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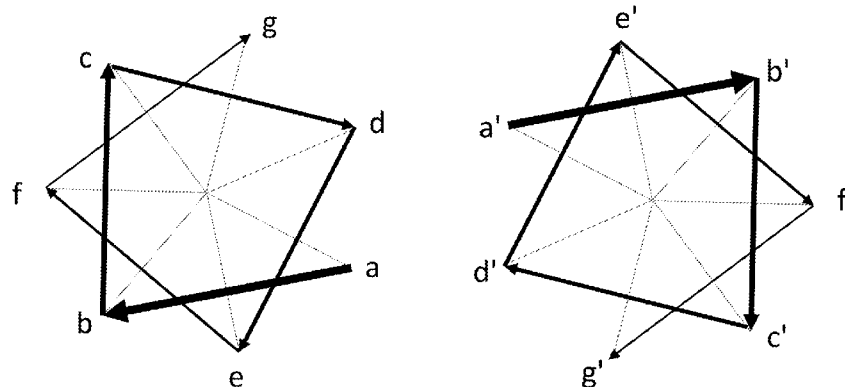
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(54) Title: DUAL LEUCINE ZIPPER KINASE INHIBITORS FOR GENE THERAPY

FIG. 1A



(57) Abstract: The disclosure provides compositions and methods useful for treating conditions related to Dual Leucine Zipper (DLK), e.g., neurodegenerative disorders including optic neuropathy. The disclosure provides gene constructs and vectors that regulate the activity of Dual Leucine Zipper Kinase (DLK) or Leucine Zipper Kinase (LZK). The disclosure also provides dominant negative DLK (DN-DLK) proteins that inhibit the kinase and/or signaling activity of DLK. The disclosure also provides vectors, pharmaceutical compositions, and methods of inhibiting DLK.

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- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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## DUAL LEUCINE ZIPPER KINASE INHIBITORS FOR GENE THERAPY

### FIELD OF THE INVENTION

[0001] The present disclosure relates generally to cellular or gene therapy using an engineered inhibitor of Dual Leucine Zipper Kinase, including for neurodegenerative disorders such as glaucoma.

### RELATED APPLICATIONS

[0002] This application claims priority to U.S. Provisional Patent Appl. No. 62/806,213, filed February 15, 2019, and U.S. Provisional Patent Appl. No. 62/806,210, filed February 15, 2019, each of which is incorporated herein by reference in its entirety.

### BACKGROUND OF THE INVENTION

[0003] Dual Leucine Zipper Kinase (DLK) a member of the serine/threonine protein kinase family encoded, in humans, by the *DLK* gene, also known as Mitogen-Activated Protein Kinase Kinase Kinase 12 or *MAP3K12*. This kinase contains a leucine-zipper domain and is predominately expressed in neuronal cells. The phosphorylation state of this kinase in synaptic terminals was shown to be regulated by membrane depolarization via calcineurin. This kinase forms homodimers, as well as heterodimers with leucine zipper containing transcription factors, such as cAMP responsive element binding protein (CREB) and MYC, and thus may play a regulatory role in PKA or retinoic acid induced neuronal differentiation. Alternatively spliced transcript variants encoding different proteins have been described. Expression of DLK with a K152A point mutation in the active site of the kinase results has a dominant negative affect on endogenous DLK. Xu et al. Mol Cell Biol. 21(14):4713-24 (2001).

[0004] DLK has been shown to be an essential component of neuronal response to axon damage. DLK protein is present in axons, and protein levels are increased in response to axonal injury (Xiong X, et al. J. Cell Biol, 2010; 191(1):211–223). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury (Watkins et al., Proc Natl Acad Sci, 2013; 110(10): 4039-4044).

[0005] Optic neuropathies are a group of vision diseases related to neuronal cell death, specifically the death of projection neurons called retinal ganglion cells (RGCs). Among the optic neuropathies, the U.S. spends \$1.9 billion per annum to treat glaucoma.

[0006] Thus, there is a long-felt yet unmet need for compositions and methods for treating diseases or disorders related to DLK and for compositions and methods for cellular or gene therapy for glaucoma. The disclosure provides such novel compositions and methods to address and solve this need.

### SUMMARY OF THE INVENTION

[0007] The present disclosure provides various embodiments of inhibitors of DLK, *e.g.*, inhibitors of DLK that act in a dominant negative fashion by forming inactive heterodimers with endogenous DLK, called Dominant-Negative Dual Leucine Zipper Kinases (DN-DLKs).

[0008] In one aspect, the disclosure provides compositions and methods useful for treating diseases or disorders related to DLK and to retinal cells. In particular, the disclosure provides vectors (including viral vectors) for delivery of DN-DLKs, methods of using such vectors in treatment, and methods of inhibiting DLK activity in cells.

[0009] In some embodiments, the disclosure provides inhibitors that comprises a sequence at least 95% identical to the leucine zipper domain of DLK (SEQ ID NO: 3) with substitutions in at least one of positions 391 and 393, using the numbering of full-length, human DLK (SEQ ID NO: 1).

[0010] The disclosure also provides recombinant virus vectors, including recombinant adeno-associated virus (rAAV) vectors, comprising an expression cassette whose polynucleotide sequence encodes one of the inhibitors of the disclosure.

[0011] The disclosure also provides methods of inhibiting DLK activity in a cell using the vectors, as well as pharmaceutical compositions and unit doses that include vector(s) of the disclosure. The disclosure further provides methods of treatment for conditions including, without limitation, diseases or disorders related to dysfunction in retinal cells is selected from the group consisting of glaucoma, wet macular degeneration, dry macular degeneration, geographic atrophy, retinal detachment, and retinal dystrophy, such as Usher's syndrome, Stargardt's disease, Leber's congenital amaurosis, or retinitis pigmentosa. In yet other embodiments, the disease is related to retinopathy, such as diabetic retinopathy, myopic maculopathy. In other embodiments, the disease is an optic neuropathy, such as optic neuritis, non-arteritic anterior ischemic optic neuropathy (NAION), arteritic anterior ischemic optic neuropathy (AION), traumatic optic neuropathy, Leber's optic neuropathy, dominant optic atrophy, recessive optic atrophy, or radiation optic neuropathy. In other embodiments, the disease may include one or more of amyotrophic lateral sclerosis (ALS), Parkinson Disease,

Alzheimer Disease, prion-related neurodegenerative disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, and multiple system atrophy.

**[0012]** In another aspect, the disclosure provides vectors that comprise a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK). The polynucleotide may be operatively linked to a promoter. The inhibitor is capable of binding either (i) an endogenous DLK protein, or (ii) an endogenous mRNA encoding an endogenous DLK. In some embodiments, the inhibitor is capable of reducing the expression, signaling activity, or kinase activity of the endogenous DLK in a retinal cell. The disclosure also provides methods of treating diseases or disorders related to retinal cells that involve administering such vectors to the retina of a subject. Diseases or disorders that can be treated include, with limitation, diseases or disorders related to dysfunction in retinal cells. Examples include glaucoma, retinopathy, retinal detachment, optic neuropathies, and retinal dystrophy.

**[0013]** The disclosure also provides a genetically modified retinal cell that includes the types of polynucleotides already described, ones encoding an inhibitor of Dual Leucine Zipper Kinase (DLK). The disclosure provides methods of making such cells, including contacting them with a viral vector. Pharmaceutical compositions are also provided herein.

**[0014]** The foregoing paragraphs are not intended to define every aspect of the invention, and additional aspects are described in other sections, such as the Detailed Description. The entire document is intended to be related as a unified disclosure, and it should be understood that all combinations of features described herein are contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document. The invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations defined by specific paragraphs above. For example, where certain aspects of the invention that are described as a genus, it should be understood that every member of a genus is, individually, an aspect of the invention.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0015] FIGs. 1A-1B** depict the leucine zipper domain of dual leucine zipper kinase (DLK). **FIG. 1A** shows a schematic depiction of the dimerization of a single heptad repeat of a leucine zipper. Amino acid residues within the heptad on one monomer are represented by letters (a) through (g) in N-terminal to C-terminal direction. The equivalent positions on the other monomer are represented by letters (a') through (g'). The pairs formed between e on one strand of the dimer, and g' on the opposite strand (called e-g' or e'-g pairs) can form electrostatic interactions and are important in determining the stability of the leucine zipper

dimer. **FIG. 1B** shows the amino-acid sequence of residues 387 to 467 of human DLK (also known as MAP3K12), according to isoform 1 numbering (UniProt Q12852). The eight heptad repeats are labeled L1 through L8, and each residues within the repeat is labeled a through g, following the convention shown in **FIG. 1A**.

[0016] **FIGs 2A-2H** depict the eight heptad repeats L1 (**FIG. 2A**), L2 (**FIG. 2B**), L3 (**FIG. 2C**), L4 (**FIG. 2D**), L5 (**FIG. 2E**), L6 (**FIG. 2F**), L7 (**FIG. 2G**), L8 (**FIG. 2H**). In the native homodimer of DLK each of the heptad repeats homodimerizes with the equivalent heptad (L1 with L1, L2 with L2, etc.). In each panel, the dimerization of one heptad is shown in the standard helical wheel representation as shown in **FIG. 1A**. Standard one-letter abbreviations are used for each amino acid position. The character of the amino acid at key positions are indicated as positive (+), negative (−), or hydrophobic (hydro). The g-e' and e-g' interactions are indicated by blocking arrows (repulsive or charged-hydrophobic interactions) or pointing arrows (charge-charge attractive forces) at the center top or center bottom of each panel.

[0017] **FIG. 3** depicts a designed dominant-negative DLK (DN-DLK) construct. Substitutions with respect to native DLK are shown underlined and bold.

[0018] **FIG. 4A-4C** depicts the homo- or hetero-dimerization interactions of DLK and the DN-DLK of **FIG. 3**. Only g and e residues are shown to highlight g-e' and g'-e interactions. Electrostatic interactions are depicted by lines. Interactions between opposite charges are shown as bolded solid lines to indicate attractive force. Interactions between like charges are shown as dashed lines to indicate repulsive force. **FIG. 4A** shows a homodimer of DLK. **FIG. 4B** shows a heterodimer of DLK and DN-DLK. **FIG. 4C** shows a homodimer of DN-DLK.

## DETAILED DESCRIPTION

### 1. Overview

[0019] The disclosure provides compositions and methods useful for treating diseases or disorders related to retinal cells. In particular, the invention provides cells and vectors comprising a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK), and method of use thereof. Advantageously, the vector is capable of reducing the expression, signaling activity, or kinase activity of the endogenous DLK in a cell, which is, in some embodiments, a retinal cell.

## **2. Definitions**

**[0020]** All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control. However, mention of any reference, article, publication, patent, patent publication, and patent application cited herein is not, and should not be taken as an acknowledgment, or any form of suggestion, that they constitute valid prior art or form part of the common general knowledge in any country in the world.

**[0021]** In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. The term “about”, when immediately preceding a number or numeral, means that the number or numeral ranges plus or minus 10%. It should be understood that the terms “a” and “an” as used herein refer to “one or more” of the enumerated components unless otherwise indicated. The use of the alternative (*e.g.*, “or”) should be understood to mean either one, both, or any combination thereof of the alternatives. The term “and/or” should be understood to mean either one, or both of the alternatives. As used herein, the terms “include” and “comprise” are used synonymously.

**[0022]** As used in this application, the terms “about” and “approximately” are used as equivalents. Any numerals used in this application with or without about/approximately are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

**[0023]** “Decrease” or “reduce” refers to a decrease or a reduction in a particular value of at least 5%, for example, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100% as compared to a reference value. A decrease or reduction in a particular value may also be represented as a fold-change in the value compared to a reference value, for example, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000-fold, or more, decrease as compared to a reference value.

**[0024]** “Increase” refers to an increase in a particular value of at least 5%, for example, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99, 100, 200, 300,

400, 500% or more as compared to a reference value. An increase in a particular value may also be represented as a fold-change in the value compared to a reference value, for example, at least 1-fold, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000-fold or more, increase as compared to the level of a reference value.

**[0025]** “Complementary” refers to the capacity for pairing, through base stacking and specific hydrogen bonding, between two sequences comprising naturally or non-naturally occurring (*e.g.*, modified as described above) bases (nucleosides) or analogs thereof. For example, if a base at one position of a nucleic acid is capable of hydrogen bonding with a base at the corresponding position of a target, then the bases are considered to be complementary to each other at that position. Nucleic acids can comprise universal bases, or inert abasic spacers that provide no positive or negative contribution to hydrogen bonding. Base pairings may include both canonical Watson-Crick base pairing and non-Watson-Crick base pairing (*e.g.*, Wobble base pairing and Hoogsteen base pairing). It is understood that for complementary base pairings, adenosine-type bases (A) are complementary to thymidine-type bases (T) or uracil-type bases (U), that cytosine-type bases (C) are complementary to guanosine-type bases (G), and that universal bases such as such as 3-nitropyrrole or 5-nitroindole can hybridize to and are considered complementary to any A, C, U, or T. Nichols *et al.*, Nature, 1994;369:492-493 and Loakes *et al.*, Nucleic Acids Res., 1994;22:4039-4043. Inosine (I) has also been considered in the art to be a universal base and is considered complementary to any A, C, U, or T. See Watkins and SantaLucia, Nucl. Acids Research, 2005; 33 (19): 6258-6267.

**[0026]** The term “subject” includes animals, such as *e.g.* mammals. In some embodiments, the mammal is a primate. In some embodiments, the mammal is a human. In some embodiments, subjects are livestock such as cattle, sheep, goats, cows, swine, and the like; or domesticated animals such as dogs and cats. In some embodiments (*e.g.*, particularly in research contexts) subjects are rodents (*e.g.*, mice, rats, hamsters), rabbits, primates, or swine such as inbred pigs and the like. The terms “subject” and “patient” are used interchangeably herein.

**[0027]** “Administration” refers herein to introducing an agent or composition into a subject.

**[0028]** “Treating” as used herein refers to delivering an agent or composition to a subject to affect a physiologic outcome. In some embodiments, treating refers to the treatment of a disease in a subject, *e.g.*, in a human, including (a) inhibiting the disease, *e.g.*, arresting disease development or preventing disease progression; (b) relieving the disease, *e.g.*, causing regression of the disease state; (c) curing the disease; and (d) preventing onset of disease,

*e.g.*, arresting disease development in an asymptomatic subject identified as a carrier of a genetic defect. In one aspect, treatment excludes prophylaxis or prevention.

**[0029]** The term “effective amount” or “therapeutically effective amount” refer to the minimum amount of an agent or composition required to result in a particular physiological effect (*e.g.*, an amount required to increase, activate, or enhance a particular physiological effect). The effective amount or therapeutically effective amount of a particular agent may be represented in a variety of ways based on the nature of the agent, such as mass/volume, # of cells/volume, particles/volume, (mass of the agent)/(mass of the subject), # of cells/(mass of subject), or particles/(mass of subject). The effective amount or therapeutically effective amount of a particular agent may also be expressed as the half-maximal effective concentration ( $EC_{50}$ ), which refers to the concentration of an agent that results in a magnitude of a particular physiological response that is half-way between a reference level and a maximum response level.

**[0030]** “Population” of cells refers to any number of cells greater than 1, but is preferably at least  $1 \times 10^3$  cells, at least  $1 \times 10^4$  cells, at least  $1 \times 10^5$  cells, at least  $1 \times 10^6$  cells, at least  $1 \times 10^7$  cells, at least  $1 \times 10^8$  cells, at least  $1 \times 10^9$  cells, at least  $1 \times 10^{10}$  cells, or more cells. A population of cells may refer to an *in vitro* population (*e.g.*, a population of cells in culture) or an *in vivo* population (*e.g.*, a population of cells residing in a particular tissue).

**[0031]** The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

**[0032]** As used herein “pharmaceutically acceptable carrier, diluent or excipient” includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, surfactant, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans and/or domestic animals.

**[0033]** As used herein “vector” refers to a nucleic acid molecule capable transferring or transporting a nucleic acid molecule to cell, along with, in a viral vector, one or more viral proteins, such as for encapsulated viruses the capsid of the virus. The transferred nucleic acid is generally linked to, *e.g.*, inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication or reverse transcription in a cell, or may

include sequences sufficient to allow integration into host cell DNA. “Vectors” include gene therapy vectors. As used herein, the term “gene therapy vector” refers to a vector capable of use in performing gene therapy, *e.g.*, delivering a polynucleotide sequence encoding a therapeutic polypeptide to a subject. Gene therapy vectors may comprise a polynucleotide (“transgene”) encoding a protein.

**[0034]** As used herein, the term “expression cassette” refers to a DNA segment that is capable in an appropriate setting of driving the expression of a polynucleotide (*e.g.*, a transgene) encoding a protein that is incorporated in said expression cassette. When introduced into a host cell, an expression cassette *inter alia* is capable of directing the cell’s machinery to transcribe the transgene into RNA, which is then usually further processed and finally translated into the therapeutically active polypeptide. The gene therapy vector can comprise, or consist essentially of expression cassette. The term expression cassette excludes polynucleotide sequences 5’ to the 5’ ITR and 3’ to the 3’ ITR. Provided herein are host cells comprising, or consisting essentially of, or yet further consisting of the vector of this disclosure. The cells can be of any appropriate species, *e.g.*, mammalian cells.

**[0035]** As used herein, the phrases “operably linked” or “under the transcriptional control” with respect to a polynucleotide refers, interchangeably, to a configuration of the promoter and the polynucleotide that enables the polynucleotide to be transcribed by a polymerase capable of binding to the promoter.

**[0036]** The term “sequence identity” refers to the percentage of bases or amino acids between two polynucleotide or polypeptide sequences that are the same, and in the same relative position. As such one polynucleotide or polypeptide sequence has a certain percentage of sequence identity compared to another polynucleotide or polypeptide sequence. For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. The term “reference sequence” refers to a molecule to which a test sequence is compared. A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) having a certain percentage (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%) of “sequence identity” to a reference sequence means that, when aligned, that percentage of bases (or amino acids) at each position in the test sequence are identical to the base (or amino acid) at the same position in the reference sequence. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Ausubel et al. eds. (2007) Current Protocols in Molecular Biology. Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and

BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: [ncbi.nlm.nih.gov/blast/Blast.cgi](http://ncbi.nlm.nih.gov/blast/Blast.cgi).

**[0037]** An “equivalent” or “functional equivalent” of a polypeptide or protein is one that has a certain sequence identity to that reference polypeptide or protein, (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% identity to the reference) and retains similar activity or function compared to the reference polypeptide or protein.

**[0038]** “Comprising” or “comprises” is intended to mean that the compositions, for example media, and methods include the recited elements, but not excluding others. “Consisting essentially of” when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination for the stated purpose. Thus, a composition consisting essentially of the elements as defined herein would not exclude other materials or steps that do not materially affect the basic and novel characteristic(s) of the claimed invention. “Consisting of” shall mean excluding more than trace elements of other ingredients and substantial method steps. Embodiments defined by each of these transition terms are within the scope of this disclosure.

**[0039]** General methods in molecular and cellular biochemistry can be found in such standard textbooks as *Molecular Cloning: A Laboratory Manual*, 3rd Ed. (Sambrook *et al.*, HaRBor Laboratory Press 2001); *Short Protocols in Molecular Biology*, 4th Ed. (Ausubel *et al.* eds., John Wiley & Sons 1999); *Protein Methods* (Bollag *et al.*, John Wiley & Sons 1996); *Nonviral Vectors for Gene Therapy* (Wagner *et al.* eds., Academic Press 1999); *Viral Vectors* (Kaplift & Loewy eds., Academic Press 1995); *Immunology Methods Manual* (I. Lefkovits ed., Academic Press 1997); and *Cell and Tissue Culture: Laboratory Procedures in Biotechnology* (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference.

**[0040]** The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

### **3.1 Vectors**

**[0041]** Various embodiments of the invention include viral vectors and non-viral vectors. Viral vectors include adeno-associated virus, adenovirus, lentivirus, Sleeping Beauty, herpesvirus, and other viruses known in the art. It is within the skill of the ordinary artisan to

adapt the polynucleotides of the invention to various viral vectors. Non-viral vectors include liposomes, lipid-like particles, and cationic polymers, as well as electroporation of naked DNA in plasmid, bacmid, linear, minicircular or other form. The polynucleotides of the disclosure may be delivered as either DNA or RNA. Modified polynucleotides are known in the art and are included methods and compositions of the present disclosure. The disclosure of particular types of viral and non-viral vectors in particular embodiments described herein is not intended as limiting the invention.

**[0042]** As used herein, the term “AAV” is a standard abbreviation for adeno-associated virus or a recombinant vector thereof. Adeno-associated virus is a single-stranded DNA parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. General information and reviews of AAV can be found in, for example, Carter, 1989, *Handbook of Parvoviruses*, Vol. 1, pp. 169-228, and Berns, 1990, *Virology*, pp. 1743-1764, Raven Press, (New York). It is fully expected that the same principles described in these reviews will be applicable to additional AAV serotypes characterized after the publication dates of the reviews because it is well known that the various serotypes are quite closely related, both structurally and functionally, even at the genetic level. (*See, for example*, Blacklowe, 1988, pp. 165-174 of *Parvoviruses and Human Disease*, J. R. Pattison, ed.; and Rose, *Comprehensive Virology* 3: 1-61 (1974)). For example, all AAV serotypes apparently exhibit very similar replication properties mediated by homologous rep genes; and all bear three related capsid proteins such as those expressed in AAV2. The degree of relatedness is further suggested by heteroduplex analysis which reveals extensive cross-hybridization between serotypes along the length of the genome; and the presence of analogous self-annealing segments at the termini that correspond to "inverted terminal repeat sequences" (ITRs). The similar infectivity patterns also suggest that the replication functions in each serotype are under similar regulatory control.

**[0043]** As used herein, an “AAV vector” or “rAAV vector” refers to a recombinant vector comprising one or more polynucleotides of interest (or transgenes) that are flanked by AAV terminal repeat sequences (ITRs). Such AAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been transfected with a vector encoding and expressing rep and cap gene products.

**[0044]** As used herein, an “AAV virion” or “AAV viral particle” or “AAV vector particle” refers to a viral particle composed of at least one AAV capsid protein and an encapsidated polynucleotide AAV vector. As used herein, if the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene

to be delivered to a mammalian cell), it is typically referred to as an “AAV vector particle” or simply an “AAV vector.” Thus, production of AAV vector particle necessarily includes production of AAV vector, as such a vector is contained within an AAV vector particle.

**[0045]** Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including two 145 nucleotide inverted terminal repeat (ITRs). There are multiple known variants of AAV, also sometimes called serotypes when classified by antigenic epitopes. The nucleotide sequences of the genomes of the AAV serotypes are known. For example, the complete genome of AAV-1 is provided in GenBank Accession No. NC\_002077; the complete genome of AAV-2 is provided in GenBank Accession No. NC\_001401 and Srivastava et al., *J. Virol.*, 45: 555-564 (1983); the complete genome of AAV-3 is provided in GenBank Accession No. NC\_1829; the complete genome of AAV-4 is provided in GenBank Accession No. NC\_001829; the AAV-5 genome is provided in GenBank Accession No. AF085716; the complete genome of AAV-6 is provided in GenBank Accession No. NC\_001862; at least portions of AAV-7 and AAV-8 genomes are provided in GenBank Accession Nos. AX753246 and AX753249, respectively; the AAV-9 genome is provided in Gao et al., *J. Virol.*, 78: 6381-6388 (2004); the AAV-10 genome is provided in *Mol. Ther.*, 13(1): 67-76 (2006); and the AAV-11 genome is provided in *Virology*, 330(2): 375-383 (2004). The sequence of the AAV rh.74 genome is provided in U.S. Patent 9,434,928, incorporated herein by reference. The sequence of ancestral AAVs including AAV.Anc80, AAV.Anc80L65 and their derivatives are described in WO2015054653A2 and Wang et al. Single stranded adeno-associated virus achieves efficient gene transfer to anterior segment in the mouse eye. *PLoS One*. 2017 Aug 1;12(8):e0182473.

**[0046]** AAV vectors may refer to either naturally occurring AAVs, such as AAV-1 to AAV-10, or non-naturally occurring vectors, such as those that have been engineered through rational design, directed evolution, and the like. Cis-acting sequences directing viral DNA replication (rep), encapsidation/packaging and host cell chromosome integration are contained within the AAV ITRs. Three AAV promoters (named p5, p19, and p40 for their relative map locations) drive the expression of the two AAV internal open reading frames encoding rep and cap genes. The two rep promoters (p5 and p9), coupled with the differential splicing of the single AAV intron (at nucleotides 2107 and 2227), result in the production of four rep proteins (rep 78, rep 68, rep 52, and rep 40) from the rep gene. Rep proteins possess multiple enzymatic properties that are ultimately responsible for replicating the viral genome. The cap gene is expressed from the p40 promoter and it encodes the three capsid proteins

VP1, VP2, and VP3. Alternative splicing and non-consensus translational start sites are responsible for the production of the three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology*, 158: 97-129 (1992).

**[0047]** AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells, for example, in gene therapy. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects many mammalian cells allowing the possibility of targeting many different tissues in vivo. Moreover, AAV transduces slowly dividing and non-dividing cells, and can persist essentially for the lifetime of those cells as a transcriptionally active nuclear episome (extrachromosomal element). The AAV proviral genome is inserted as cloned DNA in plasmids, which makes construction of recombinant genomes feasible. Furthermore, because the signals directing AAV replication and genome encapsidation are contained within the ITRs of the AAV genome, some or all of the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may be replaced with foreign DNA. To generate AAV vectors, the rep and cap proteins may be provided in trans. Another significant feature of AAV is that it is an extremely stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56° to 65°C for several hours), making cold preservation of AAV less critical. AAV may even be lyophilized. Finally, AAV-infected cells are not resistant to superinfection.

### **3.2 AAV Serotypes and Genomes**

**[0048]** AAV DNA in the rAAV genomes may be from any AAV variant or serotype for which a recombinant virus can be derived including, but not limited to, AAV variants or serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV- 10, AAV-11, AAV- 12, AAV-13 and Anc80L65. Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692. Other types of rAAV variants, for example rAAV with capsid mutations, are also contemplated. *See, for example*, Marsic et al., *Molecular Therapy*, 22(11): 1900-1909 (2014). The nucleotide sequences of the genomes of various AAV serotypes are known in the art. To promote eye-specific expression, AAV6, AAV8 or AAV9 may be used.

**[0049]** In some cases, the rAAV comprises a self-complementary genome. As defined herein, an rAAV comprising a “self-complementary” or “double stranded” genome refers to an

rAAV which has been engineered such that the coding region of the rAAV is configured to form an intra-molecular double-stranded DNA template, as described in McCarty et al. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Therapy*. 8 (16): 1248–54 (2001). The present disclosure contemplates the use, in some cases, of an rAAV comprising a self-complementary genome because upon infection (such transduction), rather than waiting for cell mediated synthesis of the second strand of the rAAV genome, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. It will be understood that instead of the full coding capacity found in rAAV (4.7-6kb), rAAV comprising a self-complementary genome can only hold about half of that amount ( $\approx 2.4$ kb).

**[0050]** In other cases, the rAAV vector comprises a single stranded genome. As defined herein, a “single standard” genome refers to a genome that is not self-complementary. In most cases, non-recombinant AAVs are have singled stranded DNA genomes. There has been some indications that rAAVs should be scAAVs to achieve efficient transduction of cells, such as ocular cells. The present disclosure contemplates, however, rAAV vectors that maybe have singled stranded genomes, rather than self-complementary genomes, with the understanding that other genetic modifications of the rAAV vector may be beneficial to obtain optimal gene transcription in target cells. In some cases, the present disclosure relates to single-stranded rAAV vectors capable of achieving efficient gene transfer to anterior segment in the mouse eye. *See* Wang et al. Single stranded adeno-associated virus achieves efficient gene transfer to anterior segment in the mouse eye. *PLoS ONE* 12(8): e0182473 (2017).

**[0051]** In some cases, the rAAV vector is of the serotype AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13, AAV2.7m8, or Anc80L65. Anc80L65 is described in Sharma et al. Transduction efficiency of AAV 2/6, 2/8 and 2/9 vectors for delivering genes in human corneal fibroblasts. *PLoS ONE* 12(8): e0182473 (2017). Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692. Other types of rAAV variants, for example rAAV with capsid mutations, are also contemplated. *See, for example*, Marsic et al., *Molecular Therapy*, 22(11): 1900-1909 (2014). In some cases, the rAAV vector is of the serotype AAV9. In some embodiments, said rAAV vector is of serotype AAV9 and comprises a single stranded genome. In some embodiments, said rAAV vector is of serotype AAV9 and comprises a self-complementary genome. In some embodiments, a rAAV vector comprises the inverted terminal repeat (ITR)

sequences of AAV2. In some embodiments, the rAAV vector comprises an AAV2 genome, such that the rAAV vector is an AAV-2/9 vector, an AAV-2/6 vector, or an AAV-2/8 vector. Other combinations of genome and serotype are contemplated by the present disclosure, including, without limitation, non-naturally occurring variants made by rational design, directed evolution, and the like, and those described in. *Brain Res Bull.* 2010 Feb 15; 81(2-3): 273; Castle et al. Controlling AAV Tropism in the Nervous System with Natural and Engineered Capsids. *Methods Mol Biol.* 2016; 1382: 133–149; and Kay et al. Targeting Photoreceptors via Intravitreal Delivery Using Novel, Capsid-Mutated AAV Vectors. *PLoS One.* 2013 Apr 26;8(4):e62097.

### **3.3 Promoters**

**[0052]** In some cases, a polynucleotide sequence encoding a transgene is operatively linked to an inducible promoter. A polynucleotide sequence operatively linked to an inducible promoters may be configured to cause the polynucleotide sequence to be transcriptionally expressed or not transcriptionally expressed in response to addition or accumulation of an agent or in response to removal, degradation, or dilution of an agent. The agent may be a drug. The agent may be tetracycline or one of its derivatives, including, without limitation, doxycycline. In some cases, the inducible promoter is a tet-on promoter, a tet-off promoter, a chemically-regulated promoter, a physically-regulated promoter (i.e. a promoter that responds to presence or absence of light or to low or high temperature). This list of inducible promoters is non-limiting.

**[0053]** In some embodiments, the polynucleotide sequence encoding the transgene is operably linked to a CMV promoter. The present disclosure further contemplates the use of other promoter sequences. Promoters useful in embodiments of the present disclosure include, without limitation, cytomegalovirus (CMV) and murine stem cell virus (MSCV), phosphoglycerate kinase (PGK), a promoter sequence comprised of the CMV enhancer and portions of the chicken beta-actin promoter and the rabbit beta-globin gene (CAG), a CBh promoter, a promoter sequence comprised of portions of the SV40 promoter and CD43 promoter (SV40/CD43), and a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer (MND). In some cases, the promoter may be a synthetic promoter. Exemplary synthetic promoters are provided by Schlachet et al. Synthetic design of strong promoters. *Proc Natl Acad Sci U S A.* 2010 Feb 9; 107(6): 2538–2543.

[0054] In some embodiments, the polynucleotide sequence encoding the transgene is operably linked to a *Nefh* promoter, as described in Hanlon et al. *Front Neurosci.* 11:521 (2017) (PMID: 28983234). In some embodiments, the polynucleotide sequence encoding the transgene is operably linked to a *DCX* promoter, as described in Smith et al. *Sci. Rep.* 8:1490 (2018) (PMID: 29367685). In some embodiments, the polynucleotide sequence encoding the transgene is operably linked to a *Thy1* promoter, a rhodopsin promoter, a rhodopsin kinase promoter, a pR2.1 promoter, or a MNTC promoter. Promoters provided by this disclosure include those described in US20180100165A1, US20180066022A1, US20180258446A1, US20150259395A1, US20140248701A1, US20120278912A1, WO2006108201A1, the disclosures of each of which are incorporated by reference herein in their entireties.

### **3.4 Dual-Leucine Zipper Kinase (DLK)**

[0055] As used herein, the terms “dual-leucine zipper kinase” or “DLK” refers to the kinase, also known in the art as Mitogen-activated protein kinase kinase kinase 12 (MAP3K12), encoded by the *DLK* gene. DLK is a member of the Mixed Lineage Kinase (MLK). MLKs were identified initially as signaling molecules in the nervous system. They were also shown to play a role in the cell cycle. Like other MLKs, DLK has a kinase catalytic domain followed C-terminally by two leucine/isoleucine zippers separated by a short space sequence. Mata et al. *J. Bio. Chem.* 271, 16888-16896 (1996).

[0056] DLK is predominately expressed in neuronal cells. The phosphorylation state of this kinase in synaptic terminals was shown to be regulated by membrane depolarization via calcineurin. This kinase forms heterodimers with leucine zipper containing transcription factors, such as cAMP responsive element binding protein (CREB) and MYC, and thus may play a regulatory role in PKA or retinoic acid induced neuronal differentiation.

[0057] **Table 1** provides the native protein sequence of human DLK, isoform 1 and isoform 2. DLKs from other species may be used in the present invention. Through the disclosure, the numbering of human isoform 1 is used. **Table 1** also provides other protein sequences. **Table 2** provides corresponding polynucleotide sequences, including expression cassettes and vectors. The polynucleotides disclosed can be used in various viral and non-viral vectors. In particular, the AAV genome provided as SEQ ID NO: 22 may be provided either as a plasmid for use in generating a viral particle or as a constituent of a pre-formed viral particle. It is well within the skill of those in the art to modify these sequences, such as by codon optimization, removal of spurious start or stop codons, reducing G/C content, removal of

CpG dinucleotides or CpG islands, substitutions of promoter or other genetic elements, use of lentiviral LTRs rather than AAV ITRs, etc. Such polynucleotides are themselves embodiments of this disclosure.

**[0058]** The AAV genome disclosed as SEQ ID NO: 22 has a total length of 4099 bp. In some embodiments, SEQ ID NO: 22 is modified by insert of other polynucleotides between the flanking ITRs to increase the length of the sequence to about 4.2 to about 4.5 kilobases, in line with the expectation that this size is optimal in some contexts for AAV vectors, provided modification of SEQ ID NO: 22 does not disrupt the function of the polynucleotide sequence as an AAV genome. The disclosure contemplates substitution of various coding regions for DLK inhibitors into SEQ ID NO: 22, or into other viral genomes, including into other AAV genomes.

**Table 1: Exemplary Dual-Leucine Zipper Kinase Sequences**

| Sequence description                     | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | SEQ ID NO |
|------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| DLK / Map3k12 (variant 2, aka isoform 1) | MACLHETRTSPSPSFGGFVSTLSEASMRKLDPDTSDCTPE<br>KDLTPTHVLQLHEQDAGGPGGAAGSPESRASRVRADEV<br>RLQCQSGSGFLEGLFGCLRPVWTMIGKAYSTEHKQQQE<br>DLWEVPFEEILDQWVGSGAQAQAVFLGRFHGEEVAVKK<br>VRDLKETDIKHLRKLKHPNIITFKGVCTQAPCYCILMEFC<br>AQQQLYEVLRAGRPVTPSLLVDWSMGIAGGMNYLHLH<br>KIIHRDLKSPNMLITYDDVVKISDFGTSKELSDKSTKMSF<br>AGTVAWMAPEVIRNEPVSEKVDIWSFGVVLWELLTGEIP<br>YKDVDSSAIWGVGSNSLHLPVPSSCPDGFKILLRQCWN<br>SKPRNRPSFRQILLHLDIASADVLSTPQETYFKSQAEWRE<br>EVKLHFEKIKSEGTCLHRLEEELVMRRREELRHLDIRE<br>HYERKLERANNLYMELNALMLQLELKERELLRREQALE<br>RRCPGLLKPHPSRGLLHGNTMEKLIKRNVPQKLSPHSK<br>RPDILKTESLLPKLDAALSGVGLPGCPKGPPSPGRSRRGK<br>TRHRKASAKGSCGDLPLRLTAVPPHEPGGPGSPGGLGG<br>GPSAWEACPPALRGLHDLRLKMSSSPDLLSAALGSR<br>GRGATGGAGDPGSPPPARGDTPPSEGSAPGSTSPDSPGG<br>AKGEPPPPVGPGEVGLLGTGREGTSGRGGSRAGSQHLT<br>PAALLYRAAVTRSQKRGISSEEEEGEVDSEVELTSSQRW<br>PQSLNMRQSLSTFSENPSDGEEGTASEPSPSGTPEVGST<br>NTDERPDERSDDMCSQGSEIPLDPPPSEVIPGPEPSSLPIPH<br>QELLRERGPPNSEDSDCDSTELDNSNSVDALRPPASLPP* | 1         |
| DLK / Map3k12 (isoform 2)                | MACLHETRTSPSPSFGGFVSTLSEASMRKLDPDTSDCTPE<br>KDLTPTQCVLRDVVPLGGQGGGGPSPSPGGEPPPEPFAN<br>SVLQLHEQDAGGPGGAAGSPESRASRVRADEVRLQCQS<br>SGSFLEGLFGCLRPVWTMIGKAYSTEHKQQQEDLWEVP<br>FEEILDQWVGSGAQAQAVFLGRFHGEEVAVKKVRDLKE<br>TDIKHLRKLKHPNIITFKGVCTQAPCYCILMEFCAQQQLY                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 2         |

|                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |   |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|
|                                                                                                                                                                                       | EVLRAGRPVTPSLLVDWSMGIAGGMNYLHLHKIIHRDL<br>KSPNMLITYDDVVKISDFGTSKELSDKSTKMSFAGTVAW<br>MAPEVIRNEPVSEKVDIWSFGVVLWELLTGEIPYKDVDSS<br>SAIHWGVGSNSLHLPVPSSCPDGFKILLRQCWNSKPRNRP<br>SFRQILLHLDIASADVLSTPQETYFKSQA EWREEVKLHFE<br>KIKSEGTC LHRLEEELVMRRREELRHALDIREHYERKLE<br>RANNLYMELNALMLQLELKERELLRREQALERRCPGLL<br>KPHPSRGLLHGNTMEKLIKRNVPQKLSPHSKRDPILKT<br>ESLLPKLDAALSGVGLPGCPKGPPSPGRSRRGKTRHRKA<br>SAKGSCGDLPLRTAVPPHEPGGPGSPGGLGGGPSAWE<br>ACPPALRGLHHDLLLRKMSSSSPDLLSAALGSRGRGATG<br>GAGDPGSPPPARGDTPPSEGSA PGSTSPDSPGGAKGEP<br>PVGPGEVGLLTGTGREGTSGRGGSRAGSQHLTPAALLY<br>RAAVTRSQKRGISSEEEEGEVDSEVELTSSQRWPQSLNM<br>RQSLSTFSSENPSDGEEGTASEPSPGTPEVGSTNTDERPD<br>ERSDDMCSQGSEIPLDPPPSEVIPGPEPSSLPIPHQELLRER<br>GPPNSEDSDCDSTELDNSNSVDALRPPASLPP |   |
| DLK leucine zipper domain fragment (Nihalani et al. <i>J. Bio Chem.</i> 275, 7273-7279 (2000))                                                                                        | <u>LSTPQETYFKSQA EWREEVKLHFEKIKSEGTC LHRLEEEL</u><br><u>VMRRREELRHALDIREHYERKLERANNLYMELNALMLQL</u><br><u>ELKERELLRREQALERRCPGLLKPHPSRGLLHGNTME</u>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | 3 |
| <u>Zipper 1</u> , <u>bridge</u> , and <u>zipper 2</u> from DLK                                                                                                                        | <u>REEVKLHFEKIKSEGTC LHRLEEELVMRRREELRHALDIR</u><br><u>EHYERKLERANNLYMELNALMLQLELKERELLRREQALE</u><br><u>RR</u>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 4 |
| Kinase domain from DLK                                                                                                                                                                | ILDLQWVGSGAQQGAVFLGRFHGEEVAVKKVRDLKETDI<br>KHLRKLKHPNIITFKGVCTQAPCYCILMEFCAQQQLYEV<br>LRAGRPVTPSLLVDWSMGIAGGMNYLHLHKIIHRDLKSP<br>NMLITYDDVVKISDFGTSKELSDKSTKMSFAGTVAWMA<br>PEVIRNEPVSEKVDIWSFGVVLWELLTGEIPYKDVDSSAI<br>WGVGSNSLHLPVPSSCPDGFKILLRQCWNSKPRNRP<br>SFRQILLHLDI                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 5 |
| Modified DLK <u>Zipper 1</u> , <u>bridge</u> , and <u>zipper 2</u> .<br><br>Modifications in bold enhance heterodimerization with DLK, compared to DLK homodimers or LZ-LZ homodimers | <u>REE<b>VE</b>LEFEKIRSEGTC LER<b>EEEE</b>LEMERREELRHALDIRE</u><br><u>HYERKLERANNLYMELNALMLQLEL<b>RERELEREE</b>QALER</u><br><u><b>R</b></u>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 6 |
| Longer LZ domain with same                                                                                                                                                            | LSTPQETYFKSQA EW <b>REEVELEFEKIRSEGTC LER<b>EEEE</b></b><br><b>EMERREELRHALDIREHYERKLERANNLYMELNALMLQL</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 7 |

|                                          |                                                                                                                                                                                                                                                                                    |    |
|------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| modifications                            | <u>ELRERELEREEQALERRCPGLLKPHPSRGLLHGNTME</u>                                                                                                                                                                                                                                       |    |
| Kinase domain of kinase-dead DLK (K152A) | ILDLQWVGSGAQGAVFLGRFHGEEVAV <u>A</u> KVRDLKETDI<br>KHLRKLKHPNIITFKGVCTQAPCYCILMEFCAQGQLYEV<br>LRAGRPVTPSLLVDWSMGIAGGMNYLHLHKIIHRDLKSP<br>NMLITYDDVVKISDFGTSKELSDKSTKMSFAGTVAWMA<br>PEVIRNEPVSEKVDIWSFGVVLWELLTGEIPYKDVDSSAI<br>WGVGSNSLHLPVPSSCPDGFKILLRQCWNSKPRNRPSFR<br>QILLHLDI | 8  |
| Zipper1                                  | REEV <u>XL</u> XFEKIYSEGTC <u>LXR</u> XEEELXMX<br><br>X = E or D<br>Y = R, H, or K                                                                                                                                                                                                 | 9  |
| Zipper2                                  | YMELNALMLQLELYEREL <u>XR</u> XEQALERR<br><br>X = E or D<br>Y = R, H, or K                                                                                                                                                                                                          | 10 |
| Bridge                                   | RREELRHALDIREHYERKLERANNL                                                                                                                                                                                                                                                          | 11 |
| Zipper1 – K391D, H393D                   | REEV <u>DL</u> DFEKIYSEGTC <u>LXR</u> XEEELXMX<br><br>X = E or D<br>Y = R, H, or K                                                                                                                                                                                                 | 12 |
| Zipper1 – K391E, H393D                   | REEV <u>EL</u> DFEKIYSEGTC <u>LXR</u> XEEELXMX<br><br>X = E or D<br>Y = R, H, or K                                                                                                                                                                                                 | 13 |
| Zipper1 – K391D, H393E                   | REEV <u>DLE</u> FEKIYSEGTC <u>LXR</u> XEEELXMX<br><br>X = E or D<br>Y = R, H, or K                                                                                                                                                                                                 | 14 |
| Zipper1 – K391E, H393E                   | REEV <u>ELE</u> FEKIYSEGTC <u>LXR</u> XEEELXMX<br><br>X = E or D<br>Y = R, H, or K                                                                                                                                                                                                 | 15 |

Table 2: Exemplary Polynucleotide Sequences

| Sequence description                  | Sequence                                                                                                                                                                                                                                                   | SEQ ID NO |
|---------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Zipper-bridge-zipper of native DLK    | GTAAAACTGCACTTTGAAAAGATTAAGTCAGAAGGGA<br>CCTGTCTGCACCGCCTAGAAGAGGAACTGGTGATGAG<br>GAGGAGGGAGGAGCTCAGACACGCCCTGGACATCAG<br>GGAGCACTATGAAAGGAAGCTGGAGAGAGCCAACAA<br>CCTGTATATGGAACCTTAATGCCCTCATGTTGCAGCTGG<br>AACTCAAGGAGAGGGAGCTGCTCAGGCGAGAGCAAG<br>CTTTA | 16        |
| <u>Zipper-bridge-zipper</u> of native | CTCTCCACACCCCAGGAGACTTACTTTAAGTCCCAGGC<br>AGAGTGGCGGGAAGAAGTAAAACTGCACTTTGAAAA                                                                                                                                                                             | 17        |

|                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| DLK with flanking sequence                                                                                                                                                                                                                                                             | <u>GATTAAGTCAGAAAGGGACCTGTCTGCACCGCCTAGAA</u><br><u>GAGGAACTGGTGATGAGGAGGAGGGAGGAGCTCAGA</u><br><u>CACGCCCTGGACATCAGGGAGCACTATGAAAGGAAGC</u><br><u>TGGAGAGAGCCAACAACCTGTATATGGAACCTTAATGC</u><br><u>CCTCATGTTGCAGCTGGAACCTCAAGGAGAGGGAGCTG</u><br><u>CTCAGGCGAGAGCAAGCTTTAGAGCGGAGGTGCCAG</u><br><u>GCCTGCTGAAGCCACACCCTTCCCGGGGCCTCCTGCA</u><br><u>TGGAAACACAATGGAG</u>                                                                                                                                            |    |
| <u>Zipper-bridge-</u><br><u>zipper</u> of native<br>DLK with flanking<br>sequence with<br>codons<br>corresponding to<br>substituted bases in<br><b>bold for K391E</b><br><b>and H393E</b>                                                                                              | CTCTCCACACCCCAGGAGACTTACTTTAAGTCCCAGGC<br><u>AGAGTGGCGGGAAGAAGTAGA<b>AACTGGA</b>ATTTGAAAA</u><br><u>GATTAAGTCAGAAAGGGACCTGTCTGCACCGCCTAGAA</u><br><u>GAGGAACTGGTGATGAGGAGGAGGGAGGAGCTCAGA</u><br><u>CACGCCCTGGACATCAGGGAGCACTATGAAAGGAAGC</u><br><u>TGGAGAGAGCCAACAACCTGTATATGGAACCTTAATGC</u><br><u>CCTCATGTTGCAGCTGGAACCTCAAGGAGAGGGAGCTG</u><br><u>CTCAGGCGAGAGCAAGCTTTAGAGCGGAGGTGCCAG</u><br><u>GCCTGCTGAAGCCACACCCTTCCCGGGGCCTCCTGCA</u><br><u>TGGAAACACAATGGAG</u>                                           | 18 |
| <u>Zipper-bridge-</u><br><u>zipper</u> of native<br>DLK with flanking<br>sequence with<br>codons<br>corresponding to<br>substituted bases in<br><b>bold for K391E,</b><br><b>H393E, K398R,</b><br><b>H405E, L407E,</b><br><b>V412E, R414E,</b><br><b>K453R, L458E,</b><br><b>R460E</b> | CTCTCCACACCCCAGGAGACTTACTTTAAGTCCCAGGC<br><u>AGAGTGGCGGGAAGAAGTAGA<b>AACTGGA</b>ATTTGAAAA</u><br><u>GATT<b>CGTT</b>CAGAAAGGGACCTGTCT<b>GGAACGCGA</b>AGA</u><br><u>AGAGGAACT<b>GGA</b>AT<b>GGA</b>AAGGAGGGAGGAGCTCAG</u><br><u>ACACGCCCTGGACATCAGGGAGCACTATGAAAGGAA</u><br><u>GCTGGAGAGAGCCAACAACCTGTATATGGAACCTTAAT</u><br><u>GCCCTCATGTTGCAGCTGGAACCT<b>CCGT</b>GAGAGGGAGC</u><br><u><b>TGGAAAGGGA</b>AGAGCAAGCTTTAGAGCGGAGGTGCC</u><br><u>CAGGCCTGCTGAAGCCACACCCTTCCCGGGGCCTCCT</u><br><u>GCATGGAAACACAATGGAG</u> | 19 |
| Above sequence<br>with <u>Kozak</u> and<br><b>stop codon</b>                                                                                                                                                                                                                           | <u>GCCGCCATGGCGCTCTCCACACCCCAGGAGACTTACT</u><br><u>TTAAGTCCCAGGCAGAGTGGCGGGAAGAAGTAGAACT</u><br><u>GGAATTTGAAAAGATTCGTT<b>CAGA</b>AGGGACCTGTCTG</u><br><u>GAACGCGAAGAAGAGGAACTGGAATGGAAAGGAGG</u><br><u>GAGGAGCTCAGACACGCCCTGGACATCAGGGAGCACT</u><br><u>ATGAAAGGAAGCTGGAGAGAGCCAACAACCTGTATAT</u><br><u>GGAACCTTAATGCCCTCATGTTGCAGCTGGAACCTCCGT</u><br><u>GAGAGGGAGCTGGAAGGGAAGAGCAAGCTTTAGAG</u><br><u>CGGAGGTGCCAGGCCTGCTGAAGCCACACCCTTCCC</u><br><u>GGGGCCTCCTGCATGGAAACACAATGGAG<b>TAA</b></u>                  | 20 |
| Expression<br>cassette including<br>above sequence<br>with Cbh<br>promoter, hybrid<br>intron, bGH polyA<br>tail, and WPRE                                                                                                                                                              | CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGA<br>CCGCCCAACGACCCCCGCCATTGACGTCAATAATGA<br>CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCAT<br>TGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCC<br>ACTTGGCAGTACATCAAGTGTATCATATGCCAAGTAC<br>GCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCC<br>TGGCATTATGCCCAGTACATGACCTTATGGGACTTTCC<br>TACTTGGCAGTACATCTACGTATTAGTCATCGCTATTA<br>CCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTC                                                                                                                                    | 21 |

CCCATCTCCCCCCCCCTCCCCACCCCCCAATTTTGTATTT  
 ATTTATTTTTTAATTATTTTGTGCGAGCGATGGGGGCGG  
 GGGGGGGGGGGGGGGCGGGGCGAGGGGCGGGGCGGG  
 GCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGC  
 GGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGC  
 GGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGC  
 GGGCGGGAGTCGCTGCGACGCTGCCTTCGCCCCGTGC  
 CCCGCTCCGCCGCCGCTCGCGCCGCCCGCCCCGGCT  
 CTGACTGACCGCGTTACTCCCACAGGTGAGCGGGCGG  
 GACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGT  
 TTAATGACGGCTTGTTTCTTTTCTGTGGCTGCGTGAAA  
 GCCTTGAGGGGCTCCGGGAGGGCCCTTTGTGCGGGGG  
 GAGCGGCTCGGGGGGTGCGTGCGTGTTGTGTGCGTG  
 GGGAGCGCCGCGTGCGGCTCCGCGCTGCCCGGCGGCT  
 GTGAGCGCTGCGGGGCGGGCGCGGGGCTTTGTGCGCT  
 CCGCAGTGTTGCGCGAGGGGAGCGCGGCCGGGGGCGG  
 TGCCCCGCGGTGCGGGGGGGGCTGCGAGGGGAACAA  
 AGGCTGCGTGCGGGGTGTGTGCGTGGGGGGGTGAGCA  
 GGGGGTGTGGGCGCGTCCGTCGGGCTGCAACCCCCC  
 TGCACCCCCCTCCCCGAGTTGCTGAGCACGGCCCGGC  
 TTCGGGTGCGGGGCTCCGTACGGGGCGTGCGCGGGG  
 CTCGCCGTGCCGGGCGGGGGGTGGCGGCAGGTGGGG  
 GTGCCGGGCGGGGCGGGGCCGCTCGGGCCGGGGAG  
 GGCTCGGGGGAGGGGCGCGGCGGCCCGGAGCGCC  
 GGCGGCTGTCGAGGCGCGGCGAGCCGACGCCATTGCC  
 TTTTATGGTAATCGTGCGAGAGGGCGCAGGGACTTCC  
 TTTGTCCCAAATCTGTGCGGAGCCGAAATCTGGGAGG  
 CGCCGCCGCACCCCCTCTAGCGGGCGCGGGGCGAAGC  
 GGTGCGGCGCCGGCAGGAAGGAAATGGGCGGGGAGG  
 GCCTTCGTGCGTCGCCGCGCCGCGTCCCCTTCTCCCT  
 CTCCAGCCTCGGGGCTGTCCGCGGGGGGACGGCTGCC  
 TTCGGGGGGGACGGGGCAGGGCGGGGTTTCGGCTTCTG  
 GCGTGTGACCGGCGGCTCTAGAGCCTCTGCTAACCAT  
 GTTCATGCCTTCTTCTTTTCTACAGCTCCTGGGCAA  
 CGTGCTGGTTATTGTGCTGTCTCATCATTTTGGCAAAG  
 AATTCGAGCGGCCGCCAGTGTGAGTTGGACCGGTGCC  
 GCCATGGCGCTCTCCACACCCCAGGAGACTTACTTTA  
 AGTCCCAGGCAGAGTGGCGGGAAGAAGTAGAACTGG  
 AATTTGAAAAGATTTCGTTTTCAGAAAGGGACCTGTCTGGA  
 ACGCGAAGAAGAGGAACTGGAAATGGAAAGGAGGGA  
 GGAGCTCAGACACGCCCTGGACATCAGGGAGCACTAT  
 GAAAGGAAGCTGGAGAGAGCCAACAACCTGTATATG  
 GAACTTAATGCCCTCATGTTGCAGCTGGAACCTCCGTG  
 AGAGGGAGCTGGAAAGGGAAGAGCAAGCTTTAGAGC  
 GGAGGTGCCCAGGCCTGCTGAAGCCACACCTTCCCCG  
 GGGCCTCCTGCATGGAAACACAATGGAGTAATGAGGC  
 ATGCTTCTATATTATTTTCTAAAAGATTTAAAGTTTTG  
 CCTTCTCCATTTAGACTTATAATTCACTGGAATTTTTT  
 GTGTGTATGGTATGACATATGGGTTCCCTTTTATTTTT  
 ACATATAAATATATTTCCCTGTTTTTCTAAAAAAGACC

|                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |    |
|------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
|                                                                                                                                    | <p>             TAGGAAAACGTGCTTCATAATCAACCTCTGGATTACA<br/>             AAATTTGTGAAAGATTGACTGGTATTCTTAACATATGTT<br/>             GCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCC<br/>             TTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTT<br/>             CTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATGA<br/>             GGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTG<br/>             TGCAGTGTGTTTGTGACGCAACCCCCACTGGTTGGGG<br/>             CATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTCG<br/>             CTTTCCCCCTCCCTATTGCCACGGCGGAACATCGCC<br/>             GCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTT<br/>             GGGCACTGACAATTCCGTGGTGTGTCGGGGAAATCA<br/>             TCGTCCTTTCCTTGGCTGCTCGCCTATGTTGCCACCTG<br/>             GATTCTGCGCGGGACGTCCTTCTGCTACGTCCCTTCGG<br/>             CCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTG<br/>             CCGGCTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCC<br/>             TCAGACGAGTCGGATCTCCCTTTGGGCCGCCTCCCCGC<br/>             ATCGATACCGAGCGCTGCTAGAGAGATCGATCTGCCT<br/>             CAACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGC<br/>             CCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCAC<br/>             TCCCAGTGTCTTTCCTAATAAAATGAGGAAATTGCAT<br/>             CGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGT<br/>             GGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA<br/>             GAGAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTA<br/>             TGGGTACCCAGGTGCTGAAGAATTGACCCGGTTCTC<br/>             CTGGG           </p>                   |    |
| <p>             Expression<br/>             cassette above with<br/>             flanking AAV<br/>             ITRs           </p> | <p>             ATGTCCTACAGCGCGCTCGCTCGCTCACTGAGGCCGC<br/>             CCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTTCG<br/>             CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGA<br/>             GTGGCCAACTCCATCACTAGGGGTTCTTGTAGTTAAT<br/>             GATTAACTCTGGAGACCGCCATGCTACTTATCTACCA<br/>             GGGTAATGGGGATCCTCTAGAACTATAGCGTTACATA<br/>             ACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAAC<br/>             GACCCCGCCCATGACGTCAATAATGACGTATGTTCC<br/>             CATAGTAACGCCAATAGGGACTTTCCATTGACGTCAA<br/>             TGGGTGGAGTATTTACGGTAACTGCCCACTTGGCAG<br/>             TACATCAAGTGTATCATATGCCAAGTACGCCCCCTATT<br/>             GACGTCAATGACGGTAAATGGCCCGCCTGGCATTATG<br/>             CCCAGTACATGACCTTATGGGACTTTCTACTTGGCAG<br/>             TACATCTACGTATTAGTCATCGCTATTACCATGGTTCGA<br/>             GGTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCC<br/>             CCCCCTCCCCACCCCAATTTTGTATTTATTTATTTT<br/>             AATTATTTTGTGTCAGCGATGGGGGCGGGGGGGGGGG<br/>             GGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAG<br/>             AGGTGCGGCGGCAGCCAATCAGAGCGGCGCGCTCCGA<br/>             AAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGC<br/>             CCTATAAAAAGCGAAGCGCGCGGCGGGCGGGAGTCG<br/>             CTGCGACGCTGCCTTCGCCCCGTGCCCCGCTCCGCCG<br/>             CGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCG<br/>             TTAATCCCACAGGTGAGCGGGCGGGACGGCCCTTCTC<br/>             CTCCGGGCTGTAATTAGCGCTTGGTTTAATGACGGCTT           </p> | 22 |

|  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |  |
|--|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
|  | <p>             GTTTCCTTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGCT<br/>             CCGGGAGGGCCCTTTGTGCGGGGGGAGCGGCTCGGGG<br/>             GGTGCGTGCGTGTGTGTGTGCGTGGGGAGCGCCGCGT<br/>             GCGGCTCCGCGCTGCCCCGGCGGCTGTGAGCGCTGCGG<br/>             GCGCGGCGCGGGGCTTTGTGCGCTCCGCAGTGTGCGC<br/>             GAGGGGAGCGCGGCCGGGGGGCGGTGCCCCGCGGTGC<br/>             GGGGGGGGCTGCGAGGGGAACAAAGGCTGCGTGCGG<br/>             GGTGTGTGCGTGGGGGGGTGAGCAGGGGGTGTGGGC<br/>             GCGTCGGTCGGGCTGCAACCCCCCTGCACCCCCCTC<br/>             CCCGAGTTGCTGAGCACGGCCCGGCTTCGGGTGCGGG<br/>             GCTCCGTACGGGGCGTGGCGCGGGGCTCGCCGTGCCG<br/>             GGCGGGGGGTGGCGGCAGGTGGGGGTGCCGGGCGGG<br/>             GCGGGGCCGCCTCGGGCCGGGGAGGGCTCGGGGGAG<br/>             GGGCGCGGCGGCCCGGAGCGCCGGCGGCTGTGCA<br/>             GGCGCGGCGAGCCGCAGCCATTGCCTTTTATGGTAAT<br/>             CGTGCGAGAGGGCGCAGGGACTTCCTTTGTCCCAAAT<br/>             CTGTGCGGAGCCGAAATCTGGGAGGCGCCGCCGCACC<br/>             CCCTCTAGCGGGCGCGGGGCGAAGCGGTGCGGCGCCG<br/>             GCAGGAAGGAAATGGGCGGGGAGGGCCTTCGTGCGT<br/>             CGCCGCGCCGCGTCCCCTTCTCCCTCTCCAGCCTCGG<br/>             GGCTGTCCGCGGGGGGACGGCTGCCTTCGGGGGGGAC<br/>             GGGGCAGGGCGGGGTTCGGCTTCTGGCGTGTGACCGG<br/>             CGGCTCTAGAGCCTCTGCTAACCATGTTTCATGCCTTCT<br/>             TCTTTTTCCTACAGCTCCTGGGCAACGTGCTGGTTATT<br/>             GTGCTGTCTCATCATTTTGGCAAAGAATTCGAGCGGCC<br/>             GCCAGTGTGAGTTGGACCGGTGCCGCCATGGCGCTCT<br/>             CCACACCCCAGGAGACTTACTTTAAGTCCCAGGCAGA<br/>             GTGGCGGGAAGAAGTAGAACTGGAATTTGAAAAGATT<br/>             CGTTCAGAAGGGACCTGTCTGGAACGCGAAGAAGAG<br/>             GAACTGGAAATGGAAAGGAGGGAGGAGCTCAGACAC<br/>             GCCCTGGACATCAGGGAGCACTATGAAAGGAAGCTGG<br/>             AGAGAGCCAACAACCTGTATATGGAACCTTAATGCCCT<br/>             CATGTTGCAGCTGGAACCTCCGTGAGAGGGAGCTGGAA<br/>             AGGGAAGAGCAAGCTTTAGAGCGGAGGTGCCAGGC<br/>             CTGCTGAAGCCACACCCTTCCCGGGGCCTCCTGCATG<br/>             GAAACACAATGGAGTAATGAGGCATGCTTCTATATTA<br/>             TTTTCTAAAAGATTTAAAGTTTTGCCTTCTCCATTTAG<br/>             ACTTATAATTCAGTGAATTTTTTTGTGTGTATGGTAT<br/>             GACATATGGGTTCCTTTTATTTTTTACATATAAATAT<br/>             ATTTCCCTGTTTTTCTAAAAAAGACCTAGGAAAACCTGT<br/>             CTTCATAATCAACCTCTGGATTACAAAATTTGTGAAAG<br/>             ATTGACTGGTATTCTTAACCTATGTTGCTCCTTTTACGCT<br/>             ATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTA<br/>             TTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGTATA<br/>             AATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCC<br/>             GTTGTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGC<br/>             TGACGCAACCCCCACTGGTTGGGGCATTGCCACCACC<br/>             TGTCAGCTCCTTTCCGGGACTTTCGCTTTCCCCCTCCCT<br/>             ATTGCCACGGCGGAACCTCATCGCCGCTGCCTTGCCC<br/>             GCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAA           </p> |  |
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|  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |  |
|--|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
|  | TTCCGTGGTGTGTCGGGGAAATCATCGTCCTTTCCTT<br>GGCTGCTCGCCTATGTTGCCACCTGGATTCTGCGCGGG<br>ACGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGC<br>GGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGGC<br>CTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGG<br>ATCTCCCTTTGGGGCCGCCTCCCCGCATCGATAACGAGC<br>GCTGCTAGAGAGATCGATCTGCCTCAACTGTGCCTTCT<br>AGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGCC<br>TTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTT<br>CCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAG<br>TAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAG<br>GACAGCAAGGGGGAGGATTGGGAAGAGAATAGCAGG<br>CATGCTGGGGATGCGGTGGGCTCTATGGGTACCCAGG<br>TGCTGAAGAATTGACCCGGTTCCTCCTGGGGAAGCAA<br>TTCGTTGATCTGAATTTTCGACCACCGATAATACCTATT<br>ACCCTGGTAGATAAGTAGCACGGCGGGTTAATGATTA<br>ACTACAGCAATTCGTTGATCTGAATTTTCGACCACCCAT<br>AATAGATCTCCCATACCTGGTAGATAAGTAGCATG<br>GCGGGACAATTAAGTACCTCAAAGAACTATTCTTGTTT<br>GCCTTATTCTATGTAAATAACTGAAATCTTTGTTTTT<br>CTTCCTAAAAGGGGTGATGTTGATTTTTACTTACAATG<br>TATTTTAAGTTTGTCACTCTAAATGGTTATGAGCAAGT<br>TTAAGAAAAATCTTCAGCAAATACTACCTTAGATTAT<br>GACCCCAAAACACATTTACTTATGATTATGTTGAAAA<br>CATAGGGTCTGGGGAAAAAGGGATTAAATTAAGAAG<br>AAAAAGAAGACTTCGGACTTAAAAAGTCTTTTAGAGG<br>CCAGCTCACCAACAACACAACACCGAGTCTGTGTTGC<br>ACAATATGTTACTTAGGTATAAATCAAGGATTCATGT<br>AATTTTGTCAATTCCTTGCGTGATATTTTAAAAAACATT<br>CTGTGTAAGGTATTTATAAAGCTCTCTTCTAAAAATAC<br>AAAAATTTGTGTCATTAATCAACAGTCAGGTTAATCAT<br>TAACTACAAGGAACCCCTAGTGATGGAGTTGGCCACT<br>CCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGC<br>GACCAAGGTGCGCCGACGCCCGGGCTTTGCCCGGGC<br>GGCCTCAGTGAGCGAGCGAGCGCGCACTGTCA |  |
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[0059] It has been shown the *DLK* is a target for at least one microRNA, miR-130a. Ye et al. MicroRNA-130a Targets MAP3K12 to Modulate Diabetic Endothelial Progenitor Cell Function. *Cell Physiol Biochem.* 2015;36(2):712-26 (2015). Other examples of microRNA inhibitors of *DLK* are hsa-miR-10a-3p, hsa-miR-155-5p, and hsa-miR-30a-5p. Hu et al. *Oncology Lett.* 15:467-474 (2018). In some embodiments the inhibitor comprises a microRNA from **Table 3** or a functional equivalent thereof.

**Table 3: Exemplary microRNA sequences**

| Sequence description | Sequence | miRBase Accession | SEQ ID NO |
|----------------------|----------|-------------------|-----------|
|----------------------|----------|-------------------|-----------|

|                |                                                                                                     |              |    |
|----------------|-----------------------------------------------------------------------------------------------------|--------------|----|
| hsa-mir-130a   | UGCUGCUGGCCAGAGCUCUUUUCAC<br>AUUGUGCUACUGUCUGCACCUGUCA<br>CUAGCAGUGCAAUGUUAAGGGC<br>AUUGGCCGUGUAGUG | MI0000448    | 19 |
| hsa-miR-10a-5p | UACCCUGUAGAUCCGAAUUUGUG                                                                             | MIMAT0000253 | 20 |
| hsa-miR-155-5p | UUA AUGCUAAUCGUGAUAGGGGUU                                                                           | MIMAT0000646 | 21 |
| hsa-mir-30a    | GCGACUGUAAACAUC CUGACUGGA<br>AGCUGUGAAGCCACAGAUGGGCUU<br>UCAGUCGGAUGUUUGCAGCUGC                     | MI0000088    | 22 |

### **3.5 In Vivo and In Vitro Assays**

[0060] The present disclosure further relates to assessment of efficacy and safety of gene therapy vectors in *in vitro* assay systems. Expression levels can be measured by immunohistochemistry of target cells or tissues. In some embodiments the vectors of the disclosure comprise nucleic acids encoding fluorescent proteins or other marker proteins used to assess transduction efficiency or gene expression levels. Functional assays include standard test of vision as well as inspection of the subjects' eye.

### **3.6 Therapeutic Compositions and Methods**

[0061] As used herein, the term "patient in need" or "subject in need" refers to a patient or subject at risk of, or suffering from, a disease, disorder or condition that is amenable to treatment or amelioration with a vector as provided herein. A patient or subject in need may, for instance, be a patient or subject diagnosed with a disease associated retinal cells. "Subject" and "patient" are used interchangeably herein.

[0062] The subject treated by the methods described herein may be a mammal. In some cases, a subject is a human, a non-human primate, a pig, a horse, a cow, a dog, a cat, a rabbit, a mouse or a rat. A subject may be a human female or a human male. Subjects may range in age. Thus, the present disclosure contemplates administering any of the vectors of the disclosure to a subject suffering optic neuropathy.

[0063] Combination therapies are also contemplated by the invention. Combination as used herein includes simultaneous treatment or sequential treatment. Combinations of methods of the invention with standard medical treatments (e.g., corticosteroids or topical pressure reducing medications) are specifically contemplated, as are combinations with novel therapies. In some cases, a subject may be treated with a steroid to prevent or to reduce an immune response to administration of a vector described herein. In certain cases, a subject

may receive topical pressure reducing medications before, during, or after administering of a vector described herein. In certain cases, a subject may receive a medication capable of causing the pupil of the eye to dilate (e.g., tropicamide and/or phenylephrine). In certain cases, the subject may receive a moisturizing gel during recovery to prevent corneal dehydration.

**[0064]** A therapeutically effective amount of the rAAV vector is a dose of rAAV ranging from about  $1e8$  to about  $1e13$  vector genomes (vg), more specifically  $1e10$ - $1e12$  vg or  $1e11$  vg.

**[0065]** In some cases, the therapeutic composition comprises more than about  $1e9$ ,  $1e10$ , or  $1e11$  genomes of the rAAV vector per volume of therapeutic composition injected. In some cases, the therapeutic composition comprises more than approximately  $1e10$ ,  $1e11$ ,  $1e12$ , or  $1e13$  genomes of the rAAV vector per mL.

### **3.7 Administration of Compositions**

**[0066]** Administration of an effective dose of the compositions may be by routes standard in the art including, but not limited to, intracameral inoculation, intravitreal inoculation, subretinal inoculation, suprachroidal inoculation, canaloplasty, or episcleral vein-mediated delivery. Route(s) of administration and serotype(s) of AAV components of the rAAV (in particular, the AAV ITRs and capsid protein) of the invention may be chosen and/or matched by those skilled in the art taking into account the disease state being treated and the target cells/tissue(s).

**[0067]** The disclosure provides for local administration and systemic administration of an effective dose of rAAV and compositions of the invention. For example, systemic administration is administration into the circulatory system so that the entire body is affected. Systemic administration includes enteral administration such as absorption through the gastrointestinal tract and parental administration through injection, infusion or implantation or intravenous administration.

**[0068]** In particular, actual administration of rAAV of the present invention may be accomplished by using any physical method that will transport the rAAV recombinant vector into the target tissue of an animal. Administration according to the invention includes, but is not limited to, injection into the bloodstream and/or directly into the eye. Simply resuspending a rAAV in phosphate buffered saline has been demonstrated to be sufficient to provide a vehicle useful for eye expression, and there are no known restrictions on the

carriers or other components that can be co-administered with the rAAV (although compositions that degrade DNA should be avoided in the normal manner with rAAV).

**[0069]** Capsid proteins of a rAAV may be modified so that the rAAV is targeted to a particular target tissue of interest such as eye. *See, for example*, WO 02/053703, the disclosure of which is incorporated by reference herein. Pharmaceutical compositions can be prepared as injectable formulations or as topical formulations to be delivered to the eyes by administration of eye drops or otherwise. Additionally, when a tetracycline-inducible promoter is used to control transgene expression, it may be advantageous to co-administer doxycycline via eyedrops. Numerous formulations of rAAV have been previously developed and can be used in the practice of the invention. The rAAV can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

**[0070]** For purposes of injection, various solutions can be employed, such as sterile aqueous solutions. Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of rAAV as a free acid (DNA contains acidic phosphate groups) or a pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. A dispersion of rAAV can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In this connection, the sterile aqueous media employed are all readily obtainable by standard techniques well-known to those skilled in the art.

**[0071]** The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating actions of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium

chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

**[0072]** Sterile injectable solutions are prepared by incorporating rAAV in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique that yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

**[0073]** Transduction with rAAV may also be carried out *in vitro*. In one embodiment, desired target cells are removed from the subject, transduced with rAAV and reintroduced into the subject. Alternatively, syngeneic or xenogeneic ocular cells can be used where those cells will not generate an inappropriate immune response in the subject.

**[0074]** Suitable methods for the transduction and reintroduction of transduced cells into a subject are known in the art. In one embodiment, cells can be transduced *in vitro* by combining rAAV with cells, e.g., in appropriate media, and screening for those cells harboring the DNA of interest using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be formulated into pharmaceutical compositions, and the composition introduced into the subject by various techniques, such as by intracameral inoculation, intravitreal inoculation, subretinal inoculation, canaloplasty, intrathecal delivery, stereotactic delivery to brain, or episcleral vein-mediated delivery. In some embodiments, transduction of cells with rAAV of the invention results in sustained expression of the inhibitor of DLK for more than 1 month, more than 2 months, more than 3 months, more than 4 months, more than 5 months, more than 6 months, more than 9 months, or more than 12 months. The present invention thus provides methods of administering/delivering rAAV which express an inhibitor of DLK to a mammalian subject, preferably a human being. These methods include transducing tissues (including the tissues of the eye, motor neurons, or brain) with one or more rAAV of the present invention. Transduction may be carried out with gene cassettes comprising tissue specific control elements. For example, one embodiment of the invention provides methods of transducing eye cells and eye tissues directed by eye specific control elements, including, promoters described herein.

#### **4.1 Inhibitors**

[0075] In some embodiments, the disclosure provides an inhibitor, comprising a protein comprising a leucine zipper domain that shares at least 95% identity to SEQ ID NO: 3, wherein the leucine zipper domain comprises at least one or at least two two substitutions at positions 391 and 393, relative to SEQ ID NO: 1. In some embodiments, said substitutions comprise K391 to D or K391 to E, and H393 to D or H393 to E. In some embodiments, said substitutions, individually or collectively, either (i) increase the binding affinity of the leucine zipper domain for endogenous DLK or (ii) decrease affinity of said inhibitor for itself, compared to the corresponding protein without said substitutions.

[0076] In some embodiments, the leucine zipper domain further comprises one or more additional substitutions at one or more positions, related to SEQ ID NO: 1 selected from one or more of K398, L407, H405, V412, R414, K453, L458, and R460. In some embodiments, the one or more substations comprise K398 to R if the one or more positions comprise K398; L407 to D or L407 to E if the one or more positions comprise L407; H405 to D or H405 to E if the one or more positions comprise H405; V412 to D or V412 to E if the one or more positions comprise V412; R414 to D or R414 to E if the one or more positions comprise R414; K453 to R if the one or more positions comprise K453; L458 to D or L458 to E if the one or more positions comprise L458; and R460 to D or R460 to E if the one or more positions comprise R460.

[0077] In some embodiments, the inhibitory domain comprises, in N terminal to C terminal order a first polypeptide having sequence REEVXLXFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 9); a polypeptide bridge comprising 20 to 30 amino-acid residues, optionally SEQ ID NO: 11; and a second polypeptide having sequence YMELNALMLQLELYERELXR XEQALERR (SEQ ID NO: 10); wherein X = E or D and Y = R, H, or K.

[0078] In some embodiments, the first polypeptide is selected from the group consisting of a polypeptide having sequence REEVDLDFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 12); a polypeptide having sequence REEVELDFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 13); a polypeptide having sequence REEVDLEFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 14); and a polypeptide having sequence REEVELEFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 15); wherein X = E or D and Y = R, H, or K.

[0079] In some embodiments, the leucine zipper domain comprises a polypeptide that shares at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identity to one of SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, the leucine zipper domain comprises a polypeptide identical to one of SEQ ID NO: 6 or SEQ ID NO: 7. In

some embodiments, the substitution either (i) increases affinity of the leucine zipper domain for endogenous DLK, or (ii) decreases affinity of the leucine zipper domain for itself; each of (i) and (ii) as compared to the same leucine zipper domain having the native residue at the position of the substitution. In some embodiments, the leucine zipper preferentially forms heterodimers with endogenous DLK compared to either one of (i) homodimers of the inhibitor or (ii) DLK homodimers.

**[0080]** In some embodiments, the inhibitor comprises a DLK kinase domain having at least 95% identity to SEQ ID NO: 5, wherein the DLK kinase domain comprises a substitution that decreases the kinase activity of the DLK kinase domain. In some embodiments, the substitution that decreases the kinase activity of the DLK kinase domain is a substitution of lysine (K) at position 152 relative to SEQ ID NO: 1, from lysine (K) to alanine (A). In some embodiments, the DLK kinase domain comprises SEQ ID NO. 8.

**[0081]** In some embodiments, the inhibitor comprises a functional fragment of LZK. In some embodiments, the inhibitor comprises a functional fragment a neurotrophic factor, optionally BDNF, GDNF, NT4, TrkB, RdCVF, or CNTF, or a functional fragment thereof. In some embodiments, the inhibitor further comprises a functional fragment capable of inhibiting cAMP-mediated signaling, optionally cAMP-mediated signalling through mAKAP. In some embodiments, the inhibitor comprises a second domain, and the inhibitor is capable of having neurotrophic activity in the cell.

## **4.2 Vectors**

**[0082]** The disclosure provides a vector, comprising a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK), the polynucleotide operatively linked to a promoter, wherein the inhibitor is capable of binding either (i) an endogenous DLK protein, or (ii) an endogenous mRNA encoding an endogenous DLK; and wherein the inhibitor is capable of reducing the expression, signaling activity, or kinase activity of the endogenous DLK in a retinal cell. As already stated, the vector may be viral or non-viral. In some embodiments, the vector is recombinant AAV.

**[0083]** In some embodiments, the promoter is a ubiquitous promoter. As used herein a “ubiquitous promoter” is a promoter that is capable of mediating expression of a polynucleotide in many, if not most, cells of a target organism. Generally ubiquitous promoters in one eukaryotic organism are capable of mediating expression in others. Ubiquitous promoters include mammalian promoters. In some embodiments, the ubiquitous promoter comprises a CMV promoter, a CAG promoter, CBA promoter, or a CBh promoter.

In some embodiments, the promoter is a retinal cell-type specific. In some embodiments, the promoter is an RGC-specific promoter. Exemplary RGC-specific promoters, *e.g.* the *Nefh* promoter, are provided by Hanlon et al. *Front Neurosci.* 2017 Sep 21;11:521.

**[0084]** In some embodiments, the promoter is a Thy1 promoter, a *Nefh* promoter, or a DCX promoter. The DCX promoter is described in Smith et al. *Sci Rep.* 8:1490 (2018); and US20140248701A1. In some embodiments, the promoter is a minipromoter. In some embodiments, said minipromoter is derived from POU4F1, TUBB3, NEFL, or NEFM. The NEFL promoter is described in Simpson et al. New MiniPromoter Ple345 (NEFL) Drives Strong and Specific Expression in Retinal Ganglion Cells of Mouse and Primate Retina. *Hum Gene Ther.* (2018). In some embodiments, the promoter is a rod photoreceptor-specific promoter. In some embodiments, the promoter is a rhodopsin promoter or an RK promoter. In some embodiments, the promoter is a cone photoreceptor-specific promoter. In some embodiments, the promoter is a pR2.1 promoter or a MNTC promoter, as disclosed in U.S. Patent No. 10,000,741, which is incorporated here in its entirety.

**[0085]** In some embodiments, the inhibitor is an RNA capable of binding an endogenous mRNA encoding an endogenous DLK, and wherein the inhibitor is capable of reducing the expression of the endogenous DLK in the cell. In some embodiments, the inhibitor is an shRNA or an siRNA. In some embodiments, the inhibitor is a microRNA. In some embodiments, the microRNA is miR-130a, miR-122-5p, miR-30a-5p, or miR-10-3p. In some embodiments, inhibitor is a protein capable of binding an endogenous DLK, and In some embodiments, wherein the inhibitor is capable of reducing the signaling activity or the kinase activity of the endogenous DLK in the cell.

**[0086]** In some embodiments, the protein comprises an inhibitory domain, the inhibitory domain comprising a leucine zipper domain. In some embodiments, the inhibitory domain is capable of binding the leucine zipper domain of endogenous DLK, thereby competitively inhibiting homodimerization of said endogenous DLK or competitively inhibiting heterodimerization of said endogenous DLK and endogenous Leucine Zipper Kinase (LZK). In some embodiments, the inhibitory domain comprises, consists essentially of, or consists of a polypeptide at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to SEQ ID NO: 3 or SEQ ID NO: 4.

**[0087]** In some embodiments, the leucine zipper domain of the inhibitory domain comprises at least one amino acid substitution relative to SEQ ID NO: 1, and In some embodiments, wherein the substitution increases affinity of the leucine zipper domain for endogenous DLK

or decreases affinity of the leucine zipper domain for itself. In some embodiments, the inhibitory domain contains a substitution selected from those listed in **Table 4**.

**[0088]** In some embodiments, the inhibitory domain comprises, in N terminal to C terminal order, a polypeptide having sequence REEVXLXFEKIYSEGTCLXRXEELXMX (SEQ ID NO: 9), a polypeptide bridge comprising 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino-acid residues, optionally SEQ ID NO: 11, and a polypeptide having sequence YMELNALMLQLELYERELXRXEALERR (SEQ ID NO: 10), wherein X = E or D and Y = R, H, or K.

**[0089]** In some embodiments, the inhibitory domain comprises a polypeptide that shares at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identity to one of SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, the inhibitory zipper domain comprises a polypeptide that is identical to one of SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, the substitution increases affinity of the leucine zipper domain for endogenous DLK or decreases affinity of the leucine zipper domain for itself compared to the same leucine zipper domain having the native residue at the position of the substitution. In some embodiments, the leucine zipper preferentially forms heterodimers with endogenous DLK compared to either one of (i) inhibitor homodimers or (ii) DLK homodimers.

**[0090]** In some embodiments, the inhibitor comprises a DLK kinase domain having at least 75% identity to SEQ ID NO: 5, wherein the DLK kinase domain comprises a substitution that decreases the kinase activity of the DLK kinase domain. In some embodiments, the inhibitor is an inactive form of DLK. In some embodiments, said inhibitor contains a substitution at position 152 relative to SEQ ID NO: 1 from lysine (K) to alanine (A). In some embodiments, the DLK kinase domain comprises SEQ ID NO: 8. In some embodiments, the inhibitor comprises a functional fragment of LZK. In some embodiments, the inhibitor comprises a functional fragment a neurotrophic factor, optionally BDNF, GDNF, NT4, TrkB, RdCVF, or CNTF, or a functional fragment thereof. In some embodiments, the inhibitor comprises a functional fragment capable of inhibiting cAMP-mediated signaling, optionally cAMP-mediated signaling through mAKAP. In some embodiments, the inhibitor comprises a second domain, and wherein the inhibitor is capable of having neurotrophic activity in the cell.

**[0091]** In some embodiments, the vector is a recombinant adeno-associated virus (rAAV) vector. In some embodiments, the vector is a non-viral vector.

**[0092]** In some embodiments, the disclosure provides, a recombinant virus vector, comprising an expression cassette comprising a polynucleotide encoding the inhibitor of claim 1 operably linked to a promoter.

**[0093]** In some embodiments, the virus is a recombinant adeno-associated virus (rAAV) vector. In some embodiments, the expression cassette is flanked by 5' ITR and 3' ITR. In some embodiments, the vector comprises a genome between 3.2 kb and 5.0 kb, 3.4 kb and 4.9 kb, 3.6 kb and 4.8 kb, 3.8 kb and 4.7 kb, 3.8 kb and 4.6 kb, 4.0 kb and 4.6 kb, 4.2 kb and 4.6 kb, 4.2 kb and 4.4 kb, or 4.4 kb and 4.6 kb. In some embodiments, the vector comprises a genome between 3.2 kb and 3.4 kb, 3.4 kb and 3.6 kb, 3.6 kb and 3.8 kb, 3.8 kb and 4.0 kb, 4.0 kb and 4.2 kb, 4.2 kb and 4.4 kb, 4.4 kb and 4.6 kb, 4.6 kb and 4.8 kb, or 4.8 kb and 5.0 kb. In some embodiments, the vector comprises a genome of about 3.2 kb, 3.3 kb, 3.4 kb, 3.5 kb, 3.6 kb, 3.7 kb, 3.8 kb, 3.9 kb, 4.0 kb, 4.1 kb, 4.2 kb, 4.3 kb, 4.4 kb, 4.5 kb, 4.6 kb, 4.7 kb, or 4.8 kb. In some embodiments, the rAAV comprises a deletion of one or more non-essential genes. In some embodiments, the rAAV comprises a deletion of one or more essential genes, provided *in trans* during production of the rAAV. In some embodiments, the rAAV comprises a deletion of *rep*, *cap*, and *rep* or *cap*. In some embodiments, the rAAV is a self-complementary AAV. In some embodiments, the rAAV comprises a deletion in the D region of either or both of the 5' ITR and 3' ITR. In some embodiments, the rAAV is a hybrid AAV. In some embodiments, the rAAV comprises a stuffer sequence. In some embodiments, the rAAV comprises the 5' ITR and 3' ITR of AAV2 and the capsid proteins of an AAV serotype other than AAV2. In some embodiments, the rAAV comprises 5' ITR and 3' ITR from different serotypes. In some embodiments the rAAV is modified by one or more of transcapsidation, adsorption modification, mosaic capsid, or chimeric capsid (*e.g.* as described in Choi et al. *Curr Gene Ther.* 2005 Jun; 5(3): 299–310).

**[0094]** In some embodiments, the vector comprises a capsid of serotype AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, or AAV2/2(7m8). In some embodiments, the rAAV selectively infects a cell type, such as neurons, retinal cells, or RGCs. Serotypes useful in the vectors of the present disclosure include those described in Lebherz et al. *J Gene Med.* 2008 Apr; 10(4): 375–382; Hanlon et al. *Front Neurosci.* 2017; 11: 521; and Hickey et al. *Gene Therapy* 24:787–800 (2017).

**[0095]** In some embodiments, the promoter is a ubiquitous promoter. As used herein a “ubiquitous promoter” is a promoter that is capable of mediating expression of a polynucleotide in many, if not most, cells of a target organism. Generally ubiquitous promoters in one eukaryotic organism are capable of mediating expression in others. Ubiquitous promoters include mammalian promoters. In some embodiments, the ubiquitous promoter comprises a CMV promoter, a CAG promoter, CBA promoter, or a CBh promoter. In some embodiments, the promoter is a retinal cell-type specific. In some embodiments, the

promoter is an RGC-specific promoter. Exemplary RGC-specific promoters, *e.g.* the *Nefh* promoter, are provided by Hanlon et al. *Front Neurosci.* 2017 Sep 21;11:521.

**[0096]** In some embodiments, the promoter is a Thy1 promoter, a *Nefh* promoter, or a DCX promoter. The DCS promoter is described in Smith et al. *Sci Rep.* 8 :1490 (2018); and US20140248701A1. In some embodiments, the promoter is a minipromoter. In some embodiments, said minipromoter is derived from POU4F1, TUBB3, NEFL, or NEFM. The NEFL promoter is described in Simpson et al. New MiniPromoter Ple345 (NEFL) Drives Strong and Specific Expression in Retinal Ganglion Cells of Mouse and Primate Retina. *Hum Gene Ther.* (2018). In some embodiments, the promoter is a rod photoreceptor-specific promoter. In some embodiments, the promoter is a rhodopsin promoter or an RK promoter. In some embodiments, the promoter is a cone photoreceptor-specific promoter. In some embodiments, the promoter is a pR2.1 promoter or a MNTC promoter.

### **4.3 Methods of Treatment**

**[0097]** In some embodiments, the disclosure provides a method of treating a disease or disorder, comprising contacting a cell with any of the vectors of the disclosure. In some embodiments, the cell is a retinal cell. In some embodiments, the retinal cell is a retinal ganglion cell (RGC), a photoreceptor cell, or a retinal neuron (*e.g.* a bipolar cell, an amacrine cell, or a horizontal cell). In some embodiments, the cell is a dopaminergic neuron, a dorsal striatum neuron, or a basal forebrain cholinergic neuron. In some embodiments, the vector is administered by injection of a volume of solution of about 10 to 100  $\mu$ L, preferably 50  $\mu$ L. In some embodiments, the disease or disorder is related to dysfunction in retinal cells. In some embodiments, the disease or disorder related to dysfunction in retinal cells is selected from the group consisting of glaucoma, wet macular degeneration, dry macular degeneration, geographic atrophy, retinal detachment, and retinal dystrophy, such as Usher's syndrome, Stargardt's disease, Leber's congenital amaurosis, or retinitis pigmentosa. In yet other embodiments, the disease is related to retinopathy, such as diabetic retinopathy, myopic maculopathy. In other embodiments, the disease is an optic neuropathy, such as optic neuritis, non-arteritic anterior ischemic optic neuropathy (NAION), arteritic anterior ischemic optic neuropathy (AION), traumatic optic neuropathy, Leber's optic neuropathy, dominant optic atrophy, recessive optic atrophy, or radiation optic neuropathy.

**[0098]** In some embodiments, the disclosure provides a method of treating a disease or disorder related to retinal cells, comprising administering to the retina of the subject any of the vectors of the disclosure. In some embodiments, the retinal cell is a retinal ganglion cell

(RGC), a photoreceptor cell, or a retinal neuron—such as a bipolar cell, an amacrine cell, or a horizontal cell. In some embodiments, the vector is administered by injection of a volume of solution of about 10 to 100  $\mu\text{L}$ , preferably 50  $\mu\text{L}$ . In some embodiments, the disease or disorder related to dysfunction in retinal cells is selected from the group consisting of glaucoma, wet macular degeneration, dry macular degeneration, geographic atrophy, retinal detachment, and retinal dystrophy, such as Usher's syndrome, Stargardt's disease, Leber's congenital amaurosis, or retinitis pigmentosa. In yet other embodiments, the disease is related to retinopathy, such as diabetic retinopathy, myopic maculopathy. In other embodiments, the disease is an optic neuropathy, such as optic neuritis, non-arteritic anterior ischemic optic neuropathy (NAION), arteritic anterior ischemic optic neuropathy (AION), traumatic optic neuropathy, Leber's optic neuropathy, dominant optic atrophy, recessive optic atrophy, or radiation optic neuropathy.

#### **4.4 Retinal Cells**

[0099] In some embodiments, the disclosure provides genetically modified retinal cells, comprising a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK), the polynucleotide operatively linked to a promoter. In some embodiments, the inhibitor is capable of binding either (i) an endogenous DLK protein, or (ii) an endogenous mRNA encoding an endogenous DLK. In some embodiments, wherein the inhibitor is capable of reducing the expression, signaling activity, or kinase activity of the endogenous DLK in the retinal cell. Any of the embodiments of features of the polynucleotide described above with respect to vectors can be incorporated in the retinal cells of the disclosure. Indeed it is contemplated that the retinal cells disclosed with have been generated directly or indirectly using the vectors of the disclosure.

[0100] In some embodiments, the disclosure provides a method of generating a genetically modified retinal cell, comprising contacting a retinal cell with any vector of the disclosure. The disclosure further provides pharmaceutical compositions comprises any of the vectors or retinal cells of the disclosure.

#### **4.5 Pharmaceutical Compositions and Methods of Treatment**

[0101] In some embodiments, the disclosure provides a pharmaceutical composition comprising the vector of any one of claims 18 to 33 and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition comprises at least  $1\text{e}9 - 1\text{e}13$  vector genomes (vg) of the vector. In some embodiments, the disclosure provides a unit dose,

comprising any of the vectors of the disclosure, wherein the unit does comprises  $1e9 - 1e13$  vg of vector in a total volume of 10-200  $\mu$ L, in some embodiments 50-100  $\mu$ L.

**[0102]** In some embodiments, the disclosure provides a method of treating a condition in subject in need thereof, comprising administering an effective amount of a pharmaceutical composition of the disclosure to the subject. In some embodiments, the condition is glaucoma, optic neuropathy, retinopathy, retinal detachment, retinitis pigmentosa, retinal dystrophy, macular degeneration, amyotrophic lateral sclerosis (ALS), Parkinson Disease, Alzheimer Disease, prior-related neurodegenerative disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, multiple system atrophy. In some embodiments, the glaucoma is primary open angle (*e.g.* chronic glaucoma), normal tension glaucoma, angle closure glaucoma (*e.g.* acute glaucoma), secondary glaucoma, inherited glaucoma (*e.g.* pigmentary glaucoma), or myocilin (*MYOC*) glaucoma. In some embodiments, the neuropathy is Leber hereditary optic neuropathy (LHON) or autosomal dominant optic atrophy (ADOA or DOA). In some embodiments, the retinopathy is retinopathy of prematurity (ROP) or diabetic retinopathy.

**[0103]** In some embodiments, the retinitis pigmentosa is related to Leber congenital amaurosis. The retinitis pigmentosa may be caused by any of the genotypes known to have causative relationship with the condition. Mutations in more than 60 genes are known to cause nonsyndromic retinitis pigmentosa. More than 20 of these genes are associated with the autosomal dominant form of the disorder. Mutations in the *RHO* gene are the most common cause of autosomal dominant retinitis pigmentosa, accounting for 20 to 30 percent of all cases. At least 35 genes have been associated with the autosomal recessive form of the disorder. The most common of these is *USH2A*; mutations in this gene are responsible for 10 to 15 percent of all cases of autosomal recessive retinitis pigmentosa. Changes in at least six genes are thought to cause the X-linked form of the disorder. Together, mutations in the *RPGR* and *RP2* genes account for most cases of X-linked retinitis pigmentosa. In some embodiments the retinitis pigmentosa is associated with inheritance of alleles of one or more of genes selected from the group consisting of *C2ORF71*, *C8ORF37*, *CA4*, *CERKL*, *CLRN1*, *CNGA1*, *CNGB1*, *CRB1*, *CRX*, *DHDDS*, *EYS*, *FAM161A*, *FSCN2*, *GUCA1B*, *IDH3B*, *IMPDH1*, *IMPG2*, *KLHL7*, *LRAT*, *MAK*, *MERTK*, *NR2E3*, *NRL*, *OFD1*, *PDE6A*, *PDE6B*, *PDE6G*, *PRCD*, *PROM1*, *PRPF3*, *PRPF6*, *PRPF8*, *PRPF31*, *PRPH2*, *RBP3*, *RDH12*, *RGR*, *RHO*, *RLBP1*, *ROM1*, *RP1*, *RP2*, *RP9*, *RPE65*, *RPGR*, *SAG*, *SEMA4A*, *SNRNP200*, *SPATA7*, *TOPORS*, *TTC8*, *TULP1*, *USH2A*, and *ZNF513*, as described in Daiger et al. *Clin Genet*. 84:132-141 (2013).

[0104] In some embodiments, the retinal dystrophy is Stargardt macular degeneration, rod-cone dystrophy, or cone-rod dystrophy.

### EXAMPLES

[0105] The invention is further described in the following Examples, which do not limit the scope of the invention described in the claims.

#### Example 1: Engineering of Improved Dominant-Negative DLK (DN-DLK)

[0106] Expression of the leucine zipper domain of DLK has been shown to have a dominant negative effect of DLK function. Nihalani et al. *J. Bio Chem.* 275, 7273-7279 (2000). To attempt to generate improved dominant negative DLKs (DN-DLKs), Applicants identified mutations in the leucine zipper domain intended to (1) increase the affinity of the DLK / DN-DLK heterodimeric interaction and/or (2) decrease the affinity of the DN-DLK / DN-DLK homodimeric interaction, with the aim of favoring disruption of DLK / DLK homodimers in cells.

[0107] Using the known pinwheel structure of leucine zippers (**FIG 1A**), the sequence DLK (**FIG 1B; SEQ ID NO: 4**) was modelled as eight pinwheels (**FIG. 2A-2H**). Amino-acid substitutions identified by analysis of these models are listed in **Table 4**.

**Table 4: Substitutions to Enhance DN-DLKs**

| Repeat | Substitutions                          | Effect                                          |
|--------|----------------------------------------|-------------------------------------------------|
| L1     | Lysine (K) 391 to glutamic acid (E)    | Create H-E pair<br>Stabilize heterodimer        |
|        | Lysine (K) 391 to aspartic acid (D)    | Create H-D pair<br>Stabilize heterodimer        |
|        | Histidine (H) 393 to glutamic acid (E) | Create K-E pair<br>Stabilize heterodimer        |
|        | Histidine (H) 393 to aspartic acid (D) | Create K-D pair<br>Stabilize heterodimer        |
| L2     | Lysine (K) 398 to glutamic acid (E)    | Create R-E pair<br>Stabilize heterodimer        |
|        | Lysine (K) 398 to aspartic acid (D)    | Create R-D pair<br>Stabilize heterodimer        |
| L3     | Lysine (K) 407 to glutamic acid (E)    | Create H-E pair<br>Stabilize heterodimer        |
|        | Lysine (K) 407 to aspartic acid (D)    | Create H-D pair<br>Stabilize heterodimer        |
| L3     | Histidine (H) 405 to glutamic acid (E) | Create L-E pair<br>Destabilize DN-DLK homodimer |
|        | Histidine (H) 405 to aspartic acid (D) | Create L-D pair<br>Destabilize DN-DLK homodimer |

|    |                                              |                                                  |
|----|----------------------------------------------|--------------------------------------------------|
| L4 | Valine (V) 412 to glutamic acid (E)          | Create E-R pair<br>Stabilize heterodimer         |
|    | Valine (V) 412 to aspartic acid              | Create D-R pair<br>Stabilize heterodimer         |
| L4 | Arginine (R) 414 to glutamic acid (E)        | Create E-R pair<br>Destabilize DN-DLK homodimer  |
|    | Arginine (R) 414 to aspartic acid (D)        | Create D-R pair<br>Destabilize DN-DLK homodimer  |
| L5 | N/A                                          | N/A                                              |
| L6 | Lysine (K) 407 to glutamic acid (E)          | Create R-E pair                                  |
|    | Lysine (K) 407 to aspartic acid (D)          | Create R-D pair                                  |
| L7 | Leucine (L) 458 to glutamic acid (E)         | Create L-E pair,<br>Destabilize DN-DLK homodimer |
|    | Leucine (L) 458 to aspartic acid (D)         | Create L-D pair<br>Destabilize DN-DLK homodimer  |
| L7 | Glutamic acid (E) 454 to A, V, I, L, M, F, W | Strengthen hydrophobic core                      |
|    | Glutamic acid (E) 454 to R, H, K             | Create electrostatic interaction.                |
| L8 | Glutamic acid (E) 461 to A, V, I, L, M, F, W | Strengthen hydrophobic core                      |
|    | Glutamic acid (E) 461 to R, H, K             | Create electrostatic interaction.                |

[0108] Polynucleotides encoding DLK fragments with various combinations of the substitutions listed in **Table 5** are generated. Polynucleotides encoding DLK fragments with combinations of two or more of the substitutions listed in **Table 5** are generated. A polynucleotide encoding a DLK fragment with ten substitutions is generated (SEQ ID NO: 6). A polynucleotide encoding a larger DLK fragment with the same ten substitutions is generated (SEQ ID NO: 7). These designs are tested by expression as isolated DLK fragments and as mutations in full-length DLK.

**Table 5: Exemplary Dominant-Negative Dual-Leucine Zipper Kinase Sequences**

| Sequence description                                                                                                                                         | Sequence                                                                                                                                                                      | SEQ ID NO |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Modified DLK Zipper 1, <i>bridge</i> , and <i>zipper 2</i> .<br><br>Modifications in bold enhance heterodimerization with DLK, compared to DLK homodimers or | <u>REEV<b>E</b>LEFEK<b>I</b>RSEGTCLER<b>E</b>EE<b>E</b>LE<b>M</b>ER<b>R</b>EE<b>L</b>RHALDIRE</u><br><u>HYERKLERANNLYMELNALMLQLE<b>L</b>RERELER<b>E</b>EQALER</u><br><u>R</u> | 6         |

|                                          |                                                                                                                                                                                                                                                                            |   |
|------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|
| LZ-LZ homodimers                         |                                                                                                                                                                                                                                                                            |   |
| Longer LZ domain with same modifications | <u>LSTPQETYFKSQA EWREEVELEFEKIRSEGTCLEREEEEEL</u><br><u>EMERREELRHALDIREHYERKLERANNLYMELNALMLQL</u><br><u>ELRERELEREEQALERRCPGLLKPHPSRGLLHGNTME</u>                                                                                                                        | 7 |
| Kinase domain of kinase-dead DLK (K152A) | ILDLQWVGSGAQQGAVFLGRFHGEEVAVAKVRDLKETDI<br>KHLRKLKHPNIITFKGVCTQAPCYCILMEFCAQGQLYEV<br>LRAGRPVTPSLLVDWSMGIAGGMNYLHLHKIIHRDLKSP<br>NMLITYDDVVKISDFGTSKELSDKSTKMSFAGTVAWMA<br>PEVIRNEPVSEKVDIWSFGVVLWELLTGEIPYKDVDSSAI<br>WVGGSNSLHLPVPSSCPDGFKILLRQCWNSKPRNRPSFR<br>QILLHLDI | 8 |

[0109] Alternatively or in conjunction with any of these mutations to the leucine zipper domain, the kinase domain of DLK may be either deleted or replaced with a kinase-dead kinase domain. One exemplary kinase-dead kinase domain is a kinase domain with the K152A substitution, which replaces lysine K152 with alanine (A), as shown in SEQ ID NO: 8.

[0110] The various constructs are tested for *in vitro* competition with native DLK and for *in vivo* inhibition of DLK kinase activity or downstream signaling.

\*\*\*\*

[0111] All, documents, patents, patent applications, publications, product descriptions, and protocols which are cited throughout this application are incorporated herein by reference in their entireties for all purposes.

[0112] The embodiments illustrated and discussed in this specification are intended only to teach those skilled in the art the best way known to the inventors to make and use the invention. Modifications and variation of the above-described embodiments of the invention are possible without departing from the invention, as appreciated by those skilled in the art in light of the above teachings. It is therefore understood that, within the scope of the claims and their equivalents, the invention may be practiced otherwise than as specifically described.

**What is claimed:**

1. An inhibitor, comprising a protein comprising a leucine zipper domain that shares at least 95% identity to SEQ ID NO: 1, wherein the leucine zipper domain comprises at least one substitution at one or more of the positions 391 and 393.
2. The inhibitor of claim 1, wherein said substitutions comprise at least one of:
  - a) K391 to D or K391 to E, and/or
  - b) H393 to D or H393 to E.
3. An inhibitor, comprising a protein comprising a leucine zipper domain that shares at least 95% identity to SEQ ID NO: 1, having at least one substitution, wherein said substitutions, individually or collectively, either (i) increase the binding affinity of the leucine zipper domain for endogenous DLK or (ii) decrease affinity of said inhibitor for itself, compared to the corresponding protein without said substitutions.
4. The inhibitor of claim 1, wherein the leucine zipper domain further comprises one or more additional substitutions at one or more positions, related to SEQ ID NO: 1 selected from one or more of (c) through (j):
  - a) K398,
  - b) L407,
  - c) H405,
  - d) V412,
  - e) R414,
  - f) K453,
  - g) L458, and
  - h) R460.
5. The inhibitor of claim 4, wherein, relative to SEQ ID NO: 1, the one or more substitutions comprise:
  - a) K398 to R if the one or more positions comprise K398,
  - b) L407 to D or L407 to E if the one or more positions comprise L407,
  - c) H405 to D or H405 to E if the one or more positions comprise H405,
  - d) V412 to D or V412 to E if the one or more positions comprise V412,

- e) R414 to D or R414 to E if the one or more positions comprise R414,
  - f) K453 to R if the one or more positions comprise K453,
  - g) L458 to D or L458 to E if the one or more positions comprise L458, and
  - h) R460 to D or R460 to E if the one or more positions comprise R460.
6. The inhibitor of claim 4, wherein the inhibitory domain comprises, in N terminal to C terminal order:
- a) a first polypeptide having sequence  
REEVXLXFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 9),
  - b) a polypeptide bridge comprising 20 to 30 amino-acid residues, optionally SEQ ID NO: 11, and
  - c) a second polypeptide having sequence  
YMELNALMLQLELYERELXRXEALERR (SEQ ID NO: 10);  
wherein X = E or D and Y = R, H, or K.
7. The inhibitor of claim 6, wherein the first polypeptide is selected from the group consisting of
- a) a polypeptide having sequence REEVDLDFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 12),
  - b) a polypeptide having sequence REEVELDFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 13),
  - c) a polypeptide having sequence REEVDLEFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 14), and
  - d) a polypeptide having sequence REEVELEFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 15);  
wherein X = E or D and Y = R, H, or K.
8. The inhibitor of claim 4, wherein the leucine zipper domain comprises a polypeptide that shares at least 95% identity to one of SEQ ID NO: 6 or SEQ ID NO: 7.
9. The inhibitor of claim 5, wherein the substitutions either (i) increase affinity of the leucine zipper domain for endogenous DLK, or (ii) decrease affinity of the leucine zipper domain for itself; each of (i) and (ii) as compared to the same leucine zipper domain having the native residue at the position of the substitution.

10. The inhibitor of claim 9, wherein the leucine zipper preferentially forms heterodimers with endogenous DLK compared to either one of (i) homodimers of the inhibitor or (ii) DLK homodimers.
11. The inhibitor of claim 1, wherein the inhibitor further comprises DLK kinase domain having at least 95% identity to SEQ ID NO: 5, wherein the DLK kinase domain comprises a substitution that decreases the kinase activity of the DLK kinase domain.
12. The inhibitor of claim 11, wherein the substitution that decreases the kinase activity of the DLK kinase domain is a substitution of lysine (K) at position 152 relative to SEQ ID NO: 1, from lysine (K) to alanine (A).
13. The inhibitor of claim 12, wherein the DLK kinase domain comprises SEQ ID NO: 8.
14. The inhibitor of claim 2, wherein the inhibitor further comprises a functional fragment of LZK.
15. The inhibitor of claim 2, wherein the inhibitor further comprises a functional fragment a neurotrophic factor, optionally BDNF, GDNF, NT4, TrkB, RdCVF, or CNTF, or a functional fragment thereof.
16. The inhibitor of claim 2, wherein the inhibitor further comprises a functional fragment capable of inhibiting cAMP-mediated signaling, for example through mAKAP.
17. The inhibitor of claim 2, wherein the inhibitor comprises a second domain, and wherein the said second domain confers neurotrophic activity.
18. A recombinant virus vector, comprising an expression cassette comprising a polynucleotide encoding the inhibitor of claim 1 operably linked to a promoter.
19. The vector of claim 18, wherein the virus is a recombinant adeno-associated virus (rAAV) vector.
20. The vector of claim 18, wherein the expression cassette is flanked by 5' ITR and 3' ITR

21. The vector of claim 18, wherein the vector comprises a genome between 4.2 kb and 4.6 kb.
22. The vector of claim 18, wherein the vector comprises a capsid of serotype AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, AAV2/2(7m8), or Anc80L65.
23. The vector of claim 18, wherein the promoter is a ubiquitous promoter.
24. The vector of claim 23, wherein the ubiquitous promoter comprises a CMV promoter, a CAG promoter, CBA promoter, or a CBh promoter.
25. The vector of claim 18, wherein the promoter is a retinal cell-type specific.
26. The vector of claim 25, wherein the promoter is an RGC-specific promoter.
27. The vector of claim 26, wherein the promoter is a Thy1 promoter, a Nefh promoter, or a DCX promoter.
28. The vector of claim 18, wherein the promoter is a minipromoter.
29. The vector of claim 1, wherein said minipromoter is derived from POU4F1, TUBB3, NEFL, or NEFM.
30. The vector of claim 25, wherein the promoter is a rod photoreceptor-specific promoter.
31. The vector of claim 30, wherein the promoter is a rhodopsin promoter or an RK promoter.
32. The vector of claim 25, wherein the promoter is a cone photoreceptor-specific promoter.
33. The vector of claim 32, wherein the promoter is a pR2.1 promoter or a MNTC promoter.
34. A method of inhibiting DLK activity in a cell, comprising contacting the cell with the vector of any one of claims 18 to 33.
35. The method of claim 34, wherein the cell is a retinal ganglion cell (RGC).

36. The method of claim 34, wherein the cell is a photoreceptor cell.
37. The method of claim 34, wherein the cell is a retinal neuron.
38. The method of claim 37, wherein the cell is a retinal neuron is a bipolar cell, an amacrine cell, or a horizontal cell.
39. The method of claim 34, wherein the cell is a dopaminergic neuron.
40. The method of claim 34, wherein the cell is a dorsal striatum neuron.
41. The method of claim 34, wherein the cell is a basal forebrain cholinergic neuron.
42. A pharmaceutical composition, comprising the vector of any one of claims 18 to 33 and a pharmaceutically acceptable carrier.
43. The pharmaceutical composition of claim 42, wherein the pharmaceutical composition comprises at least  $1e9 - 1e13$  vector genomes (vg) of the vector.
44. A unit dose, comprising the vector of claim 43, wherein the unit dose comprises  $1e9 - 1e13$  vg of vector in a total volume of 10-200  $\mu\text{L}$ , preferably 50-100  $\mu\text{L}$ .
45. A method of treating a condition in subject in need thereof, comprising administering to the subject an effective amount of the pharmaceutical composition of claim 42.
46. The method of claim 45, wherein the condition is glaucoma.
47. The method of claim 45, wherein the condition is optic neuropathy.
48. The method of claim 45, wherein the condition is retinopathy.
49. The method of claim 45, wherein the condition is retinal detachment.
50. The method of claim 45, wherein the condition is retinitis pigmentosa.
51. The method of claim 45, wherein the condition is retinal dystrophy.
52. The method of claim 45, wherein the condition is macular degeneration.

53. The method of claim 45, wherein the condition is amyotrophic lateral sclerosis (ALS).
54. The method of claim 45, wherein the condition is Parkinson Disease.
55. The method of claim 45, wherein the condition is Alzheimer Disease.
56. The method of claim 45, wherein the condition is prion-related neurodegenerative disease.
57. The method of claim 45, wherein the condition is Huntington's disease.
58. The method of claim 45, wherein the condition is spinocerebellar ataxia.
59. The method of claim 45, wherein the condition is spinal muscular atrophy.
60. The method of claim 45, wherein the condition is multiple system atrophy.
61. A genetically modified retinal cell, comprising a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK), the polynucleotide operatively linked to a promoter,  
wherein the inhibitor is capable of binding either (i) an endogenous DLK protein, or (ii) an endogenous mRNA encoding an endogenous DLK; and  
wherein the inhibitor is capable of reducing the expression, signaling activity, or kinase activity of the endogenous DLK in the retinal cell.
62. The cell of claim 61, wherein the retinal cell is a retinal ganglion cell (RGC).
63. The cell of claim 61, wherein the retinal cell is a photoreceptor cell.
64. The cell of claim 61, wherein the retinal cell is a retinal neuron.
65. The cell of claim 64, wherein the retinal neuron is a bipolar cell, an amacrine cell, or a horizontal cell.
66. The cell of claim 61, wherein the promoter is a ubiquitous promoter.
67. The cell of claim 66, wherein the ubiquitous promoter comprises a CMV promoter, a CAG promoter, CBA promoter, or a CBh promoter.

68. The cell of claim 61, wherein the promoter is a retinal cell-type specific.
69. The cell of claim 68, wherein the promoter is an RGC-specific promoter.
70. The cell of claim 69, wherein the promoter is a Thy1 promoter, a Nefh promoter, or a DCX promoter.
71. The cell of claim 69, wherein the promoter is a minipromoter.
72. The cell of claim 71, wherein said minipromoter is derived from POU4F1, TUBB3, NEFL, or NEFM.
73. The cell of claim 68, wherein the promoter is a rod photoreceptor-specific promoter.
74. The cell of claim 73, wherein the promoter is a rhodopsin promoter or an RK promoter.
75. The cell of claim 68, wherein the promoter is a cone photoreceptor-specific promoter.
76. The cell of claim 75, wherein the promoter is a pR2.1 promoter or a MNTC promoter.
77. The cell of claim 61, wherein the inhibitor is an RNA capable of binding an endogenous mRNA encoding an endogenous DLK, and wherein the inhibitor is capable of reducing the expression of the endogenous DLK in the cell.
78. The cell of claim 77, wherein the inhibitor is an shRNA or an siRNA.
79. The cell of claim 77, wherein the inhibitor is a microRNA.
80. The cell of claim 79, wherein the microRNA is miR-130a, miR-122-5p, miR-30a-5p, or miR-10-3p.
81. The cell of claim 61, wherein the inhibitor is a protein capable of binding an endogenous DLK, and wherein the inhibitor is capable of reducing the signaling activity or the kinase activity of the endogenous DLK in the cell.

82. The cell of claim 81, wherein the protein comprises an inhibitory domain, the inhibitory domain comprising a leucine zipper domain,  
wherein the inhibitory domain is capable of binding the leucine zipper domain of endogenous DLK, thereby competitively inhibiting homodimerization of said endogenous DLK or competitively inhibiting heterodimerization of said endogenous DLK and endogenous Leucine Zipper Kinase (LZK).
83. The cell of claim 82, wherein the inhibitory domain comprises a polypeptide at least 95% identical to SEQ ID NO: 3 or SEQ ID NO: 4.
84. The cell of claim 82, wherein the leucine zipper domain of the inhibitory domain comprises at least one amino acid substitution relative to SEQ ID NO: 1, and  
wherein the substitution increases affinity of the leucine zipper domain for endogenous DLK or decreases affinity of the leucine zipper domain for itself.
85. The cell of claim 82, wherein the inhibitory domain contains a substitution selected from one or more of (a) through (j), relative to SEQ ID NO: 1:
- a) K391 to D or K391 to E,
  - b) H393 to D or H393 to E,
  - c) K398 to R,
  - d) L407 to D or L407 to E,
  - e) H405 to D or H405 to E,
  - f) V412 to D or V412 to E,
  - g) R414 to D or R414 to E,
  - h) K453 to R,
  - i) L458 to D or L458 to E, and
  - j) R460 to D or R460 to E.
86. The cell of claim 82, wherein the inhibitory domain comprises, in N terminal to C terminal order:
- a) a polypeptide having sequence REEVXLXFEKIYSEGTCLXRXEEELXX (SEQ ID NO: 9),
  - b) a polypeptide bridge comprising 20 to 30 amino-acid residues, optionally SEQ ID NO: 11, and

- c) a polypeptide having sequence YMELNALMLQLELYERELXRXEQUALERR (SEQ ID NO: 10);  
wherein X = E or D and Y = R, H, or K.
87. The cell of claim 82, wherein the inhibitory domain comprises a polypeptide that shares at least 95% identity to one of SEQ ID NO: 6 or SEQ ID NO: 7.
88. The cell of claim 82, wherein the inhibitory zipper domain comprises a polypeptide that is identical to one of SEQ ID NO: 6 or SEQ ID NO: 7.
89. The cell of claim 82, wherein the substitution increases affinity of the leucine zipper domain for endogenous DLK or decreases affinity of the leucine zipper domain for itself compared to the same leucine zipper domain having the native residue at the position of the substitution.
90. The cell of claim 82, wherein the leucine zipper preferentially forms heterodimers with endogenous DLK compared to either one of (i) inhibitor homodimers or (ii) DLK homodimers.
91. The cell of claim 82, wherein the inhibitor comprises a DLK kinase domain having at least 75% identity to SEQ ID NO: 5, wherein the DLK kinase domain comprises a substitution that decreases the kinase activity of the DLK kinase domain.
92. The cell of claim 82, wherein the inhibitor is an inactive form of DLK.
93. The cell of claim 92, wherein said inhibitor contains a substitution at position 152 relative to SEQ ID NO: 1 from lysine (K) to alanine (A).
94. The cell of claim 93, wherein the DLK kinase domain comprises SEQ ID NO: 8.
95. The cell of claim 82, wherein the inhibitor comprises a functional fragment of LZK.
96. The cell of claim 82, wherein the inhibitor comprises a functional fragment a neurotrophic factor, optionally BDNF, GDNF, NT4, TrkB, RdCVF, or CNTF.

97. The cell of claim 82, wherein the inhibitor comprises a functional fragment capable of inhibiting cAMP-mediated signaling, optionally cAMP-mediated signaling through mAKAP.
98. The cell of claim 82, wherein the inhibitor comprises a second domain, and wherein the inhibitor is capable of having neurotrophic activity in the cell.
99. A method of generating a genetically modified retinal cell of any one of claims 61 to 98, comprising contacting a retinal cell with a vector comprising a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK).
100. A vector, comprising a polynucleotide encoding an inhibitor of Dual Leucine Zipper Kinase (DLK), the polynucleotide operatively linked to a promoter,  
wherein the inhibitor is capable of binding either (i) an endogenous DLK protein, or (ii) an endogenous mRNA encoding an endogenous DLK; and  
wherein the inhibitor is capable of reducing the expression, signaling activity, or kinase activity of the endogenous DLK in a retinal cell.
101. The vector of claim 100, wherein the promoter is a ubiquitous promoter.
102. The vector of claim 101, wherein the ubiquitous promoter comprises a CMV promoter, a CAG promoter, CBA promoter, or a CBh promoter.
103. The vector of claim 100, wherein the promoter is a retinal cell-type specific.
104. The vector of claim 103, wherein the promoter is an RGC-specific promoter.
105. The vector of claim 104, wherein the promoter is a Thy1 promoter, a Nefh promoter, or a DCX promoter.
106. The vector of claim 100, wherein the promoter is a minipromoter.
107. The vector of claim 106, wherein the minipromoter is derived from POU4F1, TUBB3, NEFL, or NEFM.
108. The vector of claim 103, wherein the promoter is a rod photoreceptor-specific promoter.

109. The vector of claim 108, wherein the promoter is a rhodopsin promoter or an RK promoter.
110. The vector of claim 25, wherein the promoter is a cone photoreceptor-specific promoter.
111. The vector of claim 110, wherein the promoter is a pR2.1 promoter or a MNTC promoter.
112. The vector of claim 100, wherein the inhibitor is an RNA capable of binding an endogenous mRNA encoding an endogenous DLK, and wherein the inhibitor is capable of reducing the expression of the endogenous DLK in the cell.
113. The vector of claim 112, wherein the inhibitor is an shRNA or an siRNA.
114. The vector of claim 112, wherein the inhibitor is a microRNA.
115. The vector of claim 114, wherein the microRNA is miR-130a, miR-122-5p, miR-30a-5p, or miR-10-3p.
116. The vector of claim 100, wherein the inhibitor is a protein capable of binding an endogenous DLK, and  
wherein the inhibitor is capable of reducing the signaling activity or the kinase activity of the endogenous DLK in the cell.
117. The vector of claim 116, wherein the protein comprises an inhibitory domain, the inhibitory domain comprising a leucine zipper domain,  
wherein the inhibitory domain is capable of binding the leucine zipper domain of endogenous DLK, thereby competitively inhibiting homodimerization of said endogenous DLK or competitively inhibiting heterodimerization of said endogenous DLK and endogenous Leucine Zipper Kinase (LZK).
118. The vector of claim 117, wherein the inhibitory domain comprises a polypeptide at least 95% identical to SEQ ID NO: 3 or SEQ ID NO: 4.

119. The vector of claim 117, wherein the leucine zipper domain of the inhibitory domain comprises at least one amino acid substitution relative to SEQ ID NO: 1, and
- wherein the substitution increases affinity of the leucine zipper domain for endogenous DLK or decreases affinity of the leucine zipper domain for itself.
120. The vector of claim 117, wherein the inhibitory domain contains a substitution selected from one or more of (a) through (j), relative to SEQ ID NO: 1:
- a) K391 to D or K391 to E,
  - b) H393 to D or H393 to E,
  - c) K398 to R,
  - d) L407 to D or L407 to E,
  - e) H405 to D or H405 to E,
  - f) V412 to D or V412 to E,
  - g) R414 to D or R414 to E,
  - h) K453 to R,
  - i) L458 to D or L458 to E, and
  - j) R460 to D or R460 to E.
121. The vector of claim 117, wherein the inhibitory domain comprises, in N terminal to C terminal order:
- a) a polypeptide having sequence REEVXLXFEKIYSEGTCLXRXEEELXMX (SEQ ID NO: 9),
  - b) a polypeptide bridge comprising 20 to 30 amino-acid residues, optionally SEQ ID NO: 11, and
  - c) a polypeptide having sequence YMELNALMLQLELYERELXRXEALERR (SEQ ID NO: 10);
- wherein X = E or D and Y = R, H, or K.
122. The vector of claim 117, wherein the inhibitory domain comprises a polypeptide that shares at least 95% identity to one of SEQ ID NO: 6 or SEQ ID NO: 7.
123. The vector of claim 117, wherein the inhibitory zipper domain comprises a polypeptide that is identical to one of SEQ ID NO. 6 or SEQ ID NO. 7.

124. The vector of claim 117, wherein the substitution increases affinity of the leucine zipper domain for endogenous DLK or decreases affinity of the leucine zipper domain for itself compared to the same leucine zipper domain having the native residue at the position of the substitution.
125. The vector of claim 117, wherein the leucine zipper preferentially forms heterodimers with endogenous DLK compared to either one of (i) inhibitor homodimers or (ii) DLK homodimers.
126. The vector of claim 117, wherein the inhibitor comprises a DLK kinase domain having at least 75% identity to SEQ ID NO: 5, wherein the DLK kinase domain comprises a substitution that decreases the kinase activity of the DLK kinase domain.
127. The vector of claim 117, wherein the inhibitor is an inactive form of DLK.
128. The vector of claim 127, wherein said inhibitor contains a substitution at position 152 relative to SEQ ID NO: 1 from lysine (K) to alanine (A).
129. The vector of claim 128, wherein the DLK kinase domain comprises SEQ ID NO. 8.
130. The vector of claim 117, wherein the inhibitor comprises a functional fragment of LZK.
131. The vector of claim 117, wherein the inhibitor comprises a functional fragment a neurotrophic factor, optionally BDNF, GDNF, NT4, TrkB, RdCVF, or CNTF.
132. The vector of claim 117, wherein the inhibitor comprises a functional fragment capable of inhibiting cAMP-mediated signaling, optionally cAMP-mediated signaling through mAKAP.
133. The vector of claim 117, wherein the inhibitor comprises a second domain, and wherein the inhibitor is capable of having neurotrophic activity in the cell.
134. The vector of any one of claims 99 to 133, wherein the vector is a recombinant adeno-associated virus (rAAV) vector.

135. The vector of any one of claims 100 to 133, wherein the vector is a non-viral vector.
136. A method of treating a disease or disorder related to retinal cells, comprising administering to the retina of the subject the vector of any one of claims 100 to 135.
137. The method of claim 136, wherein the retinal cell is a retinal ganglion cell (RGC).
138. The method of claim 136, wherein the retinal cell is a photoreceptor cell.
139. The method of claim 136, wherein the retinal cell is a retinal neuron.
140. The method of claim 139, wherein the retinal neuron is a bipolar cell, an amacrine cell, or a horizontal cell.
141. The method of any one of claims 136 to 140, wherein the vector is administered by injection of a volume of solution of about 10 to 100  $\mu\text{L}$ , preferably 50  $\mu\text{L}$ .
142. The method of any one of claims 136 to 141, wherein the disease or disorder related to dysfunction in retinal cells is selected from the group consisting of glaucoma, retinopathy, retinal detachment, and retinal dystrophy.
143. A method of generating a genetically modified retinal cell, comprising contacting a retinal cell with the vector of any one of any one of claims 100 to 135.
144. A pharmaceutical composition, comprising the vector of any one of claims 100 to 135.
145. A pharmaceutical composition, comprising the cell of any one of claims 61 to 98.

FIG. 1A

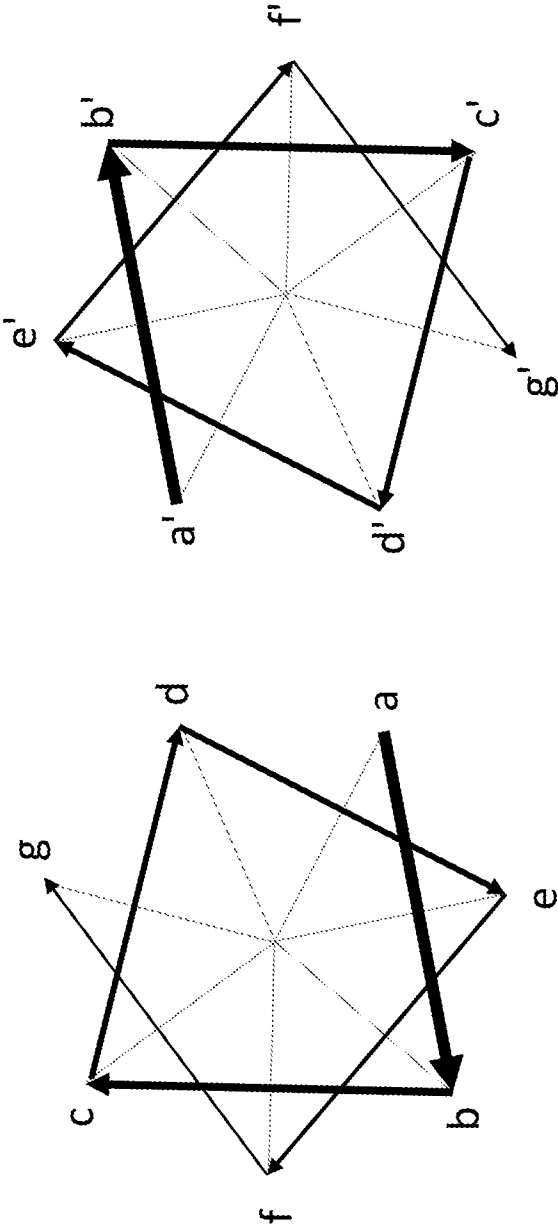


FIG. 1B

|                                                                                     |                               |
|-------------------------------------------------------------------------------------|-------------------------------|
| ---L1-----L2-----L3-----L4--                                                        | ---L5-----L6-----L7-----L8--- |
| abcdefgabcdefgabcdefgabcdefg                                                        | abcdefgabcdefgabcdefgabcdefg  |
| REEVKLHFEKIKSEGTCLHRLEEELVMRRREELRHALDIREHYERKLERANNLYMELNALMLQLLELKERELLRRREQALERR |                               |

FIG. 2A

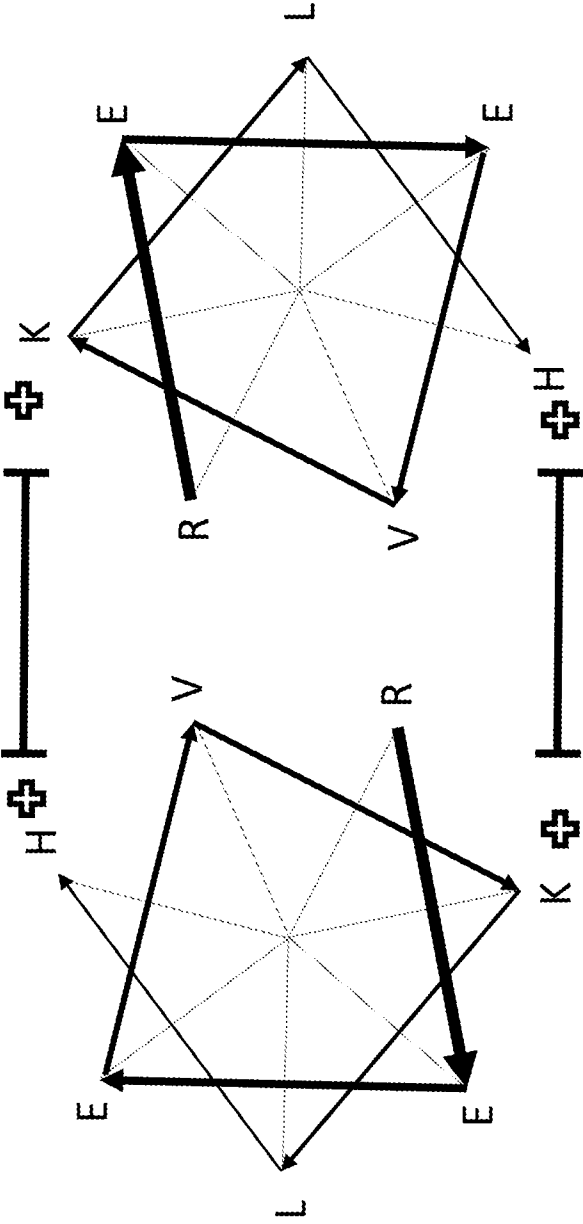


FIG. 2B

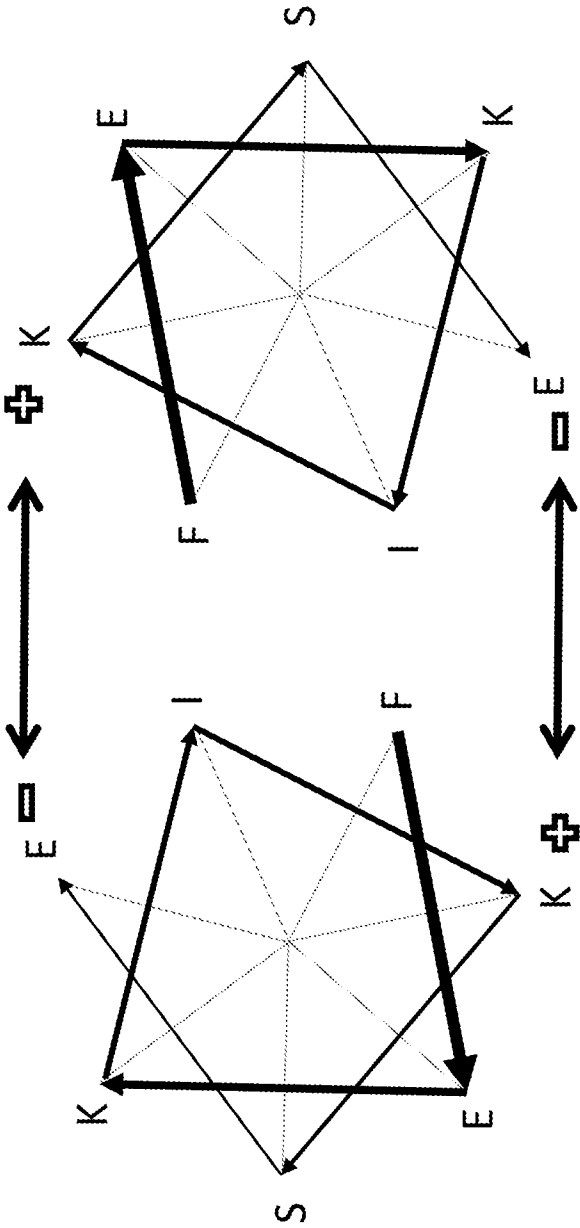
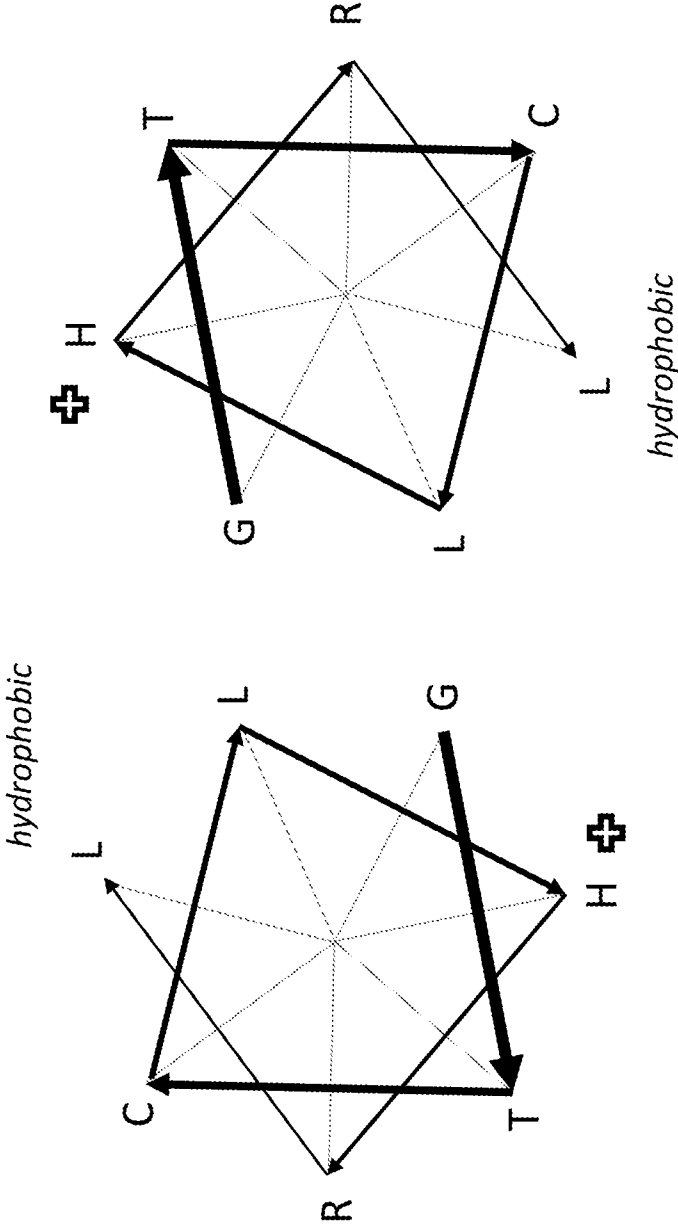


FIG. 2C



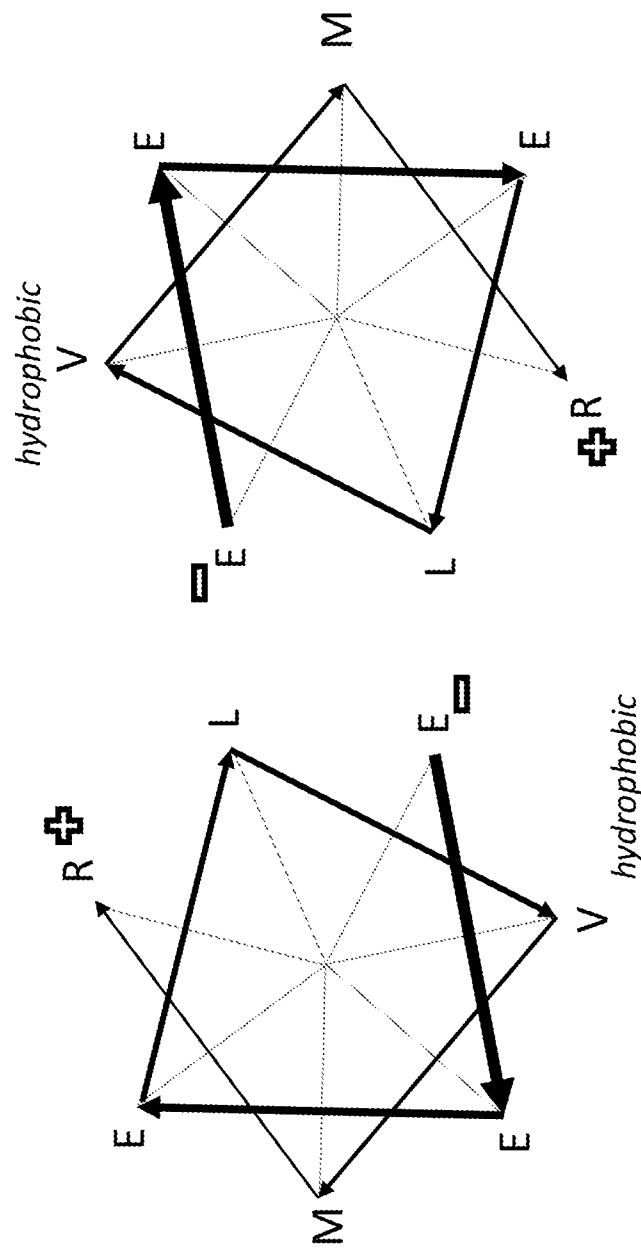


FIG. 2D

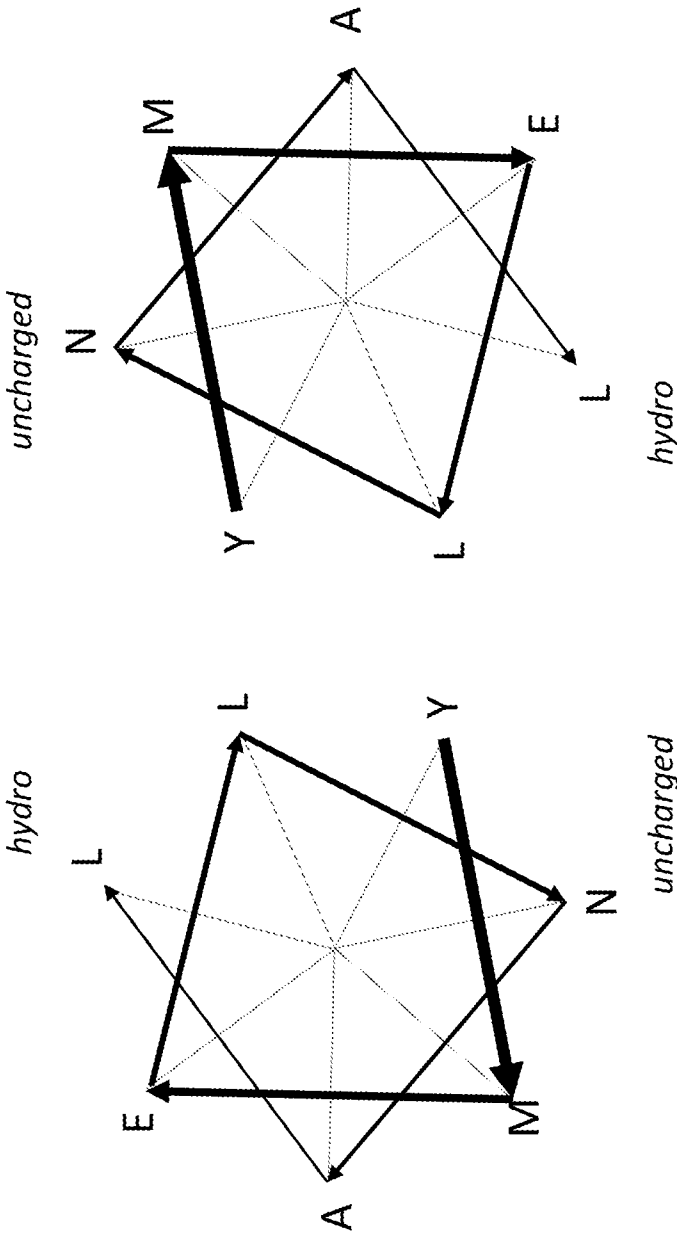


FIG. 2E

FIG. 2F

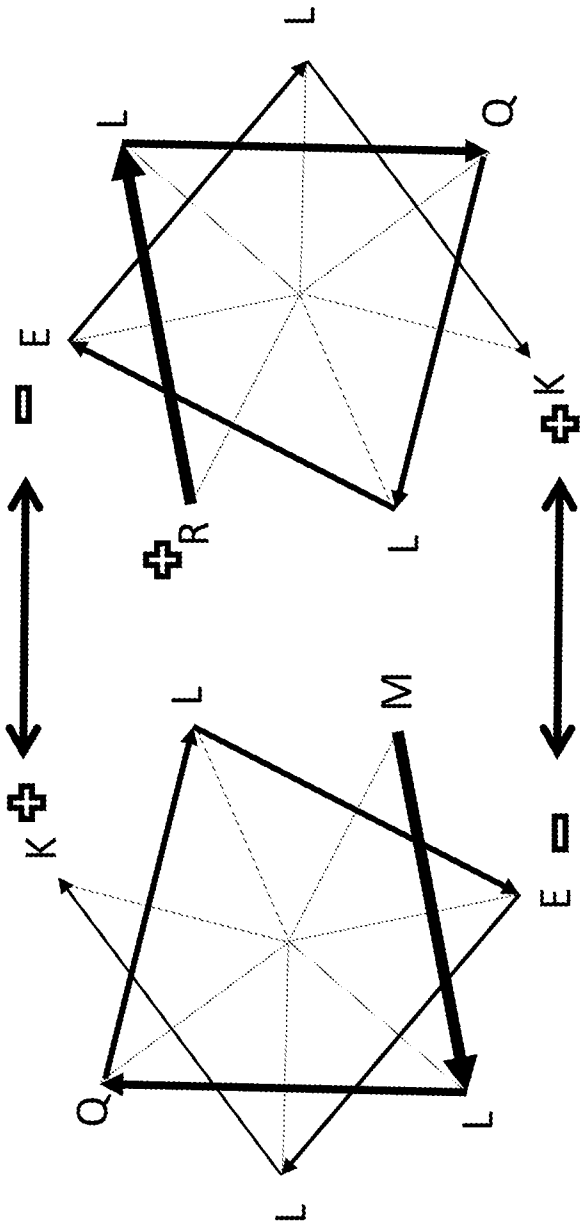


FIG. 2G

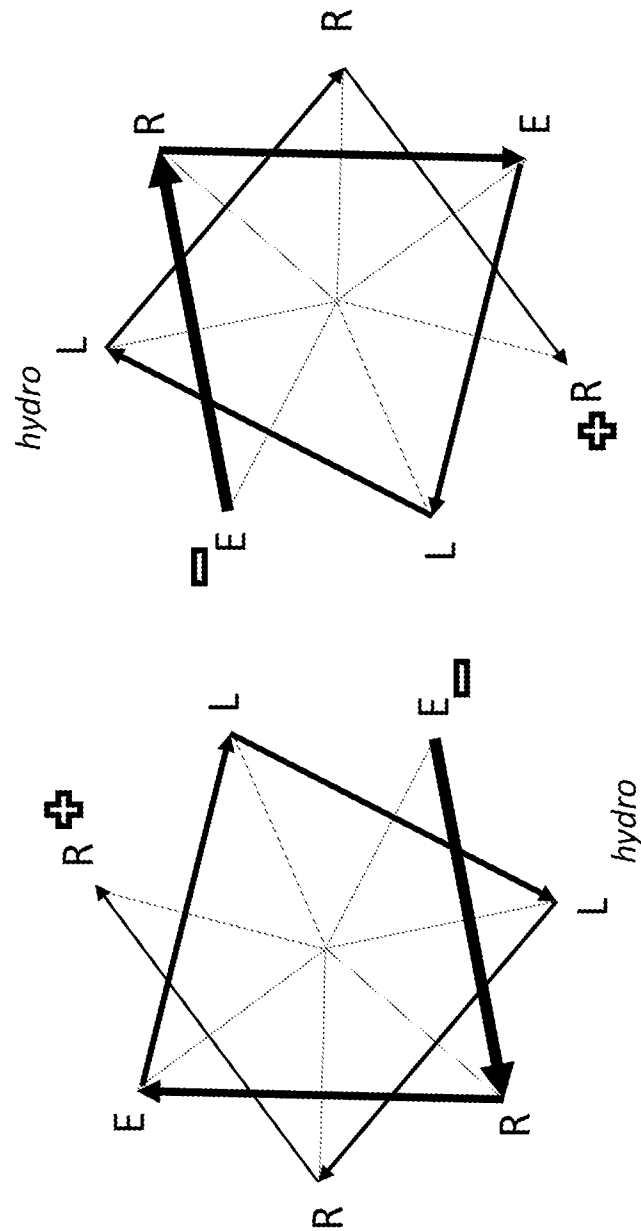


FIG. 2H

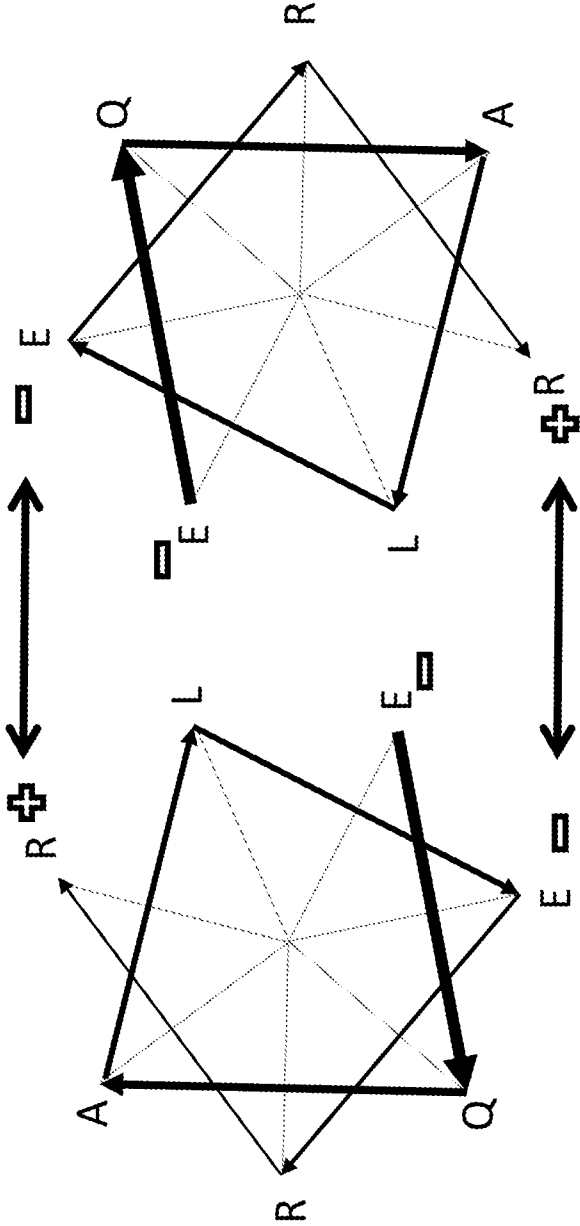




FIG. 4A

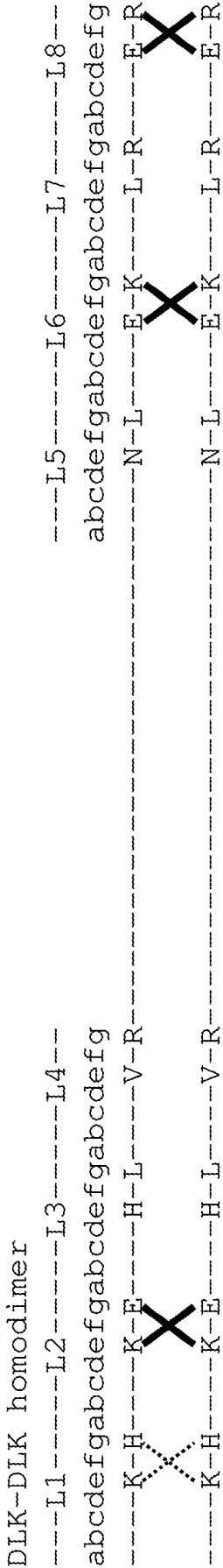


FIG. 4B



FIG. 4C



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/018161

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 7/01; C12N 9/16; C12N 9/22; C12N 15/09; C12N 15/11; C12N 15/113 (2020.01)

CPC - A61K 9/0048; A61K 38/465; A61K 48/00; A61K 48/005; A61K 48/0075; A61P 27/02 (2020.05)

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

see Search History document

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

see Search History document

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

see Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                | Relevant to claim No.                                            |
|-----------|-------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|
| X         | WO 2018/009562 A1 (THE JOHNS HOPKINS UNIVERSITY) 11 January 2018 (11.01.2018) entire document                     | 61-64, 66-68, 73, 74, 77-79, 99-103, 108, 109, 112-114, 135, 145 |
| --        |                                                                                                                   |                                                                  |
| Y         |                                                                                                                   | 65, 69-72, 75, 76, 81, 104-107, 116                              |
| Y         | US 2015/0376240 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 31 December 2015 (31.12.2015) entire document | 65                                                               |
| Y         | WO 2018/013932 A1 (SALK INSTITUTE FOR BIOLOGICAL STUDIES et al) 18 January 2018 (18.01.2018) entire document      | 69-72, 106, 107                                                  |
| Y         | WO 2011/106783 A2 (CORNELL UNIVERSITY et al) 01 September 2011 (01.09.2011) entire document                       | 70, 104, 105                                                     |
| Y         | US 2015/0259395 A1 (AVALANCHE BIOTECHNOLOGIES, INC et al) 17 September 2015 (17.09.2015) entire document          | 75, 76                                                           |
| Y         | US 9,101,645 B2 (WATTS et al) 11 August 2015 (11.08.2015) entire document                                         | 81, 116                                                          |
| A         | WO 1995/023849 A1 (THE CHILDREN'S HOSPITAL OF PHILADELPHIA) 08 September 1995 (08.09.1995) entire document        | 1-133, 135, 145                                                  |
| A         | US 9,708,604 B2 (COLLARD et al) 18 July 2017 (18.07.2017) entire document                                         | 1-133, 135, 145                                                  |

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

\* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

26 May 2020

Date of mailing of the international search report

17 JUL 2020

Name and mailing address of the ISA/US

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Blaine R. Copenheaver

Telephone No. PCT Helpdesk: 571-272-4300

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/018161

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                         | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | SIU et al. "Dual Leucine Zipper Kinase Inhibitors for the Treatment of Neurodegeneration," Journal of Medicinal Chemistry, J Med Chem, 04 June 2018 (04.06.2018), Vol. 61, Pgs. 8078-8087. entire document | 1-133, 135, 145       |
| P, A      | WO 2019/195765 A1 (UNIVERSITY OF MASSACHUSETTS) 10 October 2019 (10.10.2019) entire document                                                                                                               | 1-133, 135, 145       |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/018161

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

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

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

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

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

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

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

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

3. Additional comments:

SEQ ID NOS: 1-15 were searched.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/018161

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

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

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 134, 136-144  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

### Remark on Protest

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