(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 February 2018 (22.02.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/035213 A1

(51) International Patent Classification:
C12N 15/63 (2006.01) *A61K 48/00* (2006.01)
C12N 15/861 (2006.01) *A61P 27/00* (2006.01)
C07K 14/075 (2006.01)

(21) International Application Number:
PCT/US2017/047123

(22) International Filing Date:
16 August 2017 (16.08.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/375,666 16 August 2016 (16.08.2016) US

(71) Applicant: **THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL** [US/US]; 109 Church St., Chapel Hill, NC 27516, Chapel Hill, North Carolina 27516 (US).

(72) Inventors: **WOODARD, Kenton**; 100 Rock Haven Road, Apt. K201, Carrboro, North Carolina 27510 (US). **SAMULSKI, Richard Jude**; 102 Darlin Circle, Chapel Hill, North Carolina 27514 (US).

(74) Agent: **MILLER, Mary L.**; MYERS BIGEL, P.A., PO Box 37428, Raleigh, North Carolina 27627 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: METHODS AND COMPOSITIONS FOR TARGETED GENE TRANSFER

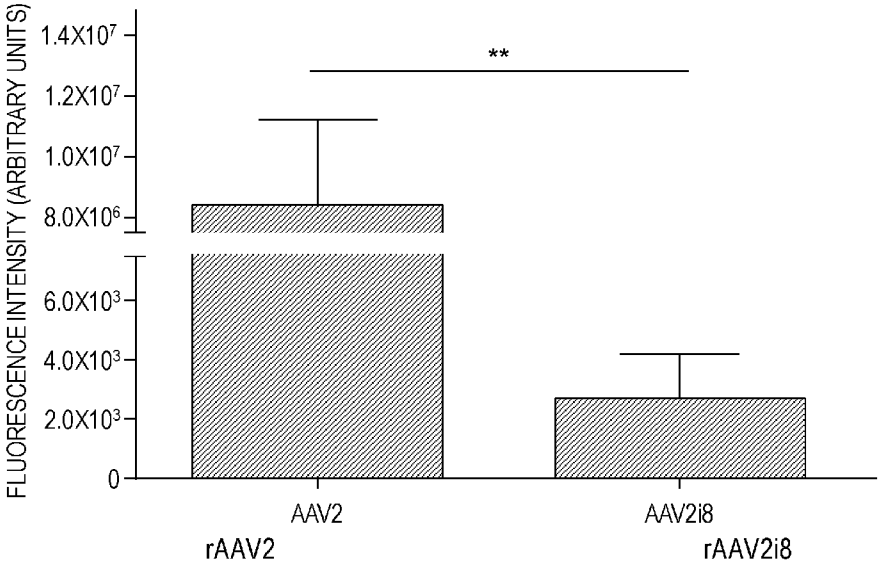

FIG. 1

(57) Abstract: The present invention provides AAV capsid proteins comprising a modification in the amino acid sequence and virus capsids and virus vectors comprising the modified AAV capsid protein. The invention also provides methods of administering the virus vectors and virus capsids of the invention to a cell or to a subject *in vivo*.

WO 2018/035213 A1

[Continued on next page]

Published:
— *with international search report (Art. 21(3))*

METHODS AND COMPOSITIONS FOR TARGETED GENE TRANSFER

STATEMENT OF PRIORITY

This application claims the benefit, under 35 U.S.C. § 119(e), of U.S. Provisional Application No. 62/375,666, filed August 16, 2016, the entire contents of which are incorporated by reference herein.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under Grant No. 5103757 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to modified capsid proteins from adeno-associated virus (AAV) and virus capsids and virus vectors comprising the same. In particular, the invention relates to modified AAV capsid proteins and capsids comprising the same that can be incorporated into virus vectors to confer a desirable transduction profile with respect to a target tissue of interest.

BACKGROUND OF THE INVENTION

New adeno-associated virus (AAV) strains isolated from animal tissues and adenoviral stocks have expanded the panel of AAV vectors available for therapeutic gene transfer applications. Comprehensive efforts to map tissue tropisms of these AAV isolates in animal models are currently underway. The ability to direct homing of AAV vectors to selective organs is useful for gene therapy and other therapeutic applications.

Adeno-associated virus (AAV) has become the vector of choice for viral gene transfer and has shown great promise in clinical trials. Of importance is the successful treatment of the retina by subretinal delivery. Development of a less invasive injection route is met by intravitreal delivery, but delivery of AAV by this route results in poor transduction outcomes. The inner limiting membrane (ILM) creates a barrier separating the vitreous and the retina.

The present invention addresses a need in the art for nucleic acid delivery vectors with desirable targeting features.

SUMMARY OF THE INVENTION

The present invention provides a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 4 (AAV4) vector comprising an AAV4 capsid protein, wherein the AAV4 capsid protein comprises a substitution at amino acid residue K530 and/or further comprises a substitution at one or more of amino acid residues S584, N585, S586 and N587 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:4 (amino acid sequence of AAV4 capsid protein).

The present invention also provides a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 5 (AAV5) vector comprising an AAV5 capsid protein, wherein the AAV5 capsid protein comprises a substitution at amino acid residue K517 and/or further comprises a substitution at one or more of amino acid residues S575, S576, T577 and T578 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:5 (amino acid sequence of AAV5 capsid protein).

Additionally provided herein is a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 7 (AAV7) vector comprising an AAV7 capsid protein, wherein the AAV7 capsid protein comprises a substitution at amino acid residue K533 and/or further comprises a substitution at one or more of amino acid residues A587, A588, N589 and R590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:7 (amino acid sequence of AAV7 capsid protein).

The present invention further provides a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 8 (AAV8) vector comprising an AAV8 capsid protein, wherein the AAV8 capsid protein comprises a substitution at amino acid residue K533 and/or further comprises a substitution at one or more of amino acid residues Q587, Q588, N589 and T590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:8 (amino acid sequence of AAV8 capsid protein).

Also provided herein is a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 9 (AAV9) vector comprising an AAV9 capsid protein, wherein the AAV9 capsid protein comprises a substitution at amino acid residue K531 and/or further comprises a substitution at one or more of amino acid residues Q587, A588, N589 and T 590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:9 (amino acid sequence of AAV9 capsid protein).

Furthermore, the present invention provides a method of treating a disorder or defect of the eye in a subject, comprising intravitreally administering to the subject the virus vector of this invention, wherein the virus vector comprises a nucleic acid molecule that encodes a therapeutic protein or therapeutic DNA effective in treating the disorder or defect of the eye in the subject.

These and other aspects of the invention are addressed in more detail in the description of the invention set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Green fluorescence protein (GFP) fluorescence following intravitreal delivery of rAAV2 vector and its HS-binding deficient variants twelve weeks post-injection. Quantification of fundus images showed a 300-fold decrease in expression between rAAV2 (HS-binding) and rAAV2i8 (ablated HS-binding). Immunohistochemistry (IHC) of rAAV2-injected retinas shows fluorescence mainly in the RGC with fewer GFP-positive somas in the INL. Graph is shown with error bars indicating the standard error mean (SEM) and significance is detected by a non-parametric t-test (**$p<0.01$).

Figure 2. Schematic of the retina depicts the trafficking of rAAV following intravitreal delivery.

Figure 3. qPCR analysis of viral binding to human retinas *ex vivo*. Results are quantified as vector genomes per cell genome. rAAV2 (HS-binding) vector shows the greatest presence at the retina with few transgenes found elsewhere. The presence of transgenes delivered by rAAV2i8 (ablated HS-binding) were low in all collected tissues but showed a significant increase compared to rAAV2-delivered transgenes in both the choroid and sclera. Error bars indicated standard deviation.

Figure 4. GFP fluorescence following intravitreal delivery of HS-binding variants of rAAV1 eight weeks post-injection. Quantification of fundus images shows a 3-

fold increase with rAAV1-E531K (HS-binding) capsid compared to rAAV1 (non HS-binding) capsid. Graph is shown with error bars indicating the SEM and significance by a non-parametric t-test (*p<0.05).

Figure 5. Chimeric capsids suggest tropism is influenced by other motifs other than HS binding. Elements of rAAV1 were applied to rAAV2 using the chimeric rAAV2.5 capsid and imaged for intravitreal delivery. Quantification of the fundus fluorescence for the collection of capsids. Error bars represent the SEM.

Figure 6. *In vitro* competition assay using soluble heparin to block the transduction of rAAV of HEK293 cells. Viruses were incubated with increasing doses of soluble heparin and applied to cell culture at a multiplicity of infection of 10,000 vg per cell. rAAV2 displayed a dose-dependent decrease in transduction which was not observed with either rAAV1 or rAAV1-E531K. The amount of transduction of rAAV1-E531K was lower than rAAV1 in all conditions. Error bars shown as SEM.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described with reference to the accompanying drawings, in which representative embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

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 this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

The present invention is based on the unexpected discovery that intravitreal transduction of cells of the retina and/or retinal pigment epithelium can be enhanced by the addition of the heparan sulfate binding motif on the AAV capsid. Thus, in one embodiment, the present invention provides a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 4 (AAV4) vector comprising an AAV4 capsid protein, wherein the AAV4 capsid protein comprises a substitution at amino acid residue K530 and/or further comprises a substitution at one or more of amino acid residues S584,

N585, S586 and N587 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:4 (amino acid sequence of AAV4 capsid protein).

The present invention also provides a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 5 (AAV5) vector comprising an AAV5 capsid protein, wherein the AAV5 capsid protein comprises a substitution at amino acid residue K517 and/or further comprises a substitution at one or more of amino acid residues S575, S576, T577 and T578 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:5 (amino acid sequence of AAV5 capsid protein).

Additionally provided herein is a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 7 (AAV7) vector comprising an AAV7 capsid protein, wherein the AAV7 capsid protein comprises a substitution at amino acid residue K533 and/or further comprises a substitution at one or more of amino acid residues A587, A588, N589 and R590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:7 (amino acid sequence of AAV7 capsid protein).

The present invention further provides a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 8 (AAV8) vector comprising an AAV8 capsid protein, wherein the AAV8 capsid protein comprises a substitution at amino acid residue K533 and/or further comprises a substitution at one or more of amino acid residues Q587, Q588, N589 and T590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:8 (amino acid sequence of AAV8 capsid protein).

Also provided herein is a method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 9 (AAV9) vector comprising an AAV9 capsid protein, wherein the AAV9 capsid protein comprises a substitution at amino acid residue K531 and/or further comprises a substitution at one or more of amino acid residues Q587, A588, N589 and T 590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:9 (amino acid sequence of AAV9 capsid protein).

Furthermore, the present invention provides a method of treating a disorder or defect of the eye in a subject, comprising intravitreally administering to the subject the virus vector of this invention, wherein the virus vector comprises a nucleic acid molecule that encodes a therapeutic protein or therapeutic DNA effective in treating the disorder or defect of the eye in the subject.

In further embodiments, the methods of this invention can be carried out with an AAV vector comprising a capsid protein that has been modified as described below. Specifically, in some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486Q, 487R, 488V, 489S, 490K, 491T, 527K, 528D, 529D, 530E, D531E, 532K, S585R, S586G, S587N, T588R) can be graphed onto an AAV1 capsid protein and/or the AAV1 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F,), galactose motif (AAV2 numbering: G469N, 470M, S471A, 472V, P474G, 500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or deletion mutation (AAV2 numbering: T265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486 Q, 487 R, 488 V, 489 S, 490 K, 491T, 527K, 528D, 529D, 530E, 531E, 532K, 585R, 586G, 587N, 588R) can be graphed onto an AAV2 capsid protein and/or the AAV2 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F), galactose motif (AAV2 numbering: D469N, I470M, R471A, D472V, S474G, Y500F), insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486Q, 487R, L488V, 489S, 490K, 491T, 527K, 528D, 529D, 530E, 531E, 532K, S585R, S586G, N587N, T588R) can be graphed onto an AAV3B capsid protein and/or the AAV3B capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F,), galactose motif (AAV2 numbering: S469N, 470M, S471A, N472V, A474G, 500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: K484R, 485Q, 486Q, G487R, F488V, 489S, 490K, 491T, G527K, P528D, A529D, D530E, S531E, 532K, S585R, N586G, S587N, N588R) can be graphed onto an AAV4 capsid protein and/or the AAV4 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F), galactose motif (AAV2 numbering: 469N, F470M, S471A, N472V, K474G, S500F), insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, T485Q, G487R, W488V, N489S, L490K, G491T, L527K, Q528D, G529D, S530E, N531E, T532K, S585R, S586G, T587N, T588R) can be graphed onto an AAV5 capsid protein and/or the AAV5 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F), galactose motif (AAV2 numbering: R469N, Y470M, 471A, N472V,Y474G, S500F), insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486Q, 487R, 488V, 489S, 490K, 491T, 527K, 528D, 529D, 530E, 531E, R532K, A585R, A586G, 587N, T588R) can be graphed onto an AAV7 capsid protein and/or the AAV7 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F,), galactose motif (AAV2 numbering: T469N, 470M, 471A, E472V, A474G, 500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486Q, 487R, 488V, 489S, T490K, 491T, 527K, 528D, 529D, 530E, 531E, R532K, Q585R, Q586G, N587N, T588R) can be graphed onto an AAV8 capsid protein and/or the AAV 8 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F,), galactose motif (AAV2 numbering: T469N, 470M, 471A, N472V, A474G, 500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or substitution mutation (AAV2 numbering:

S265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486Q, 487R, 488V, 489S, T490K, 491T, 527K, E528D, G529D, 530E, D531E, R532K, S585R, A586G, Q587N, A588R) can be graphed onto an AAV9 capsid protein for and/or the AAV9 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F), galactose motif (AAV2 numbering: 469N, 470M, 471A, 472V, 474G, 500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or substitution mutation (AAV2 numbering: S265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: 484R, 485Q, 486Q, 487R, 488V, 489S, T490K, 491T, 527 K, 528D, 529D, 530E, 531E, R532K, Q585R, A586G, 587N, T588R) can be graphed onto an AAV10 capsid protein and/or the AAV10 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F,), galactose motif (AAV2 numbering: 469N, 470M, S471A, A472V, A474G, 500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or substitution mutation (AAV2 numbering: S265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: K484R, 485Q, 486Q, 487R, F488V, 489S, 490K, 491T, G527K, P528D, S529D, D530E, G531E, D532K, N585R, A586G, T587N, T588R) can be graphed onto an AAV11 capsid protein in any combination and/or the AAV11 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F), galactose motif (AAV2 numbering: D469N, F470M, 471A, F472V, R474G, A500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587). or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In some embodiments, the heparan sulfate binding motif (AAV2 numbering: K484R, 485Q, 486Q, K487R, F488V, 489S, 490K, N491T, G527K, A528D, G529D, D530E, S531E, D532K, N585R, A586G, T587N, T588R) can be graphed onto an AAV12 capsid protein and/or the AAV12 capsid protein can comprise Tyr mutations (AAV2 numbering: Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, Y730F), galactose motif (AAV2 numbering:

D469N, F470M, 471A, F472V, R474G, A500F) insertion of peptide with the amino acid abbreviation LALGETTRPA (AAV2 numbering: 587), or insertion mutation (AAV2 numbering: 265), in any combination. Amino acid residue numbering is based on the amino acid sequence of AAV2 (SEQ ID NO:2).

In the methods of this invention, the viral vector can comprise a nucleic acid molecule that encodes a therapeutic protein and/or therapeutic DNA.

The present invention further provides a method of treating a disorder or defect of the eye in a subject, comprising the intravitreally administering viral vector of this invention to the subject receiving a therapeutic protein or therapeutic DNA effective in treating the disorder or defect of the eye in the subject.

Nonlimiting examples of a disorder or defect of the eye that can be treated according to the methods of this invention include age-related macular degeneration, Lebers congenital amarousis type 1, Lebers, congenital amarousis type 2, retinitis pigmentosa, retinoschosis, achromatopsia, color blindness, congenital stationary night blindness or any combination thereof.

Definitions.

The following terms are used in the description herein and the appended claims:

The singular forms “a,” “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Furthermore, the term "about," as used herein when referring to a measurable value such as an amount of the length of a polynucleotide or polypeptide sequence, dose, time, temperature, and the like, is meant to encompass variations of ±20%, ±10%, ±5%, ±1%, ±0.5%, or even ±0.1% of the specified amount.

Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

As used herein, the transitional phrase "consisting essentially of" (and grammatical variants) means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim, "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. Thus, the term "consisting essentially of" when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination.

Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted.

To illustrate further, if, for example, the specification indicates that a particular amino acid can be selected from A, G, I, L and/or V, this language also indicates that the amino acid can be selected from any subset of these amino acid(s) for example A, G, I or L; A, G, I or V; A or G; only L; etc. as if each such subcombination is expressly set forth herein. Moreover, such language also indicates that one or more of the specified amino acids can be disclaimed. For example, in particular embodiments the amino acid is not A, G or I; is not A; is not G or V; etc. as if each such possible disclaimer is expressly set forth herein.

As used herein, the terms "reduce," "reduces," "reduction" and similar terms mean a decrease of at least about 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97% or more.

As used herein, the terms "enhance," "enhances," "enhancement" and similar terms indicate an increase of at least about 5%, 10%, 20%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more. These terms can also be used in reference to fold increases, e.g., one-fold, two-fold, three-fold, four-fold, five-fold, six-fold, seven-fold, eight-fold, nine-fold, ten-fold, etc.

The term "parvovirus" as used herein encompasses the family *Parvoviridae*, including autonomously replicating parvoviruses and dependoviruses. The autonomous parvoviruses include members of the genera *Parvovirus*, *Erythrovirus*, *Densovirus*, *Iteravirus*, and *Contravirus*. Exemplary autonomous parvoviruses include, but are not limited to, minute virus of mouse, bovine parvovirus, canine parvovirus, chicken parvovirus, feline panleukopenia virus, feline parvovirus, goose parvovirus, H1 parvovirus, muscovy duck parvovirus, B19 virus, and any other autonomous parvovirus now known or later discovered. Other autonomous parvoviruses are known to those skilled in the art. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers).

As used herein, the term "adeno-associated virus" (AAV), includes but is not limited to, AAV type 1, AAV type 2, AAV type 3 (including types 3A and 3B), AAV type 4, AAV type 5, AAV type 6, AAV type 7, AAV type 8, AAV type 9, AAV type 10, AAV type 11, AAV type 12, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, and any other AAV now known or later discovered. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers). A number of

relatively new AAV serotypes and clades have been identified (*see, e.g.*, Gao et al. (2004) *J. Virology* 78:6381-6388; Moris et al. (2004) *Virology* 33-:375-383; and **Table 1**).

The genomic sequences of various serotypes of AAV and the autonomous parvoviruses, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Such sequences may be found in the literature or in public databases such as the GenBank® Database. *See, e.g.*, GenBank Accession Numbers NC_044927, NC_002077, NC_001401, NC_001729, NC_001863, NC_001829, NC_001862, NC_000883, NC_001701, NC_001510, NC_006152, NC_006261, AF063497, U89790, AF043303, AF028705, AF028704, J02275, J01901, J02275, X01457, AF288061, AH009962, AY028226, AY028223, NC_001358, NC_001540, AF513851, AF513852, AY530579; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also, e.g.*, Srivistava et al. (1983) *J. Virology* 45:555; Chiorini et al. (1998) *J. Virology* 71:6823; Chiorini *et al.* (1999) *J. Virology* 73:1309; Bantel-Schaal et al. (1999) *J. Virology* 73:939; Xiao et al. (1999) *J. Virology* 73:3994; Muramatsu et al. (1996) *Virology* 221:208; Shade et al. (1986) *J. Virol.* 58:921; Gao et al. (2002) *Proc. Nat. Acad. Sci. USA* 99:11854; Moris et al. (2004) *Virology* 33-:375-383; international patent publications WO 00/28061, WO 99/61601, WO 98/11244; and U.S. Patent No. 6,156,303; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also* **Table 1**.

The capsid structures of autonomous parvoviruses and AAV are described in more detail in BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapters 69 & 70 (4th ed., Lippincott-Raven Publishers). *See also*, description of the crystal structure of AAV2 (Xie et al. (2002) *Proc. Nat. Acad. Sci.* 99:10405-10), AAV4 (Padron et al. (2005) *J. Virol.* 79: 5047-58), AAV5 (Walters et al. (2004) *J. Virol.* 78: 3361-71) and CPV (Xie et al. (1996) *J. Mol. Biol.* 6:497-520 and Tsao et al. (1991) *Science* 251: 1456-64).

The term "tropism" as used herein refers to preferential entry of the virus into certain cells or tissues, optionally followed by expression (*e.g.*, transcription and, optionally, translation) of a sequence(s) carried by the viral genome in the cell, *e.g.*, for a recombinant virus, expression of a heterologous nucleic acid(s) of interest. Those skilled in the art will appreciate that transcription of a heterologous nucleic acid sequence from the viral genome may not be initiated in the absence of trans- acting factors, *e.g.*, for an inducible promoter or otherwise regulated nucleic acid sequence. In the case of a rAAV genome, gene expression from the viral genome may be from a stably integrated provirus, from a non-integrated episome, as well as any other form the virus may take within the cell.

Unless indicated otherwise, "efficient transduction" or "efficient tropism," or similar terms, can be determined by reference to a suitable control (*e.g.*, at least about 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, 99%, 100% or more of the transduction or tropism, respectively, of the control). Suitable controls will depend on a variety of factors including the desired tropism profile.

As used herein, the term "polypeptide" encompasses both peptides and proteins, unless indicated otherwise.

A "polynucleotide" is a sequence of nucleotide bases, and may be RNA, DNA or DNA-RNA hybrid sequences (including both naturally occurring and non-naturally occurring nucleotides), but in representative embodiments are either single or double stranded DNA sequences.

As used herein, an "isolated" polynucleotide (e.g., an "isolated DNA" or an "isolated RNA") means a polynucleotide at least partially separated from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polynucleotide. In representative embodiments an "isolated" nucleotide is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

Likewise, an "isolated" polypeptide means a polypeptide that is at least partially separated from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polypeptide. In representative embodiments an "isolated" polypeptide is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

As used herein, by "isolate" or "purify" (or grammatical equivalents) a virus vector, it is meant that the virus vector is at least partially separated from at least some of the other components in the starting material. In representative embodiments an "isolated" or "purified" virus vector is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

A "therapeutic protein" is a protein that can alleviate, reduce, prevent, delay and/or stabilize symptoms that result from an absence or defect in a protein in a cell or subject and/or is a protein that otherwise confers a benefit to a subject.

A "therapeutic RNA molecule" or "functional RNA molecule" as used herein can be an antisense nucleic acid, a ribozyme (*e.g.*, as described in U.S. Patent No. 5,877,022), an

RNA that effects spliceosome-mediated *trans*-splicing (*see*, Puttaraju *et al.* (1999) *Nature Biotech.* 17:246; U.S. Patent No. 6,013,487; U.S. Patent No. 6,083,702), an interfering RNA (RNAi) including siRNA, shRNA or miRNA, which mediate gene silencing (*see*, Sharp et al., (2000) *Science* 287:2431), and any other non-translated RNA, such as a "guide" RNA (Gorman *et al.* (1998) *Proc. Nat. Acad. Sci. USA* 95:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*) and the like as are known in the art.

By the terms "treat," "treating" or "treatment of" (and grammatical variations thereof) it is meant that the severity of the subject's condition is reduced, at least partially improved or stabilized and/or that some alleviation, mitigation, decrease or stabilization in at least one clinical symptom is achieved and/or there is a delay in the progression of the disease or disorder.

The terms "prevent," "preventing" and "prevention" (and grammatical variations thereof) refer to prevention and/or delay of the onset of a disease, disorder and/or a clinical symptom(s) in a subject and/or a reduction in the severity of the onset of the disease, disorder and/or clinical symptom(s) relative to what would occur in the absence of the methods of the invention. The prevention can be complete, *e.g.*, the total absence of the disease, disorder and/or clinical symptom(s). The prevention can also be partial, such that the occurrence of the disease, disorder and/or clinical symptom(s) in the subject and/or the severity of onset is less than what would occur in the absence of the present invention.

A "treatment effective" amount as used herein is an amount that is sufficient to provide some improvement or benefit to the subject. Alternatively stated, a "treatment effective" amount is an amount that will provide some alleviation, mitigation, decrease or stabilization in at least one clinical symptom in the subject. Those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

A "prevention effective" amount as used herein is an amount that is sufficient to prevent and/or delay the onset of a disease, disorder and/or clinical symptoms in a subject and/or to reduce and/or delay the severity of the onset of a disease, disorder and/or clinical symptoms in a subject relative to what would occur in the absence of the methods of the invention. Those skilled in the art will appreciate that the level of prevention need not be complete, as long as some benefit is provided to the subject.

The terms "heterologous nucleotide sequence" and "heterologous nucleic acid molecule" are used interchangeably herein and refer to a nucleic acid molecule and/or nucleotide sequence that is not naturally occurring in the virus. Generally, the heterologous

nucleic acid comprises an open reading frame that encodes a protein, protein fragment, peptide or nontranslated RNA of interest (*e.g.*, for delivery to a cell or subject).

As used herein, the terms "virus vector," "vector" or "gene delivery vector" refer to a virus (*e.g.*, AAV) particle that functions as a nucleic acid delivery vehicle, and which comprises the vector genome (*e.g.*, viral DNA [vDNA]) packaged within a virion. Alternatively, in some contexts, the term "vector" may be used to refer to the vector genome/vDNA alone.

A "rAAV vector genome" or "rAAV genome" is an AAV genome (*i.e.*, vDNA) that comprises one or more heterologous nucleic acid sequences. rAAV vectors generally require only the terminal repeat(s) (TR(s)) in *cis* to generate virus. All other viral sequences are dispensable and may be supplied in *trans* (Muzyczka (1992) *Curr. Topics Microbiol. Immunol.* 158:97). Typically, the rAAV vector genome will only retain the one or more TR sequence so as to maximize the size of the transgene that can be efficiently packaged by the vector. The structural and non-structural protein coding sequences may be provided in *trans* (*e.g.*, from a vector, such as a plasmid, or by stably integrating the sequences into a packaging cell). In embodiments of the invention, the rAAV vector genome comprises at least one terminal repeat (TR) sequence (e.g., AAV TR sequence), optionally two TRs (*e.g.*, two AAV TRs), which typically will be at the 5' and 3' ends of the vector genome and flank the heterologous nucleic acid sequence, but need not be contiguous thereto. The TRs can be the same or different from each other.

The term "terminal repeat" or "TR" includes any viral terminal repeat or synthetic sequence that forms a hairpin structure and functions as an inverted terminal repeat (*i.e.*, mediates the desired functions such as replication, virus packaging, integration and/or provirus rescue, and the like). The TR can be an AAV TR or a non-AAV TR. For example, a non-AAV TR sequence such as those of other parvoviruses (e.g., canine parvovirus (CPV), mouse parvovirus (MVM), human parvovirus B-19) or any other suitable virus sequence (*e.g.*, the SV40 hairpin that serves as the origin of SV40 replication) can be used as a TR, which can further be modified by truncation, substitution, deletion, insertion and/or addition. Further, the TR can be partially or completely synthetic, such as the "double-D sequence" as described in US Patent No. 5,478,745 to Samulski *et al.*

An "AAV terminal repeat" or "AAV TR" may be from any AAV, including but not limited to serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 or any other AAV now known or later discovered (*see, e.g.*, **Table 1**). An AAV terminal repeat need not have the native terminal repeat sequence (*e.g.*, a native AAV TR sequence may be altered by insertion, deletion,

truncation and/or missense mutations), as long as the terminal repeat mediates the desired functions, *e.g.*, replication, virus packaging, integration, and/or provirus rescue, and the like.

The virus vectors of the invention can further be "targeted" virus vectors (*e.g.*, having a directed tropism) and/or a "hybrid" parvovirus (*i.e.*, in which the viral TRs and viral capsid are from different parvoviruses) as described in international patent publication WO 00/28004 and Chao et al. (2000) *Molecular Therapy* 2:619.

The virus vectors of the invention can further be duplexed parvovirus particles as described in international patent publication WO 01/92551 (the disclosure of which is incorporated herein by reference in its entirety). Thus, in some embodiments, double stranded (duplex) genomes can be packaged into the virus capsids of the invention.

Further, the viral capsid or genomic elements can contain other modifications, including insertions, deletions and/or substitutions.

As used herein, the term "amino acid" encompasses any naturally occurring amino acid, modified forms thereof, and synthetic amino acids.

Naturally occurring, levorotatory (L-) amino acids are shown in **Table 2**.

Alternatively, the amino acid can be a modified amino acid residue (nonlimiting examples are shown in **Table 3**) and/or can be an amino acid that is modified by post-translation modification (*e.g.,* acetylation, amidation, formylation, hydroxylation, methylation, phosphorylation or sulfatation).

Further, the non-naturally occurring amino acid can be an "unnatural" amino acid as described by Wang et al. *Annu Rev Biophys Biomol Struct.* 35:225-49 (2006)). These unnatural amino acids can advantageously be used to chemically link molecules of interest to the AAV capsid protein.

Modified AAV Capsid Proteins and Virus Capsids and Virus Vectors Comprising the Same.

The present invention provides AAV capsid proteins comprising a mutation (i.e., a modification) in the amino acid sequence and virus capsids and virus vectors comprising the modified AAV capsid protein. The inventors have discovered that modifications such as substitutions at the amino acid positions described herein can confer one or more desirable properties to virus vectors comprising the modified AAV capsid protein including without limitation selective transduction of cells having heparin sulfate on the surface and enhanced transduction of cells of the retina and/or retinal pigment epithelium.

In particular embodiments, the modified AAV capsid protein of the invention comprises one or more mutations (e.g., substitutions) in the amino acid sequence of the native

AAV4 capsid protein or the corresponding region of a capsid protein from another AAV, including but not limited to AAV5. AAV7, AAV8 and AAV9.

As used herein, a "mutation" or "modification" in an amino acid sequence includes substitutions, insertions and/or deletions, each of which can involve one, two, three, four, five, six, seven, eight, nine, ten or more amino acids. In particular embodiments, the modification is a substitution. For example, in particular embodiments, the AAV4 capsid protein sequence is modified at amino acid positions 530, 584, 585, 586 and/or 587, in any combination.

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV4 capsid protein having GenBank Accession No. NP_044927 (SEQ ID NO:4):

MTDGYLPDWLEDNLSEGVREWWALQPGAPKPKANQQHQDNARGLVLPGYKYLGP
GNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQQRLQGDTS
FGGNLGRAVFQAKKRVLEPLGLVEQAGETAPGKKRPLIESPQQPDSSTGIGKKGKQP
AKKKLVFEDETGAGDGPPEGSTSGAMSDDSEMRAAAGGAAVEGGQGADGVGNAS
GDWHCDSTWSEGHVTTTSTRTWVLPTYNNHLYKRLGESLQSNTYNGFSTPWGYFD
FNRFHCHFSPRDWQRLINNNWGMRPKAMRVKIFNIQVKEVTTSNGETTVANNLTST
VQIFADSSYELPYVMDAGQEGSLPPFPNDVFMVPQYGYCGLVTGNTSQQQTDRNAF
YCLEYFPSQMLRTGNNFEITYSFEKVPFHSMYAHSQSLDRLMNPLIDQYLWGLQSTT
TGTTLNAGTATTNFTKLRPTNFSNFKKNWLPGPSIKQQGFSKTANQNYKIPATGSDSL
IKYETHSTLDGRWSALTPGPPMATAGPADSKFSNSQLIFAGPKQNGNTATVPGTLIFT
SEEELAATNATDTDMWGNLPGGDQSNSNLPTVDRLTALGAVPGMVWQNRDIYYQG
PIWAKIPHTDGHFHPSPLIGGFGLKHPPPQIFIKNTPVPANPATTFSSTPVNSFITQYSTG
QVSVQIDWEIQKERSKRWNPEVQFTSNYGQQNSLLWAPDAAGKYTEPRAIGTRYLT
HHL.

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV2 capsid protein having GenBank Accession No. YP_680426 (SEQ ID NO:2):

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPF
NGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFG
GNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTGKAGQQPA
RKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVG
NSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTP
WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIAN

NLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRT
NTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTG
ATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITD
EEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPI
WAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTG
QVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTR
NL

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV9 capsid protein having GenBank Accession No. AAS99264 (SEQ ID NO:9):

MAADGYLPDLEDNLSEGIEWWALKPGAQPKANQQHQNARGLVLPGKYLGPGNGL
DKGEPVNAADAALEHDKAYQQLKAGDNPLKYNHADAEQERLKEDTSGGNLGRAV
FQAKKRLLEPLLVEEAAKTAGKKRPVEQSQEPDSSAGIKSGAQPAKKLNFGQTGDTE
SVPDPQPIGPPAAPSGVGLTMASGGGAVADNNEGADVGSSSGNWHDSQWLGDRVIT
TSTRTWALTYNNHLYKQSNSTSGGSSDNAYFGYSTWGYFDFNRFCHFSPRDWQRLI
NNNWGFRKRLNFKLFNQVKEVTDNNVKTIANNLTTVQVFTDSDQLPYVLGSAHEGC
LPPFPAVFMIPQYGYTLNDGSQAVRSSFYCLEYPSQMLRTGNFQFSYEFENVPFHSSY
AHSSLDRLMNPLDQYLYYLSKINGSGQNQQLKFSVAGPSMAVQGRNYIPGPSYRQQ
RVTTVTQNNNSFAWPGASSWLNGRNSLMNGPAMASHKEEDRFFPLSGSLIFGKQGT
GDNVDADKVMTNEEEIKTTPVATESYGQATNHQSAQAAQTGWVQNQGILPGMVW
QDDVYLQGPIWKIPHTDGNFPSPLMGGFGKHPPPQILINTPVPADPPTAFNKDKLNSIT
QYSTGQVVEIEWELQKNSKRWNPEIYTSNYYKSNVEFAVNTEGVYSEPRPIGTYLTR
NL

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV5 capsid protein having GenBank Accession No. YP_068409 (SEQ ID NO:5):

MSFVDHPPDWLEEVGEGLREFLGLEAGPPKPKPNQQHQDQARGLVLPGYNYLGPGN
GLDRGEPVNRADEVAREHDISYNEQLEAGDNPYLKYNHADAEFQEKLADDTSFGGN
LGKAVFQAKKRVLEPFGLVEEGAKTAPTGKRIDDHFPKRKKARTEEDSKPSTSSDAE
AGPSGSQQLQIPAQPASSLGADTMSAGGGGPLGDNNQGADGVGNASGDWHCDSTW
MGDRVVTKSTRTWVLPSYNNHQYREIKSGSVDGSNANAYFGYSTPWGYFDFNRFHS
HWSPRDWQRLINNYWGFRPRSLRVKIFNIQVKEVTVQDSTTTIANNLTSTVQVFTDD
DYQLPYVVGNGTEGCLPAFPPQVFTLPQYGYATLNRDNTENPTERSSFFCLEYFPSK

MLRTGNNFEFTYNFEEVPFHSSFAPSQNLFKLANPLVDQYLYRFVSTNNTGGVQFNK
NLAGRYANTYKNWFPGPMGRTQGWNLGSGVNRASVSAFATTNRMELEGASYQVPP
QPNGMTNNLQGSNTYALENTMIFNSQPANPGTTATYLEGNMLITSESETQPVNRVAY
NVGGQMATNNQSSTTAPATGTYNLQEIVPGSVWMERDVYLQGPIWAKIPETGAHFH
PSPAMGGFGLKHPPPMMLIKNTPVPGNITSFSDVPVSSFITQYSTGQVTVEMEWELKK
ENSKRWNPEIQYTNNYNDPQFVDFAPDSTGEYRTTRPIGTRYLTRPL

In certain embodiments as described herein, amino acid numbering is based on the amino acid sequence of an AAV1 capsid protein having GenBank Accession No. NP_049542 (SEQ ID NO:1):

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQP
AKKRLNFGQTGDSESVPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGV
GNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIA
NNLTSTVQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEEVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRT
QNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWT
GASKYNLNGRESIINPGTAMASHKDDEDKFFPMSGVMIFGKESAGASNTALDNVMIT
DEEEIKATNPVATERFGTVAVNFQSSSTDPATGDVHAMGALPGMVWQDRDVYLQG
PIWAKIPHTDGHFHPSPLMGGFGLKNPPPQILIKNTPVPANPPAEFSATKFASFITQYST
GQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYL
TRPL

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV7 capsid protein having GenBank Accession No. YP_077178 (SEQ ID NO:7):

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPAKKRPVEPSPQRSPDSSTGIGKKGQQ
PARKRLNFGQTGDSESVPDPQPLGEPPAAPSSVGSGTVAAGGGAPMADNNEGADGV
GNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISSETAGSTNDNTYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKKLRFKLFNIQVKEVTTNDGVTTIA
NNLTSTIQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQSVGRSS
FYCLEYFPSQMLRTGNNFEFSYSFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLARTQ

SNPGGTAGNRELQFYQGGPSTMAEQAKNWLPGPCFRQQRVSKTLDQNNNSNFAWT
GATKYHLNGRNSLVNPGVAMATHKDDEDRFFPSSGVLIFGKTGATNKTTLENVLMT
NEEEIRPTNPVATEEYGIVSSNLQAANTAAQTQVVNNQGALPGMVWQNRDVYLQGP
IWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPANPPEVFTPAKFASFITQYSTG
QVSVEIEWELQKENSKRWNPEIQYTSNFEKQTGVDFAVDSQGVYSEPRPIGTRYLTR
NL

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV8 capsid protein having GenBank Accession No. YP_077180 (SEQ ID NO:8):

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQ
PARKRLNFGQTGDSESVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADG
VGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFG
YSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKT
IANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
SSFYCLEYFPSQMLRTGNNFQFTYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSR
TQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAW
TAGTKYHLNGRNSLANPGIAMATHKDDEERFFPSNGILIFGKQNAARDNADYSDVM
LTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRDVYLQ
GPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQYS
TGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYL
TRNL.

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV10 capsid protein having GenBank Accession No. AAT46337 (SEQ ID NO:10):

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLD61KGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQED
TSFGGNLGRAVFQ121AKKRVLEPLGLVEEAAKTAPGKKRPVEPSPQRSPDSSTGIGK
KGQQPAKKRLNFGQTGES181ESVPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNN
EGADGVGSSSGNWHCDSTWLGDRV241ITTSTRTWALPTYNNHLYKQISNGTSGGST
NDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ301RLINNNWGFRPKRLSFKLFNIQVK
EVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSA361HQGCLPPFPADVFMIPQYGY
LTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFEFSYTFED421VPFHSSYAHSQSLDR

LMNPLIDQYLYYLSRTQSTGGTQGTQQLLFSQAGPANMSAQAKNW481LPGPCYRQ
QRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSS541G
VLMFGKQGAGRDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQANTGPIVGN
VNS601QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILI
KNTPVPADP661PTTFSQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNY
YKSTNVDFAVNTE721GTYSEPRPIGTRYLTRNL.

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV11 capsid protein having GenBank Accession No. AAT46339 (SEQ ID NO:11):

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPLESPQEPDSSSGIGKKGKQPA
RKRLNFEEDTGAGDGPPEGSDTSAMSSDIEMRAAPGGNAVDAGQGSDGVGNASGD
WHCDSTWSEGKVTTTSTRTWVLPTYNNHLYLRLGTTSSSNTYNGFSTPWGYFDFNR
FHCHFSPRDWQRLINNNWGLRPKAMRVKIFNIQVKEVTTSNGETTVANNLTSTVQIF
ADSSYELPYVMDAGQEGSLPPFPNDVFMVPQYGYCGIVTGENQNQTDRNAFYCLEY
FPSQMLRTGNNFEMAYNFEKVPFHSMYAHSQSLDRLMNPLLDQYLWHLQSTTSGET
LNQGNAATTFGKIRSGDFAFYRKNWLPGPCVKQQRFSKTASQNYKIPASGGNALLK
YDTHYTLNNRWSNIAPGPPMATAGPSDGDFSNAQLIFPGPSVTGNTTTSANNLLFTSE
EEIAATNPRDTDMFGQIADNNQNATTAPITGNVTAMGVLPGMVWQNRDIYYQGPIW
AKIPHADGHFHPSPLIGGFGLKHPPPQIFIKNTPVPANPATTFTAARVDSFITQYSTGQV
AVQIEWEIEKERSKRWNPEVQFTSNYGNQSSMLWAPDTTGKYTEPRVIGSRYLTNHL

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV3B capsid protein having GenBank Accession No. NC_001863 (SEQ ID NO:3):

MAADGYLPDWLEDNLSEGIREWWALKPGVPQPKANQQHQDNRRGLVLPGYKYLG
PGNGLDKGEPVNEADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRILEPLGLVEEAAKTAPGKKRPVDQSPQEPDSSSGVGKSGKQP
ARKRLNFGQTGDSESVPDPQPLGEPPAAPTSLGSNTMASGGGAPMADNNEGADGVG
NSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTP
WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKKLSFKLFNIQVKEVTQNDGTTTIAN
NLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFQFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRT
QGTTSGTTNQSRLLFSQAGPQSMSLQARNWLPGPCYRQQRLSKTANDNNNSNFPWT

AASKYHLNGRDSLVNPGPAMASHKDDEEKFFPMHGNLIFGKEGTTASNAELDNVMI
TDEEEIRTTNPVATEQYGTVANNLQSSNTAPTTRTVNDQGALPGMVWQDRDVYLQ
GPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQIMIKNTPVPANPPTTFSPAKFASFITQY
STGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRY
LTRNL.

In certain embodiments as described herein, amino acid residue numbering is based on the amino acid sequence of an AAV12 capsid protein having GenBank Accession No. ABI16639 (SEQ ID NO:12):

MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NGRGLVLPGY
KYLGPFNGLD KGEPVNEADA AALEHDKAYD KQLEQGDNPY LKYNHADAEF
QQRLATDTSF GGNLGRAVFQ AKKRILEPLG LVEEGVKTAP GKKRPLEKTP
NRPTNPDSGK APAKKKQKDG EPADSARRTL DFEDSGAGDG PPEGSSSGEM
SHDAEMRAAP GGNAVEAGQG ADGVGNASGD WHCDSTWSEG RVTTTSTRTW
VLPTYNNHLY LRIGTTANSN TYNGFSTPWG YFDFNRFHCH FSPRDWQRLI
NNNWGLRPKS MRVKIFNIQV KEVTTSNGET TVANNLTSTV QIFADSTYEL
PYVMDAGQEG SFPPFPNDVF MVPQYGYCGV VTGKNQNQTD RNAFYCLEYF
PSQMLRTGNN FEVSYQFEKV PFHSMYAHSQ SLDRMMNPLL DQYLWHLQST
TTGNSLNQGT ATTTYGKITT GDFAYYRKNW LPGACIKQQK FSKNANQNYK
IPASGGDALL KYDTHTTLNG RWSNMAPGPP MATAGAGDSD FSNSQLIFAG
PNPSGNTTTS SNNLLFTSEE EIATTNPRDT DMFGQIADNN QNATTAPHIA
NLDAMGIVPG MVWQNRDIYY QGPIWAKVPH TDGHFHPSPL MGGFGLKHPP
PQIFIKNTPV PANPNTTFSA ARINSFLTQY STGQVAVQID WEIQKEHSKR
WNPEVQFTSN YGTQNSMLWA PDNAGNYHEL RAIGSRFLTH HL.

The modified virus capsid proteins of the invention can be but are not limited to AAV capsid proteins in which amino acids from one AAV capsid protein are substituted into another AAV capsid protein, and the substituted and/or inserted amino acids can be from any source, and can further be naturally occurring or partially or completely synthetic. Furthermore, the AAV capsid proteins of this invention can have a native amino acid sequence or a synthetic amino acid sequence.

As described herein, the nucleic acid and amino acid sequences of the capsid proteins from a number of AAVs are known in the art. Thus, for example, the amino acid(s) "corresponding" to amino acid positions 530, 584, 585, 586 and 587 of the reference AAV4 capsid protein can be readily determined for any other AAV capsid protein, including, for example, AAV5, AAV7, AAV8 and AAV9 (*e.g.*, by using sequence alignments as are well

known in the art). The amino acid positions in other AAV serotypes or modified AAV capsids that "correspond to" these positions in the native AAV4 capsid protein will be apparent to those skilled in the art and can be readily determined using sequence alignment techniques (see, e.g., Figure 7 of WO 2006/066066) and/or crystal structure analysis (Padron et al. (2005) *J. Virol.* 79:5047-58). Examples of amino acid residues that can be substituted for the native amino acid at these respective positions in other AAV serotype are set forth in **Tables 2** and **3**.

The invention contemplates that the modified capsid proteins of the invention can be produced by modifying the capsid protein of any AAV now known or later discovered. Further, the AAV capsid protein that is to be modified can be a naturally occurring AAV capsid protein (*e.g.*, an AAV4, AAV5, AAV7, AAV8, or AAV9 capsid protein or any of the AAV shown in **Table 1**) but is not so limited. Those skilled in the art will understand that a variety of manipulations to the AAV capsid proteins are known in the art and the invention is not limited to modifications of naturally occurring AAV capsid proteins. For example, the capsid protein to be modified may already have alterations as compared with naturally occurring AAV (*e.g.*, is derived from a naturally occurring AAV capsid protein, *e.g.*, AAV1, AAV2, AAV3a, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 and/or AAV12 or any other AAV serotype now known or later discovered). Such AAV capsid proteins are also within the scope of the present invention.

Thus, in particular embodiments, the AAV capsid protein to be modified can be derived from a naturally occurring AAV but further comprise one or more foreign sequences (*e.g.*, that are exogenous to the native virus) that are inserted and/or substituted into the capsid protein and/or has been altered by deletion of one or more amino acids.

Accordingly, when referring herein to a specific AAV capsid protein (*e.g.*, an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12 capsid protein or a capsid protein from any of the AAV shown in **Table 1**, *etc.*), it is intended to encompass the native capsid protein as well as capsid proteins that have alterations other than the modifications of the invention. Such alterations include substitutions, insertions and/or deletions. In particular embodiments, the capsid protein comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, less than 20, less than 30, less than 40 less than 50, less than 60, or less than 70 amino acids inserted therein (other than the insertions of the present invention) as compared with the native AAV capsid protein sequence. In embodiments of the invention, the capsid protein comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, less than 20, less than 30, less than 40 less than 50, less than

60, or less than 70 amino acid substitutions (other than the amino acid substitutions according to the present invention) as compared with the native AAV capsid protein sequence. In embodiments of the invention, the capsid protein comprises a deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, less than 20, less than 30, less than 40 less than 50, less than 60, or less than 70 amino acids (other than the amino acid deletions of the invention) as compared with the native AAV capsid protein sequence.

Thus, for example, the term "AAV4 capsid protein" includes AAV capsid proteins having the native AAV4 capsid protein sequence (*see* GenBank Accession No. NC_044927) as well as those comprising substitutions, insertions and/or deletions (as described herein) in the native AAV4 capsid protein sequence.

In particular embodiments, the AAV capsid protein has the native AAV capsid protein sequence or has an amino acid sequence that is at least about 90%, 95%, 97%, 98% or 99% similar or identical to a native AAV capsid protein sequence. For example, in particular embodiments, an "AAV4" capsid protein encompasses the native AAV4 capsid protein sequence as well as sequences that are at least about 90%, 95%, 97%, 98% or 99% similar or identical to the native AAV4 capsid protein sequence.

Methods of determining sequence similarity or identity between two or more amino acid sequences are known in the art. Sequence similarity or identity may be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, *Adv. Appl. Math.* 2, 482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch *J. Mol. Biol.* 48,443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85,2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux *et al. Nucl. Acid Res.* 12, 387-395 (1984), or by inspection.

Another suitable algorithm is the BLAST algorithm, described in Altschul et al. *J. Mol. Biol.* 215, 403-410, (1990) and Karlin et al. *Proc. Natl. Acad. Sci. USA* 90, 5873-5787 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al. *Methods in Enzymology*, 266, 460-480 (1996); blast.wustl/edu/blast/ README.html. WU-BLAST-2 uses several search parameters, which are optionally set to the default values. The parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence

and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

Further, an additional useful algorithm is gapped BLAST as reported by Altschul *et al.*, (1997) *Nucleic Acids Res.* 25, 3389-3402.

The modified virus capsids can be used as "capsid vehicles," as has been described, for example, in US Patent No. 5,863,541. Molecules that can be packaged by the modified virus capsid and transferred into a cell include heterologous DNA, RNA, polypeptides, small organic molecules, metals, or combinations of the same.

Heterologous molecules are defined as those that are not naturally found in an AAV infection, *e.g.*, those not encoded by a wild-type AAV genome. Further, therapeutically useful molecules can be associated with the outside of the chimeric virus capsid for transfer of the molecules into host target cells. Such associated molecules can include DNA, RNA, small organic molecules, metals, carbohydrates, lipids and/or polypeptides. In one embodiment of the invention the therapeutically useful molecule is covalently linked (*i.e.*, conjugated or chemically coupled) to the capsid proteins. Methods of covalently linking molecules are known by those skilled in the art.

In other embodiments, the virus capsids can be administered to block certain cellular sites prior to and/or concurrently with (*e.g.*, within minutes or hours of each other) administration of a virus vector delivering a nucleic acid encoding a polypeptide or functional RNA of interest. For example, the inventive capsids can be delivered to block cellular receptors on particular cells and a delivery vector can be administered subsequently or concurrently, which may reduce transduction of the blocked cells, and enhance transduction of other targets (*e.g.*, CNS progenitor cells and/or neuroblasts).

According to representative embodiments, modified virus capsids can be administered to a subject prior to and/or concurrently with a modified virus vector according to the present invention. Further, the invention provides compositions and pharmaceutical formulations comprising the inventive modified virus capsids; optionally, the composition also comprises a modified virus vector of the invention.

Further provided herein is an AAV capsid comprising the AAV capsid protein described herein as well as a virus vector comprising the AAV capsid. Also provided herein is a composition comprising the virus vector of this invention in a pharmaceutically acceptable carrier.

The invention also provides nucleic acid molecules (optionally, isolated nucleic acid molecules) encoding the modified virus capsids and capsid proteins of the invention. Further provided are vectors comprising the nucleic acid molecules and cells (*in vivo* or in culture) comprising the nucleic acid molecules and/or vectors of the invention. Suitable vectors include without limitation viral vectors (*e.g.*, adenovirus, AAV, herpesvirus, vaccinia, poxviruses, baculoviruses, and the like), plasmids, phage, YACs, BACs, and the like. Such nucleic acid molecules, vectors and cells can be used, for example, as reagents (*e.g.*, helper packaging constructs or packaging cells) for the production of modified virus capsids or virus vectors as described herein.

Virus capsids according to the invention can be produced using any method known in the art, *e.g.*, by expression from a baculovirus (Brown et al. (1994) *Virology* 198:477-488).

The modifications to the AAV capsid protein according to the present invention are "selective" modifications. This approach is in contrast to previous work with whole subunit or large domain swaps between AAV serotypes (*see, e.g.*, international patent publication WO 00/28004 and Hauck et al. (2003) *J. Virology* 77:2768-2774). In particular embodiments, a "selective" modification results in the insertion and/or substitution and/or deletion of less than about 20, 18, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 contiguous amino acids.

The modified capsid proteins and capsids of the invention can further comprise any other modification, now known or later identified.

For example, the AAV capsid proteins and virus capsids of the invention can be chimeric in that they can comprise all or a portion of a capsid subunit from another virus, optionally another parvovirus or AAV, *e.g.*, as described in international patent publication WO 00/28004.

The virus capsid can be a targeted virus capsid comprising a targeting sequence (*e.g.*, substituted or inserted in the viral capsid) that directs the virus capsid to interact with cell-surface molecules present on a desired target tissue(s) (*see, e.g.*, international patent publication WO 00/28004 and Hauck et al. (2003) *J. Virology* 77:2768-2774); Shi et al. *Human Gene Therapy* 17:353-361 (2006) [describing insertion of the integrin receptor binding motif RGD at positions 520 and/or 584 of the AAV capsid subunit]; and US Patent No. 7,314,912 [describing insertion of the P1 peptide containing an RGD motif following amino acid positions 447, 534, 573 and 587 of the AAV2 capsid subunit]). Other positions within the AAV capsid subunit that tolerate insertions are known in the art (*e.g.*, positions 449 and 588 described by Grifman et al. *Molecular Therapy* 3:964-975 (2001)).

In representative embodiments, the targeting sequence may be a virus capsid sequence (*e.g.*, an autonomous parvovirus capsid sequence, AAV capsid sequence, or any other viral capsid sequence) that directs infection to a particular cell type(s).

As another nonlimiting example, a heparin binding domain (*e.g.*, the respiratory syncytial virus heparin binding domain) may be inserted or substituted into a capsid subunit that does not typically bind HS receptors (*e.g.*, AAV 4, AAV5) to confer heparin binding to the resulting mutant.

In representative embodiments, the exogenous targeting sequence may be any amino acid sequence encoding a peptide that alters the tropism of a virus capsid or virus vector comprising the modified AAV capsid protein. In particular embodiments, the targeting peptide or protein may be naturally occurring or, alternately, completely or partially synthetic. Exemplary targeting sequences include ligands and other peptides that bind to cell surface receptors and glycoproteins, such as RGD peptide sequences, bradykinin, hormones, peptide growth factors (*e.g.*, epidermal growth factor, nerve growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factors I and II, *etc.*), cytokines, melanocyte stimulating hormone (*e.g.*, α, β or γ), neuropeptides and endorphins, and the like, and fragments thereof that retain the ability to target cells to their cognate receptors. Other illustrative peptides and proteins include substance P, keratinocyte growth factor, neuropeptide Y, gastrin releasing peptide, interleukin 2, hen egg white lysozyme, erythropoietin, gonadoliberin, corticostatin, β-endorphin, leu-enkephalin, rimorphin, α-neo-enkephalin, angiotensin, pneumadin, vasoactive intestinal peptide, neurotensin, motilin, and fragments thereof as described above. As yet a further alternative, the binding domain from a toxin (*e.g.*, tetanus toxin or snake toxins, such as α-bungarotoxin, and the like) can be substituted into the capsid protein as a targeting sequence. In a yet further representative embodiment, the AAV capsid protein can be modified by substitution of a "nonclassical" import/export signal peptide (*e.g.*, fibroblast growth factor-1 and –2, interleukin 1, HIV-1 Tat protein, herpes virus VP22 protein, and the like) as described by Cleves (*Current Biology* 7:R318 (1997)) into the AAV capsid protein. Also encompassed are peptide motifs that direct uptake by specific cells, *e.g.*, a FVFLP peptide motif triggers uptake by liver cells.

Phage display techniques, as well as other techniques known in the art, may be used to identify peptides that recognize any cell type of interest.

The targeting sequence may encode any peptide that targets to a cell surface binding site, including receptors (*e.g.*, protein, carbohydrate, glycoprotein or proteoglycan).

Examples of cell surface binding sites include, but are not limited to, heparan sulfate, chondroitin sulfate, and other glycosaminoglycans, sialic acid moieties, polysialic acid moieties, glycoproteins, and gangliosides, MHC I glycoproteins, carbohydrate components found on membrane glycoproteins, including, mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, fucose, galactose, and the like.

As yet a further alternative, the targeting sequence may be a peptide that can be used for chemical coupling (*e.g.*, can comprise arginine and/or lysine residues that can be chemically coupled through their R groups) to another molecule that targets entry into a cell.

The foregoing embodiments of the invention can be used to deliver a heterologous nucleic acid to a cell or subject as described herein. Thus, in one embodiment, the present invention provides a method of introducing a nucleic acid molecule into a cell, comprising contacting the cell with the virus vector and/or composition of this invention.

Further provided herein is a method of delivering a nucleic acid molecule to a subject, comprising administering to the subject the virus vector of this invention and/or the composition of this invention. In some embodiments, the virus vector or composition is administered to the central nervous system of the subject.

Additionally provided herein is a method of selectively transducing a cell having heparan sulfate on the surface, comprising contacting the cell with the virus vector of this invention and/or the composition of this invention.

The present invention further provides a method of delivering a nucleic acid molecule of interest to a cell of a retina and/or retinal pigment epithelium, comprising contacting the cell with the virus vector of this invention, wherein the virus vector comprises the nucleic acid molecule of interest. In some embodiments of this method, the nucleic acid molecule of interest encodes a therapeutic protein or therapeutic RNA.

In some embodiments of the methods described above, the cell of a retina and/or retinal pigment epithelium can be in a subject and in some embodiments, the subject can be a human subject.

The present invention further provides a method of treating a disorder or defect in the eye of a subject, comprising intravitreally administering to the subject the virus vector of this invention, wherein the virus vector comprises a nucleic acid molecule that encodes a therapeutic protein or therapeutic RNA effective in treating the disorder or defect in the eye of the subject.

In further embodiments, the present invention provides a method of selectively transducing a cell of a retina and/or retinal pigment epithelium, comprising contacting the cell with a virus vector comprising an AAV capsid protein as described herein.

Those skilled in the art will appreciate that for some AAV capsid proteins the corresponding modification will be an insertion and/or a substitution, depending on whether the corresponding amino acid positions are partially or completely present in the virus or, alternatively, are completely absent. Likewise, when modifying AAV other than AAV4, the specific amino acid position(s) may be different than the position in AAV4 (*see, e.g.*, **Table 4**, which shows a representative example of amino acid residues corresponding to S257 in AAV4). As discussed elsewhere herein, the corresponding amino acid position(s) will be readily apparent to those skilled in the art using well-known techniques.

The modifications described above can be incorporated into the capsid proteins or capsids of the invention in combination with each other and/or with any other modification now known or later discovered.

The invention also encompasses virus vectors comprising the modified capsid proteins and capsids of the invention. In particular embodiments, the virus vector is a parvovirus vector (*e.g.*, comprising a parvovirus capsid and/or vector genome), for example, an AAV vector (*e.g.*, comprising an AAV capsid and/or vector genome). In representative embodiments, the virus vector comprises a modified AAV capsid comprising a modified capsid subunit of the invention and a vector genome.

For example, in representative embodiments, the virus vector comprises: (a) a modified virus capsid (*e.g.*, a modified AAV capsid) comprising a modified capsid protein of the invention; and (b) a nucleic acid comprising a terminal repeat sequence (*e.g.*, an AAV TR), wherein the nucleic acid comprising the terminal repeat sequence is encapsidated by the modified virus capsid. The nucleic acid can optionally comprise two terminal repeats (*e.g.*, two AAV TRs).

In representative embodiments, the virus vector is a recombinant virus vector comprising a heterologous nucleic acid molecule encoding a protein, peptide and/or functional RNA of interest.

It will be understood by those skilled in the art that the modified capsid proteins, virus capsids and virus vectors of the invention exclude those capsid proteins, capsids and virus vectors that have the indicated amino acids at the specified positions in their native state (i.e., are not mutants).

Recombinant Virus Vectors.

The virus vectors of the present invention are useful for the delivery of nucleic acids to cells *in vitro*, *ex vivo*, and *in vivo*. In particular, the virus vectors can be advantageously employed to deliver or transfer nucleic acids to animal, including mammalian, cells.

Any heterologous nucleic acid sequence(s) of interest may be delivered in the virus vectors of the present invention. Nucleic acids of interest include nucleic acids encoding polypeptides, including therapeutic (*e.g.*, for medical or veterinary uses) or immunogenic (*e.g.*, for vaccines) proteins and/or functional or therapeutic RNA molecules.

Heterologous nucleic acid sequences encoding polypeptides include those encoding reporter polypeptides (*e.g.*, an enzyme). Reporter polypeptides are known in the art and include, but are not limited to, green fluorescent protein (GFP), β-galactosidase, alkaline phosphatase, luciferase, and chloramphenicol acetyltransferase gene.

Optionally, the heterologous nucleic acid encodes a secreted polypeptide (*e.g.*, a polypeptide that is a secreted polypeptide in its native state or that has been engineered to be secreted, for example, by operable association with a secretory signal sequence as is known in the art).

Alternatively, in particular embodiments of this invention, the heterologous nucleic acid may encode an antisense nucleic acid, a ribozyme (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (*see*, Puttaraju *et al.* (1999) *Nature Biotech.* 17:246; U.S. Patent No. 6,013,487; U.S. Patent No. 6,083,702), interfering RNAs (RNAi) including siRNA, shRNA or miRNA that mediate gene silencing (*see*, Sharp et al. (2000) *Science* 287:2431), and other non-translated RNAs, such as "guide" RNAs (Gorman *et al.* (1998) *Proc. Nat. Acad. Sci. USA* 95:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*), and the like. Exemplary untranslated RNAs include RNAi against a multiple drug resistance (MDR) gene product (*e.g.*, to treat and/or prevent tumors and/or for administration to the heart to prevent damage by chemotherapy), RNAi against myostatin (e.g., for Duchenne muscular dystrophy), RNAi against VEGF (*e.g.*, to treat and/or prevent tumors), RNAi against phospholamban (*e.g.*, to treat cardiovascular disease, *see, e.g.*, Andino et al. *J. Gene Med.* 10:132-142 (2008) and Li et al. *Acta Pharmacol Sin.* 26:51-55 (2005)); phospholamban inhibitory or dominant-negative molecules such as phospholamban S16E (*e.g.*, to treat cardiovascular disease, *see*, *e.g.*, Hoshijima et al. *Nat. Med.* 8:864-871 (2002)), RNAi to adenosine kinase (*e.g.*, for epilepsy), and RNAi directed against pathogenic

organisms and viruses (*e.g.*, hepatitis B and/or C virus, human immunodeficiency virus, CMV, herpes simplex virus, human papilloma virus, *etc.*).

Further, a nucleic acid sequence that directs alternative splicing can be delivered. To illustrate, an antisense sequence (or other inhibitory sequence) complementary to the 5' and/or 3' splice site of dystrophin exon 51 can be delivered in conjunction with a U1 or U7 small nuclear (sn) RNA promoter to induce skipping of this exon. For example, a DNA sequence comprising a U1 or U7 snRNA promoter located 5' to the antisense/inhibitory sequence(s) can be packaged and delivered in a modified capsid of the invention.

The virus vector may also comprise a heterologous nucleic acid that shares homology with and recombines with a locus on a host chromosome. This approach can be utilized, for example, to correct a genetic defect in the host cell.

As a further alternative, the heterologous nucleic acid can encode any polypeptide that is desirably produced in a cell *in vitro*, *ex vivo*, or *in vivo*. For example, the virus vectors may be introduced into cultured cells and the expressed gene product isolated therefrom.

It will be understood by those skilled in the art that the heterologous nucleic acid(s) of interest can be operably associated with appropriate control sequences. For example, the heterologous nucleic acid can be operably associated with expression control elements, such as transcription/translation control signals, origins of replication, polyadenylation signals, internal ribosome entry sites (IRES), promoters, and/or enhancers, and the like.

Further, regulated expression of the heterologous nucleic acid(s) of interest can be achieved at the post-transcriptional level, *e.g.*, by regulating selective splicing of different introns by the presence or absence of an oligonucleotide, small molecule and/or other compound that selectively blocks splicing activity at specific sites (*e.g.*, as described in WO 2006/119137).

Those skilled in the art will appreciate that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter/enhancer can be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced.

In particular embodiments, the promoter/enhancer elements can be native to the target cell or subject to be treated. In representative embodiments, the promoters/enhancer element can be native to the heterologous nucleic acid sequence. The promoter/enhancer element is generally chosen so that it functions in the target cell(s) of interest. Further, in particular

embodiments the promoter/enhancer element is a mammalian promoter/enhancer element. The promoter/enhancer element may be constitutive or inducible.

Inducible expression control elements are typically advantageous in those applications in which it is desirable to provide regulation over expression of the heterologous nucleic acid sequence(s). Inducible promoters/enhancer elements for gene delivery can be tissue-specific or –preferred promoter/enhancer elements, and include muscle specific or preferred (including cardiac, skeletal and/or smooth muscle specific or preferred), neural tissue specific or preferred (including brain-specific or preferred), eye specific or preferred (including retina-specific and cornea-specific), liver specific or preferred, bone marrow specific or preferred, pancreatic specific or preferred, spleen specific or preferred, and lung specific or preferred promoter/enhancer elements. Other inducible promoter/enhancer elements include hormone-inducible and metal-inducible elements. Exemplary inducible promoters/enhancer elements include, but are not limited to, a Tet on/off element, a RU486-inducible promoter, an ecdysone-inducible promoter, a rapamycin-inducible promoter, and a metallothionein promoter.

In embodiments wherein the heterologous nucleic acid sequence(s) is transcribed and then translated in the target cells, specific initiation signals are generally included for efficient translation of inserted protein coding sequences. These exogenous translational control sequences, which may include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

The virus vectors according to the present invention provide a means for delivering heterologous nucleic acids into a broad range of cells, including dividing and non-dividing cells. The virus vectors can be employed to deliver a nucleic acid of interest to a cell *in vitro*, *e.g.*, to produce a polypeptide *in vitro* or for *ex vivo* gene therapy. The virus vectors are additionally useful in a method of delivering a nucleic acid to a subject in need thereof, *e.g.*, to express an immunogenic or therapeutic polypeptide or a functional RNA. In this manner, the polypeptide or functional RNA can be produced *in vivo* in the subject. The subject can be in need of the polypeptide because the subject has a deficiency of the polypeptide. Further, the method can be practiced because the production of the polypeptide or functional RNA in the subject may impart some beneficial effect.

The virus vectors can also be used to produce a polypeptide of interest or functional RNA in cultured cells or in a subject (*e.g.*, using the subject as a bioreactor to produce the polypeptide or to observe the effects of the functional RNA on the subject, for example, in connection with screening methods).

In general, the virus vectors of the present invention can be employed to deliver a heterologous nucleic acid encoding a polypeptide or functional RNA to treat and/or prevent any disease state for which it is beneficial to deliver a therapeutic polypeptide or functional RNA. Illustrative disease states of this invention include, but are not limited to, age-related macular degeneration, Lebers congenital amarousis type 1, Lebers, congenital amarousis type 2, retinitis pigmentosa, retinoschosis, achromatopsia, color blindness, congenital stationary night blindness or any combination thereof.

Gene transfer has substantial potential use for understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, which may involve regulatory or structural proteins, and which are typically inherited in a dominant manner. For deficiency state diseases, gene transfer can be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer can be used to create a disease state in a model system, which can then be used in efforts to counteract the disease state. Thus, virus vectors according to the present invention permit the treatment and/or prevention of genetic diseases.

The virus vectors according to the present invention may also be employed to provide a functional RNA to a cell *in vitro* or *in vivo*. Expression of the functional RNA in the cell, for example, can diminish expression of a particular target protein by the cell. Accordingly, functional RNA can be administered to decrease expression of a particular protein in a subject in need thereof. Functional RNA can also be administered to cells *in vitro* to regulate gene expression and/or cell physiology, *e.g.*, to optimize cell or tissue culture systems or in screening methods.

In addition, virus vectors according to the instant invention find use in diagnostic and screening methods, whereby a nucleic acid of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

In some embodiments, the virus vectors of the present invention can be used to induce an immune response in a subject and the virus vector can comprise a nucleotide sequence that encodes an immunogen.

The virus vectors of the present invention can also be used for various non-therapeutic purposes, including but not limited to use in protocols to assess gene targeting, clearance, transcription, translation, *etc.*, as would be apparent to one skilled in the art. The virus

vectors can also be used for the purpose of evaluating safety (spread, toxicity, immunogenicity, *etc.*). Such data, for example, are considered by the United States Food and Drug Administration as part of the regulatory approval process prior to evaluation of clinical efficacy.

Alternatively, the virus vector may be administered to a cell *ex vivo* and the altered cell is administered to the subject. The virus vector comprising the heterologous nucleic acid is introduced into the cell, and the cell is administered to the subject, where the heterologous nucleic acid can be expressed in the subject.

Subjects, Pharmaceutical Formulations, and Modes of Administration.

Virus vectors and capsids according to the present invention find use in both veterinary and medical applications. Suitable subjects include both avians and mammals. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys, pheasant, parrots, parakeets, and the like. The term "mammal" as used herein includes, but is not limited to, humans, non-human primates, rodents, bovines, ovines, caprines, equines, felines, canines, lagomorphs, etc. Human subjects include neonates, infants, juveniles, adults and geriatric subjects.

In representative embodiments, the subject is "in need of" the methods of the invention.

In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus vector and/or capsid of the invention in a pharmaceutically acceptable carrier and, optionally, other medicinal agents, pharmaceutical agents, stabilizing agents, buffers, carriers, adjuvants, diluents, *etc.* For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and optionally can be in solid or liquid particulate form.

By "pharmaceutically acceptable" it is meant a material that is not toxic or otherwise undesirable, *i.e.*, the material may be administered to a subject without causing any undesirable biological effects.

One aspect of the present invention is a method of transferring a nucleic acid to a cell *in vitro*. The virus vector may be introduced into the cells at the appropriate multiplicity of infection according to standard transduction methods suitable for the particular target cells. Titers of virus vector to administer can vary, depending upon the target cell type and number, and the particular virus vector, and can be determined by those of skill in the art without

undue experimentation. In representative embodiments, at least about 10^3 infectious units, optionally at least about 10^5 infectious units are introduced to the cell.

The cell(s) into which the virus vector is introduced can be of any type, including but not limited to cells of the eye (including retinal cells, retinal pigment epithelium, and corneal cells).

The virus vector can be introduced into cells *in vitro* for the purpose of administering the modified cell to a subject. In particular embodiments, the cells have been removed from a subject, the virus vector is introduced therein, and the cells are then administered back into the subject. Methods of removing cells from subject for manipulation *ex vivo*, followed by introduction back into the subject are known in the art (*see, e.g.*, U.S. patent No. 5,399,346). Alternatively, the recombinant virus vector can be introduced into cells from a donor subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof (*i.e.*, a "recipient" subject).

Suitable cells for *ex vivo* nucleic acid delivery are as described above. Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about 10^2 to about 10^8 cells or at least about 10^3 to about 10^6 cells will be administered per dose in a pharmaceutically acceptable carrier. In particular embodiments, the cells transduced with the virus vector are administered to the subject in a treatment effective or prevention effective amount in combination with a pharmaceutical carrier.

A further aspect of the invention is a method of administering the virus vector and/or virus capsid to a subject. Administration of the virus vectors and/or capsids according to the present invention to a human subject or an animal in need thereof can be by any means known in the art, but in particular embodiments, administration is intravitreal. Optionally, the virus vector and/or capsid is delivered in a treatment effective or prevention effective dose in a pharmaceutically acceptable carrier.

Dosages of the virus vector and/or capsid to be administered to a subject depend upon the mode of administration, the disease or condition to be treated and/or prevented, the individual subject's condition, the particular virus vector or capsid, and the nucleic acid to be delivered, and the like, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^3, 10^{14}, 10^{15} transducing units, optionally about $10^8 - 10^{13}$ transducing units.

In particular embodiments, more than one administration (*e.g.*, two, three, four or more administrations) may be employed to achieve the desired level of gene expression over a period of various intervals, *e.g.*, daily, weekly, monthly, yearly, *etc.*

Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus vector and/or virus capsids of the invention in a local rather than systemic manner, for example, in a depot or sustained-release formulation. Further, the virus vector and/or virus capsid can be delivered adhered to a surgically implantable matrix (*e.g.*, as described in U.S. Patent Publication No. US-2004-0013645-A1).

Disorders of the CNS include ophthalmic disorders involving the retina, posterior tract, and optic nerve (*e.g.*, retinitis pigmentosa, diabetic retinopathy and other retinal degenerative diseases, uveitis, age-related macular degeneration, glaucoma).

Most, if not all, ophthalmic diseases and disorders are associated with one or more of three types of indications: (1) angiogenesis, (2) inflammation, and (3) degeneration. The delivery vectors of the present invention can be employed to deliver anti-angiogenic factors; anti-inflammatory factors; factors that retard cell degeneration, promote cell sparing, or promote cell growth and combinations of the foregoing.

Diabetic retinopathy, for example, is characterized by angiogenesis. Diabetic retinopathy can be treated by delivering one or more anti-angiogenic factors either intraocularly (*e.g.*, in the vitreous) or periocularly (*e.g.*, in the sub-Tenon's region). One or more neurotrophic factors may also be co-delivered, either intraocularly (*e.g.*, intravitreally) or periocularly.

Uveitis involves inflammation. One or more anti-inflammatory factors can be administered by intraocular (*e.g.*, vitreous or anterior chamber) administration of a delivery vector of the invention.

Retinitis pigmentosa, by comparison, is characterized by retinal degeneration. In representative embodiments, retinitis pigmentosa can be treated by intraocular (*e.g.*, vitreal administration) of a delivery vector encoding one or more neurotrophic factors.

Age-related macular degeneration involves both angiogenesis and retinal degeneration. This disorder can be treated by administering the inventive deliver vectors encoding one or more neurotrophic factors intraocularly (*e.g.*, vitreous) and/or one or more anti-angiogenic factors intraocularly or periocularly (*e.g.*, in the sub-Tenon's region).

Glaucoma is characterized by increased ocular pressure and loss of retinal ganglion cells. Treatments for glaucoma include administration of one or more neuroprotective agents that protect cells from excitotoxic damage using the inventive delivery vectors. Such agents include N-methyl-D-aspartate (NMDA) antagonists, cytokines, and neurotrophic factors, delivered intraocularly, optionally intravitreally.

In particular embodiments, the vector can comprise a secretory signal as described in U.S. Patent No. 7,071,172.

The virus vector and/or capsid may also be administered to different regions of the eye such as the retina, cornea and/or optic nerve.

In particular embodiments, the virus vector and/or capsid is administered in a liquid formulation by direct injection (*e.g.*, stereotactic injection) to the desired region or compartment in the CNS. In other embodiments, the virus vector and/or capsid may be provided by topical application to the desired region or by intra-nasal administration of an aerosol formulation. Administration to the eye, may be by topical application of liquid droplets. As a further alternative, the virus vector and/or capsid may be administered as a solid, slow-release formulation (*see, e.g.,* U.S. Patent No. 7,201,898).

Having described the present invention, the same will be explained in greater detail in the following examples, which are included herein for illustration purposes only, and which are not intended to be limiting to the invention.

EXAMPLES

EXAMPLE 1.

Recombinant adeno-associated virus (rAAV) has become the preferred vector for retinal gene transfer. Delivery of rAAV to the retina through the vitreous results in few serotypes efficiently transducing the retina. These few serotypes have capsid proteins which bind to heparan sulfate proteoglycan (HSPG). The interaction between capsid and receptor was evaluated using rAAV capsid with modified receptor interactions. Viruses were delivered intravitreally in adult mice and evaluated eight weeks later. Mutations in heparan sulfate (HS) binding residues of rAAV2 led to a dramatic decrease in the transduction of the inner retina. Elements of the non-HS binding rAAV1 were added to rAAV2 using the designer chimeric capsid, rAAV2.5. rAAV2.5 transduced along retinal vessels and showed greater expression in Muller glia cells. The addition of HS binding to rAAV1 showed an increase in fluorescence which resembled the expression pattern observed with rAAV2.5. The dual receptor interaction of HS and galactose was evaluated using a recently designed

chimeric capsid, rAAV2G9, to determine if the addition of galactose binding would enhance capsid transduction. rAAV2G9 revealed an altered fluorescence pattern by fundus compared to rAAV2. The transduction profile of rAAV2G9 showed a shift in tropism to Muller glia. Taken together, HS binding is essential for successful intravitreal transduction, and this transduction can be skewed to specific retinal cells by the addition of galactose binding.

EXAMPLE 2. Heparan Sulfate Binding Promotes Accumulation of Intravitreally-Delivered Adeno-Associated Viral Vectors at the Retina for Enhanced Transduction

Many adeno-associated virus (AAV) serotypes efficiently transduce the retina when delivered to the subretinal space, but show limited success when delivered to the vitreous due to the inner limiting membrane (ILM). Subretinal delivery of rAAV2 and its HS-binding-deficient capsid indicated rAAV2 transduction of the outer retina occurred by HS-independent mechanisms. However, intravitreal delivery of HS-ablated rAAV2 lead to a 300-fold decrease in transduction compared to rAAV2. Fluorescence *in situ* hybridization (FISH) of AAV retinal trafficking revealed a mechanism of AAV2 accumulation at the ILM was influenced by HS binding. This mechanism was tested on human *ex vivo* retinas and showed similar accumulation only with HS-binding AAV2 capsid. To evaluate if HS binding could be applied to other AAV serotypes to enhance their transduction, AAV1 and AAV8 were modified to bind HS with a single amino acid mutation and tested in mice. Both HS-binding mutants of AAV1 and AAV8 had higher intravitreal transduction over their non-HS-binding parent capsid. To understand the influence HS binding has on AAV2 tropism, chimeric capsids with dual glycan usage were tested intravitreally in mice. Compared to HS binding alone, these chimeric capsids displayed enhanced transduction that was correlated to a change in tropism. Taken together, this indicates that HS-binding serves to sequester AAV capsids from the vitreous to the ILM, but does not influence tropism. The enhanced retinal transduction of HS-binding capsids provides a rational design strategy for engineering capsids across species for intravitreal delivery.

The present studies show that HSPG binding is correlated in greater accumulation and transduction in the retina. We validated that this accumulation is conserved in mouse and human retinas. The addition of HSPG binding to any AAV capsid can increase the number of serotypes which show efficient intravitreal transduction.

Adeno-associated virus (AAV) is a small (25 nm), non-pathogenic virus that has been extensively studied as a vector for gene transfer applications. The virus consists of two parts: the viral genome and the protein capsid. The viral genome can be largely replaced with a

desired transgene to create recombinant AAV (rAAV) vectors used for gene delivery. The protein capsid is responsible for cell attachment and entry via a variety of glycans and cell surface receptors. There exist eleven naturally-occurring serotypes of AAV, denoted as AAV1 to AAV11. Glycans and receptors have been elucidated for several AAV serotypes. Heparan sulfate proteoglycan (HSPG) has been shown to be used for both rAAV2 and rAAV3 cell entry. rAAV6 displays dual glycan interaction with HSPG and sialic acid; however, HSPG binding alone is insufficient for cellular entry. Various linkages of sialic acid are important for the transduction of rAAV1, rAAV4, and rAAV5 serotypes. N-linked galactose is used for the transduction of rAAV9 serotype. Glycans expressed on the cell surface dictate the tissue and cellular tropism observed with the various AAV capsids. In addition to the attachment to these glycans, AAV serotypes interact with cell receptors for entry, including human growth factor receptor, integrins, and laminin receptors.

rAAV has shown promise for retinal gene transfer. In addition to the influence of the capsid as discussed above, the route of administration to the retina determines the transgene expression profile and efficacy. Subretinal (SR) delivery deposits vector between the outer nuclear layer (ONL) and retinal pigment epithelium (RPE), which causes a detachment of these two layers to accommodate the injected solution. Many serotypes display transduction in the ONL and RPE layers with some serotypes showing restricted tropism. Of the natural serotypes, rAAV8 is one of the best for SR delivery based on its rapid transgene expression and transduction of all retinal layers. In addition to SR delivery, vectors can be administered to the vitreous. Intravitreal delivery of rAAV vectors has become the preferred route to subretinal for several reasons, including 1) technical ease of injection, 2) the potential to deliver vector to a greater area of the retina, and 3) it's less damaging to the retina. For clinical applications, intravitreal delivery could be performed as an outpatient procedure and circumvent the retinal disruption may exclude applicable to patients with severe retinal degeneration. However, few serotypes exhibit efficient transduction by this route. rAAV2 is one of the few serotypes tested in multiple animal models, typically resulting in the transduction of retinal ganglion cells (RGC). In rodent models, transduction with this serotype has been seen in occasional Müller glia, amacrine, and horizontal cells. In addition, rAAV6 expression in the RGC and inner nuclear layer (INL) has been seen in rodent models. Understanding viral trafficking and barriers to efficient intravitreal transduction provides opportunities to rationally design capsids to overcome current limitations.

At the vitreoretinal junction, the inner limiting membrane (ILM) has been implicated as the barrier responsible for the inefficiency of most rAAV vectors to transduce the retina.

Despite the limited transduction, several AAV serotypes are capable of accumulating at the vitreoretinal junction following delivery. Injections of fluorescently-labeled capsids (rAAV1, 2, 5, 8, and 9) into the vitreous of adult rodents showed rAAV2, rAAV8, and rAAV9 accumulated at the ILM, but only rAAV2 resulted in transduction. With a degenerated ILM, all of these AAV serotypes were capable of transducing the retina. The ILM is composed of the extracellular matrix of the Müller glial endfeet which displays an array of glycans similar to other basement membranes and prevents access to cells needed for AAV transduction. The binding of rAAV2, rAAV8, and rAAV9 is likely explained by laminin interaction; however, accumulation by laminin is not sufficient for transduction of rAAV8 and rAAV9. HSPG seems to explain the rAAV2 transduction, but enzymatic digestion of HSPG increases transduction and penetration of rAAV2 in the retina. Because rAAV2 has shown HSPG-independent transduction in other tissue, it is possible that rAAV2 does not need HSPG binding for retinal transduction and that HSPG may prevent the spread of rAAV2 particles to the outer retina. To this end, rAAV2 capsid interactions with HSPG at the ILM pose the rate-limiting step to efficient intravitreal transduction of the retina and understanding these interactions will help guide rational design of vectors for more efficient intravitreal delivery.

We used a self-complementary CBh-GFP cassette for optimized transgene performance. The CBh promoter has shown exceptional activity in other neuronal tissue compared to CMV or CBA promoter activity without potential silencing issues, and its small size is beneficial for maximizing the limited transgene capacity of rAAV. The self-complementary form of the transgene facilitates faster expression that is more robust than the classic single-stranded form. This self-complementary form can also facilitate production of transgene product in cells that do not provide second-strand synthesis. In addition to optimizing GFP production, we used FISH to track rAAV capsids following intravitreal delivery and obtain an accurate picture of the trafficking. Genetic capsid mutations were used to understand the role of HS binding to rAAV transduction of the mouse retina without modifying the ILM structure. We used known capsids mutations in the HS-binding footprint of rAAV2 to ablate HS binding. The motif on the rAAV2 capsid consists of a basic patch of residues (R484, R487, K532, R585, and R588) at the base of the three-fold spike. Capsid mutants, like rAAV2i8, replace residues 585Q and 588T to alter tropism away from HS-rich liver tissue and become more systemic when delivered intravenously. Using this capsid, we investigated the unilateral necessity for HS binding for retinal transduction.

Vector production and purification. Self-complementary rAAV carrying the GFP gene under the control of the ubiquitous CBh promoter was produced by a triple transfection

method using polyethylenimine. Viruses were harvested. Lysate was clarified by centrifugation at 6200x*g* and purified by iodixanol gradient ultracentrifugation at 402,000x*g* for 1 hour. Viruses were pulled from the 40%/60% interface, purified by ion-exchange chromatography on a 1-ml Q HyperD F column (Pall) and eluted with 200 mM NaCl, 25 mM Tris [pH 9.0]. AAV8-E533K vector was difficult to produce in significant yield by iodixanol. Therefore, AAV8 and AAV8-E533K were purified by CsCl and then by sucrose to obtain pure vector. Viruses were dialyzed against 350mM NaCl, 5% sorbitol in 1xPBS before being aliquoted and frozen at -80°C. Viral titer was determined by qPCR against wild-type ITR of DNase-resistant vector genomes relative to a virus standard. Viruses underwent electrophoresis on a 1% Bis-Tris gel (Novex) and silver stain (Life Technologies) to assess purity.

Animal injections. Adult C57BL/6 mice were used for this study. All animals were housed under 12/12 hours light/dark cycle in the University of North Carolina Division of Laboratory Animal Medicine facilities and were handled in accordance within the guidelines of the Institutional Animal Care and Use Committee at the University of North Carolina. Prior to vector delivery, animals were anesthetized with ketamine (75 mg/kg), xylazine (10 mg/kg), acepromazine (1.5 mg/kg), and dilated with 1% tropicamide and 2.5% phenylephrine. Proparacaine-HCl was applied to eyes as a local anesthetic. Intraocular needles were constructed using 32G canula connected to a Hamilton syringe via tubing filled with water. An air bubble separated the water from the viral suspension. Freshly thawed viruses were diluted to working stock and incubated in the intraocular needle at room temperature for 10 minutes prior to injection. Needles were evacuated and loaded with fresh suspension. Viral suspension was mixed with fluorescein sodium salt (Sigma) to confirm successful injection. All injections were carried out by the same surgeon. For intravitreal injections, a pilot hole was made with the tip of a beveled 30G needle in the superior portion of the eye approximately 0.5 mm posterior to the limbus. The intraocular needle was inserted through this hole into the vitreous under direct observation through the microscope. A volume of 1 microliter was delivered at a constant rate over 30 seconds using a syringe pump. The needle was held in place for 20 seconds to allow for intraocular pressure equilibration before removal. For subretinal injection, the intraocular needle was inserted tangential to the eye. Delivery of fluid was immediate and characterized for success by optical coherence tomography (OCT) and fundoscopy using the Micron IV (Phoenix Research Laboratories).

GENTEAL eye drops were applied to eyes to prevent corneal drying, and mice were allowed to recover on heating pads.

***In vivo* imaging.** Fundus images and OCT were carried out by dilating and sedating animals as described herein. All fluorescence images were taken under the same settings and similar retinal position using the Micron IV. The green channel of the fluorescent fundus image was isolated, converted to grayscale, and quantified by integrated density measurements using ImageJ software (National Institutes of Health).

Enucleation and histology. Animals were sedated and perfused with PBS containing 1 unit heparin per ml, followed by 4% paraformaldehyde (PFA) in PBS. Eyes were enucleated and a puncture was made anterior to the limbus using an 18G needle before incubating in 4% PFA for 10 minutes. The anterior segment, musculature, and lens were removed and eyecups were placed in 10% sucrose at 4°C overnight followed by 20% and 30% sucrose incubations. Eyecups were embedded in OCT cutting media (Sakura), frozen at -20°C, and stored at -80°C. Ten micron transverse sections were collected on precleaned Superfrost Plus slides (Fisher) and stored at -80°C until further processing.

Immunohistochemistry and analysis. Sections were washed in TBS containing 0.3% Tween-20 (TBS-T) and incubated in blocking buffer (10% normal goat serum, 0.1% Triton-x 100 in PBS) for 1 hour in a humid chamber. Slides were incubated in antibody solution (3% NGS, 0.1% TRITON-X100 in PBS) with primary antibodies in a humid chamber overnight at room temperature. Primary antibodies used were rabbit anti-GFP (1:500; Millipore), mouse anti-glutamine synthetase (1:100; Abcam), mouse anti-heparan sulfate 10E4 epitope (1:70; Amsbio), mouse anti-rhodopsin (1:100; Rockland), and mouse anti-PKC alpha [H-7](1:250; Santa Cruz). After three washes with TBS-T, secondary fluorescent antibodies were applied in antibody solution for 2 hours in a humid chamber. Secondary antibodies were Alexa-Fluor 488 goat anti-rabbit (1:1000; Molecular Probes), Alexa-Fluor 568 goat anti-mouse (1:1000; Molecular Probes), or Alexa Fluor 568 rabbit anti-goat (1:1000; Molecular Probes). Slides were mounted in Prolong Gold Antifade with DAPI (Molecular Probes) as stated by manufacturer's protocol. Images were taken on a LeicaSP2 AOBS Upright Laser Scanning Confocal microscope or Olympus IX83 fluorescence microscope.

Soluble HS analog assays. For *in vitro* studies, HEK293 cells were plated in a 24-well dish at a density of 10^5 cells per well and allowed to adhere overnight at 37°C, 5% CO_2. Viruses were pre-incubated with soluble heparin at the specified concentrations for 1 hour

prior to the addition to cells at a concentration of 10,000 vg per cell. Cells were harvested 48 hours later and quantified by flow cytometry.

Fluorescence *in situ* hybridization. The GFP gene was cloned into the pSPT18 vector (Roche RNA in vitro transcription kit) at the HindIII and EcoRI sites and sequenced for confirmation. Plasmids were linearized with these restriction enzymes and purified by phenol-chloroform extraction/ethanol precipitation and resuspended in water. Linearized plasmids were quantified by spectrophotometry and verified by gel electrophoresis before *in vitro* transcription of antisense and sense riboprobes were carried as described by the manufacturer (Roche). Aliquots of riboprobes were frozen in water and were quantified as described by the manufacturer and analyzed by gel electrophoresis and SYBR Gold staining (Invitrogen). Riboprobe functionality was assayed for sensitivity and selectivity by dot blot of virus controls to a positively-charged nitrocellulose membrane (Roche). Both sense and antisense probe were able to detect viral GFP transgene equally.

Frozen slides were heated to 55°C for 10 minutes and pre-treated. Slides were then incubated in hybridization buffer (50% formamide, 10 mM Tris [pH 7.6], 200 µg/ml yeast tRNA, 1x Denhardt's solution, 10% dextran sulfate, 600mM NaCl, 0.25% SDS, 1mM EDTA [pH 8]) without probe at hybridization temperature of 65°C for 2 to 4 hours. Slides were transferred to prehybridization buffer containing 50 ng/ml of sense riboprobe to specifically detect DNA and not mRNA transcripts. Slides were heated to 80°C for 20 minutes, snap chilled on ice, and incubated overnight at 65°C. Slides were washed in 50% formamide/2x SSC at 65°C for 30 minutes, 2x SSC at 55°C for 20 minutes, and two washes of 0.2x SSC at 55°C for 20 minutes. Slides were washed in 1x Washing Buffer (Roche) followed by incubation of 10% sheep serum in 1x Blocking Buffer for 1 hour in a humid chamber. Sheep anti-DIG-AP antibody (1:1000; Roche) was applied and incubated 2 to 3 hours in a humid chamber. Slides were washed three times in Washing Buffer with gentle agitation for 10 minutes followed by two incubations in Detection Buffer (100 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$ [pH 8.0]) for 10 minutes. HNPP/Fast Red detection substrate was prepared and applied as directed by the manufacturer (Roche) for two to three applications. Following the detection reaction, slides were rinsed in distilled water and coverslipped using Prolong Gold antifade reagent with DAPI. Images were taken on a LeicaSP2 AOBS Upright Laser Scanning Confocal microscope or an Olympus IX83 fluorescence microscope.

***Ex vivo* human retinal binding assay.** Human whole globes were procured immediately after death and placed in a moist chamber on ice for 4 days. The anterior

chamber, iris, and lens were removed and the globe was quartered with some vitreous remaining attached to the retina. Ten µl of vector was applied into the vitreous at a titer of $2x10^9$ vg/µl and allowed to bind to the retina for 2 hours at 4°C. The quartered retinas were kept out of media to prevent the dispersion of vector solution. Following the incubation, PBS was washed over the tissue, collected, and stored at -80°C. Vitreous, retina, choroid, and sclera were collected separately and stored at -80°C. Tissue samples were digested and purified using the DNeasy Blood & Tissue kit (Qiagen). Virus in the collected samples was quantified by qPCR using primers against GFP and hGAPDH housekeeping genes.

HS binding on rAAV2 is not required for subretinal transduction. A variety of AAV serotypes, both HS binding and non-HS binding, work effectively in retinal transduction when delivered subretinally. To determine if rAAV2 requires HS binding in the transduction of the outer retina, HS-deficient rAAV2i8 and rAAV2 were subretinally delivered. Transduction between them was similar by fundoscopy. The strongest signal of the GFP fluorescence with rAAV2i8 was seen within the detached area but expression could be seen outside the bleb area. Similarly, rAAV2-injected eyes showed that transduction occurred mainly in the area of the detachment. Both vectors resulted in large areas of transduction that appeared to be the RPE. For rAAV2, transduction of ganglion cells was evident by the fluorescent axons leading to the optic head from the site of injection.

Immunohistochemistry (IHC) was used to evaluate the cell tropism of both vectors. The RPE and ONL were the major cell layers transduced by both vectors. Areas could be seen where high RPE transduction but low ONL transduction occurred, indicating that RPE may be the predominant cell type to be transduced. Transduction of the ONL occurred predominately in rods for both rAAV2 and rAAV2i8 capsids. rAAV2i8 transduction of cells in the INL was identified as rod bipolar cells and Müller glia. These results confirm that HS-deficient rAAV2 capsid is infectious in the retina by subretinal delivery.

HS binding of rAAV2 is required for intravitreal transduction. To assess whether HS-binding is necessary for intravitreal transduction, AAV2 and AAV2i8 capsids were delivered to adult mice at a titer of 10^8 vg. rAAV2-injected eyes were fluorescent at the first imaging time point of two weeks whereas rAAV2i8 showed no expression. Eyes were evaluated for up to twelve weeks for the possibility of slower expression kinetics. During that time, rAAV2 fluorescence continued to increase, but no fluorescence was detected with rAAV2i8. By twelve weeks, rAAV2 capsid leads to a diffuse pattern of fluorescence over the neural retina as seen by fundus imaging. The rAAV2i8 capsid did not yield observable GFP

fluorescence by fundoscopy and resulted in a 300-fold reduction in GFP fluorescence (**Fig. 1**). We confirmed that the vitreous was not inhibitory to the transduction of the HS-ablated rAAV2 capsid by mixing vitreous and virus before injecting subretinally. Again, rAAV2 and rAAV2i8 had intense expression throughout most of the retina observed on fundoscopy. The transduced cells for both capsids appeared to be RPE, but the addition of RGCs can be seen with rAAV2.

Eyes injected intravitreally with rAAV were further evaluated by IHC. rAAV2 transduction was mainly detected in the RGC and INL, and in some sections, transduction of photoreceptors could be observed. The histology of HS-ablated rAAV2 capsids revealed very few GFP-positive rods, but most of the retina remained negative. We tried a higher titer of $2x\ 10^9$ vg for both rAAV2 and its HS-binding mutant to maximize the chance to observe expression. The expression was much greater with higher titered rAAV2 virus compared to what was observed with the lower titer-injected eyes, but the pattern of transduction was unchanged.

Fluorescence *in situ* hybridization (FISH) was used to determine the distribution of transgenes between the two capsids after intravitreal delivery. Studies of subretinally-injected virus have shown that virus distribution and transgene expression are not synonymous; therefore, we wanted to determine how HS binding affected rAAV2 distribution irrespective of expression. Similar to the IHC expression, FISH signal for rAAV2-delivered transgenes was detected mainly in the RGC and INL, with fewer transgenes in the ONL. rAAV2i8-injected eyes showed GFP transgenes present in the ONL, but none were detected in the RGC or INL. Because histology was performed months after injection, these transgenes most likely represent episomes that are stable following entry into the retina by intravitreal delivery.

HS binding is necessary for the vitreal accumulation of rAAV2 at the ILM in mice. To better understand the trafficking differences between the capsids following intravitreal delivery, eyes were enucleated soon after injection for FISH analysis. Because we have established that exposure of charged residues on the capsid surface are important in transduction, we used FISH as an alternative to modifying the capsid with fluorescent particles for trafficking experiments. That AAV particles accumulate at the ILM 24 hours post-injection was confirmed using our FISH protocol and this indicated we could detect transgenes carried by capsids that were still intact. The time point was extended to three days post-injection to allow sufficient time for capsid accumulation at the ILM and observe trafficking differences between the rAAV2 capsid and its HS-binding mutants. A range of

doses was used to capture any concentration effect in accumulation and the enzymatic time for FISH signal was shortened to give a more dynamic range. PBS-injected eyes served as the negative control which had minimal background labeling. With the shortened detection time, a dose of $1x10^8$ vg had only weak signal in retinas for both rAAV2 and HS-deficient rAAV2-R585E capsids. At a dose of $5x10^8$ vg, transgenes delivered by rAAV2 showed an accumulation at the ILM, as well as being present in all retinal layers. Without HS binding, rAAV2-R585E had only minimal signal. At the highest dose tested of $2x10^9$ vg, rAAV2 resulted in even greater signal intensity at the ILM with sporadic transgenes detected in multiple retinal layers. At the same dose, few rAAV2-R585E-delivered transgenes were detected in the retina but did not result in any accumulation at the ILM.

Taken together, these results indicate 1) that HS binding on rAAV2 helps to accumulate vectors at the ILM, 2) that this accumulation increases the number of transgenes residing in the retina, 3) that capsids can penetrate the retina from the vitreous without binding to HS but to a far less extent, and 4) that HS binding is not required for rAAV2 transduction of the retina when vector is delivered subretinally. The FISH data indicate that of the capsids that quickly pass through the ILM barrier, they seem capable of trafficking rapidly to distal layers of the outer retina (**Fig. 2**). This highlights that rAAV's rate-limiting step to efficient intravitreal transduction of the retina lies with the interaction between capsid and ILM.

HS binding is necessary for the vitreal accumulation of rAAV2 on human retinas. The abundant HSPG staining at the ILM is present in many animal models, including humans. This suggests that this mechanism may translate across species for human clinical applications. A viral binding assay was done on human retinas *ex vivo* by quartering the eye and leaving a small amount of vitreous attached to the retina to maintain the ILM. Vectors were applied into the vitreous and then the various retinal layers were harvested. Transgenes carried by rAAV2 were bound to the retina, unlike those of the rAAV2i8 capsid. The HS-deficient rAAV2i8 had relatively low vector binding in any of the collected tissue, but did show a significant increase in binding to the choroid and sclera compared to rAAV2 (**Fig. 3**). Together with the mouse data, these results corroborate the mechanism of HS binding promoting the accumulation of AAV vector out of the vitreous and onto the ILM and later serves to enhance the transduction in the retina.

HS binding increases the intravitreal transduction of other rAAV serotypes. Intravitreal transduction of other serotypes may benefit from the addition of a HS-binding motif and carried out this selection in mice. The rAAV1 and rAAV6 serotypes differ by only

six amino acids, with a single residue responsible for their difference in HSPG binding. To evaluate the influence of HS binding between rAAV1 and rAAV6 retinal transduction for intravitreal transduction, the single residue mutant capsids were tested intravitreally. Although rAAV1 and the HS binding rAAV1-E531K had similar patterns of expression, rAAV1-E531K had 3-fold greater GFP fluorescence compared to rAAV1 (**Fig. 4**). The removal of HS-binding in rAAV6 using the rAAV6-K531E capsid led to a reduction in retinal fluorescence by fundoscopy. Both rAAV1 and rAAV6 capsids displayed a punctate expression pattern around the retinal vessels. Because of the homology between rAAV1 and rAAV6, only rAAV1 and rAAV1-E531K were further evaluated for possible differences in cell tropism. Immunohistochemistry showed the transduction of mainly Müller glia for both capsids by the colocalization of GFP and glutamine synthetase, although additional cells of the INL appear to be transduced. In addition, both rAAV1 and rAAV1-E531K showed transduction of a few RGC and photoreceptors. The similar transduction patterns of rAAV1 and rAAV1-E531K indicate that HS-binding has not altered the tropism of rAAV1.

To confirm that the HS-binding mutation on rAAV1 does not convey use of HSPG for transduction, soluble heparin was mixed with capsids and applied to cells for an *in vitro* competition assay. rAAV2 requires HSPG for *in vitro* transduction and showed a dose dependent decrease in transduction. Neither rAAV1 nor rAAV1-E531K transduction were affected at any heparin dose, indicating that the rAAV1-E531K capsid does not depend on HS binding for transduction (**Fig. 6**). On average, transduction with rAAV1-E531K led to fewer GFP-positive cells compared to rAAV1, indicating that the single amino acid change alone does not provide an enhancement in transduction. A single amino acid mutant has been identified on rAAV8 providing HS-binding ability. Titers of rAAV8 and rAAV8-E533K were matched to $1x10^8$ vg and injected intravitreally. At eight weeks post-administration, fundus images were taken of the injected eyes. Transduction of rAAV8 was very low. Eyes injected with rAAV8-E533K resulted in hazy fluorescence over the retina, which when quantified, was higher compared to the non-binding serotype. We used FISH analysis to observe trafficking differences soon after injection and found again that HS-binding promoted the accumulation of vector at the ILM and within the retina. These results suggest that HS binding alone is sufficient to enhance the transduction of intravitreally-delivered AAV capsids by increasing the amount of vector that accumulate onto the retina.

A double chimera of a rAAV2.5G9 capsid was used to determine which of rAAV1 or rAAV9 capsid elements were dominant. The intravitreal delivery of rAAV2.5G9 led to expression similar to the rAAV2.5 parent when imaged by fundoscopy. The fluorescence

around the retinal vessels was very evident with punctate expression found around the vessels. Quantification of this expression when compared all together showed rAAV2.5 and rAAV2.5G9 to have the highest transduction and non-HS-binding capsids showing the lowest transduction (**Fig. 5**).

All together, the binding to HSPG at the ILM promotes rAAV accumulation from the vitreous onto the retina, but motifs from other non-HS-binding capsids can be used to influence retinal transduction patterns. This would explain how rAAV1-E531K maintained the transduction profile of rAAV1 despite having HS-binding. It would be difficult to accurately compare rAAV2 to rAAV1-E531K as these two capsids differ in residue composition and affinity to HS. Therefore, to assess the influence that rAAV1 can have on intravitreal transduction, we used the rAAV2.5 chimeric capsid. This capsid showed greater transduction than either parent and can be skewed to transduce Müller glia by the addition of galactose binding. Although the retinal glycan staining does not readily suggest the tropism, these chimeric capsids are reagents for targeted transduction in the retina. Because HSPG is abundant at the ILM in several animal models, this mechanism could be applied across multiple species, including humans. Once at the ILM, other glycan interactions with the rAAV capsid promote the tropism profile observed with the various capsid mutants.

The interaction of rAAV capsids with the ILM poses the rate-limiting step to retinal trafficking. Through the use of FISH, a small number of capsids pass through the ILM and traffic to the ONL and outer segments rapidly. Once in the outer retina, these vectors can transduce the photoreceptors but this transduction is very rare. Perhaps the majority of the capsids that enter the retina do not successfully traffic to the nucleus once in the cell to establish latency. Increasing the viral concentration is more likely to evoke an immune response as the vitreal space is not immune privileged like the subretinal space. The number of transgenes making it to the nucleus could be increased by using a higher titer. This enhanced intracellular trafficking may help to increase the transgene expression of intravitreally-delivered rAAV2i8 and rAAV2-R585E capsids. Accumulation at the ILM can be a function of vector concentration in that high doses of vector can lead to the increased transgenes found at the ILM and in the retina. To take advantage of the retinal structure, binding to the ILM can be used to accumulate vectors at the retina for transduction. This is likely why rAAV2 has been successful at intravitreal retinal transduction.

The charged sulfate groups facilitate the interaction of HSPG with the rAAV2 capsid for transduction. The transgenes observed by FISH indicate that HS-deficient rAAV2 particles are able to traffic to distal layers of the retina, which indicate that these charged

residue changes on the capsid do not prevent the vector from entering the retina, but just limit the number of vectors accumulating on the retina. This is also demonstrated by the rare transduction of rods with HS-deficient capsid. To test if the capsid interaction with the sulfate is necessary, we mixed rAAV2 with sulfated and unsulfated HS and tested intravitreally. We found both forms could inhibit transduction with the sulfated heparin leading to greater inhibition. This experiment only suggests the importance of the interaction with the heparan chain to intravitreal transduction. The genetic capsid mutants seem to validate this chemical inhibition assay, but again, the single and double amino acid modifications that disrupt the basic patch of residues involved in HSPG binding rely on a charge interaction. Nanoparticles coated with specific charged formulations indicated cationic charges (basic) to be successful in promoting penetration in the retina from the vitreous. This may help to explain how glycans present at the ONL can influence the transduction of intravitreally delivered rAAV vectors.

The similar transduction profiles of SR-delivered rAAV2 and rAAV2i8 indicate that rAAV2 does not require HS binding for retinal transduction. It may be that the subretinal delivery effectively concentrates the vector, thereby stoichiometrically skewing capsids towards expression. In addition to transduction by receptor-mediated endocytosis, SR provides abundant rAAV vectors to the phagocytic RPE and may explain why the RPE appears to be the primary cell target by both rAAV2 and rAAV2i8. Regardless of the affinity towards any particular cell type, rAAV2 and rAAV2i8 vectors lead to transduction of RPE, rods, cones, rod bipolar cells, and Müller glia. The majority of these transduced cells are located within the injection bleb, but transduction of the RPE can be seen far outside the detached area. FISH could be used to map the trafficking of SR-delivered vector.

The ILM structure is found across multiple species and could serve to attract and concentrate rAAV capsids out of the vitreous. Indeed, HS binding led to a greater presence of transgenes in the retina compared to the parent capsid when assessed by FISH soon after injection. Transgenes of non-HS-binding rAAV1 and rAAV8 could still be detected in the retina, but to a less extent, similar to the data observed between rAAV2 and rAAV2-R585E capsids. The lack of expression with HS-binding rAAV3 when injected intravitreally was expected because this serotype is inefficient in the transduction of most cell types and may encounter additional barriers for efficient transduction of cells. rAAV6 is a serotype of interest for retinal transduction and has been modified to increase the specific transduction of Müller glia using the ShH10 capsid. Without HS binding, the ShH10 vector may reveal a much weaker fluorescence. Because both rAAV6 and rAAV1-E531K do not rely on HSPG-

mediated transduction, simply adding the ability to bind to HS to any capsid serotype could enhance its transgene expression. We tested this by using rAAV8-E533K mutant capsid. The addition of HS binding could be applied to other serotypes for a greater breath of AAV used for intravitreal delivery.

Although HSPG is abundant at the ILM, other receptors can play a role in the transduction of the retina from the vitreous. The transduction by rAAV1 and rAAV6 suggest the presence of 2,3- or 2,6- N-linked sialic acid at the ILM despite the lack of staining in that region. The pattern of transduction observed by fundus may indicate a distinct pattern of this sialic acid in the retina that is not visible by histology by could be seen by flat mount. Other forms of sialic acid known to interact with rAAV4 and rAAV5 may not be expressed abundantly at the ILM or could be masked by other glycans. This would explain the lack of transduction by these serotypes when compared in a normal mouse retina. In addition to sialic acid, laminin staining is abundant at the ILM and restricted to the blood vessels. Laminin receptor is known to interact with rAAV2, rAAV3, rAAV8, and rAAV9 serotypes. Although these capsids can interact with laminin receptors at the vitreoretinal junction, this interaction seems insufficient to promote efficient intravitreal retinal transduction.

It is important to remember that the transduction observed in a mouse model may not be indicative to other models. While the mouse has become a standard model for retinal gene transfer, certain size and anatomical differences exist between them and primates. Other models, such as rabbit or pig, have similarly sized globes and vitreal volumes compared to primates. In addition, the thickness differences of the ILM between mouse and primates may lead to selection of capsids that are not efficient across species. The over abundance of HSPG at the ILM in multiple animal species, including avian, rodent, rabbit, primate, and human makes studying the influence of capsid interaction with HS important for the rational design of efficient vectors for intravitreal retinal gene transfer.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

All publications, patent applications, patents, sequences and other references mentioned herein are incorporated by reference herein in their entirety.

Table 1

	GenBank Accession Number			GenBank Accession Number			GenBank Accession Number
Complete Genomes			**Hu S17**	AY695376		Hu66	AY530626
Adeno-associated virus 1	NC_002077, AF063497		**Hu T88**	AY695375		Hu42	AY530605
Adeno-associated virus 2	NC_001401		**Hu T71**	AY695374		Hu67	AY530627
Adeno-associated virus 3	NC_001729		**Hu T70**	AY695373		Hu40	AY530603
Adeno-associated virus 3B	NC_001863		**Hu T40**	AY695372		Hu41	AY530604
Adeno-associated virus 4	NC_001829		**Hu T32**	AY695371		Hu37	AY530600
Adeno-associated virus 5	Y18065, AF085716		Hu T17	AY695370		Rh40	AY530559
Adeno-associated virus 6	NC_001862		Hu LG15	AY695377		Rh2	AY243007
Avian AAV ATCC VR-865	AY186198, AY629583, NC_004828		**Clade C**			Bb1	AY243023
Avian AAV strain DA-1	NC_006263, AY629583		Hu9	AY530629		Bb2	AY243022
Bovine AAV	NC_005889, AY388617, AAR26465		Hu10	AY530576			
AAV11	AAT46339, AY631966		Hu11	AY530577		Rh10	AY243015
AAV12	ABI16639, DQ813647					Hu17	AY530582
Clade A			Hu53	AY530615		Hu6	AY530621
AAV1	NC_002077, AF063497		Hu55	AY530617		Rh25	AY530557
AAV6	NC_001862		Hu54	AY530616		Pi2	AY530554
Hu.48	AY530611		Hu7	AY530628		Pi1	AY530553
Hu 43	AY530606		Hu18	AY530583		Pi3	AY530555
Hu 44	AY530607		Hu15	AY530580		Rh57	AY530569
Hu 46	AY530609		Hu16	AY530581		Rh50	AY530563
Clade B			Hu25	AY530591		Rh49	AY530562
Hu. 19	AY530584		Hu60	AY530622		Hu39	AY530601
Hu. 20	AY530586		Ch5	AY243021		Rh58	AY530570
Hu 23	AY530589		Hu3	AY530595		Rh61	AY530572

Hu22	AY530588		Hu1	AY530575		Rh52	AY530565
Hu24	AY530590		Hu4	AY530602		Rh53	AY530566
Hu21	AY530587		Hu2	AY530585		Rh51	AY530564
Hu27	AY530592		Hu61	AY530623		Rh64	AY530574
Hu28	AY530593		**Clade D**			Rh43	AY530560
Hu 29	AY530594		Rh62	AY530573		**AAV8**	AF513852
Hu63	AY530624		Rh48	AY530561		Rh8	AY242997
Hu64	AY530625		Rh54	AY530567		Rh1	AY530556
Hu13	AY530578		Rh55	AY530568		**Clade F**	
Hu56	AY530618		Cy2	AY243020		Hu14 (**AAV9**)	AY530579
Hu57	AY530619		**AAV7**	AF513851		Hu31	AY530596
Hu49	AY530612		Rh35	AY243000		Hu32	AY530597
Hu58	AY530620		Rh37	AY242998		**Clonal Isolate**	
Hu34	AY530598		Rh36	AY242999		**AAV5**	Y18065, AF085716
Hu35	AY530599		Cy6	AY243016		**AAV 3**	NC_001729
AAV2	NC_001401		Cy4	AY243018		**AAV 3B**	NC_001863
Hu45	AY530608		Cy3	AY243019		**AAV4**	NC_001829
Hu47	AY530610		Cy5	AY243017		Rh34	AY243001
Hu51	AY530613		Rh13	AY243013		Rh33	AY243002
Hu52	AY530614		**Clade E**			Rh32	AY243003
Hu T41	AY695378		Rh38	AY530558			

TABLE 2

Amino Acid Residue	Abbreviation	
	Three-Letter Code	One-Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid (Aspartate)	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid (Glutamate)	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

TABLE 3

Modified Amino Acid Residue	Abbreviation
Amino Acid Residue Derivatives	
2-Aminoadipic acid	Aad
3-Aminoadipic acid	bAad
beta-Alanine, beta-Aminoproprionic acid	bAla
2-Aminobutyric acid	Abu
4-Aminobutyric acid, Piperidinic acid	4Abu
6-Aminocaproic acid	Acp
2-Aminoheptanoic acid	Ahe
2-Aminoisobutyric acid	Aib
3-Aminoisobutyric acid	bAib
2-Aminopimelic acid	Apm
t-butylalanine	t-BuA
Citrulline	Cit
Cyclohexylalanine	Cha
2,4-Diaminobutyric acid	Dbu
Desmosine	Des
2,2'-Diaminopimelic acid	Dpm
2,3-Diaminoproprionic acid	Dpr
N-Ethylglycine	EtGly
N-Ethylasparagine	EtAsn
Homoarginine	hArg
Homocysteine	hCys
Homoserine	hSer
Hydroxylysine	Hyl
Allo-Hydroxylysine	aHyl
3-Hydroxyproline	3Hyp
4-Hydroxyproline	4Hyp
Isodesmosine	Ide
allo-Isoleucine	aIle
Methionine sulfoxide	MSO
N-Methylglycine, sarcosine	MeGly

N-Methylisoleucine	MeIle
6-N-Methyllysine	MeLys
N-Methylvaline	MeVal
2-Naphthylalanine	2-Nal
Norvaline	Nva
Norleucine	Nle
Ornithine	Orn
4-Chlorophenylalanine	Phe(4-Cl)
2-Fluorophenylalanine	Phe(2-F)
3-Fluorophenylalanine	Phe(3-F)
4-Fluorophenylalanine	Phe(4-F)
Phenylglycine	Phg
Beta-2-thienylalanine	Thi

Table 4

Serotype	Position 1	Position 2
AAV1	**A263X**	**T265X**
AAV2	**Q263X**	**-265X**
AAV3a	**Q263X**	**-265X**
AAV3b	**Q263X**	**-265X**
AAV4	**S257X**	**-259X**
AAV5	**G253X**	**V255X**
AAV6	**A263X**	**T265X**
AAV7	**E264X**	**A266X**
AAV8	**G264X**	**S266X**
AAV9	**S263X**	**S265X**
Where, (X) → mutation to any amino acid **(-) → insertion of any amino acid** **Note: Position 2 inserts are indicated by the site of insertion**		

That which is claimed is:

1. A method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 4 (AAV4) vector comprising an AAV4 capsid protein, wherein the AAV4 capsid protein comprises a substitution at amino acid residue K530 and/or further comprises a substitution at one or more of amino acid residues S584, N585, S586 and N587 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:4 (amino acid sequence of AAV4 capsid protein).

2. A method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 5 (AAV5) vector comprising an AAV5 capsid protein, wherein the AAV5 capsid protein comprises a substitution at amino acid residue K517 and/or further comprises a substitution at one or more of amino acid residues S575, S576, T577 and T578 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:5 (amino acid sequence of AAV5 capsid protein).

3. A method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 7 (AAV7) vector comprising an AAV7 capsid protein, wherein the AAV7 capsid protein comprises a substitution at amino acid residue K533 and/or further comprises a substitution at one or more of amino acid residues A587, A588, N589 and R590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:7 (amino acid sequence of AAV7 capsid protein).

4. A method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 8 (AAV8) vector comprising an AAV8 capsid protein, wherein the AAV8 capsid protein comprises a substitution at amino acid residue K533 and/or further comprises a substitution at one or more of amino acid residues Q587, Q588, N589 and T590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:8 (amino acid sequence of AAV8 capsid protein).

5. A method of introducing a nucleic acid molecule into a cell of a retina and/or retinal pigment epithelium of a subject, comprising intravitreally administering an adeno-associated virus (AAV) serotype 9 (AAV9) vector comprising an AAV9 capsid protein, wherein the AAV9 capsid protein comprises a substitution at amino acid residue K531 and/or further comprises a substitution at one or more of amino acid residues Q587, A588, N589 and T 590 in any combination, wherein the numbering of the residues is based on the amino acid sequence of SEQ ID NO:9 (amino acid sequence of AAV9 capsid protein).

6. The method of any of claims 1-5, wherein the vector comprises a nucleic acid molecule that encodes a therapeutic protein or therapeutic DNA.

7. A method of treating a disorder or defect of the eye in a subject, comprising intravitreally administering to the subject the virus vector of any of claims 1-6, wherein the virus vector comprises a nucleic acid molecule that encodes a therapeutic protein or therapeutic DNA effective in treating the disorder or defect of the eye in the subject.

8. The method of claim 7, wherein the disorder or defect of the eye not limited to age-related macular degeneration, Lebers congenital amarousis type 1, Lebers, congenital amarousis type 2, retinitis pigmentosa, retinoschosis, achromatopsia, color blindness, congenital stationary night blindness or any combination thereof.

9. The method of any preceding claim, wherein the subject is a human.

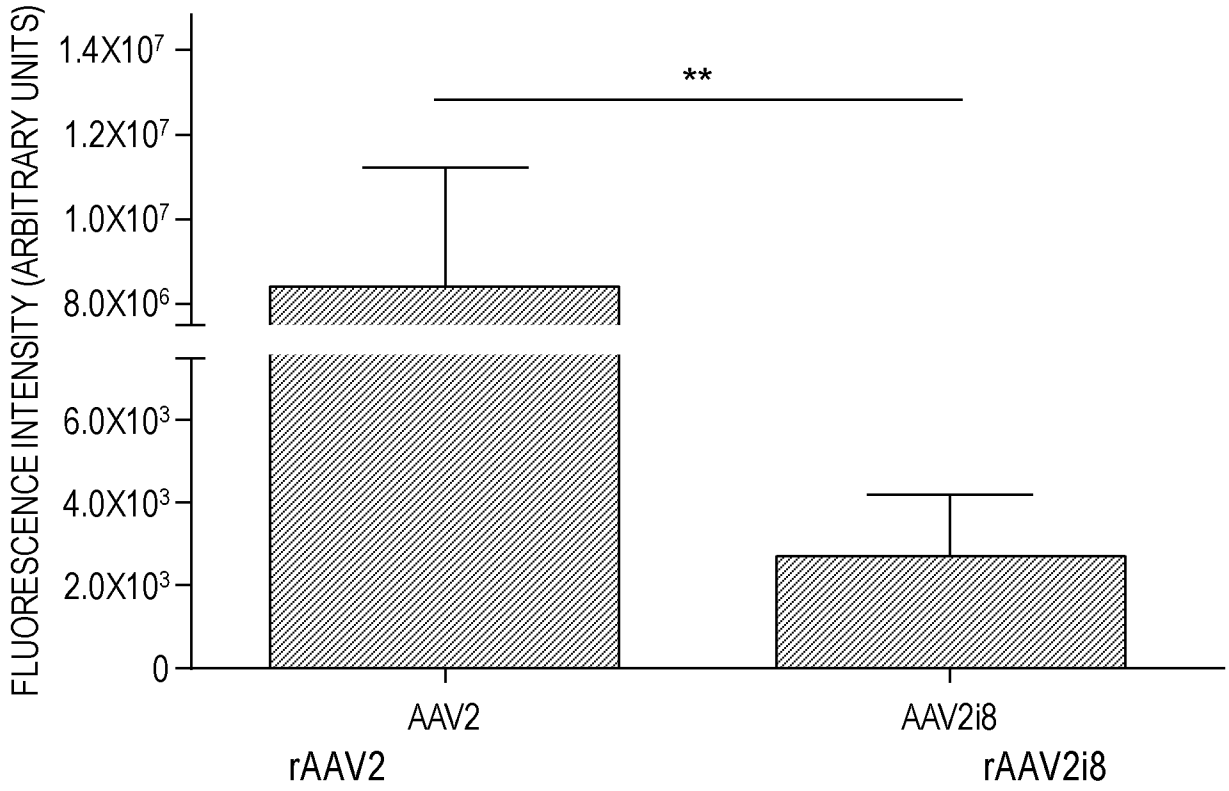
FLUORESCENCE INTENSITY (ARBITRARY UNITS)
1.4X10^7
1.2X10^7
1.0X10^7
8.0X10^6
6.0X10^3
4.0X10^3
2.0X10^3
0
**
AAV2
AAV2i8
rAAV2
rAAV2i8

FIG. 1

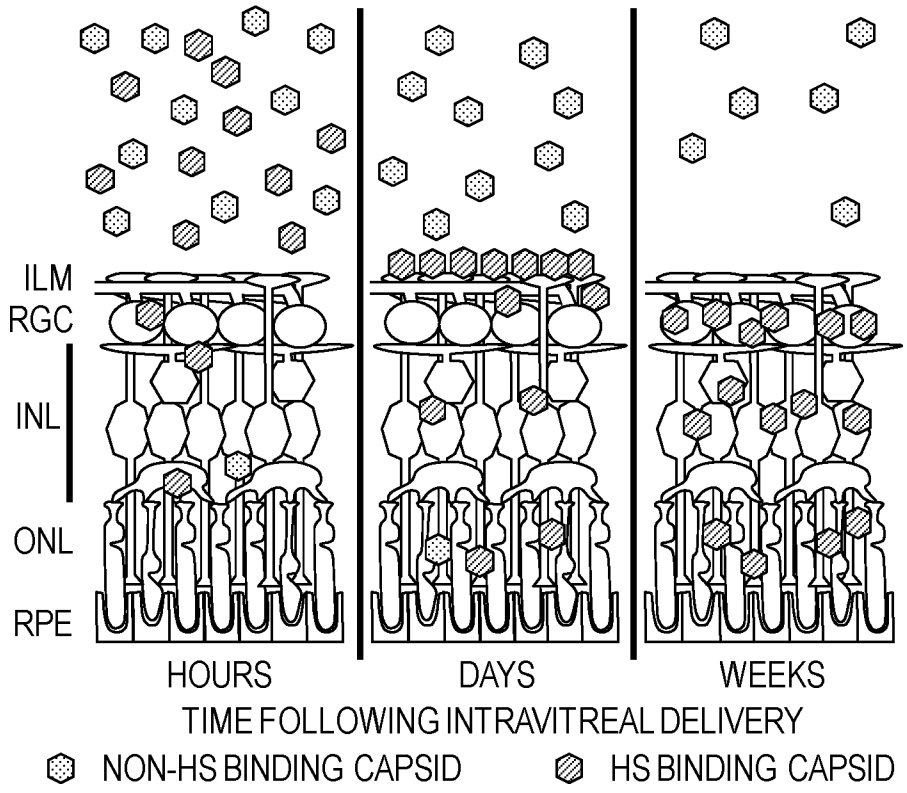
ILM
RGC
INL
ONL
RPE
HOURS
DAYS
WEEKS
TIME FOLLOWING INTRAVITREAL DELIVERY
NON-HS BINDING CAPSID
HS BINDING CAPSID

FIG. 2

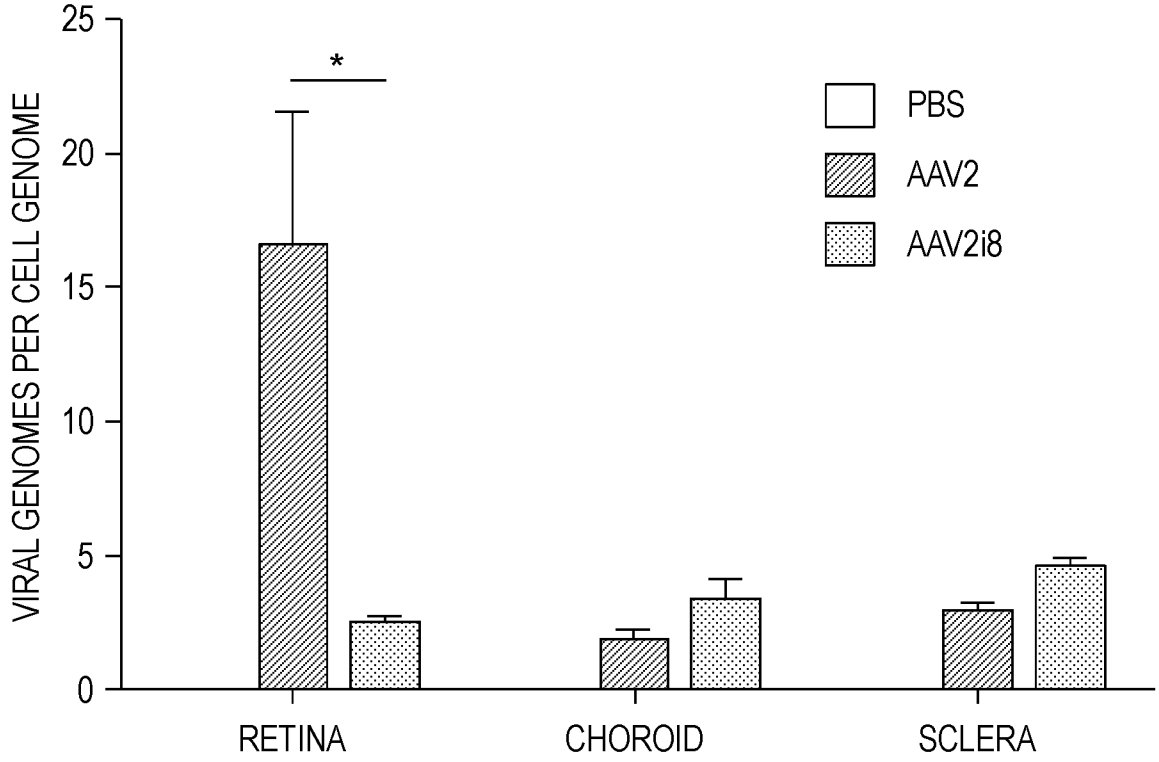
25
20
15
10
5
0
VIRAL GENOMES PER CELL GENOME
*
PBS
AAV2
AAV2i8
RETINA
CHOROID
SCLERA

FIG. 3

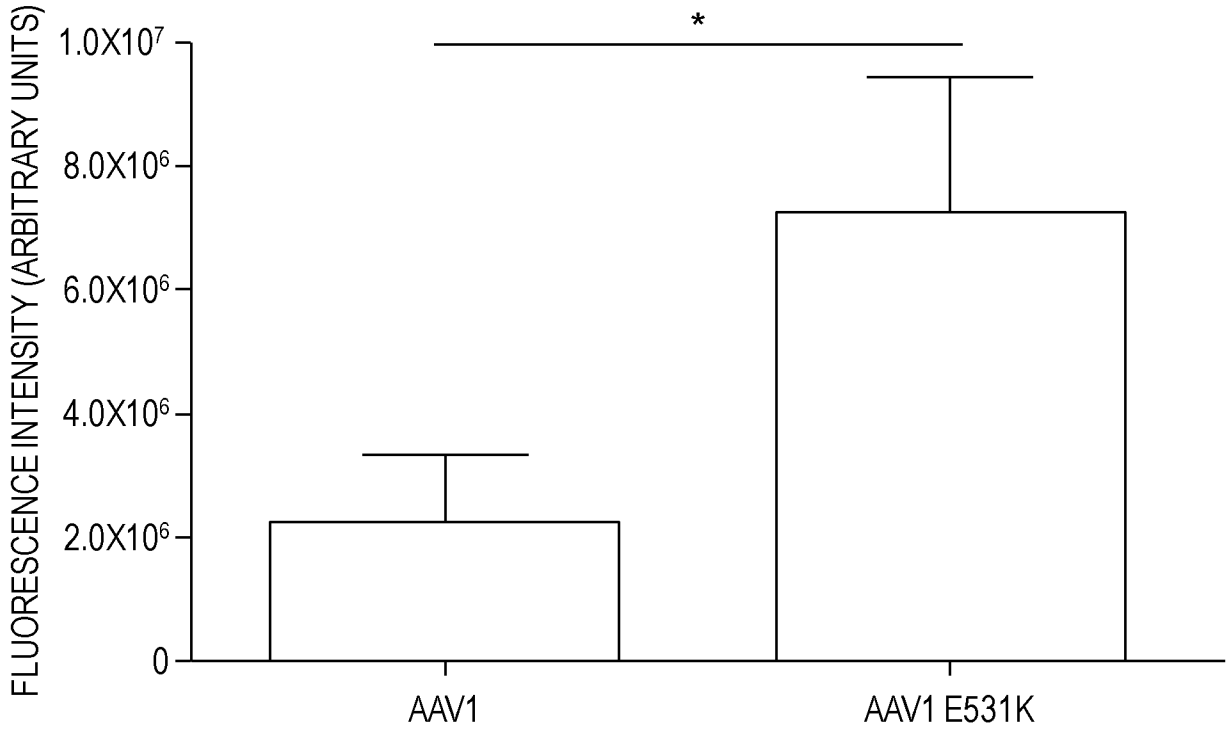
1.0X10^7
8.0X10^6
6.0X10^6
4.0X10^6
2.0X10^6
0
FLUORESCENCE INTENSITY (ARBITRARY UNITS)
*
AAV1
AAV1 E531K

FIG. 4

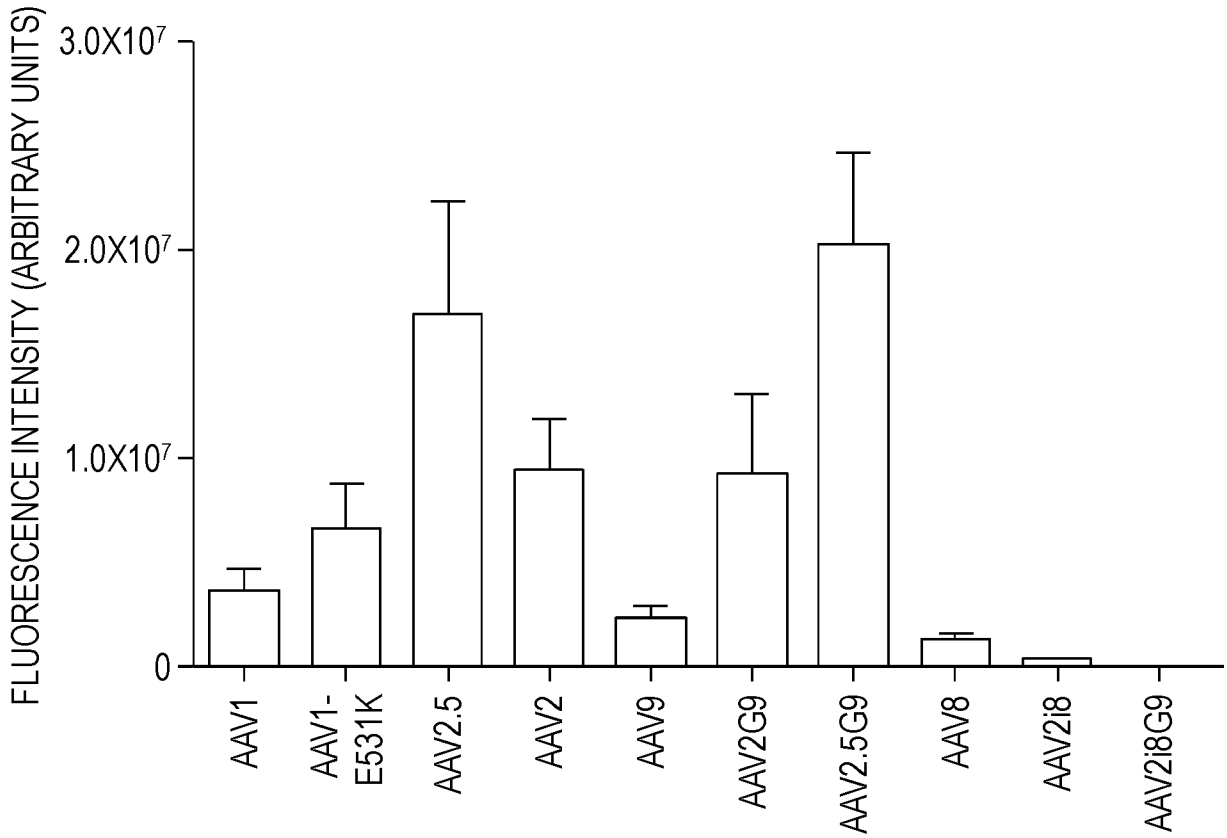
FLUORESCENCE INTENSITY (ARBITRARY UNITS)
3.0X10^7
2.0X10^7
1.0X10^7
0
AAV1
AAV1-E531K
AAV2.5
AAV2
AAV9
AAV2G9
AAV2.5G9
AAV8
AAV2i8
AAV2i8G9

FIG. 5

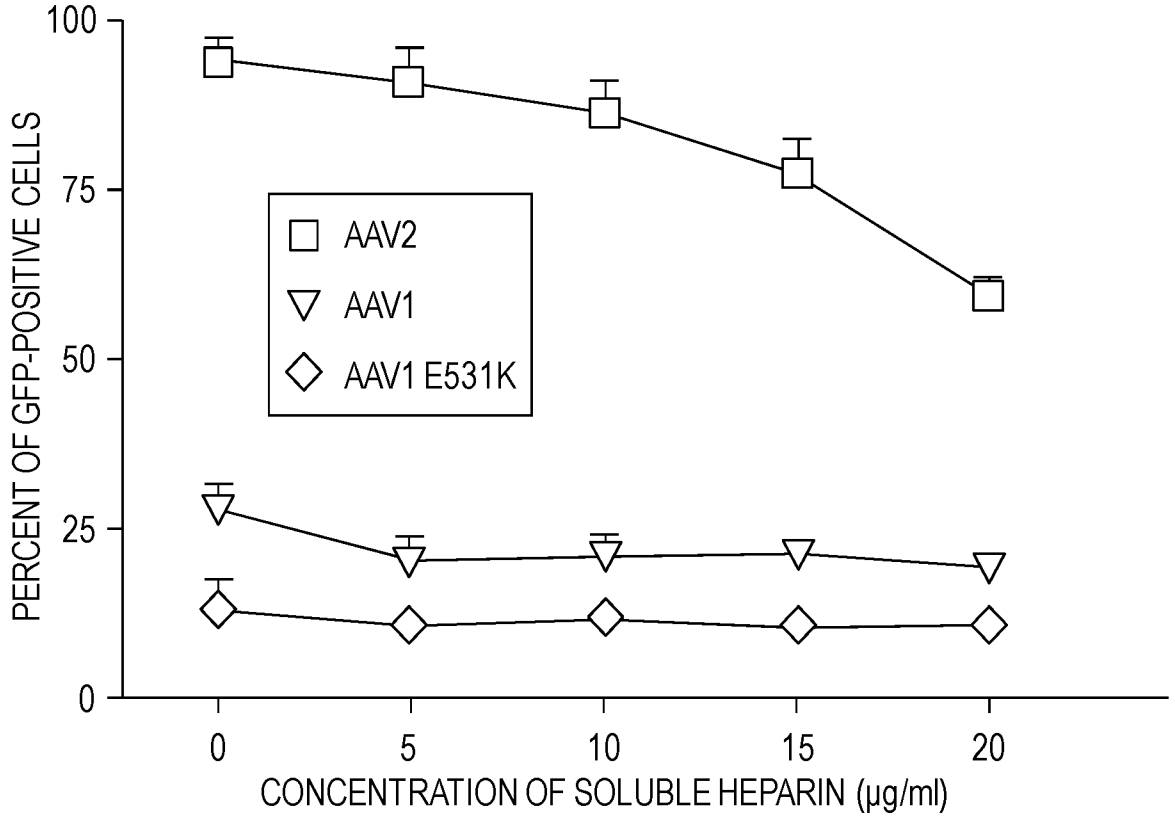
PERCENT OF GFP-POSITIVE CELLS
100
75
50
25
0
AAV2
AAV1
AAV1 E531K
0
5
10
15
20
CONCENTRATION OF SOLUBLE HEPARIN (µg/ml)

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2017/047123

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [X] on paper or in the form of an image file.

   b. [X] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2017/047123

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.: 5
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: Claim 5 is unclear, since the claimed amino acid sequence of SEQ ID NO:9 is not according to amino acid sequence of an AAV9 capsid protein having GenBank Accession No.AAS99264, and other amino acid residues are situated in positions 587, 588, 589 and 590 of said SEQ ID NO:9 and amino acid sequence of an AAV9 capsid protein having GenBank Accession No. AAS99264.

3. ☐ Claims Nos.:
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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2017/047123

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/63 *(2006.01)*
C12N 15/861 *(2006.01)*
C07K14/075 *(2006.01)*
A61K 48/00 *(2006.01)*
A61P 27/00 *(2006.01)*

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)

C12N 15/63, 15/861, C07K 14/075, A61K 48/00, A61P 27/00

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

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

Esp@cenet, USPTO, PubMed, RUPAT, PatSearch (RUPTO internal), PAJ, NCBI, DWPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/115382 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 21.07.2016, p. 1, lines 30-34, p. 2, lines 1-21, p. 3, lines 8-11, p. 15, lines 20-26, p.15, lines 25-40, p. 17, lines 1-7, p. 19, lines 12-15, p. 21, lines 17-30, p. 24, lines 24-26, p. 43, lines 1-5, p. 44, lines 6-10, p. 46, lines 10-12, 26-32	1, 6-9
X	WO 2015/121501 A1 (KING 'S COLLEGE LONDON et al.) 20.08.2015, p. 3 , paragraphs 5-6, p. 5, paragraphs 2, 5, 6, p. 6, paragraphs 3-4, 5-6, p. 7, paragraph 1, p. 8 , paragraphs 3-4, p. 37, paragraph 2-p. 38, paragraph 1, p. 36, paragraph 3, p. 35, paragraph 4,p. 54, paragraph 4, claims 9, 12-15, 18, 25, 28	2, 4, 6-9
A	US 2016/0017005 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 21.01.2016, paragraphs [0031], [0067], [0122], [0174], [0176], [0180]	3

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"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)

"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

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 2017 (23.10.2017)	16 November 2017 (16.11.2017)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer L.Sonina Telephone No. 495 531 65 15