp™ SYBR™Green Master Mix (Applied Biosystems) and intron, intron-spanning, or GAPDH primers to detect unspliced, spliced, or housekeeping gene products.

25

#### Example 17—Transmission Electron Microscopy

For negative staining on chromatography-purified AAV3 and AAV3stop, vector samples (5 µL or 50 µL drop) and a blank buffer control were adsorbed onto 200 mesh carbon and formvar coated nickel grids, rinsed, and stained with 2% aqueous uranyl acetate for 30 seconds then absorbed off on filter paper and air dried. All grids were imaged using a FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, Oregon) at 100 kV accelerating voltage, interfaced with an AMT XR41 digital CCD camera (Advanced Microscopy Techniques, Woburn, Massachusetts) for digital TIFF file

image acquisition. TEM imaging of AAV samples were assessed and digital images captured at 2k x 2k pixel, 16-bit resolution.

Example 18—*In Vitro* Transduction

5           The appropriate serotype and GC particles of AAV-CMV-EGFP-T2A-Luc was added to HEK-293 cells on a 96-well plate pre-infected with human adenovirus 5 (hAd5) 24 h prior at a multiplicity of infection of 20 particles/cell. The cells were imaged with an EVOS® FL Imaging System at 24 and 48 h, after which D-luciferin containing buffer was added and luminescence was measured using Synergy H1 microplate reader (BioTek; 10 Winooski, VT).

Example 19—Tables

**Table 1**

		AAV2		AAV3	
		WT	AAPstop60	WT	AAPstop60
Trial 1	GC/mL: qPCR	1.34E+10	2.64E+07	1.72E+10	2.89E+09
	particles/mL: ELISA	2.48E+11	2.95E+08	6.14E+11	1.37E+11
	%WT: qPCR	100	0.197	100	16.813
	ELISA	100	0.119	100	22.298
Trial 2	GC/mL: qPCR	8.25E+10	2.09E+08	1.76E+11	3.88E+10
	particles/mL: ELISA	6.78E+12	1.29E+09	2.23E+13	3.83E+12
	%WT: qPCR	100	0.253	100	22.045
	ELISA	100	0.019	100	16.278
Average %wt:		AAV2		AAV3	
qPCR	100	0.225	100	19.43	
ELISA	100	0.069	100	19.29	

15

**Table 2**

**A**

Figure 1C	Serotype	rh32.33	AAV3	AAV3	AAV5	AAV1	AAV4	rh8	AAV6	AAV2	AAV7	rh10	AAV8
	p-value	0.013	0.038	0.033	0.090	0.075	0.114	0.004	ND	ND	0.002	0.017	ND

Figure 3A	Serotype	Anc113	Anc110	Anc126	Anc127	Anc81	Anc84	Anc80	Anc83	Anc85
	p-value	0.918	0.603	0.078	0.046	ND	ND	ND	ND	ND

Figure 4B	Serotype	Anc82	Anc82	82B1
	Anc82	-	ND	0.03
	82B1	0.94	ND	0.028

**B**

Figure 2C		minor	major	unspliced
	WT/AAPstop60	0.581	0.298	0.075
	WT/average	0.324	0.188	0.113

**Table 3**

		% WT titers		
		AAPstop60	AAPstop20	AAPstop20 +AAP
Trial 1	Anc113	126.757	-0.030	61.254
	rh32.33	52.415	96.577	114.124
	AAV3	27.085	0.050	61.983
	Anc110	20.791	-0.037	34.955
	AAV9	23.145	-0.013	28.922
	AAV5	22.512	6.297	83.410
	AAV4	5.429	5.662	54.170
Trial 2	Anc113	253.371	0.359	46.539
	rh32.33	39.059	89.731	105.907
	AAV3	28.319	0.840	78.249
	Anc110	23.771	0.281	39.816
	AAV9	8.769	-0.010	26.927
	AAV5	14.108	6.912	79.285
	AAV4	4.204	5.080	5.109
Average	Anc113	190.064	0.165	53.897
	rh32.33	45.737	93.154	110.016
	AAV3	27.702	0.445	70.116
	Anc110	22.281	0.122	37.386
	AAV9	15.957	-0.012	27.924
	AAV5	18.310	6.605	81.348
	AAV4	4.816	5.371	29.639

Table 4

		% DI WT titer					
		Trial 1		Trial 2		Average	
site		WT	AAPstop	WT	AAPstop	WT	AAPstop
	82DI	100.000	12.047	100.000	16.941	100.000	14.494
1	S163K	140.290	0.158	91.977	-0.008	116.134	0.075
2	S206A	128.656	-0.013	56.219	-0.009	92.438	0.011
3	R478K	154.220	-0.035	76.595	-0.008	115.408	-0.022
4	V481L	95.214	0.033	56.655	-0.009	75.935	0.012
5	M520V	7.970	0.017	0.382	-0.010	4.176	0.003
6	S528T	123.339	-0.064	83.723	-0.019	103.531	0.042
7	H586L	44.444	-0.035	18.062	-0.008	31.253	-0.021
8	QA592AP	157.428	-0.024	122.904	-0.013	140.166	-0.019
9	QN599NS	120.802	0.029	83.228	-0.019	102.015	0.005
10	I603A	110.992	0.019	90.107	-0.024	100.549	-0.003

#### 5 Example 20—Requirement for AAP Ranges Broadly Across all AAV Clades

To test whether AAP is required to assemble capsids from the full complement of VP proteins (i.e., VP1, 2, and 3), AAP expression was abolished from *rep-cap* trans plasmids by an early stop codon in the AAP reading frame, a silent mutation in VP (FIG. 1A, AAPstop60). AAPstop60s were generated for 12 serotypes, including at least one member of each AAV clade, with the aim of a comprehensive assessment of AAP requirement across mammalian AAV serotypes. Considering AAP's non-canonical CTG start codon, AAPstop60 mutations were positioned such that they would be sufficiently downstream of potential alternate start codons, yet upstream of regions shown to be essential for AAP2 function (Naumer et al., 2012, J. Virol., 86:13038-48). To verify loss of AAP protein, a Hemagglutinin tag was inserted in the C-terminal region of the AAP ORF in two representative serotypes (FIG. 1A, AAP-HA). Whole cell lysates transfected with these constructs were analyzed by Western Blot (FIG. 1B), confirming that AAPstop60 results in loss of full-length AAP or any shorter protein product translated from alternate starts. A double band in the AAP-HA lane supports the likelihood of additional downstream start codons and corroborates the late placement of the stop codons (FIG. 1B).

Recombinant AAV vectors were produced from AAPstop60 and wildtype AAP (WT) plasmids, and titrated by qPCR quantifying DNase resistant particles (DRP).

AAPstop60 vector titers reveal that when all three VP proteins are present, AAP is not strictly required to assemble the virion in several serotypes (FIG. 1C). Rather, AAP requirement ranges broadly across serotypes, with AAPstop60 vectors producing as high as 39% of WT titer for rh32.33, and as low as 0.035% WT titer for AAV8. This observation is in contrast with previous findings demonstrating AAP's absolute requirement for assembling VP3-only capsids, in particular AAV9 and AAV1 (Sonntag et al., 2011, J. Virol., 85:12686-97).

The advantage of DRP titration is the ability to quantify virus of any capsid serotype with the same vector genome absent of differential bias in measurement. However, DRP measures the amount of assembled particles that also underwent viral genome packaging, a process that occurs downstream of capsid assembly. Moreover, DRP does not assess for non-packaged, empty AAV virions. To directly assay capsid assembly and rule out the possibility that serotypes with low AAPstop60 titers were due to a packaging defect, an A20 capsid ELISA was performed, which recognizes a conformational epitope only present in assembled AAV2 capsids. A20 cross-reacts with AAV3, allowing us to assay assembly directly for an AAV that requires AAP (AAV2) and one that accomplishes assembly in the AAPstop60 context (AAV3). A20 ELISA data for both serotypes corroborated the DRP indirect measure of assembly (FIG. 1D).

#### 20 Example 21—A Role for AAP in VP Protein Stability

To ensure that the observed range of AAP dependence for assembly was not due to variation in VP translation efficiencies imposed by alternate codon usage in the AAPstop60 mutants, VP protein levels produced by WT and AAPstop60 constructs were interrogated. The B1 monoclonal antibody detects a conserved linear epitope on VP proteins in denaturing conditions across all AAVs tested in this study except AAV4 and rh32.33, allowing nearly all serotypes to be assayed. No appreciable difference in VP2 or VP3 protein levels, and a slight decrease in VP1 levels, was observed for AAPstop60s that produce 10% or higher of their respective WT titers (FIG. 2A), whereas AAPstop60s with titers below this threshold show a dramatic decrease in VP protein levels (FIG. 2B). To examine whether the observed decreases in VP levels were due to a potential translational defect, AAP2 was co-expressed *in trans* with AAPstop60 (rescue; FIG. 2B). Appreciable restoration of VP protein was observed for all affected serotypes. Furthermore, no difference was observed between WT, AAPstop60, or rescue transcript

levels (FIG. 2C), indicating that VP protein loss in the absence of AAP most likely occurs post- (or co-) translationally.

To interrogate degradation as the mechanism for instability, AAV8 AAPstop60 transfected cells were treated with increasing concentrations of the proteasome inhibitor Bortezomib, the E1 inhibitor MLN7243, or the vacuolar specific H<sup>+</sup> ATPase inhibitor Bafilomycin (FIG. 2D). This allowed the examination of the earliest and latest steps of the Ubiquitin-Proteasome Pathway, as well as late steps of lysosomal degradation or autophagy by inhibiting the required acidification. Inhibiting lysosomal acidification resulted in a mild yet dose-dependent rescue of AAV8 VP3 protein. Proteasomal inhibition is accompanied by a robust rescue in AAV8 VP proteins in a dose dependent manner, but this was not concomitant with rescue of assembled capsids (FIG. 2E). E1 inhibition provided an equally mild to moderate VP rescue independent of drug concentration. Collectively, these results suggest that instability of VP proteins in the absence of AAP can primarily be attributed to proteasomal degradation, and that this may in part be Ubiquitin-independent. Lysosomal or autophagosomal degradation may also degrade a proportion of VP proteins.

In an attempt to examine the rate of AAV8 VP degradation, protein synthesis was blocked with Cycloheximide (CHX) and protein lysates were harvested at progressive time points (FIG. 8A). As expected in AAPstop60 lysates, VP protein levels were too low to detect even without CHX treatment and despite long exposure times with highly sensitive detection reagents. However, in the presence of AAP, VP protein levels remain consistent over all time points of CHX treatment. This is likely because capsids are assembled rapidly in the presence of AAP, and because assembled VP proteins are not susceptible to degradation, a VP band persists.

Given the spectrum of AAP phenotypes observed across the major clades, 9 putative evolutionary intermediates (AncAAVs) to the major AAV serotypes also were tested in order to gain insight into what elements of VP structure either impose the observed requirement for AAP or impart an ability for some VPs to perform these functions independently (Zinn et al., 2015, Cell Rep., 12:1056-68). As with the natural serotypes, a broad range of requirement for AAP was observed for the AAPstop60 AncAAVs (FIG. 3A).

Although the AAPstop60 early stop codon is placed upstream of domains shown to be required for AAP2 function, it is downstream of a highly conserved region (residues

52-57) in AAP (FIG. 9, conserved core). This domain was shown by deletion analyses to be important for AAP2 function, but not sufficient to assemble VP3 only capsids without more C-terminal portions of AAP present (Naumer et al., 2012, *J. Virol.*, 86:13038-48). Because AAPs in other serotypes have not yet been tested by deletion analyses, and  
5 because algorithms that generated the AncAAVs were applied only to the VP ORF and may have unpredictable consequences on AAP (Zinn et al., 2015, *Cell Rep.*, 12:1056-68), we wanted to examine whether a partially functional, N-terminal AAP (AAPN) was expressed from some AAPstop60 constructs, contributing to the observed varying requirement for AAP across the 21 AAVs that were examined. For the 6 AAVs whose  
10 AAPstop60 produces at least 10% of WT titer and for AAV4, recently demonstrated to assemble VP3-only capsids without AAP (Earley et al., 2017, *J. Virol.*, 91:e01980-16), further upstream stop constructs (AAPstop20) were generated, placing the early stop codon at residue ~23 in the AAP ORF (silent mutations in VP). Of these, AAV5, rh32.33, and AAV4 AAPstop20 produce virus, while AAV3, AAV9, Anc110, and  
15 Anc113 do not (FIG. 3B). Although the B1 antibody does not detect AAV4 and rh32.33, levels of VP protein produced from the remaining AAPstop20 constructs mirror the titer (FIG. 3C). Taken together with FIG. 2, these results demonstrate that stability is a serotype-specific property of VP proteins that fall into one of three categories: (i) independently stable, (ii) require only AAPN for stability, or (iii) requiring full-length  
20 AAP. These results clearly illustrate a role for AAP in VP stability and provide an explanation for the broad range of requirement for AAP. The discrepancy between AAPstop60 and WT titers, particularly for serotypes requiring only AAPN, points to additional shortcomings in some serotypes' VPs for which AAP compensates, and potentially discrete functions contained primarily in AAPN versus the C-terminal two-thirds of AAP (AAPC). Taking into consideration VP stability, AAPstop60 titers, and  
25 AAPstop20 titers, for clarity, the AAP phenotypes were categorized as (i) AAP-independent, (ii) AAPC-independent, and (iii) AAP-dependent (FIG. 3D). Additionally, it was shown that the AAPC-independent phenotype is a property of the VP proteins and not a result of a fully functional AAPN by demonstrating that AAPN of an AAPC-independent serotype cannot rescue viral production of an AAP-dependent serotype (FIG.  
30 10).

To examine whether serotypes with different AAP phenotypes' VPs are subject to the same mechanisms of degradation, degradation of AAV3 proteins were blocked in the

same manner as previously performed for AAV8 (FIG. 8B). Proteasome inhibition with Bortezomib provided a dose-responsive, robust rescue as with AAV8, and E1 inhibition with MLN7243 rescued VP at the highest dose. Unlike AAV8, Inhibiting Lysosomal acidification with Bafilomycin robustly rescued AAV3 VP levels in a dose dependent fashion, indicating that AAPC-independent serotypes (or at least AAV3) may be more susceptible to Lysosomal degradation or autophagy. These results could also suggest that AAPN somehow promotes the proteasome as the primary means of degradation, whether by blocking lysosomal degradation or by other means, because AAPN is present in the AAV8 AAPstop60 lysates but is absent in the AAV3 AAPstop20 lysates.

10

#### Example 22—AAPC Does Not Impact Virion Morphology, Infectivity, or Stability

While AAPN alone facilitates the production of appreciable quantities of virus for many serotypes, whether AAPN-assembled particles retain the proper morphology as well as infectivity functions was next addressed. TEM imaging of AAV3 WT and AAV3AAPstop60 vectors indicate identical gross particle morphologies (FIG. 11A). To examine whether AAPC loss affected infection capabilities, AAV9, AAV3, and their AAPstop60 vectors were tested on HEK293 cells in culture (FIG. 11B, C), demonstrating that virus assembled without AAPC retain infection capabilities. Additionally, the melting temperature of these particles were tested, and no appreciable difference was observed (FIG. 11D).

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#### Example 23—Requirement for AAP Exhibits Branch Specificity in the Context of a Putative AAV Phylogeny

As a next step toward identifying VP structure responsible for assembly functions, an overview of how AAP phenotypes diverge across the wide genetic range of AAV capsids tested was sought, aiming to identify phenotypic differences across small genetic distances. AAP phenotypes of the 12 natural serotypes and the nine ancestral variants were correlated to the reconstructed phylogeny (FIG. 3E). This revealed branch-specific AAP dependence profiles, with phylogenetic nodes illustrating clear divergence in AAP phenotype. Among other apparent trends, Anc80, Anc81, Anc82, Anc83, and Anc84 comprise a fully dependent lineage that terminates in AAV8 and rh.10, and will thus be referred to as Branch D (FIG. 3E, red arrow). At the Anc82 node, a phenotypic switch from AAP-dependent to AAPC-independent is observed in its successor Anc110. The

25  
30

serotypes that diverge from Anc110, rh8 and AAV9, are also AAPC-independent; this branch was named Branch I (FIG. 3E, green arrow).

Example 24—Phenotype-to-Phylogeny Mapping Analysis Reveals a Set of Residues Functioning in AAPC-Independent Assembly

5 The observation that AAP phenotypes have branch-specific trends within the phylogeny presented the opportunity for a facile method to identify elements of VP structure critical for assembly functions. Given that VP sequence diverges by small increments along each of these branches, it was hypothesized that a set of residues  
10 homologous only within the members of their respective branch were likely to functionally contribute to capsid assembly. To this end, a multiple sequence alignment was generated with the Branch D and Branch I members. Within the alignment, a total number of 149 positions varied, however, at only twelve positions, the residue is conserved within Branch I, with a different yet shared identity on Branch D. Of these  
15 twelve, eight individual residues and two pairs of adjacent residues comprise 10 sites on VP. At some of these sites, residue identity diverges within Branch I members; however, they share a chemical property that contrasts with Branch D. For example, site 1 is a basic lysine in Branch D serotypes, compared to a threonine in Branch I for Anc110 and rh8, and a serine in AAV9, both hydroxylic residues. The approach to identify a  
20 phenotypic switch along a reconstructed phylogeny and then interrogate the conserved differences across the two diverging lineages for the phenotype of interest in order to map the structural determinant(s) responsible was named phenotype-to-phylogeny mapping.

Example 25—A Functional Motif Conferring AAPC-Independent Assembly and VP Protein Stability is Transferable to a Heterologous Capsid

25 To test whether the 10 sites constitute a motif that functions in capsid assembly, the Branch I identities were engrafted onto a member of Branch D and tested to determine whether the resulting hybrid gains AAPC-independent assembly function. Anc82, the node from which Branch I diverges, was chosen as the background for these mutations; as  
30 the closest relative to the Branch I serotypes it is more likely to tolerate several targeted mutations and retain functionality than a more distant relative. All ten sites in Anc82 were mutated to Branch I identities en masse by site-directed mutagenesis, creating a variant named 82DI (FIG. 4A). 82DIAAPstop60 gained AAPC-independent assembly

function (FIG. 4B). To determine the minimal motif required to confer this phenotype, each site was individually reverted back to its Branch D identity. All revertants are AAP-dependent (FIG. 4C), corroborating the 10 sites identified using phenotype-to-phylogeny mapping not only constitute a functional motif critical for capsid assembly, but also  
5 comprise a minimal motif required for AAPC-independent assembly in this subset of serotypes.

Whether this DI motif affects VP protein stability was next assessed. Consistent with other AAP-dependent serotypes (FIG. 2B), Anc82 exhibits a dramatic decrease in VP levels in the AAPstop60 condition, whereas 82DIAAPstop60 does not (FIG. 4D). To  
10 properly categorize 82DI's AAP phenotype, AAPstop20 for Anc82 and DI were generated, and loss of protein in 82DIstop20 was observed, indicating that 82DI is AAPC-independent (FIG. 4D).

To assess the broader impact of AAPC-independent assembly on the capsid as a whole, 82DI's biophysical properties and transduction capabilities was further  
15 characterized compared to its parental strain, Anc82. The  $T_m$  of 82DI is 5°C lower than Anc82 (FIG. 4E), a primary indication of a biophysically distinct entity (Pacouret et al., 2017, Mol. Ther., 25:1375-86). Considering the marked changes in Anc82 vs. 82DI's  $T_m$  and AAP phenotypes, the infectivity of both variants was tested. 82DI retains infectivity and transduction may be increased moderately compared to Anc82 both *in vitro* (FIG.  
20 12A) and *in vivo* (FIGs 4F & 12B).

#### Example 26—Candidate Residues Contributing to AAP-Independent Assembly Lie at the VP Trimer Interface

To examine how this motif influences particle assembly, where these residues lie  
25 within the 3-dimensional fold of VP and within an assembled capsid were mapped. Although crystal structures of AncAAVs are not available, the terminal Branch D (AAV8) and Branch I serotypes (AAV9) have been solved (DiMattia et al., 2012, J. Virol., 86:6947-58; Nam et al., 2007, J. Virol., 81:12260-71), and were used as surrogates to map the DI motif. Two of the 10 sites lie in the unstructured region of the VP N-  
30 terminus, but only site 1 is outside of VP3. Of the eight sites within the structured region of VP, seven of them map to the three-fold interface of a VP trimer and contact a neighboring monomer (FIGs. 5A-C). Comparing an AAV8 (AAP-dependent) trimer to an AAV9 (AAPC-independent) trimer at the atomic level, most of these sites exhibit

compelling evidence for stronger inter-monomeric interactions within an AAPC-independent trimer than in the AAP-dependent trimer (FIGs. 5D-F). For example, a conserved glutamic acid forms a salt bridge with an adjacent monomer's histidine at site 7 in the AAV9 trimer that cannot form with the leucine at site 7 in AAV8 (FIG. 5D). On AAV9, a hydrogen bond forms between a conserved asparagine and a glutamine at site 8 of an adjacent VP monomer. On AAV8, this bond is unable to materialize due to a Gln to Ala substitution (FIG. 5E). Site 10 lies at the 3-fold axis, and beneath a conserved phenylalanine, the valine residues in AAV9 create a much larger network of hydrophobic interactions than the alanines in AAV8 (FIG. 5F). Moreover, the site 10 interaction exists between all three monomers of the trimer simultaneously. These observations suggest that in AAPC-independent serotypes, this motif aids in trimer stabilization and possibly nucleates capsid assembly in the absence of a full length AAP.

#### Example 27—AAPC-Independent Capsomer Nucleation

Next, it was hypothesized that VPs of AAPC-independent AAVs are able to associate into oligomers in the absence of AAPC, whereas AAP-dependent serotypes' VPs do not strongly associate unless a full-length AAP is present. To test this theory, VP-VP interaction of AAP-dependent and AAPC-independent AAVs were evaluated by co-immunoprecipitation of VP3 with HA-tagged VP1 as bait (FIGs. 6A-B). The AAPC-independent VP1s tested, AAV3 and 82DI, were able to co-precipitate VP3 in the absence of a full-length AAP, despite low VP3 levels in the input. Conversely, neither AAP-dependent AAV2 nor Anc82 VP1 co-precipitated significant VP3 despite appreciable input levels (FIG. 6B). Addition of AAP2 allowed VP3 co-precipitation in AAP-dependent serotypes, and co-precipitated an unknown VP species between VP2 and VP3's predicted molecular weights in the AAPC-independent serotypes (marked with \*, FIG. 6B). These may be VP2-like proteins translated from an alternate start codon, or VP1 N-terminal cleavage/degradation products stabilized by AAP. Despite the appreciable increase in Anc82 and AAV3 VP3 input levels in the +AAP condition, these data support the above hypothesis.

To begin examining whether AAP is promoting oligomerization into species of defined geometry such as trimers or pentamers, or whether the increase in VP-VP interactions observed by co-IP are more randomized associations, crosslinking agents were added to transfected cell lysates and VP species interrogated by Western Blot (FIG.

6C). In the presence of AAP, a single supershifted VP band appears around 97 kDa when DSG (7 angstrom crosslinking arm) was added, and a slightly larger supershifted doublet appears when DSS (11 angstrom crosslinking arm) was added. Although it is difficult to determine the molecular weight of crosslinked species due to unpredictable migration patterns, the discrete banding suggests that AAP promotes VP-VP interactions of defined geometry or number of monomers, but may also be indicative of increased association of VP with a host protein(s) involved in capsid assembly, an association promoted by AAP.

To further ensure that the VP oligomerization process was being interrogated separately from their assembly into full capsids, the IP experiment was repeated with AAV2 VPs, adding *rep*, *aap2*, helper, and ITR-flanked reporter genome plasmids *in trans* to allow quantification of assembled DRPs (FIG. 13A). Appreciable quantities of genomes were detected only in the input and supernatant fractions, but absent from the IP fraction. ELISA to quantify fully assembled capsids mirrored these results (FIG. 13B), indicating only oligomerized VPs were precipitated. Taken together, these data support that in addition to a role for AAP in VP protein stability, AAP also promotes oligomerization of VP proteins to nucleate assembly of the icosahedron, which could potentially increase the efficiency of the capsid assembly process.

### OTHER EMBODIMENTS

It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.

The disclosed methods, compositions, and other materials are disclosed as described herein, but it is understood that combinations, subsets, interactions, groups, etc. of these methods, compositions, and other materials are also disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated

to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

**WHAT IS CLAIMED IS:**

1. An adeno-associated virus (AAV) capsid polypeptide comprising an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3.  
5
2. The AAV capsid polypeptide of claim 1 having at least 99% sequence identity to the amino acid sequence of SEQ ID NO:3.  
10
3. The AAV capsid polypeptide of claim 1 having the amino acid sequence of SEQ ID NO:3.
4. The AAV capsid polypeptide of claim 1, encoded by the nucleic acid sequence of SEQ ID NO: 4.  
15
5. A virus particle comprising the adeno-associated virus (AAV) capsid polypeptide of any of claims 1-4.
- 20 6. The virus particle of claim 5, further comprising a transgene.
7. A nucleic acid molecule comprising a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4 and encoding an adeno-associated virus (AAV) capsid polypeptide.  
25
8. The nucleic acid molecule of claim 7 having at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:4.
9. The nucleic acid molecule of claim 7, having the nucleic acid sequence of SEQ ID NO:4.  
30
10. The nucleic acid molecule of claim 7, encoding the amino acid sequence of SEQ ID NO: 3.

11. A vector comprising the nucleic acid molecule of any of claims 7-10.
12. A host cell comprising the nucleic acid molecule of any of claims 7-10 or  
5 the vector of claim 11.
13. The host cell of claim 12, wherein the host cell is a packaging cell.
14. A packaging cell comprising a nucleic acid molecule encoding an adeno-  
10 associated virus (AAV) capsid polypeptide, wherein the AAV capsid polypeptide has at  
least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3.
15. The packaging cell of claim 14, wherein the packaging cell lacks the  
assembly activating protein (AAP).
16. A method of reducing the assembly activating protein (AAP)-dependence  
of an adeno-associated virus (AAV), the method comprising providing an AAV having a  
capsid polypeptide that has at least 95% sequence identity to the amino acid sequence of  
20 SEQ ID NO: 3.
17. A method of relieving, at least partially, the assembly activating protein  
(AAP)-dependence of an adeno-associated virus (AAV), the method comprising  
incorporating a capsid polypeptide into the AAV that has at least 95% sequence identity  
to the amino acid sequence of SEQ ID NO: 3.
25. A method of engineering an adeno-associated virus (AAV) to reduce its  
dependence on assembly activating protein (AAP), comprising engineering an AAV that  
comprises a capsid polypeptide that has at least 95% sequence identity to the amino acid  
sequence of SEQ ID NO: 3.
30. The method of any of claims 16-18, further comprising culturing the  
adeno-associated virus (AAV) in the absence of the assembly activating protein (AAP).

20. The method of claim 18, further comprising sequencing the engineered adeno-associated virus (AAV).

5 21. The method of claim 18, further comprising comparing the assembly activating protein (AAP)-dependence of the engineered adeno-associated virus (AAV) relative to a non-engineered or wild type AAV.

10 22. The method of claim 18, further comprising aligning the engineered adeno-associated virus (AAV) with the non-engineered or wild type AAV.

23. An AAV capsid polypeptide comprising the amino acid sequence of SEQ ID NO:1 and further comprising the amino acid residues at the indicated positions shown in Table 6 for AAP-dependence.

15

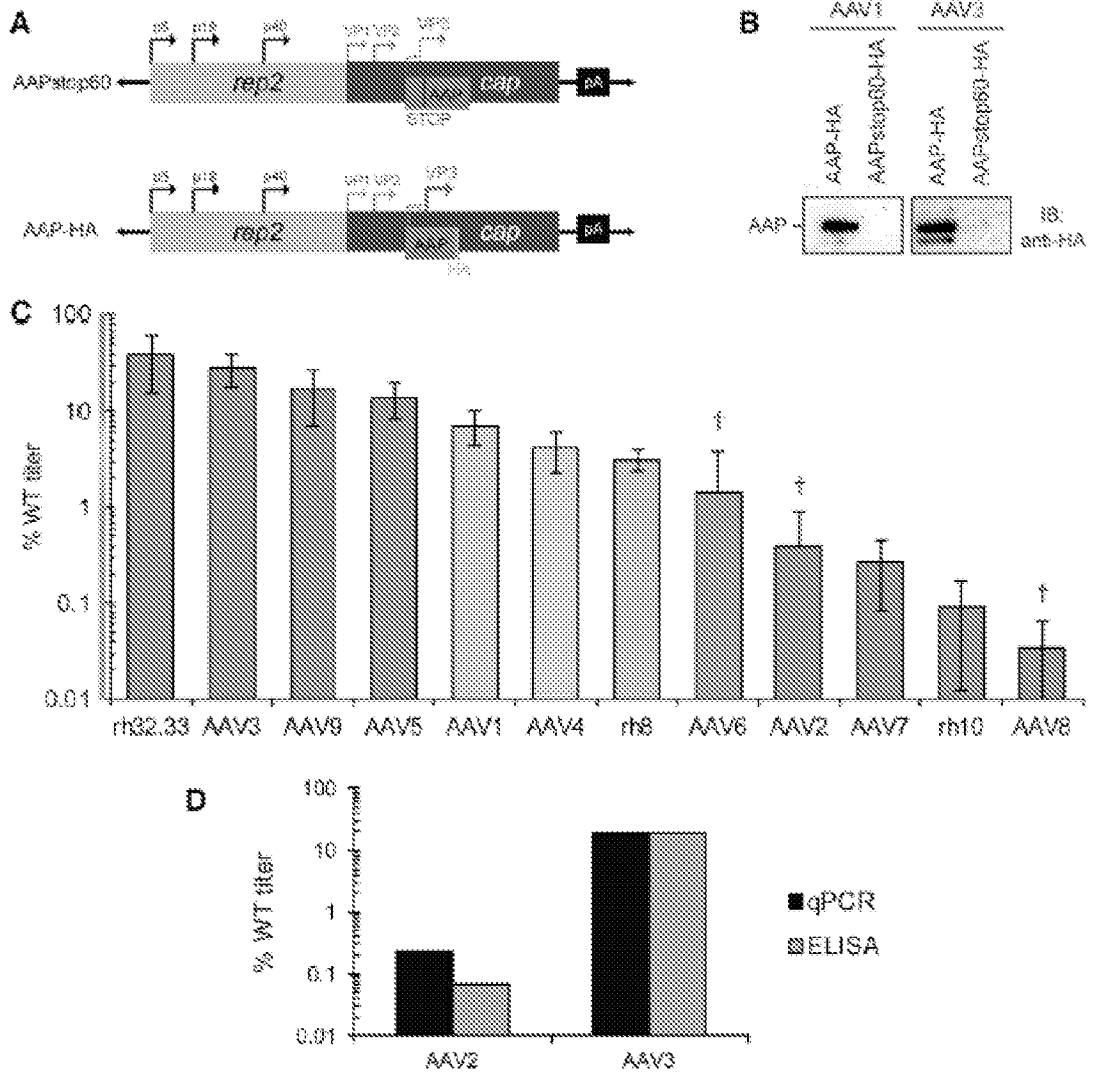


FIG. 1

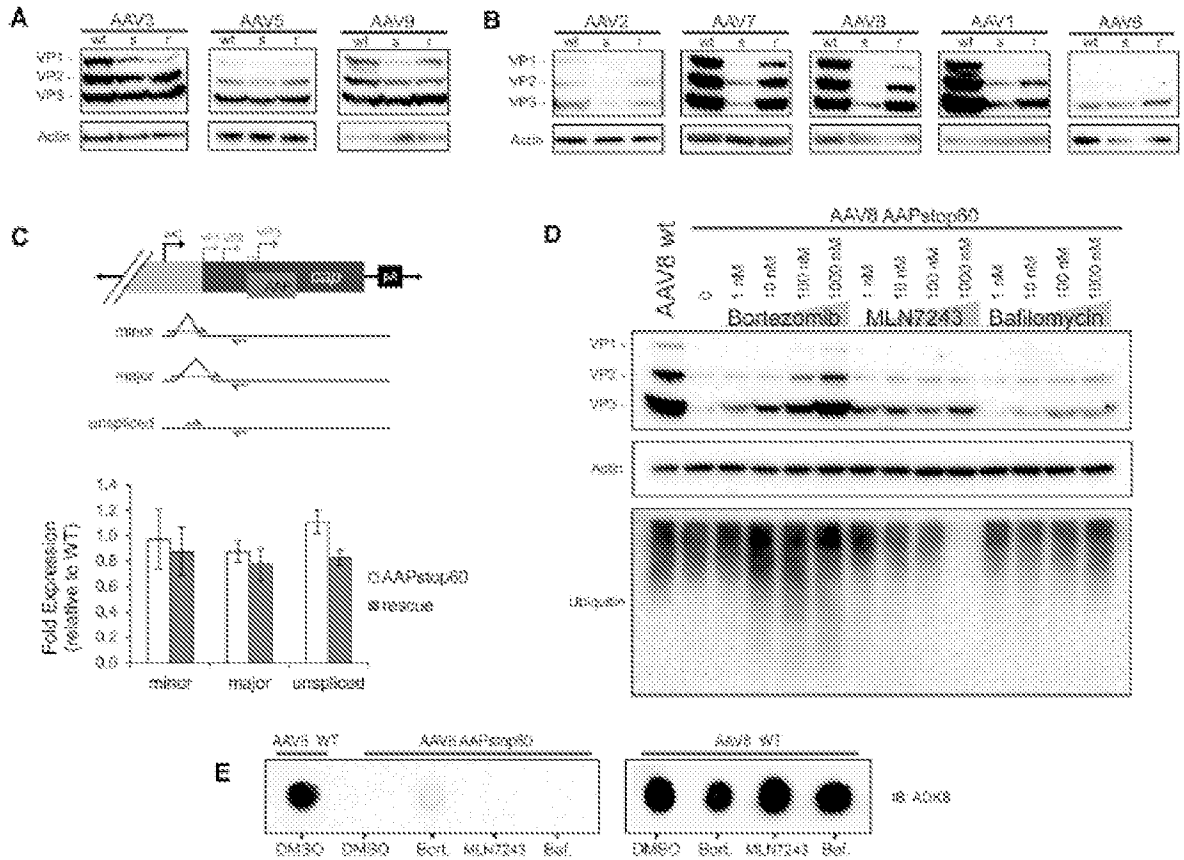


FIG. 2

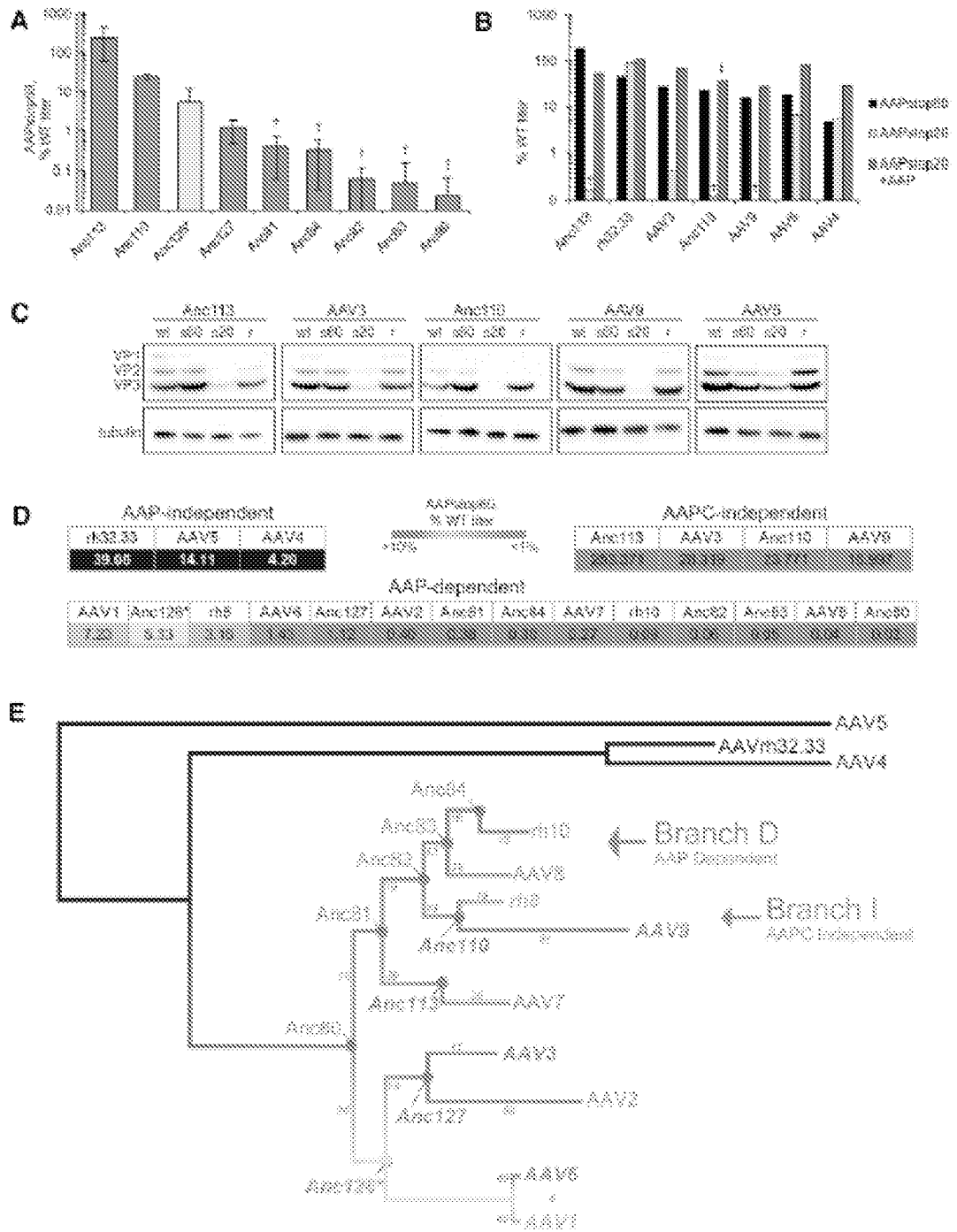


FIG. 3

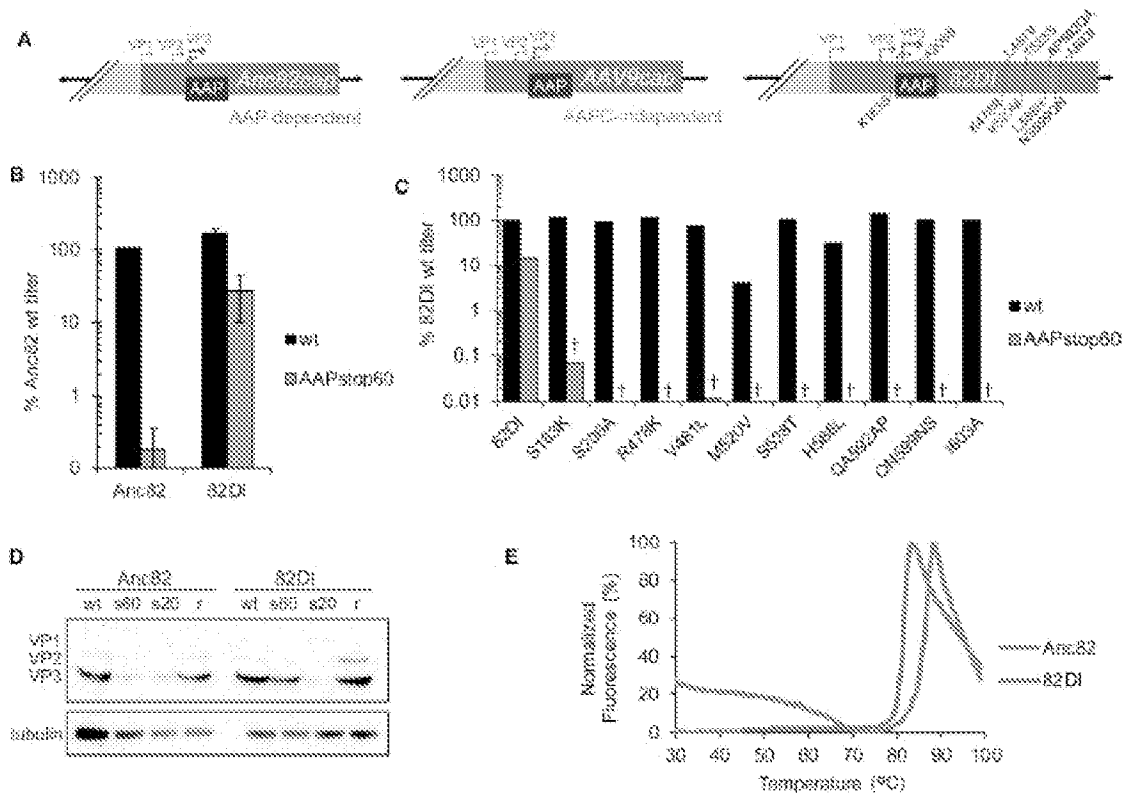


FIG. 4-1

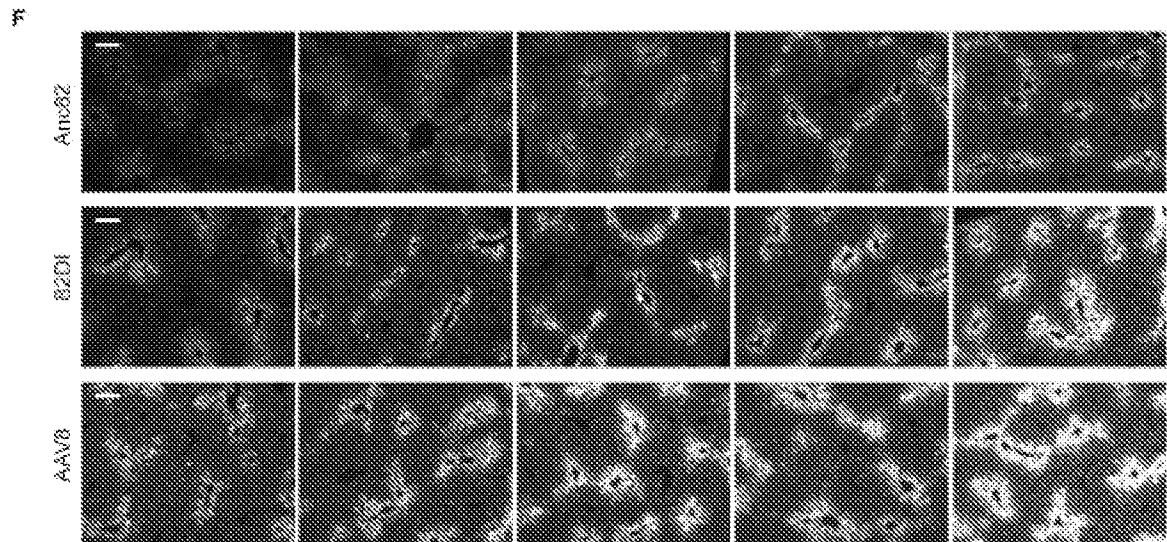


FIG. 4-2

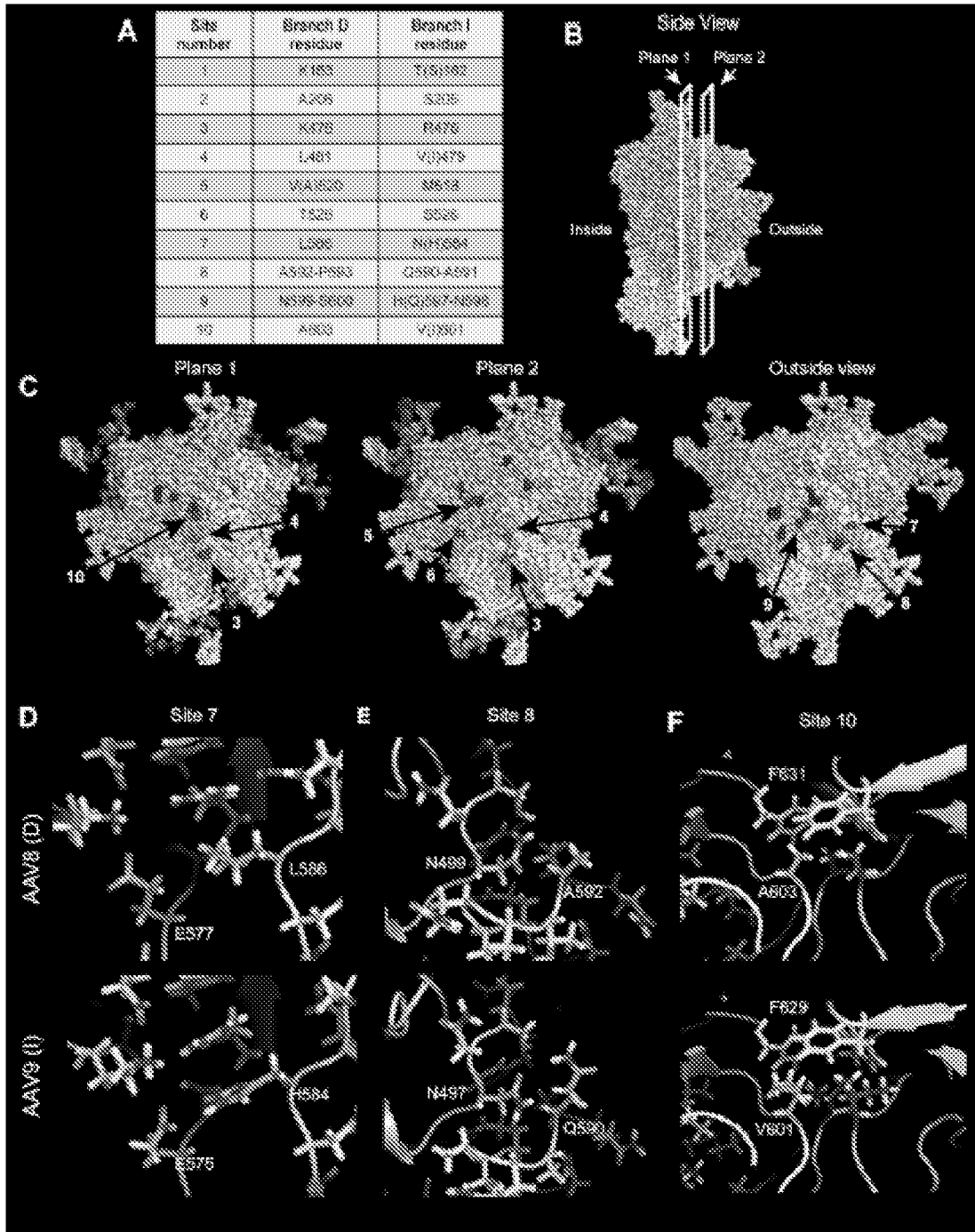
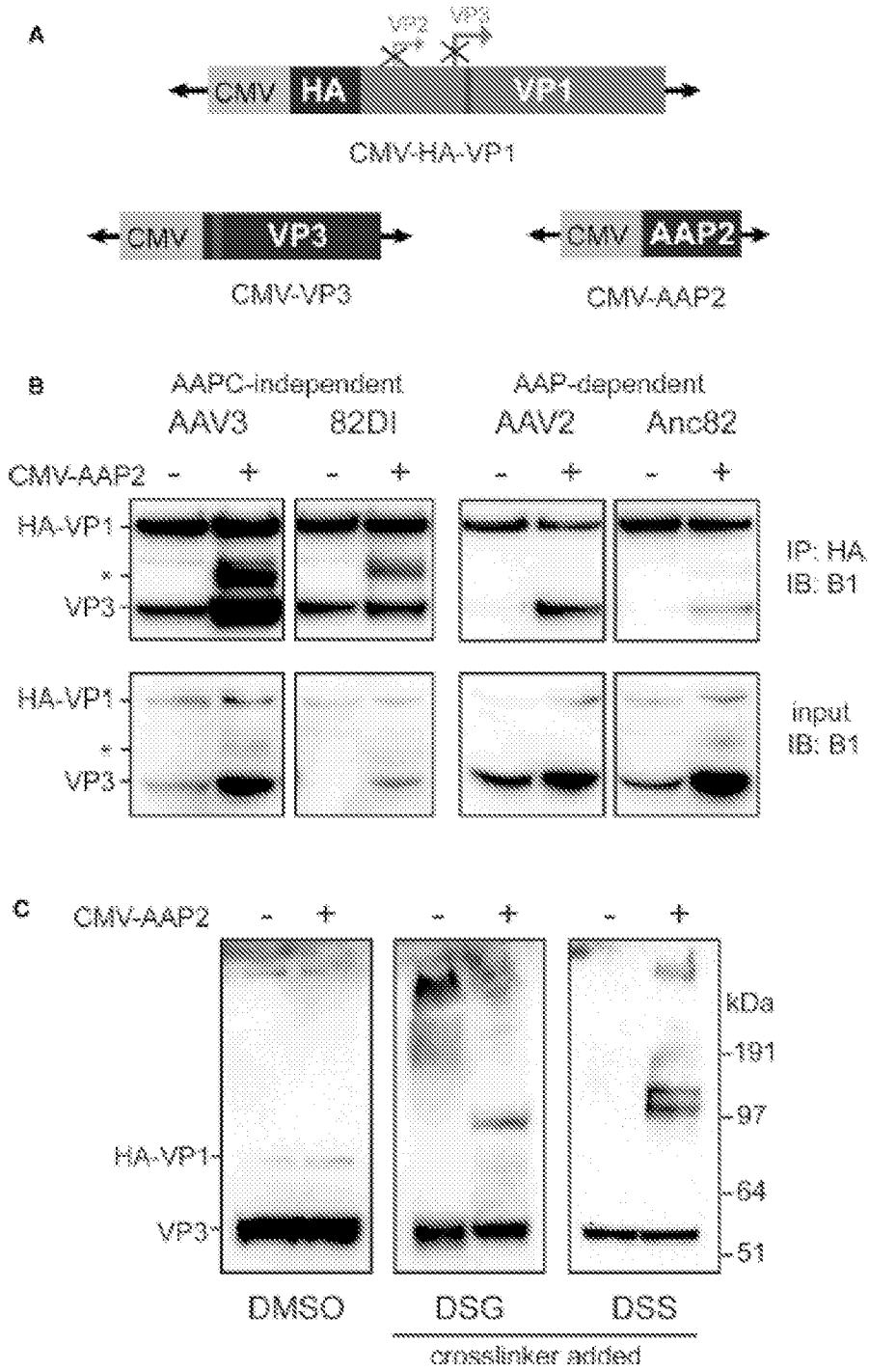
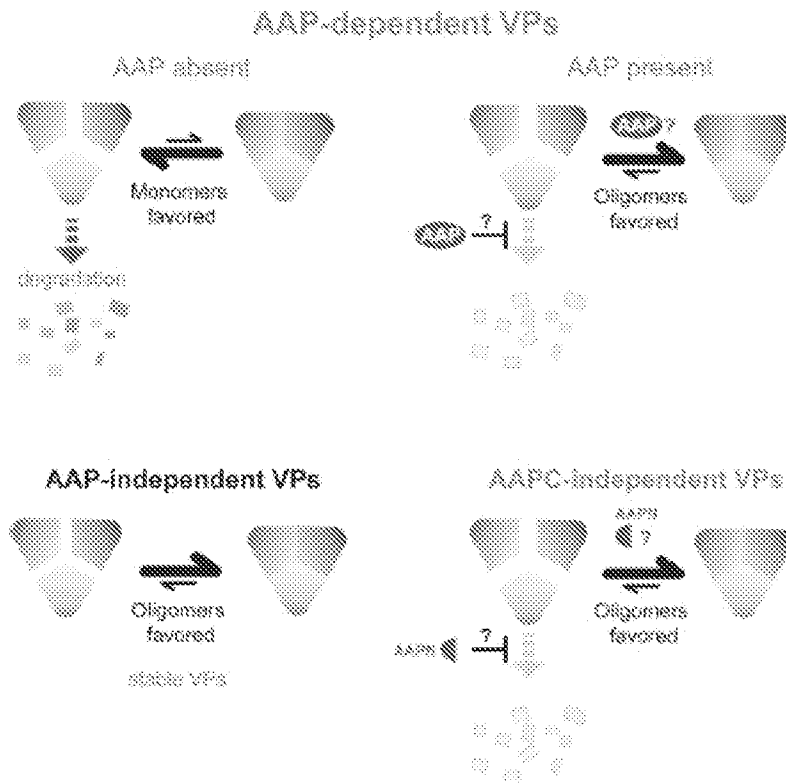


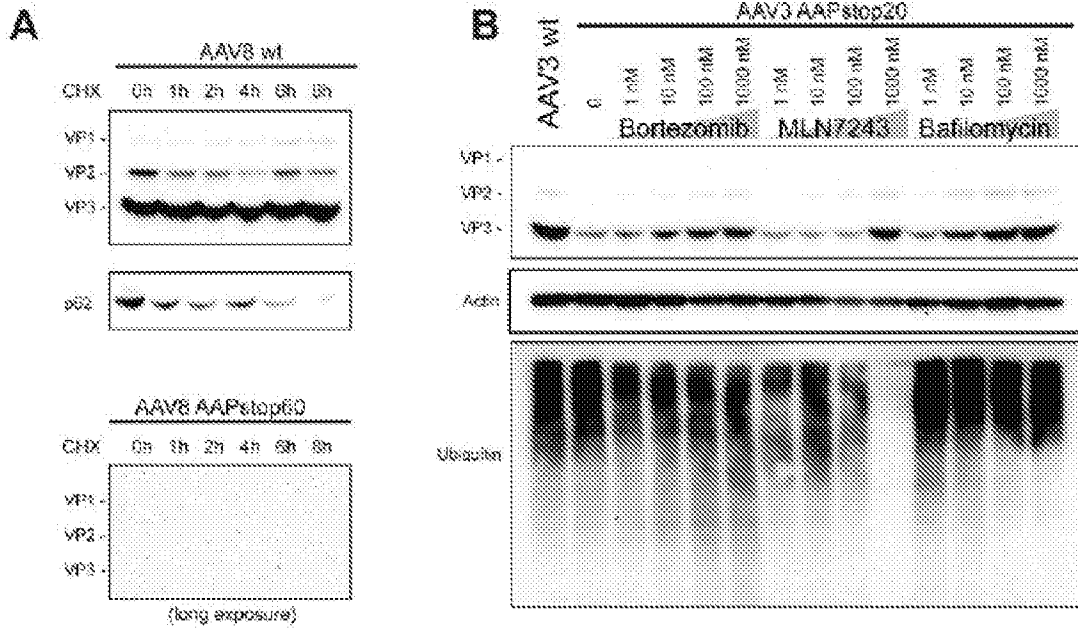
FIG. 5



**FIG. 6**



**FIG. 7**



**FIG. 8**

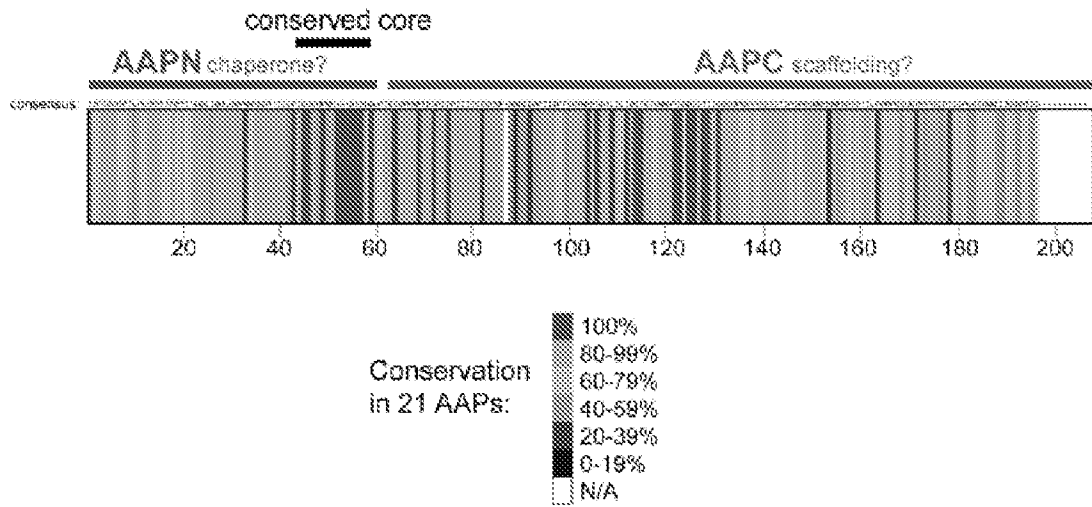


FIG. 9

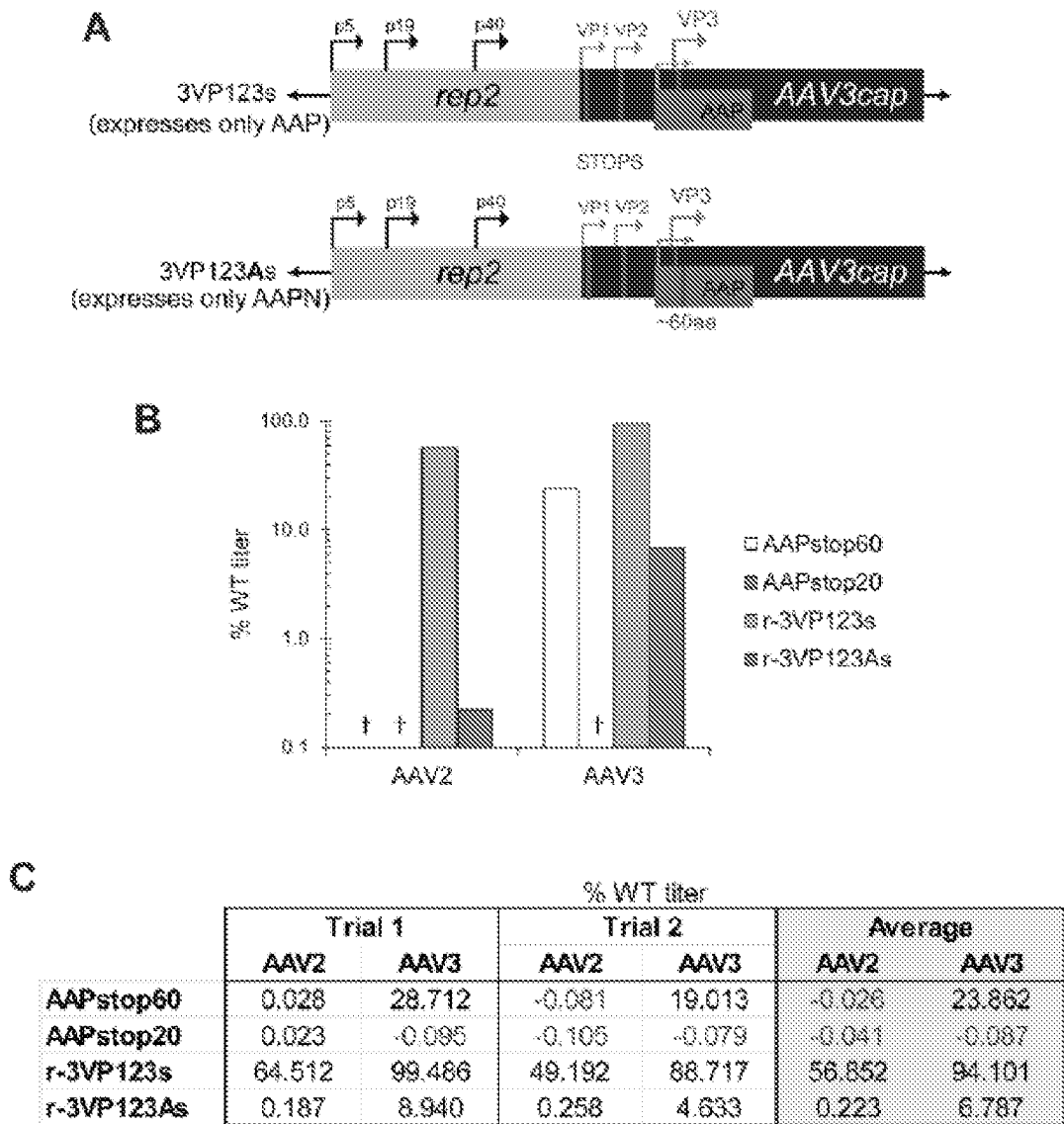


FIG. 10

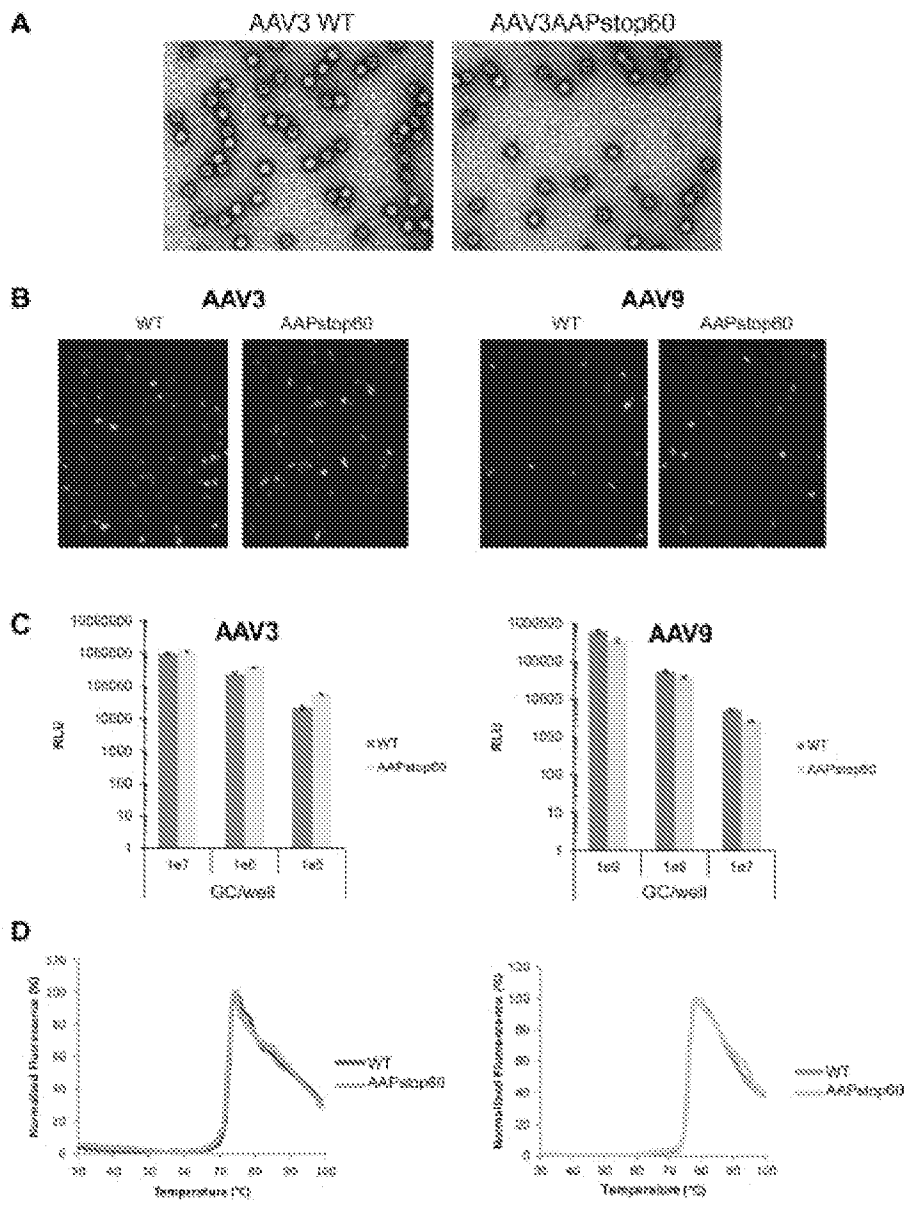


FIG. 11

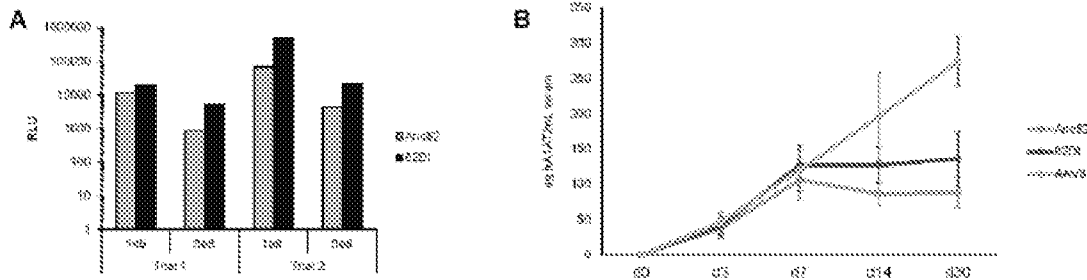


FIG. 12

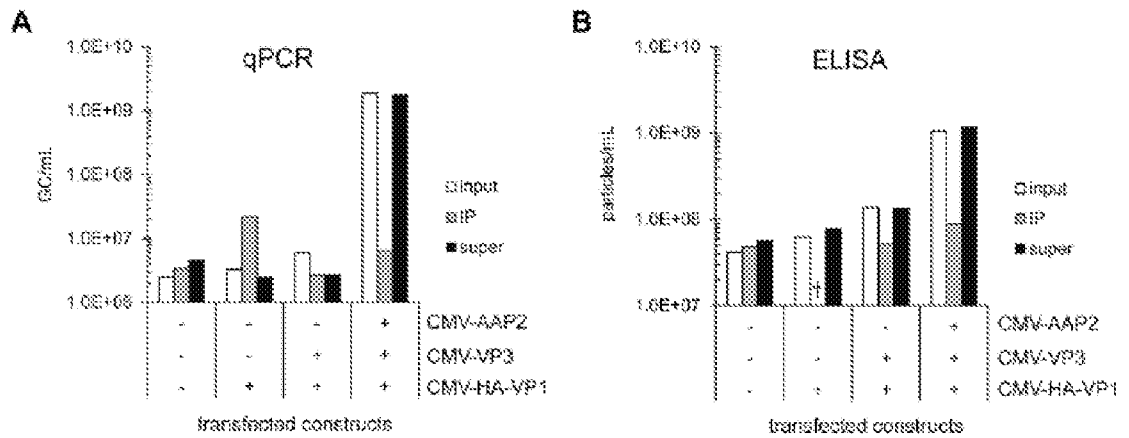


FIG. 13

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/032166

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC(8) - A61K 35/761; C07K 14/005; C07K 14/075; C12N 15/00; C12N 15/09; C12N 15/86 (2018.01)  
 CPC - C07K 14/005; C12N 15/86; C12N 15/861; C12N 2750/14121; C12N 2750/14141; C12N 2750/14143; C12N 2750/14151 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/93.2; 424/233.1; 435/91.4; 435/235.1; 435/320.1; 530/350; 536/23.72 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016/0215024 A1 (MASSACHUSETTS EYE & EAR INFIRMARY et al) 28 July 2016 (28.07.2016) entire document	1, 5-7, 11, 14, 15
A	US 2017/0096683 A1 (GENZYME CORPORATION) 06 April 2017 (06.04.2017) entire document	1-11, 14-23
A	WO 2017/019994 A2 (MASSACHUSETTS EYE AND EAR INFIRMARY et al) 02 February 2017 (02.02.2017) entire document	1-11, 14-23
A	WO 2016/144892 A1 (MASSACHUSETTS EYE AND EAR INFIRMARY) 15 September 2016 (15.09.2016) entire document	1-11, 14-23
A	US 2017/0028082 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 02 February 2017 (02.02.2017) entire document	1-11, 14-23
P, A	MAURER et al. "The Assembly-Activating Protein Promotes Stability and Interactions between AAV's Viral Proteins to Nucleate Capsid Assembly," Cell Rep, 08 May 2018 (08.05.2018), Vol. 23, Pgs. 1817-1830. entire document	1-11, 14-23

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 August 2018

Date of mailing of the international search report

27 SEP 2018

Name and mailing address of the ISA/US

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Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300  
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/032166

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: 12, 13  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
  - The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
  - No protest accompanied the payment of additional search fees.