(56) Related Art
WU, Z. et al., "Preclinical development of AAV vectors for the treatment of X- linked retinitis pigmentosa caused by RPGR mutations", Investigative Ophthalmology & Visual Science, (2014), vol. 55, no. 13, XP 055310512
WU, Z. et al., "Preclinical development of an AAV vector for the treatment of X-linked retinitis pigmentosa due to RPGRorf15 mutation", Molecular Therapy, (2013), vol. 21, no. 9, page 709, XP 055258076
BELTRAN, W. et al., Proceedings of the National Academy of Sciences, (2012), vol. 109, no. 6, pages 2132 - 2137, XP 055155762
WU, Z. et al., "Gene therapy for X-linked retinitis pigmentosa: A long-term efficacy study in a mouse model of RPGR deficiency", Molecular Therapy, (2014), vol. 22, no. supplement 1, page s209, XP 055310541
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MOOKHERJEE, S. et al., "A low dose AAV vector administration preserves cone function in an RP2 knockout mouse model for retinitis pigmentosa", Investigative Ophthalmology & Visual Science, (2014), vol. 55, no. 13, page 3310, XP 055310549

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(54) Title: RP2 AND RPGR VECTORS FOR TREATING X-LINKED RETINITIS PIGMENTOSA

(57) Abstract: Disclosed are adeno-associated virus (AAV) vectors comprising a nucleotide sequence encoding RP2 or RPGR-ORF15 and related pharmaceutical compositions. Also disclosed are methods of treating or preventing X-linked retinitis pigmentosa, increasing photoreceptor number in a retina of a mammal, and increasing visual acuity of a mammal using the vectors and pharmaceutical compositions.

RP2 VECTORS FOR TREATING X-LINKED RETINITIS PIGMENTOSA

CROSS-REFERENCE TO RELATED APPLICATION

[0001]   This patent application claims the benefit of U.S. Provisional Patent Application No. 62/131,661, filed March 11, 2015, which is incorporated by reference in its entirety herein.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002]   Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 77,632 Byte ASCII (Text) file named "723551_ST25.txt," dated March 11, 2016.

BACKGROUND OF THE INVENTION

[0003]   X-linked retinitis pigmentosa (XLRP) is an X-linked, hereditary retinal dystrophy characterized by a progressive loss of photoreceptor cells, leading to vision impairment or blindness. XLRP may involve rod photoreceptor death, followed by cone cell death. As a result, an XLRP patient usually experiences an early onset of night-blindness, followed by a gradual but progressive loss of peripheral vision, and an eventual loss of central vision. There is currently no treatment for XLRP. Accordingly, there exists a need for compositions and methods for treating XLRP.

BRIEF SUMMARY OF THE INVENTION

[0004]   An embodiment of the invention provides an adeno-associated virus (AAV) vector comprising (a) a nucleotide sequence encoding RP2 or a functional fragment thereof and (b) an AAV2 Inverted Terminal Repeat (ITR) or a functional fragment thereof.
Another embodiment of the invention provides an AAV vector comprising a nucleotide sequence encoding RPGR-ORF15 or a functional fragment or functional variant thereof, wherein (i) the vector further comprises a CMV/human β-globin intron and/or a human β-globin polyadenylation signal; and (ii) the nucleotide sequence encoding RPGR-

ORF15 or a functional fragment or a functional variant thereof is optionally under the transcriptional control of a rhodopsin kinase promoter.

[0006]   Additional embodiments of the invention provide related pharmaceutical compositions and methods of making the AAV vector comprising a nucleotide sequence encoding RPGR-ORF15 or a functional fragment or functional variant thereof.

[0007]   Another embodiment of the invention provides a method of treating or preventing X-linked retinitis pigmentosa (XLRP) in a mammal in need thereof, the method comprising administering to the mammal the inventive vector or pharmaceutical composition in an amount effective to treat or prevent XLRP in the mammal.

[0008]   Still another embodiment of the invention provides a method of increasing photoreceptor number in a retina of a mammal, the method comprising administering to the mammal the inventive vector or pharmaceutical composition in an amount effective to increase photoreceptor number in the retina of the mammal.

DETAILED DESCRIPTION OF THE INVENTION

[0009]   In accordance with an embodiment of the invention, it has been discovered that an AAV vector comprising a nucleotide sequence encoding RP2 or RPGR-ORF15 effectively preserved the function and viability of photoreceptors in mouse models of XLRP.

[0010]   Approximately 15% of XLRP patients have a mutation in the Retinitis Pigmentosa 2 (*RP2*) gene. The human *RP2* gene has five exons and encodes a protein of 350 amino acid residues. RP2 protein is a GTPase activating protein (GAP) for arginine adenosine-5'-diphosphoribosylation (ADP-ribosylation) factor-like 3 (ARL3), a microtubule-associated small GTPase that localizes to the connecting cilium of photoreceptors. An example of a complementary DNA (cDNA) sequence encoding the human RP2 protein is the nucleotide sequence of SEQ ID NO: 1. An example of a protein sequence encoding the human RP2 protein is the amino acid sequence of SEQ ID NO: 2.

[0011]   An embodiment of the invention provides an AAV vector comprising a nucleic acid comprising (a) a nucleotide sequence encoding RP2 or a functional fragment thereof and (b) an AAV2 ITR or a functional fragment thereof. The nucleotide sequence encoding RP2 may be any suitable nucleotide sequence that encodes RP2 from any species. In a preferred embodiment, the RP2 is human RP2. In an embodiment of the invention, the nucleotide sequence encoding human RP2 comprises a nucleotide sequence that encodes a human RP2 protein comprising the amino acid sequence of SEQ ID NO: 2. In an embodiment of the

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invention, the nucleotide sequence encoding human RP2 comprises the nucleotide sequence of SEQ ID NO: 1.

[0012]   Approximately 75% of XLRP patients have a mutation in the Retinitis Pigmentosa GTPase Regulator (RPGR) gene. Multiple RPGR transcripts have been detected in the retina. A majority of the disease-causing mutations have been detected in a variant isoform RPGR-ORF15, which is expressed in the retina. RPGR-ORF15 protein interacts with centrosome-cilia proteins and localizes to the connecting cilia in both rod and cone photoreceptors. An example of a cDNA sequence encoding the wild-type human RPGR-ORF15 protein is the nucleotide sequence of SEQ ID NO: 27. An example of a protein sequence encoding the wild-type human RPGR-ORF15 protein is the amino acid sequence of SEQ ID NO: 4. An example of a cDNA sequence encoding a functional variant of the wild-type human RPGR-ORF15 protein is the nucleotide sequence of SEQ ID NO: 3. An example of a protein sequence encoding a functional variant of the wild-type human RPGR-ORF15 protein is the amino acid sequence of SEQ ID NO: 25.

[0013]   Another embodiment of the invention provides an AAV vector comprising a nucleic acid comprising a nucleotide sequence encoding RPGR-ORF15 or a functional fragment or functional variant thereof, wherein (i) the vector further comprises a CMV/human β-globin intron and/or a human β-globin polyadenylation signal; and (ii) the nucleotide sequence encoding RPGR-ORF15 or a functional fragment or a functional variant thereof is optionally under the transcriptional control of a rhodopsin kinase promoter. In an embodiment of the invention, the AAV vector comprising a nucleic acid comprising a nucleotide sequence encoding RPGR-ORF15 or a functional fragment or functional variant thereof, wherein (i) the nucleotide sequence encoding human RPGR-ORF15 or a functional fragment or functional variant thereof is under the transcriptional control of a rhodopsin kinase promoter, and/or (ii) the vector further comprises a CMV/human β-globin intron and/or a human β-globin polyadenylation signal. The nucleotide sequence encoding wild-type RPGR-ORF15 may be any suitable nucleotide sequence that encodes wild-type RPGR-ORF15 from any species. In a preferred embodiment, the RPGR-ORF15 is human RPGR-ORF15. In an embodiment of the invention, the nucleotide sequence encoding wild-type human RPGR-ORF15 comprises a nucleotide sequence that encodes a wild-type human RPGR-ORF15 protein comprising the amino acid sequence of SEQ ID NO: 4. In an embodiment of the invention, the nucleotide sequence encoding wild-type human RPGR-ORF15 protein comprises the nucleotide sequence of SEQ ID NO: 27. The nucleotide

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sequence encoding a functional variant of a wild-type RPGR-ORF15 may be any suitable nucleotide sequence that encodes a functional variant of the wild-type RPGR-ORF15. In a preferred embodiment, the functional variant of the RPGR-ORF15 is a functional variant of human RPGR-ORF15. In an embodiment of the invention, the nucleotide sequence encoding a functional variant of the wild-type human RPGR-ORF15 comprises a nucleotide sequence that encodes a functional variant of the wild-type human RPGR-ORF15 protein comprising the amino acid sequence of SEQ ID NO: 25. In an embodiment of the invention, the nucleotide sequence encoding a functional variant of the wild-type human RPGR-ORF15 protein comprises the nucleotide sequence of SEQ ID NO: 3. Hereinafter, wild-type RPGR-ORF15 and functional variants of wild-type RPGR-ORF15 will be collectively referred to as "RPGR-ORF15," unless specified otherwise.

[0014]   The AAV vector may be suitable for packaging into any AAV serotype or variant thereof that is suitable for administration to ocular cells. Examples of suitable AAV serotypes may include, but are not limited to, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and any variant thereof. Preferably, the AAV vector is packaged into serotype AAV8 or AAV9.

[0015]   The AAV vector may be packaged in a capsid protein, or fragment thereof, of any of the AAV serotypes described herein. Preferably, the vector is packaged in an AAV8 capsid. In an embodiment of the invention, the AAV8 capsid comprises the amino acid sequence of SEQ ID NO: 5.

[0016]   A suitable recombinant AAV may be generated by culturing a packaging cell which contains a nucleic acid sequence encoding an AAV serotype capsid protein, or fragment thereof, as defined herein; a functional *rep* gene; any of the inventive vectors described herein; and sufficient helper functions to permit packaging of the inventive vector into the AAV capsid protein. The components required by the packaging cell to package the inventive AAV vector in an AAV capsid may be provided to the host cell in *trans*. Alternatively, any one or more of the required components (e.g., inventive vector, *rep* sequences, capsid sequences, and/or helper functions) may be provided by a stable packaging cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art.

[0017]   In an embodiment of the invention, the AAV vector is self-complementary. Self-complementary vectors may, advantageously, overcome the rate-limiting step of second-strand DNA synthesis and confer earlier onset and stronger gene expression. Preferably, the

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AAV vector comprising a nucleotide sequence encoding RP2 is self-complementary. In an embodiment, the vector comprises single-stranded DNA.

[0018]   By "nucleic acid" as used herein includes "polynucleotide," "oligonucleotide," and "nucleic acid molecule," and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide. It is generally preferred that the nucleic acid does not comprise any insertions, deletions, inversions, and/or substitutions. However, it may be suitable in some instances, as discussed herein, for the nucleic acid to comprise one or more insertions, deletions, inversions, and/or substitutions.

[0019]   In an embodiment, the nucleic acids of the invention are recombinant. As used herein, the term "recombinant" refers to (i) molecules that are constructed outside living cells by joining natural or synthetic nucleic acid segments to nucleic acid molecules that can replicate in a living cell, or (ii) molecules that result from the replication of those described in (i) above. For purposes herein, the replication can be *in vitro* replication or *in vivo* replication.

[0020]   The nucleic acids can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. See, for example, Green et al. (eds.), *Molecular Cloning, A Laboratory Manual*, 4th Edition, Cold Spring Harbor Laboratory Press, New York (2012). For example, a nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed upon hybridization (e.g., phosphorothioate derivatives and acridine substituted nucleotides). Examples of modified nucleotides that can be used to generate the nucleic acids include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N^6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-substituted adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-

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methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N^6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine. Alternatively, one or more of the nucleic acids of the invention can be purchased from companies, such as Macromolecular Resources (Fort Collins, CO) and Synthegen (Houston, TX).

[0021]   In an embodiment of the invention, the vector is a recombinant expression vector. For purposes herein, the term "recombinant expression vector" means a genetically-modified oligonucleotide or polynucleotide construct that permits the expression of an mRNA, protein, polypeptide, or peptide by a host cell, when the construct comprises a nucleotide sequence encoding the mRNA, protein, polypeptide, or peptide, and the vector is contacted with the cell under conditions sufficient to have the mRNA, protein, polypeptide, or peptide expressed within the cell. The vectors of the invention are not naturally-occurring as a whole. However, parts of the vectors can be naturally-occurring. The inventive recombinant expression vectors can comprise any type of nucleotides, including, but not limited to DNA and RNA, which can be single-stranded or double-stranded, synthesized or obtained in part from natural sources, and which can contain natural, non-natural or altered nucleotides. The recombinant expression vectors can comprise naturally-occurring, non-naturally-occurring internucleotide linkages, or both types of linkages. Preferably, the non-naturally occurring or altered nucleotides or internucleotide linkages do not hinder the transcription or replication of the vector.

[0022]   The recombinant expression vectors of the invention can be prepared using standard recombinant DNA techniques described in, for example, Green et al., *supra*. Constructs of expression vectors, which are circular or linear, can be prepared to contain a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived, e.g., from ColEI, 2 μ plasmid, λ, SV40, bovine papilloma virus, and the like.

[0023]   The recombinant expression vector can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to antibiotics, heavy metals, etc., complementation in an auxotrophic host to provide prototrophy, and the like. Suitable marker genes for the inventive expression vectors include, for instance, neomycin/G418 resistance genes, hygromycin resistance genes, histidinol resistance genes, tetracycline resistance genes, and ampicillin resistance genes.

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[0024]   The vector may further comprise regulatory sequences which are operably linked to the nucleotide sequence encoding RP2 or RPGR-ORF15 in a manner which permits one or more of the transcription, translation, and expression of RP2 or RPGR-ORF15 in a cell transfected with the vector or infected with a virus that comprises the vector. As used herein, "operably linked" sequences include both regulatory sequences that are contiguous with the nucleotide sequence encoding RP2 or RPGR-ORF15 and regulatory sequences that act in *trans* or at a distance to control the nucleotide sequence encoding RP2 or RPGR-ORF15.

[0025]   The regulatory sequences may include appropriate transcription initiation, termination, promoter and enhancer sequences; RNA processing signals such as splicing and polyadenylation (polyA) signal sequences; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. PolyA signal sequences may be synthetic or may be derived from many suitable species, including, for example, SV-40, human and bovine. Preferably, the vector comprises a full-length or truncated human beta (β)-globin polyA signal sequence. In an embodiment of the invention, the human β-globin polyA signal sequence comprises the nucleotide sequence of SEQ ID NO: 6 (full-length) or SEQ ID NO: 7 (truncated).

[0026]   The regulatory sequences may also include an intron. Preferably, the intron is positioned between the promoter sequence and the nucleotide sequence encoding RP2 or RPGR-ORF15. Examples of suitable intron sequences include a cytomegalovirus (CMV)/human β-globin intron and an intron derived from SV-40 (referred to as SD-SA). Preferably, the intron is a CMV/human β-globin intron. In an embodiment of the invention, the CMV/human β-globin intron comprises the nucleotide sequence of SEQ ID NO: 8 or 9.

[0027]   The regulatory sequences may also include a promoter. The promoter may be any promoter suitable for expressing RP2 or RPGR-ORF15 in a target cell, e.g., an ocular cell. The promoter may be inducible or constitutive. In an embodiment of the invention, the promoter is suitable for expressing RP2 or RPGR-ORF15 in a particular ocular cell type. In this regard, the promoter may be cell-specific. For example, the promoter may be specific for expression in any one or more of ocular cells, retinal pigment epithelium (RPE) cells, photoreceptor cells, in rods, or in cones. Examples of suitable promoters include, but are not limited to, the human G-protein-coupled receptor protein kinase 1 (GRK1) promoter (also referred to as the human rhodopsin kinase promoter), the human interphotoreceptor retinoid-binding protein proximal (IRBP) promoter, the native promoter for RP2 or RPGR-ORF15,

the RPGR proximal promoter, the rod opsin promoter, the red-green opsin promoter, the blue opsin promoter, the cGMP-β-phosphodiesterase promoter, the mouse opsin promoter, the rhodopsin promoter, the alpha-subunit of cone transducin, beta phosphodiesterase (PDE) promoter, the retinitis pigmentosa (RP1) promoter, the NXNL2/NXNL1 promoter, the RPE65 promoter, the retinal degeneration slow/peripherin 2 (Rds/perphZ) promoter, the VMD2 promoter, and functional fragments of any of the foregoing. Preferably, the nucleotide sequence encoding RP2 or RPGR-ORF15 is under the transcriptional control of the GRK1 promoter (also referred to as human rhodopsin kinase promoter). In an embodiment of the invention, the human rhodopsin kinase promoter comprises the nucleotide sequence of SEQ ID NO: 10.

[0028]   In an embodiment of the invention, the vector comprises an ITR or a functional fragment thereof. Preferably, the vector comprises a 5' and a 3' AAV ITR. The ITRs may be of any suitable AAV serotype, including any of the AAV serotypes described herein. The ITRs may be readily isolated using techniques known in the art and may be isolated or obtained from public or commercial sources (e.g., the American Type Culture Collection, Manassas, VA). Alternatively, the ITR sequences may be obtained through synthetic or other suitable means by reference to published sequences. Preferably, the vector comprises a 5' and a 3' AAV2 ITR. In an embodiment of the invention, the vector comprises a truncated 5' AAV2 ITR. In an embodiment of the invention, the vector comprises a 5' AAV2 ITR comprising the nucleotide sequence of SEQ ID NO: 11 and a 3' AAV2 ITR comprising the nucleotide sequence of SEQ ID NO: 12. In another embodiment of the invention, the vector comprises a truncated 5' AAV2 ITR comprising the nucleotide sequence of SEQ ID NO: 13 and a 3' AAV2 ITR comprising the nucleotide sequence of SEQ ID NO: 12.

[0029]   Included in the scope of the invention are vectors comprising functional fragments of any of the nucleotide sequences described herein that encode functional fragments of the proteins described herein. The term "functional fragment," when used in reference to a RP2 or RPGR-ORF15 protein, refers to any part or portion of the protein, which part or portion retains the biological activity of the protein of which it is a part (the parent protein). Functional protein fragments encompass, for example, those parts of a protein that retain the ability to recognize treat or prevent XLRP, increase photoreceptor number, decrease retinal detachment in a mammal, increase the electrical response of a photoreceptor in a mammal, increase protein expression in a retina of a mammal, localize protein to rod outer segments in a retina of a mammal, or increase visual acuity in a mammal, to a similar extent, the same

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extent, or to a higher extent, as the parent protein. For example, a functional fragment of a nucleotide sequence encoding RPGR-ORF15 may be a cDNA encoding RPGR-ORF15 but shortened by 654 base pairs (bp) in the repetitive region, which has been shown to reconstitute RPGR function in mice (Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 46(2): 435-441 (2005)).

[0030] The term "functional fragment" when used in reference to a polyA signal sequence or an ITR, refers to any part or portion of the nucleotide sequence, which part or portion retains the biological activity of the nucleotide sequence of which it is a part (the parent nucleotide sequence). Functional protein fragments encompass, for example, those parts of a polyA signal sequence that retain the ability to be recognized by a RNA cleavage complex or those parts of an ITR that retain the ability to allow for replication to a similar extent, the same extent, or to a higher extent, as the parent nucleotide sequence. In reference to the parent nucleotide sequence or protein, the functional fragment can comprise, for instance, about 10%, 25%, 30%, 50%, 68%, 80%, 90%, 95%, or more, of the parent nucleotide sequence or protein.

[0031] Included in the scope of the invention are vectors encoding functional variants of the RP2 and RPGR-ORF15 proteins described herein. The term "functional variant," as used herein, refers to a protein having substantial or significant sequence identity or similarity to a parent protein, which functional variant retains the biological activity of the protein of which it is a variant. Functional variants encompass, for example, those variants of the RP2 or RPGR-ORF15 proteins described herein (the parent protein) that retain the ability to treat or prevent XLRP, increase photoreceptor number, decrease retinal detachment in a mammal, increase the electrical response of a photoreceptor in a mammal, increase protein expression in a retina of a mammal, localize protein to rod outer segments in a retina of a mammal, and/or increase visual acuity in a mammal to a similar extent, the same extent, or to a higher extent, as the parent RP2 or RPGR-ORF15 protein. In reference to the parent RP2 or RPGR-ORF15 protein, the functional variant can, for instance, be at least about 30%, about 50%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or more identical in amino acid sequence to the parent RP2 or RPGR-ORF15 protein.

[0032] A functional variant can, for example, comprise the amino acid sequence of the parent RP2 or RPGR-ORF15 protein with at least one conservative amino acid substitution. Alternatively or additionally, the functional variants can comprise the amino acid sequence of

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the parent RP2 or RPGR-ORF15 protein with at least one non-conservative amino acid substitution. In this case, it is preferable for the non-conservative amino acid substitution to not interfere with or inhibit the biological activity of the functional variant. The non-conservative amino acid substitution may enhance the biological activity of the functional variant, such that the biological activity of the functional variant is increased as compared to the parent RP2 or RPGR-ORF15 protein.

[0033]   Amino acid substitutions of the parent RP2 or RPGR-ORF15 protein are preferably conservative amino acid substitutions. Conservative amino acid substitutions are known in the art, and include amino acid substitutions in which one amino acid having certain physical and/or chemical properties is exchanged for another amino acid that has the same or similar chemical or physical properties. For instance, the conservative amino acid substitution can be an acidic/negatively charged polar amino acid substituted for another acidic/negatively charged polar amino acid (e.g., Asp or Glu), an amino acid with a nonpolar side chain substituted for another amino acid with a nonpolar side chain (e.g., Ala, Gly, Val, Ile, Leu, Met, Phe, Pro, Trp, Cys, Val, etc.), a basic/positively charged polar amino acid substituted for another basic/positively charged polar amino acid (e.g. Lys, His, Arg, etc.), an uncharged amino acid with a polar side chain substituted for another uncharged amino acid with a polar side chain (e.g., Asn, Gln, Ser, Thr, Tyr, etc.), an amino acid with a beta-branched side-chain substituted for another amino acid with a beta-branched side-chain (e.g., Ile, Thr, and Val), an amino acid with an aromatic side-chain substituted for another amino acid with an aromatic side chain (e.g., His, Phe, Trp, and Tyr), etc.

[0034]   The RP2 or RPGR-ORF15 protein or functional variant can consist essentially of the specified amino acid sequence or sequences described herein, such that other components, e.g., other amino acids, do not materially change the biological activity of the RP2 or RPGR-ORF15 protein or functional variant.

[0035]   In an embodiment of the invention, the RP2 vector comprises a nucleotide sequence comprising the components set forth in Table 1. In this regard, the full-length RP2 vector may comprise the nucleotide sequence of SEQ ID NO: 14.

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TABLE 1

RP2 vector (SEQ ID NO: 14)		
Nucleotide Position of SEQ ID NO: 14	SEQ ID NO:	Component
1-106	13	5' AAV2 ITR (truncated)
137-432	10	human rhodopsin kinase promoter
446-730	9	CMV/human β-globin intron
759-1811	1	human RP2 cDNA
1817-1930	7	human β-globin polyadenylation signal (truncated)
1943-2087	12	3' AAV2 ITR

[0036]   In an embodiment of the invention, the vector encoding a functional variant of wild-type human RPGR-ORF15 comprises a nucleotide sequence comprising the components set forth in Table 2. In this regard, the full-length vector encoding a functional variant of wild-type human RPGR-ORF15 (SEQ ID NO: 25) may comprise the nucleotide sequence of SEQ ID NO: 15.

TABLE 2

Functional Variant of Wild-Type Human RPGR-ORF15 vector (SEQ ID NO: 15)		
Nucleotide Position of SEQ ID NO: 15	SEQ ID NO:	Element
1-130	11	5' AAV2 Inverted Terminal Repeat (ITR)
140-434	10	human rhodopsin kinase promoter
449-668	8	cytomegalovirus (CMV)/human β-globin intron
686-4144	3	functional variant of wild-type human RPGR-ORF15 cDNA
4194-4403	6	human β-globin polyadenylation signal
4417-4561	12	3' AAV2 ITR

[0037]   In an embodiment of the invention, the vector encoding wild-type human RPGR-ORF15 comprises a nucleotide sequence comprising the components set forth in Table 3. In this regard, the full-length vector encoding wild-type human RPGR-ORF15 (SEQ ID NO: 4) may comprise the nucleotide sequence of SEQ ID NO: 26.

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TABLE 3

Wild-Type Human RPGR-ORF15 vector (SEQ ID NO: 26)		
Nucleotide Position of SEQ ID NO: 26	SEQ ID NO:	Element
1-130	11	5' AAV2 Inverted Terminal Repeat (ITR)
140-434	10	human rhodopsin kinase promoter
449-668	8	cytomegalovirus (CMV)/human β-globin intron
686-4144	27	wild-type human RPGR-ORF15 cDNA
4194-4403	6	human β-globin polyadenylation signal
4417-4561	12	3' AAV2 ITR

[0038] An embodiment of the invention provides an AAV vector comprising a nucleic acid comprising a nucleotide sequence encoding mouse RPGR-ORF15 or a functional fragment thereof. The nucleotide sequence encoding mouse RPGR-ORF15 may comprise a nucleotide sequence encoding a mouse RPGR-ORF15 protein comprising the amino acid sequence of SEQ ID NO: 23. The vector may further comprise regulatory sequences which are operably linked to the nucleotide sequence encoding mouse RPGR-ORF15 as described herein with respect to other aspects of the invention. In an embodiment of the invention, the AAV vector comprising a nucleic acid comprising a nucleotide sequence encoding mouse RPGR-ORF15 comprises the nucleotide sequence of SEQ ID NO: 24.

[0039] In an embodiment of the invention, the vector may also comprise a nucleotide sequence that is about 70% or more, e.g., about 80% or more, about 90% or more, about 91% or more, about 92% or more, about 93% or more, about 94% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more identical to any of the nucleotide sequences described herein.

[0040] An embodiment of the invention provides a method of making any of the AAV vectors comprising a nucleotide sequence encoding RPGR-ORF15 or a functional fragment or functional variant thereof described herein. The method may comprise amplifying the purine-rich region of RPGR-ORF15 or a functional variant thereof using genomic DNA as a template. Amplifying may be carried out by any suitable method known in the art. For example, the amplifying may be carried out by PCR. The method may comprise ligating the purine-rich region to a nucleotide sequence encoding exons 1 to 14 of RPGR-ORF15 or a functional variant thereof. Ligating may be carried out by any suitable method known in the art (see, e.g., Greene *supra*). The method may further comprise propagating the vector in a XL10-gold bacterial strain.

[0041]   The inventive vectors can be formulated into a composition, such as a pharmaceutical composition. In this regard, the invention provides a pharmaceutical composition comprising any of the vectors described herein, and a pharmaceutically acceptable carrier. Any suitable carrier can be used within the context of the invention, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition is to be administered (e.g., ocular cells, RPE cells, photoreceptor cells, rods, and cones) and the particular method used to administer the composition. The pharmaceutical composition can optionally be sterile or sterile with the exception of the one or more adeno-associated viral vectors.

[0042]   Suitable formulations for the pharmaceutical composition include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain anti-oxidants, buffers, and bacteriostats, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets. Preferably, the carrier is a buffered saline solution. More preferably, the pharmaceutical composition for use in the inventive method is formulated to protect the adeno-associated viral vectors from damage prior to administration. For example, the pharmaceutical composition can be formulated to reduce loss of the adeno-associated viral vectors on devices used to prepare, store, or administer the expression vector, such as glassware, syringes, or needles. The pharmaceutical composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the adeno-associated viral vectors. To this end, the pharmaceutical composition preferably comprises a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. Use of such a composition may extend the shelf life of the vector, facilitate administration, and increase the efficiency of the inventive method. A pharmaceutical composition also can be formulated to enhance transduction efficiency of the adeno-associated viral vector. In addition, one of ordinary skill in the art will appreciate that the pharmaceutical composition can comprise other therapeutic or biologically-active agents. For example, factors that control inflammation, such as

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ibuprofen or steroids, can be part of the pharmaceutical composition to reduce swelling and inflammation associated with *in vivo* administration of the adeno-associated viral vectors. Antibiotics, i.e., microbicides and fungicides, can be present to treat existing infection and/or reduce the risk of future infection, such as infection associated with gene transfer procedures.

[0043]   It is contemplated that the inventive vectors and pharmaceutical compositions (hereinafter referred to collectively as "inventive AAV vector materials") can be used in methods of treating or preventing XLRP. In this regard, an embodiment of the invention provides a method of treating or preventing XLRP in a mammal comprising administering to the mammal any of the inventive AAV vector materials described herein, in an amount effective to treat or prevent the XLRP in the mammal.

[0044]   The terms "treat" and "prevent" as well as words stemming therefrom, as used herein, do not necessarily imply 100% or complete treatment or prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the inventive methods can provide any amount or any level of treatment or prevention of XLRP in a mammal. Furthermore, the treatment or prevention provided by the inventive method can include treatment or prevention of one or more conditions, symptoms, or signs of XLRP. In some cases, the inventive methods may cure XLRP. Also, for purposes herein, "prevention" can encompass delaying the onset of XLRP, or a symptom, sign, or condition thereof.

[0045]   For example, the inventive methods may ameliorate, correct or stop the progression of any one or more of a loss of photoreceptor structure and/or function; thinning or thickening of the outer nuclear layer (ONL); thinning or thickening of the outer plexiform layer (OPL); shortening of the rod and cone inner segments; retraction of bipolar cell dendrites; thinning or thickening of the inner retinal layers including inner nuclear layer, inner plexiform layer, ganglion cell layer and nerve fiber layer; opsin mislocalization; overexpression of neurofilaments; retinal detachment in a mammal, decrease in the electrical response of a photoreceptor in a mammal, loss of electroretinography (ERG) function; loss of visual acuity and contrast sensitivity; loss of visually guided behavior; decreased peripheral vision, decreased central vision, decreased night vision, loss of contrast sensitivity, and loss of color perception.

[0046]   The inventive methods, vectors and pharmaceutical compositions may provide any one or more advantages. For example, the inventive methods, vectors and pharmaceutical compositions may improve the health or quality of the retina and may reduce

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or prevent vision impairment. Accordingly, the inventive methods, vectors and pharmaceutical compositions may, advantageously, improve a patient's ability to carry out vision-guided activities such as, for example, driving an automobile and living independently.

[0047]   It is contemplated that the inventive vectors and pharmaceutical compositions can be used in methods of increasing photoreceptor number in a retina of a mammal. In this regard, an embodiment of the invention provides a method of increasing photoreceptor number in a retina of a mammal, the method comprising administering to the mammal any of the inventive AAV vector materials described herein, in an amount effective to increase photoreceptor number in the retina of the mammal.

[0048]   The inventive vectors and pharmaceutical compositions may also be useful for increasing visual acuity in a mammal. In this regard, an embodiment of the invention provides a method of increasing visual acuity of a mammal, the method comprising administering to the mammal any of the inventive AAV vector materials described herein, in an amount effective to increase visual acuity in the mammal.

[0049]   The inventive vectors and pharmaceutical compositions may also be useful for decreasing retinal detachments in a mammal. In this regard, an embodiment of the invention provides a method of decreasing retinal detachment in a mammal, the method comprising administering to the mammal any of the inventive AAV vector materials described herein, in an amount effective to decrease retinal detachment in the mammal.

[0050]   The inventive vectors and pharmaceutical compositions may also be useful for increasing the electrical response of a photoreceptor in a mammal. In this regard, an embodiment of the invention provides a method of increasing the electrical response of a photoreceptor in a mammal, the method comprising administering to the mammal any of the inventive AAV vector materials described herein, in an amount effective to increase the electrical response of a photoreceptor in the mammal. The photoreceptor may include, for example, one or both of rods and cones. The electrical response of a photoreceptor may be measured by any suitable method known in the art such as, for example, electroretinography (ERG).

[0051]   Another embodiment of the invention provides a method of increasing expression of a protein in a retina of a mammal. The method may comprise administering to the mammal any of the inventive AAV RP2 vectors described herein or a pharmaceutical composition comprising the vector in an amount effective to increase expression of the

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protein in the retina of the mammal. In an embodiment of the invention, the protein is RP2, cone opsin, or cone PDE6.

[0052]   Another embodiment of the invention provides a method of increasing expression of a protein in a retina of a mammal, the method comprising administering to the mammal any of the inventive RPGR vectors described herein or a pharmaceutical composition comprising the vector in an amount effective to increase expression of a protein in the retina of the mammal. In an embodiment of the invention, the protein is RPGR.

[0053]   Another embodiment of the invention provides a method of localizing a protein to the rod outer segments in the retina of a mammal. The method may comprise administering to the mammal any of the inventive RPGR vectors described herein or a pharmaceutical composition comprising the vector in an amount effective to localize the protein to the rod outer segments in the retina of the mammal. In an embodiment of the invention, the protein is rhodopsin or PDE6.

[0054]   The inventive methods may comprise administering the AAV vector material to the eye of the mammal, for example, intraocularly, subretinally, or intravitreally. Preferably, the AAV vector material is administered subretinally.

[0055]   For purposes of the invention, the amount or dose of the inventive AAV vector material administered should be sufficient to effect a desired response, e.g., a therapeutic or prophylactic response, in the mammal over a reasonable time frame. For example, the dose of the inventive AAV vector material should be sufficient to treat or prevent XLRP, increase photoreceptor number, and/or increase visual acuity, in a period of from about 2 hours or longer, e.g., 12 to 24 or more hours, from the time of administration. In certain embodiments, the time period could be even longer. The dose will be determined by the efficacy of the particular inventive AAV vector material and the condition of the mammal (e.g., human), as well as the body weight of the mammal (e.g., human) to be treated.

[0056]   Many assays for determining an administered dose are known in the art. An administered dose may be determined *in vitro* (e.g., cell cultures) or *in vivo* (e.g., animal studies). For example, an administered dose may be determined by determining the IC_{50} (the dose that achieves a half-maximal inhibition of signs of disease), LD_{50} (the dose lethal to 50% of the population), the ED_{50} (the dose therapeutically effective in 50% of the population), and the therapeutic index in cell culture, animal studies, or combinations thereof. The therapeutic index is the ratio of LD_{50} to ED_{50} (i.e., LD_{50}/ED_{50}).

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[0057]   The dose of the inventive AAV vector material also may be determined by the existence, nature, and extent of any adverse side effects that might accompany the administration of a particular inventive AAV vector material. Typically, the attending physician will decide the dosage of the inventive AAV vector material with which to treat each individual patient, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, inventive AAV vector material to be administered, route of administration, and the severity of the condition being treated. By way of example and not intending to limit the invention, the dose of the inventive AAV vector material can be about 1 x 10^8 to about 2.5 x 10^8 vector genomes (vg) per eye, about 1 x 10^8 to about 1 x 10^9 vector genomes (vg) per eye, or about 1 x 10^6 to about 1 x 10^{13} vg per eye. In an embodiment of the invention, the dose of the inventive RP2 vector is about 5 x 10^6 to about 5 x 10^{12}, about 5 x 10^6 to about 5 x 10^8, or about 5 x 10^7 to about 5 x 10^8 vector genomes (vg) per eye. In another embodiment of the invention, the dose of the inventive RPGR-ORF15 vector is about 5 x 10^6 to about 5 x 10^{12}, about 1 x 10^8 to about 5 x 10^9 vg per eye, preferably about 5 x 10^8 to about 2 x 10^9 vg per eye. A dose of the inventive RPGR-ORF15 vector of about 1 x 10^9 vg per eye is especially preferred. In another embodiment of the invention, the dose of the inventive mouse RPGR-ORF15 vector is about 1 x 10^8 to about 5 x 10^8 vg per eye, preferably about 3 x 10^8 vg per eye.

[0058]   As used herein, the term "mammal" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

[0059]   The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES 1-6

[0060]   The following materials and methods were employed for Examples 1-6:

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Mouse line and husbandry

[0061]   *Rpgr*-knockout (KO) mice were maintained in National Institutes of Health (NIH) animal care facilities in controlled ambient illumination on a 12 hour (h) light/12 h dark cycle. Studies conform to Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocols were approved by National Eye Institute (NEI) Animal Care and Use Committee.

AAV vector construction and production

[0062]   The purine-rich region of the mouse or human *RPGR-ORF15* exon was polymerase chain reaction (PCR)-amplified from the genomic DNA of a male C57 mouse or a healthy adult male donor, respectively. The 3' DNA of exon ORF15 including a Sap I restriction enzyme site and the adjacent purine-rich region was PCR amplified from genomic DNA of a healthy adult male donor. Sequences of the primers are as follows:

*   *mRpgr* forward (F): SEQ ID NO: 16;
*   *mRpgr* reverse (R): SEQ ID NO: 17;
*   *hRPGR* F: SEQ ID NO: 18; and
*   *hRPGR* R: SEQ ID NO: 19.

[0063]   PCR was performed with PRIMESTAR HS DNA Polymerase (Clontech Laboratories, Inc., Mountain View, CA). The PCR conditions were 94 °C for 1 minute followed by 30 cycles at 98 °C for 10 seconds and 72 °C for 80 seconds; followed by 7 minutes of extension at 72 °C and hold at 4 °C. The PCR products were verified by sequencing (Sequetech Inc., Redwood City, CA) and ligated to the synthetic upstream exons to generate a full length human or mouse *RPGR-ORF15* cDNA. Exons 1 to 14 and 5' part of exon ORF15 with the Sap I site was synthesized. The PCR-amplified and the synthesized DNA fragments were digested with Sap I respectively, and then ligated to assemble the full-length human *RPGR-ORF15* cDNA (SEQ ID NO: 3). Mouse full-length *Rpgr-ORF15* cDNA was generated using the same strategy.

[0064]   AAV type 2 inverted terminal repeats (ITRs) (SEQ ID NOs: 11 and 12) were used in the AAV vector construction. The RPGR-ORF15 expression cassettes included a human rhodopsin kinase promoter (SEQ ID NO: 10) (Khani et al., *Invest. Ophthalmol. Vis. Sci.*, 48: 3954-3961 (2007)), a chimeric CMV/human β-globin intron (SEQ ID NO: 8), the human

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(SEQ ID NO: 3) or mouse *RPGR-ORF15* cDNA and a human β-globin polyadenylation site (SEQ ID NO: 6). The vector plasmids were propagated in a XL10-gold bacterial strain (Agilent Technologies, Inc., Santa Clara, CA).

[0065]   AAV vectors were produced by triple-plasmid transfection to HEK293 cells, as described in (Grimm et al., *Blood*, 102: 2412-2419 (2003)). The human *RPGR-ORF15* AAV construct was packaged into AAV8, while the mouse *Rpgr-ORF15* construct was packaged into both AAV8 and AAV9. The vectors were purified by polyethylene glycol precipitation followed by cesium chloride density gradient fractionation, as described in Grimm, *supra*. Purified vectors were formulated in 10 mM Tris–HCl, 180 mM NaCl, pH 7.4, quantified by real-time PCR using linearized plasmid standards, and stored at −80 °C until use. Integrity of the vectors was examined each time after purification by amplifying the purine-rich region of the RPGR-ORF15 cDNA.

Subretinal injections

[0066]   AAV vectors were injected subretinally, as described in Sun et al., *Gene Ther.*, 17: 117-131 (2010) but with some modifications. Briefly, mice were anesthetized by intra-peritoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Pupils were dilated with topical atropine (1%) and tropicamide (0.5%). Surgery was performed under an ophthalmic surgical microscope. A small incision was made through the cornea adjacent to the limbus using 18-gauge needle. A 33-gauge blunt needle fitted to a Hamilton syringe was inserted through the incision while avoiding the lens and pushed through the retina. All injections were made subretinally in a location within the nasal quadrant of the retina. Each animal received 1 μl of AAV vector at the concentration of 1×10^{11} to 1×10^{13} vector genomes per ml. Treatment vectors were given in the right eye, and control vehicle was injected in the fellow eye. Visualization during injection was aided by addition of fluorescein (100 mg /ml AK-FLUOR, Alcon, Fort Worth, TX) to the vector suspensions at 0.1% by volume. The dose efficacy studies were carried out on more than 100 *Rpgr*-knockout (KO) mice.

Immunoblot analysis

[0067]   Mouse retinas were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing $1 \times$ proteinase inhibitor by brief sonication. The tissue debris was removed by a brief centrifugation. Retinal protein was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis and transferred to nitrocellulose membranes. After

pre-adsorption with 5% nonfat dry milk for 1 h at room temperature, the membrane blots were incubated overnight at 4°C with the primary antibody. The blots were then washed with Tris buffered saline with the TWEEN 20 detergent (TBST: 137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20, pH 7.6), incubated for 1 h at room temperature with the secondary antibody-horseradish peroxidase conjugated goat anti-rabbit or anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and developed by SUPERSIGNAL West Pico Chemiluminescent (Thermo Fisher Scientific Inc., Rockford, IL). The primary antibodies used in this study were: rabbit anti-mouse RPGR-ORF15 antibody C100 and rabbit anti-human RPGR antibody 643, which recognize the C-terminal of the mouse RPGR-ORF15 and a common region of human RPGR-ORF15 and RPGR[1-19] isoforms, respectively. Mouse monoclonal anti-β-actin antibody (Sigma) was used for loading controls.

Tissue processing, immunofluorescence and morphometric analysis

[0068]   After euthanasia, mouse eyes were harvested. A blue dye was used to mark the orientation of the eye before enucleation to ensure that immunostaining was performed on equivalent areas on vector-treated and vehicle-treated eyes. For fixation, eyes were immediately placed in 4% paraformaldehyde for 1 h. The fixed tissues were soaked in 30% sucrose/PBS overnight, quickly frozen and sectioned at 10-μm thickness using cryostat. An alternative protocol was used to detect RPGR localization to the connecting cilia, as described in Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 44: 2413-2421 (2003). Briefly, eyes were embedded in optimal cutting temperature compound (OCT) without fixation and quick-frozen in liquid nitrogen. Cryosections were cut at 10 μm and collected on pretreated glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Sections were stored at −80°C and used within 2 to 3 days. Just before use, sections were fixed on slides for 2 min with 1% formaldehyde in phosphate-buffered saline (PBS) at pH 7.0. If sections were stored for longer than 1 week, an additional treatment was performed in 0.1% 2-mercaptomethanol (in PBS) for 5 minutes (min), followed by 1% formaldehyde fixation for 5 min. Sections were then washed once in PBS and carried through to immunofluorescence staining.

[0069]   For immunofluorescence staining, the cryosections were pre-adsorbed in 5% goat serum in PBS containing 0.1% Triton X-100 (PBST) for 1 h, and then incubated overnight at 4°C in primary antibody diluted in 5% goat serum, as described in Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 44: 2413-2421 (2003). Sections were washed three times in PBST and incubated with fluorochrome-conjugated secondary antibodies and 0.2 μg/ml DAPI (4',6-

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diamidino-2-phenylindole) for 1 h. Sections were washed again and mounted in
FLUOROMOUNT-G mounting medium (SouthernBiotech, Birmingham, AL). Images were
captured using a fluorescence microscope AXIO IMAGER Z1 or a confocal scanning
microscope LSM700 (Zeiss, Germany).

[0070] The primary antibodies included the poly-clonal rabbit anti-human RPGR-ORF15
antibody 636 and rabbit anti-mouse RPGR-ORF15 antibody S1 (Hong et al., *Invest.
Ophthalmol. Vis. Sci.*, 43: 3373-3382 (2002)), which recognize the common region of RPGR-
ORF15 and RPGR[1-19] isoforms in human and mouse, respectively. Other primary antibodies
used in this study include monoclonal antibody for rhodopsin (1D4, Santa Cruz
Biotechnology, Dallas, TX) and M-cone opsin (Millipore, Billerica, MA). Secondary
antibodies included goat anti-rabbit and anti-mouse antibodies conjugated with ALEXA
FLUOR 555 and 568 dyes (Life Technologies, Grand Island, NY).

[0071] For morphometric analyses of outer nuclear layer (ONL) thickness, measurements
were made along the vertical meridian at four locations to each side of the optic nerve head
separated by 500 μm each. Measurements began at about 500 μm from the optic nerve head
itself.

Electroretinogram (ERG)

[0072] Mice were dark-adapted overnight. Anesthesia and pupil dilation were conducted
as described above. A computer-based system (ESPION E2 electroretinography system,
Diagnosys LLC, Lowell, MA) was used for ERG recordings in response to flashes produced
with light-emitting diode (LED) or Xenon bulbs. Corneal ERGs were recorded from both
eyes using gold wire loop electrodes with a drop of 2.5% hypromellose ophthalmic
demulcent solution. A gold wire loop placed in the mouth was used as reference, and a
ground electrode was on the tail. The ERG protocol consisted of recording dark-adapted
ERGs using brief flashes of -2 to +3 log sc cd.s.m^{-2}/flash. Responses were computer
averaged and recorded at 3 to 60 second (s) intervals depending upon the stimulus intensity.
Light-adapted ERGs were recorded after 2 min of adaptation to a white 32 cd.m^{-2} rod-
suppressing background. ERGs were recorded for stimulus intensities of -0.52 to +2 log sc
cd.s.m^{-2}.

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Optical Coherence Tomography (OCT)

[0073]   OCT volume scan images were acquired with a spectral domain (SD) OCT system (SPECTRALIS system, Heidelberg Engineering, Carlsbad, CA). Mice were anesthetized and pupils were dilated as described above. The optic nerve head was centered within ~1.0 mm diameter field of view. Retinal thickness maps were generated by Heidelberg Eye Explorer software.

Statistical analysis

[0074]   Two-tailed paired t-test was used to compare outcomes in vector-treated versus vehicle-treated eyes. GRAPHPAD Prism 6 software (GraphPad Software, La Jolla, CA) was used for statistical analysis.

EXAMPLE 1

[0075]   This example demonstrates the generation of mouse and human *RPGR*-ORF15 AAV vectors.

[0076]   AAV vectors carrying either a mouse or a human RPGR-ORF15 expression cassette were constructed. Previous efforts to obtain a full-length *RPGR-ORF15* cDNA using reverse transcription PCR had not been successful due to the purine-rich region of the terminal ORF15 exon. To overcome this problem, regular PCR was conducted using genomic DNA as a template to amplify the purine-rich region, and then the purine-rich region was ligated to a synthetic DNA fragment encoding the upstream exons. This strategy was adopted for obtaining both mouse and human *RPGR-ORF15* cDNA. Sequencing of the complete cDNA was performed to validate the products. A human rhodopsin kinase (RK) promoter (SEQ ID NO: 10), which shows rod and cone cell specificity (Khani et al., *Invest. Ophthalmol. Vis. Sci.*, 48: 3954-3961 (2007)), was used to drive RPGR-ORF15 expression. These two vectors were packaged into AAV type 8 and are hereafter referred to as AAV8-*mRpgr* and AAV8-*hRPGR*, respectively. The mouse *RPGR-ORF15* vector was also packaged into AAV type 9 (hereafter referred to AAV9-*mRpgr*), a serotype that transduces cones of non-human primate efficiently (Vandenberghe et al., *PLoS Pne*, 8: e53463 (2013)).

[0077]   The vector plasmids containing the purine-rich region of *RPGR-ORF15* and two AAV inverted terminal repeats (ITRs) were prone to deletions or rearrangements when the plasmid clones were propagated in commonly used bacterial strains. After extensive testing,

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it was observed that the vector plasmids maintained their integrity in XL10 Gold cells. PCR amplification of the region spanning the repetitive glutamic acid-glycine coding sequence in the mouse or human *RPGR-ORF15* cDNA produced the expected 1.3 kb or 1.6 kb fragment in the vector plasmids and all vector preparations. The PCR assay did not identify visible deletion in most AAV vector preparations. However, minor deletions were detected in two vector preparations. The full-length human *RPGR-ORF15* vector comprised SEQ ID NO: 15 and encoded the amino acid sequence of SEQ ID NO: 25 (functional variant of wild-type human RPGR). The full-length mouse *RPGR-ORF15* vector comprised SEQ ID NO: 24 and encoded the amino acid sequence of SEQ ID NO: 23 (mouse RPGR).

EXAMPLE 2

[0078]   This example demonstrates the AAV vector-mediated expression of RPGR-ORF15 proteins.

[0079]   To test whether the vectors of Example 1 mediate full-length RPGR-ORF15 protein expression in mouse retina, immunoblot analyses of the retinal lysates from vector-treated *Rpgr*-KO mice were performed. Using an antibody against the C-terminus of the mouse RPGR-ORF15, RPGR protein was identified in the *Rpgr*-KO retina treated with the AAV8-*mRpgr* vector. The retinal lysate from an *Rpgr*-KO mouse injected subretinally with 1×10^9 vg AAV8-*mRpgr* vector revealed a ~200 kDa protein band corresponding to the full length RPGR-ORF15 protein, which is identical to that detectable in a wild-type (WT) C57/Bl6 mouse retina. This protein was identical in size to the wild type (WT) RPGR protein, suggesting the ability of the vector to produce a full-length mouse RPGR-ORF15 protein. Similarly, AAV8-*hRPGR* vector was able to generate the full-length human RPGR-ORF15 protein in the *Rpgr*-KO retina. The retinal lysate from an *Rpgr*-KO mouse injected subretinally with 1×10^9 vg AAV8-*hRPGR* vector revealed a ~200 kDa protein band corresponding to the full length RPGR-ORF15 protein, identical to that from a commercially sourced human retinal lysate. No signal was detected in the lane that included 1/10th the amount of retinal lysate from the vector-injected eye, revealing the sensitivity limits of the assay. A set of lower molecular weight proteins in the AAV8-*hRPGR*-treated retina were also detected when an antibody against the epitopes upstream of the ORF15 exon was employed. Although the possibility that these shorter proteins were caused by the deletions in the ORF15 exon in AAV vector preparations cannot be ruled out, these shorter proteins could also be alternatively spliced or C-terminal truncated forms of RPGR-ORF15, as

observed in WT mouse retina (Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 43: 3373-3382 (2002)). Shorter proteins have also been identified in AAV8-*mRpgr*-treated *Rpgr*-KO retina when an antibody against the epitopes upstream of the ORF15 exon of mouse RPGR was used.

[0080]   RPGR-ORF15 protein localizes to the connecting cilia of the photoreceptors in mouse and other mammalian species (Hong et al., *Proc. Natl. Acad. Sci.*, 97: 3649-3654 (2000); Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 44: 2413-2421 (2003)). To test whether the vector-expressed mouse and human RPGR-ORF15 also localize to the connecting cilia, a brief on-slide fixation of frozen retinal section using 1% formaldehyde instead of the conventional 4% paraformaldehyde (PFA) fixation was employed for immunofluorescence assay, since the latter blocks antibody penetration to this region (Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 44: 2413-2421 (2003)). Similar to the WT protein, the vector-expressed RPGR-ORF15 mainly appeared as dots between the inner (IS) and outer segments (OS) corresponding to the location of the connecting cilia. In addition to its connecting cilia localization, the vector-expressed RPGR-ORF15 was frequently observed at the IS, and sometimes at the nuclei and the synaptic terminals of the photoreceptors when the conventional 4% PFA fixation was used. When the modified fixation method was used, RPGR immunostaining was observed at the connecting cilia region in both WT and the AAV8-*mRpgr* treated retina. When the regular fixation method was used, RPGR was undetectable in the WT retina, but the staining was observed at the IS, ONL and the synaptic region in the AAV8-*mRpgr* treated retina. This apparent mis-localization of RPGR-ORF15 seems to be vector-related as it was also observed in AAV5 RPGR vector-treated canine retina (Beltran et al., *Proc. Natl. Acad. Sci.*, 109: 2132-2137 (2012)) but not in the WT mouse retina. Without being bound to a particular theory or mechanism, it is believed that over-expression of the protein by using a relatively high vector dose (1×10^9 vector genomes (vg)) and a strong RK promoter may account for this observation. No detectable RPGR-ORF15 expression was observed in other retinal layers owing to the photoreceptor specificity of the RK promoter. The mouse *Rpgr-ORF15* delivered by the AAV9 vector expressed the protein at a similar level as the AAV8 vector when same dose was used, and the protein was targeted to identical subcellular localization.

EXAMPLE 3

[0081]   This example demonstrates the short-term dose-toxicity profile of the mouse and human *RPGR-ORF15* vectors.

[0082]   To define the dose range for a long–term efficacy study, a short-term vector toxicity study was conducted over a 4-month period. Eight week-old *Rpgr*-KO mice were unilaterally injected with 1×10^{10} or 1×10^9 vg of AAV8-*hRPGR* or AAV8-*mRpgr* vector per eye through sub-retinal injection. The fellow eye was used as control by injecting the same volume of vehicle. Dark- and light-adapted ERG were recorded to evaluate responses from rod and cone photoreceptors at 4 months post-injection (PI) before sacrificing the mice for immunofluorescence analyses. Due to the slow retinal degeneration in the *Rpgr*-KO mouse line, a therapeutic effect at 4 months after vector treatment was not expected.

[0083]   No statistically significant difference was observed between the vector and the vehicle-treated eyes in ERG amplitudes of dark-adapted a-, b- and light-adapted b-waves in mice that received 1×10^9 vg AAV8-*hRPGR* or AAV8-*mRpgr* vector. However, remarkably lower amplitudes of all three ERG components were observed in eyes receiving 1×10^{10} vg vectors, while 1×10^9 vg/eye vector administration did not cause significant ERG change, indicating the vector toxicity at the high dose. This observation was corroborated by immunofluorescence analyses of the vector-treated retinas. Since the retina sections were fixed in 4% PFA before freezing, only the pool of mis-localized recombinant RPGR at IS was detected (as explained above). More intensive RPGR-ORF15 expression was observed in 1×10^{10} vg vector-treated retina, accompanied by a much thinner outer nuclear layer (ONL) and shorter IS. In contrast, the ONL thickness of the 1×10^9 vg vector-treated retina did not reveal marked difference from the vehicle-treated retina. Both the ERG and immunofluorescence analyses indicate that the dose of 1×10^9 vg per eye is well tolerated, while 1×10^{10} vg is toxic to the mouse retina. Without being bound to a particular theory or mechanism, it is believed that a combinational effect of overexpressing the RPGR-ORF15 protein, the large amount of AAV capsid protein and vector DNA that exceeds the processing capacity of the retinal cells might account for the toxicity of the high vector dose. Therefore, the dose of 1×10^{10} vg per eye was not included in the subsequent long-term efficacy study.

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EXAMPLE 4

[0084]   This example demonstrates the treatment effect in the *Rpgr*-KO mice following gene delivery of mouse *Rpgr-ORF15*.

[0085]   To test whether the mouse *Rpgr-ORF15* cDNA delivered by AAV8 or AAV9 vector was efficacious, the vectors were injected in the subretinal space of 6-8 week-old mice at doses ranging from 1×10^8 to 1×10^9 vg per eye. Unilateral vector injection was performed on each mouse and the contralateral eye was injected with the vehicle. Due to the slow progression of retinal degeneration in the *Rpgr*-KO mice (Hong et al., *Proc. Natl. Acad. Sci.*, 97: 3649-3654 (2000); Hong et al., *Invest. Ophthalmol. Vis. Sci.*, 46: 435-441 (2005)), a longitudinal ERG monitoring was performed during the 18-month follow-up period. Given the large variation in ERG amplitudes among individual mice, paired t-test was employed throughout the study to compare the vector- and the vehicle-treated eyes. Among all cohorts, mice receiving 3×10^8 vg AAV9-*mRpgr* displayed the strongest therapeutic effect in vector-treated eyes. Although only a slight improvement was observed in the vector-treated eyes at 12 months PI, the therapeutic effect became more pronounced at 18 months PI, in which significantly larger amplitudes were observed for dark-adapted a-wave and light-adapted b-wave in response to increasing intensities of flash stimuli. These eyes also displayed significantly larger dark-adapted b-wave amplitude, which was not seen at 12 months PI, reflecting a better preservation of visual signaling to the bipolar cells. In all seven mice that survived 18 month monitoring, each individual animal exhibited greater dark-adapted a-, b- and light-adapted b- wave amplitudes elicited from the highest flash intensity in the vector-treated eye. Cohorts receiving other vector doses (1×10^9 vg/eye AAV8-, 1×10^8 vg/eye AAV8-, or 1×10^9 vg/eye AAV9-*mRpgr* vector) displayed suboptimal rescue at 18 months PI compared with the one receiving 3×10^8 vg AAV9-*mRpgr* vector.

[0086]   Functional rescue of the vector-treated retinas was correlated with their structural improvement. Much thicker ONL was observed in 3×10^8 vg AAV9-*mRpgr* treated eyes than the control eyes, as revealed by optical coherence tomography (OCT) retinal imaging at 18 months PI. The increased retinal thickness in the vector-treated eye was observed within a ~1.0 mm^2 diameter field of view, except for the central area where optic nerve head (ONH) was located. Subsequent to OCT, immunofluorescence analyses of treated mouse retina showed that AAV-mediated RPGR expression spanned roughly half of the cross-section. Vector-treated eyes preserved significantly more rows of photoreceptors than control eyes, consistent with the OCT findings. Seven to ten rows of photoreceptors were maintained in a

majority of the vector-treated eyes, compared with four to six rows in the control eyes. The measurements of ONL thickness at 500 μm intervals along the vertical (dorsal-ventral) meridian on retinal sections further corroborated these findings. The average ONL thickness at different locations of the vector-treated retinas ranged between 31.7μm and 43.5μm, while it ranged between 19.0 μm and 28.3μm in the control retinas. The treatment effect appeared to be even more pronounced at 24 months PI in one group of mice receiving 1×10^9 vg AAV8-*mRPGR* injection. While the ONL of the control retina almost disappeared in the superior portion and only 1 to 3 rows of photoreceptors remained in the inferior retina, 6-8 rows of photoreceptors survived in inferior areas of the vector-treated retina where RPGR was expressed.

[0087] Opsin mis-localization (because of altered transport/targeting to outer segments) is detectable in animal models and in a human carrier with *RPGR* mutations. Immuno-staining was performed on the 3×10^8 vg AAV9-*mRpgr*-treated retina at 18 months PI to assess whether opsin transport could be corrected by *Rpgr* gene delivery. In the WT retina, M-cone opsin is found exclusively in the outer segments of cone cells. In the vehicle-treated *Rpgr*-KO retina, M-opsin was detected in inner segments as well as in the perinuclear and synaptic regions in addition to the outer segments. More M-opsin was present in photoreceptor inner segments in the superior retina compared with the inferior retina. Without being bound to a particular theory or mechanism, it is believed that this is probably due to the superior to inferior gradient of M-opsin expression. This M-opsin transport to outer segments was partially rescued in the vector-treated retina at 18 months PI.

[0088] Rhodopsin localized only to the rod outer segments in WT retina. This mis-localization was not seen in the RPGR-expressed area in the vector-injected KO retina. Rhodopsin was additionally observed at the IS and perinuclears in the vehicle-injected KO retina. In the young *Rpgr*-KO mouse retina, rhodopsin was appropriately localized; however, rhodopsin immunostaining was detected in the inner segments and perinuclear region in 20 month-old vehicle-injected *Rpgr*-KO retina. Rhodopsin localization was corrected in the areas expressing RPGR-ORF15 in the vector-treated retina of *Rpgr*-KO mice.

EXAMPLE 5

[0089] This example demonstrates the treatment effect in the *Rpgr*-KO mice following gene delivery of human *RPGR-ORF15*.

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[0090]   As a potential vector candidate for future human trials, AAV8-*hRPGR* was tested for its efficacy in *Rpgr*-KO mice with four different doses; 3×10^9, 1×10^9, 3×10^8 and 1×10^8 vg per eye. Six to 8 week-old mice were injected with the vector subretinally and ERG was performed at 12 and 18 months PI. Among the four dose groups, optimal outcome was observed in 1×10^9 vg-treated group. At this dose, the vector-treated eyes displayed significantly higher amplitudes dark-adapted a-, b-wave and light-adapted b-wave were observed in response to increasing intensities of flash stimuli at 18 month PI, indicating the rescue of retinal function in the *Rpgr*-KO mouse following human *RPGR-ORF15* gene delivery. All 11 mice that survived the 18 month monitoring period exhibited higher light-adapted b-wave amplitudes in vector-treated eyes; of these, 10 and 9 mice respectively displayed higher dark-adapted b-wave and dark-adapted a-wave.

[0091]   Mice receiving 3×10^9 vg vector demonstrated much lower ERG amplitudes in the vector-treated eyes than control eyes at 18 month PI; however, this difference was not statistically significant at 12 month PI indicating the long-term toxicity at this dose. The 3×10^8 vg and 1×10^8 vg vector-treated eyes did not show a difference from control eyes for ERG amplitudes. To investigate whether the therapeutic effect was too small to be detected, ERG was performed again 6 months later when these mice were almost 26 month-old. ERG improvement was still not observed in the vector-treated eyes in both dose groups, indicating that these two vector doses were too low to achieve functional rescue in the *Rpgr*-KO mice.

[0092]   OCT retinal imaging was performed on the *Rpgr*-KO mice treated with 1×10^9 vg AAV8-*hRPGR* vector at 18 months PI. Much thicker ONL was observed in the vector-treated retinas than the controls, and the thickness of the whole retina in the vector-treated eyes was greater than the controls within ~ 1.0 mm^2 diameter field of view. By immunofluorescence analyses, vector-treated retina revealed hRPGR expression in about half of the area of the cross section. Consistent with the OCT findings, more rows of photoreceptors were preserved in the vector-treated retina than in the control and measurements of ONL thickness across 4 mm of retina along the vertical (dorsal-ventral) meridian corroborated these observations. The average ONL thickness at different locations of the vector-treated retinas ranged between 21.2 µm and 33.4 µm, while vehicle-treated retinas had ONL between 14.3 µm and 24.1 µm. Immunofluorescence analyses of the retina receiving a lower vector dose (3×10^8 vg) revealed hRPGR staining in a smaller area; however, more photoreceptors were preserved in this area compared to the adjacent region. Therefore, preservation of photoreceptors in vector-transduced areas was still achieved in the

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lower dose groups despite the lack of overall functional preservation as evaluated by full-field ERG. Rhodopsin was only observed in rod OS of WT retina, but additional staining was observed at the IS, perinuclear and synaptic terminals in the vehicle-treated *Rpgr*-KO retina. This rhodopsin mis-localization was not detected in areas with appropriate hRPGR expression in the vector-treated (1 x 10^9 vg AAV8-*hRPGR*) retina, while it was apparent in the control retina.

EXAMPLE 6

[0093]   This example demonstrates the rescue of retinal function and structure following *RPGR-ORF15* gene delivery to older *Rpgr*-KO mice.

[0094]   To assess whether retina with more substantial degeneration would still benefit from the treatment, 3×10^8 vg AAV8-*mRPGR* was subretinally injected into 1 year-old *Rpgr*-KO mice. No appreciable difference was observed between vector- and vehicle-treated eyes when tested at 5 months PI. However, the ERG rescue became apparent in vector-treated eyes at 11 months PI, when mice were 23 month-old. OCT imaging revealed much thicker ONL in the vector-injected retina than in the vehicle-injected control; this finding was subsequently confirmed by morphology analyses. Substantially more rows of photoreceptors were observed in the area with RPGR expression in the vector-injected eye as compared to control retina. These results suggest that the *Rpgr*-KO mouse could still respond favorably to *Rpgr* gene delivery even when treated at an advanced age with active degeneration in the retina.

EXAMPLES 7-12

[0095]   The following materials and methods were employed for Examples 7-12:

Generation of the Rp2-KO mouse line and animal husbandry

[0096]   An *Rp2-KO* mouse line was created by crossing an *Rp2*^{flox/flox} line with a ubiquitous Cre expressing line (*CAG cre and Zp3 Cre* line). All of the mice were maintained as described in the "mouse line and husbandry" section of the methods described for Examples 1-6 above.

AAV Vector construction and production

[0097]   A synthetic human *RP2 cDNA* (SEQ ID NO: 1) with ClaI and XhoI sites was cloned in a vector with a rhodopsin kinase promoter (SEQ ID NO: 10), a chimeric *(β-globin/CMV)* intron (SEQ ID NO: 9), and a *β-globin poly-A* tail (SEQ ID NO: 7). AAV type 2 *inverted terminal repeats (ITRs)* (SEQ ID NOs: 12 and 13) were used in the AAV vector construction. The left *ITR* (*ITR* near the promoter region) (SEQ ID NO: 13) was mutated to eliminate the terminal resolution site and AAV D sequence to make it a self-complementary AAV vector.

[0098]   Triple-plasmid transfection to HEK293 cells was used to produce AAV vectors as described in Grimm et al., *Blood*, 102: 2412-2419 (2003). The self-complementary human RP2 construct (SEQ ID NO: 14) was packaged into an AAV8 capsid (SEQ ID NO: 5). The amount of virus was measured by real time PCR using the following primer and fluorescent labeled probes:

- Forward primer (5'-3'):-GCACCTTCTTGCCACTCCTA (SEQ ID NO: 20);
- Reverse primer (5'-3'):- GACACAGCACCAGGCTAAATCC (SEQ ID NO: 21); and
- Probe (5'-3'):- CGTCCTCCGTGACCCCGGC (SEQ ID NO: 22).

Sub-retinal injection

[0099]   Subretinal injection was performed as described in Sun et al., *Gene Therapy*, 17: 117-131 (2010) with some modifications. Mice were anesthetized with an intra-peritoneal injection of ketamine (80 mg/Kg) and xylazine (8 mg/Kg). The pupils were dilated with topical atropine (1%) and tropicamide (0.5%). Proparacaine (0.5%) was used as topical anesthesia. Surgery was performed under an ophthalmic surgical microscope. An 18 gauge hypodermic needle was used to make a small incision in the cornea adjacent to the limbus. A 33 gauge blunt needle fitted to a Hamilton syringe was inserted through the incision while avoiding the lens and pushed through the retina. A 1 µl of sample containing either therapeutic vector or a saline solution was delivered subretinally. Therapeutic vectors were given in the right eye and vehicle was given in the fellow eye. Visualization during injection was aided by addition of fluorescein (100 mg/ml AK-FLUOR (fluorescein injection, USP), Alcon, Fort Worth, TX, USA) to the vector suspensions at 0.1% by volume.

ERG

[0100]   ERGs were performed using ESPIONE E2 electroretinography system. Mice were dark adapted overnight. Pupils were dilated with topical atropine (1%) and tropicamide (0.5%). The mice were anesthetized with an intra-peritoneal injection of ketamine (80 mg/Kg) and xylazine (8 mg/Kg). All the above procedures were done in dim red light. ERGs were recorded from both eyes using gold wire loops with 0.5% proparacaine topical anesthesia and a drop of 2% methylcellulose for corneal hydration. A gold wire loop placed in the mouth was used as reference, and a ground electrode was placed on the tail. Dark-adapted ERG was done in the dark with brief white flash intensity ranging from -4 log cd-s/m^2 to +3 log cd-s/m^2. Light-adapted ERG was recorded after light adaptation of 2 min with white light. ERG recording was done with brief white flash intensity ranging from -0.53 log cd-s/m^2 to +2 log cd-s/m^2 with a background white light of 20 cd/m^2 intensity. The flicker response was taken with 10 Hz light flicks. For recording M and S-opsin mediated ERG response, the mice were first light adapted for 2 minutes in a green light with 20 cd/m^2 light intensity. ERG was recorded by alternating green and ultraviolet (UV) flash with intensity ranging from -0.52 to +2 log cd-s/m^2 for green flash and -4 to -0.52 log cd-s/m^2 for UV flash with a background green illumination of 20 cd/m^2. ERG was recorded from *Rp2-KO* mice treated with different vector doses and littermate wild type mice.

Determination of visual acuity

[0101]   Visual acuity of the mice was determined by an optokinetic test in an optokinetic reflex (OKR) arena developed by Cerebral Mechanics following the protocol described in Douglas et al., *Vis. Neurosci.*, 22: 677-684 (2005) and Prusky et al., *Invest. Ophthalmol. Vis. Sci.*, 45: 4611-4616 (2004). Briefly, the mouse was placed in the center of a closed OKR arena surrounded by four computer screens and a camera on top to monitor the movement of the animal. The computer screens created a virtual image of a rotating drum with sine waves grating in a 3D confirmation. The tracking of the gratings by the mouse was scored by its head and neck movement. The spatial frequency of the grating was controlled and monitored by OPTOMOTRY software (Version 14). The maximum spatial frequency in a 100% background contrast which generated a tracking movement by the animal was recorded for each eye.

Immunoblotting

[0102]   Whole retinal lysate was prepared in RIPA buffer with protease inhibitor cocktail by sonication. The lysate was cleared by centrifugation, and the protein was estimated using Bradford reagent. Approximately 20 µg of protein was used in every lane of 10% denaturing protein gel (BioRad, Hercules, CA). Immunoblotting was performed by a standard procedure using the primary antibody against human *RP2* and β-actin. The proteins were visualized with peroxidase-conjugated secondary antibody with appropriate reagents.

Immunohistochemistry

[0103]   For immunohistochemistry, mice were euthanized and eyes were enucleated. The eyes were fixed in 4% PFA solution for 1-2 hours, passed through a series of sucrose solution for cryo-protection, and were flash frozen in OCT solution. A series of retinal sections having a thickness of 12 µm was cut through the eyes in a superior-inferior pole orientation by cryostat. The sections were stained with specific antibody (M & S cone opsin, rhodopsin, PNA, RP2) using the protocol described below. Briefly, sections were blocked in 5% goat serum in PBS containing 0.1% Triton X-100 (PBST) for 1 h, followed by incubation in primary antibodies diluted in 2% goat serum at 4 °C overnight. Sections were washed three times in PBST and incubated with fluorochrome-conjugated secondary antibodies and 0.2 µg/ml DAPI for 1 h. Sections were washed again with PBS and mounted in FLUOROMOUNT-G mounting medium (SouthernBiotech, Birmingham, Alabama). Sections were visualized, and images were captured on a confocal scanning microscope LSM700 (Zeiss, Germany).

[0104]   To prepare a flat mount, retina enucleated eyes from euthanized mice were first incubated in chilled PBS solution for 15 minutes over ice. Afterwards, eyeballs were then squeezed gently several times to detach the retina. The eyeballs were then fixed in 4% PFA for 1 hour, and the retina was separated from other parts of eye, washed in PBS containing 0.1% Triton, blocked in 5% goat serum in PBST for 4 hrs, followed by incubation in primary antibodies diluted in 2% goat serum at 4°C overnight. The retina was again washed 3 times (2 for 45 mins each and 1 for 1 hr) in PBST, and incubated with fluorochrome-conjugated secondary antibodies for 4 hrs. The sections were again washed in PBST as described above and mounted in FLUOROMOUNT-G mounting medium (SouthernBiotech, Birmingham,

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Alabama) with the photoreceptor layers facing up. Images were captured on a confocal scanning microscope LSM700 (Zeiss, Germany).

Statistical analysis

[0105] Two-tailed paired and unpaired t-test was used to compare outcomes in vector-treated versus vehicle-treated eyes. GRAPHPAD Prism 6 software (GraphPad Software, La Jolla, CA) was used for statistical analysis.

EXAMPLE 7

[0106] This example demonstrates that a *Rp2*-KO mouse exhibits a progressive degeneration of cone photoreceptors.

[0107] An *Rp2*-KO mouse model was generated by crossing *Rp2*$^{flox/flox}$ mice with either a *CAG Cre* transgenic mouse line as reported in Li et al., *Invest. Ophthalmol. Vis. Sci.*, 54: 4503-4511 (2013) or a *ZP3 Cre* mouse line. In *ZP3-Cre* line, Cre is expressed specifically in oocytes. Although Cre is ubiquitously expressed in *CAG-Cre* line, Cre expression on its own does not affect retinal function, as shown in Li et al., *Invest. Ophthalmol. Vis. Sci.*, 54: 4503-4511 (2013). Addition of *CAG-Cre* transgene even in the Rp2-KO line has no further impact of the retina. *RP2* exon 2 was deleted in the resulting *Rp2*-KO mouse line, and no RP2 protein was detectable in the retina and other tissues (Li et al., *Invest. Ophthalmol. Vis. Sci.*, 54: 4503-4511 (2013)). To evaluate the progression of retinal degeneration in this model, a large cohort of the mice was monitored along with their wild-type (WT) littermates by electroretinogram (ERG) during an 18-month period. Amplitude of dark-adapted a-wave is mainly contributed by rods. Though cone-derived a-wave is relatively small under light-adapted conditions, b-wave is produced by the inner retina neurons and reflects the activity of cone system. Therefore, dark-adapted a-wave and light-adapted b-wave were used to represent rod and cone functions, respectively. Consistent with previous observations (Li et al., *Invest. Ophthalmol. Vis. Sci.*, 54: 4503-4511 (2013); Zhang et al., *FASEB J.*, 29: 932-942 (2014)), the *Rp2*-KO mice exhibited significantly reduced amplitudes of dark-adapted a-wave and light-adapted b-wave through the entire duration of the experiments. The stimulus intensities for dark- and light-adapted ERGs were −4.0 to 3.0 and −1.0 to 2.0 log cd s/m^2, respectively. This ERG amplitude reduction happened even as early as 1 month of age in a small group of monitored mice, indicating functional impairment of both rods and cones at an early age. However, measurement of the ratio of KO to WT for ERG amplitudes revealed

distinct dynamics between rod and cone functions in the KO mice over the 18-month period. The dark-adapted a-wave amplitude of KO relative to that of WT remained stable after 4 months of age without additional reduction, whereas the KO to WT ratio of light-adapted b-wave amplitude continuously declined at a nearly constant rate between 4 and 18 months. As a result, about 78% of rod ERG amplitude was preserved at 18 months compared with only 33% of cone ERG amplitude, demonstrating a more severe impairment of cone function in the KO mice. Additionally, the relatively mild impairment in rod function did not significantly impact the inner retina function since no difference was observed between KO and WT mice for dark-adapted b-wave with dim flash intensity. The progressive worsening of cone function in the KO retina was also reflected by the pronounced reduction in the flicker response.

[0108]   A significant alteration in light-adapted b-wave kinetics was observed in *Rp2*-KO mice when compared with their WT littermates, consistent with the findings in Zhang et al., *FASEB J.*, 29: 932-942 (2014). To assess the response kinetics, the time it took the b-wave to rise to 50% of its peak amplitude ($T_{50\ rise}$), the time it took to reach the peak amplitude (T_{max}, same as implicit time) and the time to fall from the peak to 50% of the peak amplitude (T_{50} decay) were measured. The 4-month-old KO mice displayed significantly longer time course in all three measurements than their WT littermates. In particular, the kinetics of the b-wave falling phase was distinctly slower in KO mice compared with WT, as reflected by much longer T_{50} decay time (40.1 ± 1.6 ms in WT versus 81.8 ± 5.5 ms in KO, mean ± SEM). This alteration in kinetics began early, as longer T_{max} and T_{50} decay were already observed in 1-month-old KO mice. The kinetics difference between KO and WT mice appeared to be specific to the cone system, as no such change was observed in dark-adapted b-wave under low flash stimulus intensity, which reflects the function of pure rod system.

[0109]   Consistent with the cone-mediated ERG findings, very few M- or S-cone photoreceptors were observed at 18 months of age compared with the WT retina, indicating a severe cone degeneration in the *Rp2*-KO retina. In contrast, no detectable change in the thickness of the rod-dominant photoreceptor layer was seen during the 18-month period. In addition, distribution of rhodopsin in the *Rp2*-KO mice remained the same as the WT mouse, with rhodopsin mainly being detected at the OS, its natural localization. The thickness of rod-dominant photoreceptor layer was not significantly altered even in the 18-month-old KO retina. The relatively mild rod dysfunction in the KO mice is likely caused by somewhat disorganized OS as revealed by ultrastructural analysis (Li et al., *Invest. Ophthalmol. Vis.*

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Sci., 54: 4503-4511 (2013)). Rod disorganization was not captured by light microscopy analyses.

EXAMPLE 8

[0110]   This example demonstrates that an AAV8 vector carrying human *RP2* cDNA mediates stable RP2 expression in mouse photoreceptors.

[0111]   To develop gene therapy for *RP2*-associated retinal degeneration, an AAV vector carrying a human RP2 expression cassette was designed and constructed. The vector (SEQ ID NO: 14) was composed of a photoreceptor-specific human rhodopsin kinase (RK) promoter (SEQ ID NO: 10), a CMV and human β-globin hybrid intron (SEQ ID NO: 9), a human *RP2* cDNA (SEQ ID NO: 1), and the human β-globin polyadenylation site (SEQ ID NO: 7), flanked by two inverted terminal repeats (ITRs) from AAV serotype 2 (AAV2) (SEQ ID NOs: 12 and 13). The RK promoter has been shown to be able to drive cell-specific transgene expression in both rods and cones in mice (Khani et al., *Invest. Ophthalmol. Vis. Sci.*, 48: 3954-3961 (2007)). The length of this human *RP2* expression cassette was smaller than 2 kilo-basepairs (kb), a size that fit well with a self-complementary (sc) AAV vector that is capable of mediating earlier onset and more efficient transgene expression than a conventional single-stranded (ss) vector. To construct a sc vector, one WT ITR was replaced with a mutant ITR in which the terminal resolution site and the AAV D sequence were deleted. The vector was packaged into AAV8 (SEQ ID NO: 5), a serotype that transduces photoreceptors of mouse and non-human primate very efficiently, and was designated as AAV8-scRK-*hRP2* vector (SEQ ID NO: 14), encoding the amino acid sequence of SEQ ID NO: 2 (human RP2).

[0112]   To test whether the vector mediates human RP2 expression, the vector was injected subretinally into *RP2*-KO mice, and the retinal extracts were subjected to immunoblot analyses 4 weeks later with a polyclonal antibody recognizing both mouse and human RP2 proteins. While the vehicle-treated retina did not reveal any RP2-specific band, the vector-treated retina exhibited a band at the expected molecular weight of ~40 kDa, identical to that of the human retinal lysate, indicating the vector's ability to express human RP2 protein. The endogenous mouse RP2 protein in the WT retina migrated slightly faster than the human counterpart. Without being bound to a particular theory or mechanism, it is believed that because mouse and human RP2 proteins contain similar numbers of amino acid residues (a.a.) (350 a.a. for human RP2 and 347 a.a. for mouse RP2), this electrophoretic

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mobility difference might reflect the different amino acid compositions and/or post-translational modifications of the two proteins.

[0113]   Immunofluorescence analysis was performed to examine the cellular and subcellular localization of the vector-expressed RP2 protein in the retina. Endogenous mouse RP2 protein was detected in multiple layers in WT retina, including the IS, outer and inner plexiform layers (OPL and IPL), which was not seen in the *Rp2*-KO retina. The vector-expressed human RP2 protein was primarily localized at the IS and nuclei of photoreceptors, but was not observed in any other layers of the retina. Without being bound to a particular theory or mechanism, it is believed that this is probably due to the specificity of the *RK* promoter and the inaccessibility of the vector to the inner retinal layers following subretinal administration. The vector-mediated RP2 expression was sustained throughout the entire 18-month study period without detectable loss. No expression of RP2 protein was detected in *Rp2*-KO mice injected with vehicle.

EXAMPLE 9

[0114]   This example demonstrates that *RP2* gene delivery with a wide dose range rescues cone function in *Rp2*-KO mice.

[0115]   To test the treatment effect of the AAV8-scRK-*hRP2* vector, 4 to 6 week-old *Rp2*-KO mice were administered subretinally with the vector at three doses; 1×10^8, 3×10^8 and 1×10^9 vector genomes (vg) per eye. The mice received unilateral vector injections, with the contralateral eyes receiving vehicle injections as controls. A longitudinal ERG monitoring was performed until the mice reached 18 months of age. Given the large variation in ERG amplitudes among individual mice, paired t-test was employed throughout the study to compare the vector- and the vehicle-treated eyes. Cone function rescue was achieved in the 1×10^8 and the 3×10^8 vg/eye dose groups as reflected by the significantly higher light-adapted ERG b-wave amplitude in vector-treated eyes as compared to vehicle-injected fellow eyes. This therapeutic effect was observed as early as at 4 months of age, the earliest time point of examination, and it lasted through the entire duration of the study period. Almost 75% (71-78%) of photopic b-wave amplitude was preserved in vector-treated eyes at 18 months in contrast to only ~28% remaining in the control eyes. In addition to preservation of light-adapted b-wave amplitude, the treatment completely corrected the alteration of b-wave kinetics in the KO retina, as revealed by nearly normal T_{50} rise, T_{max} and T_{50} decay measured in the vector-treated eyes of 4-month-old mice. The 1×10^8 vg/eye vector treatment appeared

not to be toxic to rods, as no significant difference was observed between vector- and vehicle-treated eyes in rod ERG response (dark-adapted a-wave) during the 18-month study period. Similarly, 3×10^8 vg/eye vector treatment had no obvious effect on rods in general, although slightly lower dark-adapted response was observed at certain time points. The lack of effect on rods may be explained by early onset (within 1 month of age and before vector administration, data not shown), through slower progression of functional impairment in rods of *Rp2*-KO mice.

[0116]   The effectiveness of 1×10^8 and 3×10^8 vg/eye vector treatment prompted exploration into whether a lower dose could still be functional. Therefore, the vector was administered to one group of mice at a dose of 5×10^7 vg/eye, and the treated mice were examined by ERG at 6.5 and 18 months of age. Significantly higher light-adapted b-wave amplitude was observed in the vector-treated eyes as compared to the control eyes, indicating the vector's potency at this low dose. The cone function rescue was not biased toward M- or S-cones, since the vector-treated eyes displayed comparable preservation of M- and S-cone-driven ERG responses.

[0117]   To determine if *RP2* gene delivery could result in a better visual acuity, mice treated with 1×10^8 vg or 3×10^8 vg vector were subjected to an optokinetic test under photopic conditions at 19 months of age. Although lower than WT controls, the visual acuity of the vector-treated eyes was significantly higher than that of the vehicle-treated eyes, indicating an improvement in cone-mediated visual behavior in *RP2*-KO mice by the treatment.

EXAMPLE 10

[0118]   This example demonstrates that *RP2* gene delivery with a wide dose range rescues cone function in *Rp2*-KO mice.

[0119]   M-opsin localized to OS in WT cones but was found mis-localized to IS, peri-nuclei, and synaptic terminals in vehicle-treated *Rp2*-KO cones, consistent with previous findings (Li et al., *Invest. Ophthalmol. Vis. Sci.*, 54: 4503-4511 (2013)). In addition, the number of M-cones in vehicle-treated KO retina appeared to be reduced at 6.5 months of age compared to WT retina, indicating substantial cone degeneration. However, in vector treated-retina, the M-opsin mis-localization was alleviated, and more M-cone cells were preserved. Normal subcellular localization of M-opsin was observed in vector-treated retina, suggesting that the treatment either prevented or reversed M-cone mis-trafficking. Similarly, more S-cones were observed in vector-treated retina, although no detectable S-opsin mis-

localization was seen in either vehicle or vector-treated KO eyes. Consistent with the findings of Zhang et al., *FASEB J.*, 29(3):932-42 (2015), cone PDE6 expression were almost undetectable in the outer segments of vehicle-treated *Rp2*-KO retina, whereas vector-treated retina retained near normal expression of the protein in the outer-segments. Localizations of two rod-specific proteins, rhodopsin and PDE6β, were also examined. These two proteins were mainly localized at the OS of photoreceptors in WT retina, and their expression or localization in KO retina was not affected by vector treatment.

[0120]   Cone rescue was more pronounced in the treated eyes at the final 18-month time point, as revealed by a significantly higher number of peanut agglutinin (PNA)-stained cells in both superior and inferior retina compared with those of the control eyes. Immunofluorescence analyses of both retinal whole-mounts and sections revealed significantly higher number of M- and S-cones in vector-treated KO retina than the vehicle-treated retina.

EXAMPLE 11

[0121]   This example demonstrates that late *RP2* gene delivery maintains cone function and viability in *Rp2*-KO mice.

[0122]   Impairment of cone function starts before 1-month of age in the *Rp2*-KO mouse model (Example 7; Li et al., *Invest. Ophthalmol. Vis. Sci.*, 54: 4503-4511 (2013)). To assess whether *Rp2*-KO mice with more advanced cone dysfunction would still benefit from the treatment, the vector was administered to 10-month old *Rp2*-KO mice at a dose of 3×10^8 vg/eye, and their retinal function and structure was examined when they reached 18-month of age. The vector-treated eyes displayed significantly higher light-adapted b-wave amplitude than vehicle-treated eyes as compared to vehicle-treated eyes, although no difference was seen in rod ERG. Consistent with this, substantial M- and S-opsin and cone PDE6-expressing cells were observed in the vector-treated retina, in contrast to the vehicle-treated retina.

EXAMPLE 12

[0123]   This example demonstrates an effective dosage of the RP2 vector for use in *Rp2*-KO mice.

[0124]   Vector doses ranging from 5×10^7 to 3×10^8 vg/eye were found to be efficacious in rescuing the function and viability of cone photoreceptors in *Rp2*-KO mice, as described

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above. These doses did not seem to affect rod function during the 18-month study period, although slight reductions were seen at 8 and 12 months in the 3×10^8 vg dose group. Most toxicity was confined to the dark-adapted ERG response, indicating transient toxicity of the vector at this dose towards rods. However, cone function was significantly improved at the dose of 3×10^8 vg. In contrast, mice that received the dose of 1×10^9 vg/eye exhibited significantly impaired rod function at all the time points of ERG examination (4 months, 8 months, and 18 months), as reflected by remarkably reduced amplitudes of dark-adapted a- and b-waves. Although this dose preserved cone function at 4 and 8 months, this treatment benefit eventually diminished at 18 months. Without being bound to a particular theory or mechanism, it is believed that this is probably due to secondary cone cell death caused by eventual loss of rods. Immunofluorescence analysis of retinal sections at the final 18-month time point revealed much thinner or even diminished outer nuclear layer at multiple regions in the 1×10^9 vg-treated eye, in contrast to no obvious changes in the 1×10^8 vg-treated eye. Therefore, the dose of 1×10^9 vg/eye was toxic to the retina.

[0125]   All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0126]   The use of the terms "a" and "an" and "the" and "at least one" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term "at least one" followed by a list of one or more items (for example, "at least one of A and B") is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly

contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0100]   Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0101]   This invention was made with Government support under project number 1ZIAEY000443 by the National Institutes of Health, National Eye Institute. The Government has certain rights in this invention.

CLAIM(S):

1. An adeno-associated virus (AAV) vector comprising a nucleic acid comprising the following components:

(a) a nucleotide sequence encoding RP2 or a functional fragment thereof;

(b) a first AAV2 Inverted Terminal Repeat (ITR) or a functional fragment thereof, and a second ITR or a functional fragment thereof;

(c) a human β-globin polyadenylation signal or a functional fragment thereof;

(d) a cytomegalovirus (CMV)/human β-globin intron; and

(e) a human rhodopsin kinase promoter comprising the nucleotide sequence of SEQ ID NO: 10; wherein the nucleotide sequence encoding RP2 or functional fragment thereof is under the transcriptional control of the rhodopsin kinase promoter; and

wherein the components of the vector are 5' to 3' in the following order: the first ITR or a functional fragment thereof, the human rhodopsin kinase promoter, the CMV/human β-globin intron, the nucleotide sequence encoding RP2 or a functional fragment thereof, the human β-globin polyadenylation signal or a functional fragment thereof, and the second ITR or a functional fragment thereof.

2. The vector according to claim 1, wherein the vector is self-complementary.

3. The vector according to claim 1 or 2, comprising the nucleotide sequence of SEQ ID NO: 14.

4. The vector according to any one of claims 1-3, wherein the vector is an AAV8 or AAV9 vector.

5. A pharmaceutical composition comprising the vector of any one of claims 1-4, further comprising a pharmaceutically acceptable carrier.

6. A method of treating or preventing X-linked retinitis pigmentosa (XLRP) in a mammal in need thereof, the method comprising administering to the mammal the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in an amount effective to treat or prevent XLRP in the mammal.

7. A method of increasing photoreceptor number in a retina of a mammal, the method comprising administering to the mammal the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in an amount effective to increase photoreceptor number in the retina of the mammal.

8. A method of increasing visual acuity of a mammal, the method comprising administering to the mammal the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in an amount effective to increase visual acuity in the mammal.

9. A method of decreasing retinal detachment in a mammal, the method comprising administering to the mammal the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in an amount effective to decrease retinal detachment in the mammal.

10. A method of increasing the electrical response of a photoreceptor in a mammal, the method comprising administering to the mammal the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in an amount effective to increase the electrical response of the photoreceptor in the mammal.

11. A method of increasing expression of a protein in a retina of a mammal, the method comprising administering to the mammal the vector of any one of claims 1-4 or a pharmaceutical composition comprising the vector in an amount effective to increase expression of the protein in the retina of the mammal, wherein the protein is RP2, cone opsin, or cone PDE6.

12. The method of any one of claims 6-11, comprising administering the vector comprising the nucleotide sequence encoding RP2 at a dose of about 5 x 10^6 to about 5 x 10^{12} vector genomes (vg) per eye.

13. The method of any one of claims 6-12, wherein the mammal is a human.

14. Use of the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in the manufacture of a medicament for the treatment or prevention of X-linked retinitis pigmentosa (XLRP) in a mammal.

15. Use of the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in the manufacture of a medicament for the increase of the number of photoreceptors in a retina of a mammal.

16. Use of the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in the manufacture of a medicament for the increase of visual acuity of a mammal.

17. Use of the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in the manufacture of a medicament for the decrease of retinal detachment in a mammal.

18. Use of the vector of any one of claims 1-4 or the pharmaceutical composition of claim 5 in the manufacture of a medicament for the increase of the electrical response of a photoreceptor in a mammal.

19. Use of the vector of any one of claims 1-4 or a pharmaceutical composition comprising the vector in the manufacture of a medicament for the increase of the expression of a protein in a retina of a mammal, wherein the protein is RP2, cone opsin, or cone PDE6.

20. The use of any one of claims 14-19, comprising the use of the nucleotide sequence encoding RP2 at a dose of about 5×10^6 to about 5×10^{12} vector genomes (vg) per eye.