

G-PROTEIN-GATED-K⁺ CHANNEL-MEDIATED ENHANCEMENTS IN LIGHT SENSITIVITY IN ROD-CONE DYSTROPHY (RCD)

DESCRIPTION

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Technical field of the invention

The present invention concerns a new gene therapy approach to increase light-sensitivity in degenerating cones in advanced stages of rod-cone dystrophy (RCD) mediated by G-protein-gated-K⁺ channel (GIRK), in particular GIRK1 F137S, activated by G proteins recruited by cone opsin expressed in degenerating cones.

In the description below, references in square brackets ([]) refer to the list of references at the end of the text.

State of the art

15 Retina is the light sensitive tissue of the eye composed of three layers of neurons interconnected by synapses. The primary neurons of the retina are the light-sensing photoreceptors (PR), which are of two types: the rods for night vision and the cones for daylight vision. Cone-mediated vision is mostly supported by the fovea and is responsible for high acuity central vision most valuable to our daily visual tasks (Sinha et al., 2017) [1]. The light sensitive G protein coupled receptors that link photon capture to intracellular signaling leading to membrane hyperpolarization in photoreceptors are called opsins (Yau and Hardie, 2009) [2]. There is one type of rod opsin found in rods and three types of cone opsins – responsible for trichromatic vision – in the primate retina. The structural properties and phototransduction cascades are similar between these opsins.

25 The phototransduction cascade is composed of several proteins that are concentrated in the photoreceptor outer-segments in normal retinas (Figure 1A). The role of the photoreceptor is to sense light via this phototransduction cascade and induce an electrical signal that is then processed and transmitted towards downstream neurons (Ebrey and Koutalos, 2001) [3].

30 The absorption of a photon activates the opsin composed of two parts: the protein part, and the light absorbing part, which is the retinal – a derivative of vitamin A. The latter isomerizes from 11-cis-retinal (dark adapted state) into all-trans-retinal configuration (light adapted state). As a result, the opsin becomes catalytically active recruiting the G protein transducin. The α -subunit of transducin is activated by the replacement of GDP by GTP. After, 35 the α -subunit dissociates from the $\beta\gamma$ -subunits to activate the membrane-associated phosphodiesterase 6 (PDE) by binding its two inhibitory γ subunits. The activated PDE

hydrolyses cGMP into GMP. The reduction of cGMP closes the nucleotide-gated channels (CNG) and this stops cation entry, resulting in PR hyperpolarization and reduction in glutamate release by the photoreceptor (Larhammar et al., 2009) [4].

In order to respond to another photon, this phototransduction cascade is deactivated
5 by two mechanisms: (i) the transducin inactivates itself by hydrolyzing the bound GTP and (ii) the rhodopsin kinase (GRK) phosphorylates the opsin that interacts with the regulatory protein arrestin, leading to opsin inactivation. Retinal is then recycled by the retinal pigment epithelium (RPE) and Müller glial cells. Each and every protein of this cascade plays an important role in converting the light signal into an electrical signal conveyed to the second and third order
10 neurons (Maeda et al., 2003) [5].

Inherited retinal degenerations are mostly due to mutations in photoreceptor or RPE cells leading to the degeneration of the rods, followed by cone outer segment degeneration eventually leading to blindness (Buch et al., 2004) [6]. Among them, rod-cone dystrophy (RCD) represents the largest category where genetic causes are highly heterogeneous. More than
15 60 different genes expressed in rod photoreceptors or the retinal pigment epithelium are involved (Wright et al., 2010) [7]. The first gene that has been linked to RCD is the rhodopsin gene RHO that counts for 25% of RCD autosomal dominant cases. Many other causative genes have also been identified: the cGMP-PDE subunit gene and the cyclic GMP gated channel protein α subunit gene. Although there are many causative genes, the resulting RCD
20 phenotype is the same across different mutations (Ferrari et al., 2011) [8]. The common RCD phenotype is characterized by the progressive rod degeneration, causing night blindness, and followed by progressive peripheral cone degeneration, causing "tunnel vision", mediated entirely by the remaining foveal cones then eventually resulting in complete blindness in the latest stages of disease. Usually, when patients are diagnosed with RCD, they already show
25 night blindness, meaning their rods have degenerated. However, the cones remain until the late stages of the disease; particularly in the foveal region responsible for high acuity leading to tunnel vision in early stages (Li et al., 1995) [9]. In later stages of the disease, these cones lose their outer segment structures leading to complete blindness before the complete loss of the cone soma and pedicle (Li et al., 1995) [9].

In order to preserve the vision in these patients presenting light sensitive cone cell
30 bodies, one innovative strategy is retinal gene therapy, which broadly refers to the transfer of a therapeutic gene into retinal cells to mediate a therapeutic effect (Bennet, 2017) [10]. Although the first successful clinical trials of gene therapy have focused on gene replacement, where a gene carrying a recessive mutation is replaced by a functional cDNA copy, this
35 strategy is limited because it cannot be used for the majority of retinal degenerations (Bennet, 2017) [10]. For example, in RCD, the huge variability of mutations makes it difficult to apply to

each specific mutation. Moreover, dominant mutations cannot be treated using this approach. Furthermore, in 50% of cases, the causative mutation is not elucidated or the rod photoreceptors bearing the most frequent mutations are already lost (Dalkara et al., 2015) [11]. For these reasons, mutation-independent gene therapies that can be applied beyond the loss of rods must be developed to treat a large number of patients without knowledge of the mutant gene.

Taking this goal into account, previous studies used the ectopic expression of microbial opsins, like channelrhodopsin in bipolar cells or halorhodopsin in cones PRs, to modulate membrane potential and induce a depolarization or hyperpolarization respectively (Busskamp et al., 2012; Dalkara and Sahel, 2014; Scholl et al., 2016) [12-14]. Thus, in RCD, optogenetics can be used as a therapeutic strategy to restore vision in blind retinas (Baker and Flannery, 2008) [15]. There are many parameters to consider: (i) the choice of cell target to make the most out of the retinal circuitry sending the most interpretable signal to the brain (ii) the choice of the appropriate optogenetic tool to obtain a close to natural electrophysiological response in these target cells. Concerning the second point, the relatively weak capacity of microbial opsins has been a major limitation: potential immunogenicity of using an opsin from prokaryotic species, high intensities required due to the lack of signal amplification by these directly light gated channels and pumps originating from single cell microorganisms, as in the case of halorhodopsin (Baker and Flannery, 2008) [15], (Cehajic-Kapetanovic et al., 2015; Gaub et al., 2015; Van Gelder and Kaur, 2015; van Wyk et al., 2015) [16-19]. Unlike rhodopsins or cone opsins, microbial opsins are not able to activate G protein coupled cascades such as the phototransduction cascade present in healthy retinas. One of the possible ways to go beyond the limits of microbial opsins is the use of animal opsins, which are all G protein coupled receptors. However all work in this field has so far been focused on inner retinal neurons (Berry et al., 2019; Cehajic-Kapetanovic et al., 2015; De Silva et al., 2017; Gaub et al., 2015; Lin et al., 2008; van Wyk et al., 2015) [20, 16, 21, 17, 22, 19].

In a previous study, it has been shown that light-activation of two animal cone opsins can stimulate the $G_{i/o}$ signalling pathways in human kidney cells and in neuronal cells *in vitro* and *in vivo* (Masseck et al., 2014) [23]. This pathway is involved in fast dampening of neuronal activity and inhibition of intrinsic ion channels. However, it has also been shown that animal cone opsins can directly modulate the G protein-gated inwardly rectifying potassium channels (GIRK) upon co-expression in any given cell (Berry et al., 2019; Masseck et al., 2014) [20, 23]. GIRK channels are composed of two subunits. There are four types of subunits: GIRK1 to 4. GIRK1 and 3 cannot form homotetramers; they have to be associated with GIRK2 to be functional (Mark and Herlitze, 2000) [24]. Conversely, GIRK2 alone can form homotetramers. A single point mutant GIRK1 F137S was suggested to form functional homomeric channels

(Chan et al., 1996) [31]. The GIRK channel is predominantly closed at resting membrane potentials. After its activation by the $\beta\gamma$ subunit of a $G_{i/o}$ protein, potassium ions flow out of the cell, thus, hyperpolarizing the neuron (Figure 1B). It has therefore been possible to use vertebrate cone opsins SWO (for short wavelength opsin) and LWO (for long wavelength opsin) for repetitive $G_{i/o}$ activation of a specific wavelength *in vivo* in the anxiety circuitry, and the combination of cone opsins with GIRK has proven more efficient than microbial opsins at low light intensities (Masseck et al., 2014) [23].

Description of the invention

10 The Inventors have thus investigated if it was possible to implement this cone opsin based system in a vision restoration setting and investigated patient retinas in preparation for clinical candidate selection. Therefore, in order to develop a light-sensitive cone reactivation strategy, the expression of the phototransduction cascade elements in cones during degeneration was first examined in two RCD mouse models. After exploring the phosphotransduction cascade in two RCD mouse models, a target molecule approach acting via $G_{i/o}$ proteins recruited by the activation of remaining cone opsin was proposed for the first time. It has thereby created a new "short phototransduction cascade" that is independent from the expression of PDE and transducin.

20 First, the state of the endogenous phototransduction cascade in degenerating cones through the progression of disease was investigated. In both mouse models, only opsin and arrestin were found to migrate to the cone cell bodies after outer segment degeneration. Thus it was hypothesized that cone reactivation based on cone opsin signaling may be feasible, which in turn will allow to recover high sensitivity vision. It was found that endogenous cone opsins were still expressed at the level of the cone cell body in rd10 mouse model (Figure 3) suggesting the possibility of linking their activity to GIRK channels, such as GIRK2 channel, even in absence of transducin and phosphodiesterase (Figure 1B). In this configuration, the opening of GIRK channel will allow the exit of potassium ions due to the resting membrane potential of dormant cones (Busskamp, 2010) [25]. K^+ efflux via the GIRK channel will hyperpolarize the cones in response to light as it was seen in the two mouse models of RCD.

30 Next, GIRK2 channel activated by G proteins recruited by cone opsin was expressed in degenerating cones. Moreover, since the remaining opsin in the cone cell bodies is still functional and sufficient to induce a light response in the degenerated cones, the insertion of GIRK2 in all cones leads to light responses following the spectral properties of each of the opsins preserving color vision. The results pointed towards enhanced light-sensitivity in cones of RCD retinas during and after degeneration of cone outer-segments. The results thus demonstrated that vertebrate light sensitive proteins combined with GIRK channel, in particular

GIRK2 channel, activated by an endogenous G protein, could improve the visual function in the two mouse models, as demonstrated by electroretinography and behaviour. This is the first time that a light insensitive mammalian ion channel has been linked to intrinsic opsins providing new avenues in vision restoration that can be implemented to increase light sensitivity even before complete outer segment degeneration. Since this new system makes use of intrinsic opsins expressed in degenerating cones, it also enables for the first time, color vision restoration/maintenance.

Similarly to the RCD mouse models, the cone opsin and the cone arrestin remain in the cone cell bodies of RP human patients (Figure 12). This result demonstrates the feasibility of reactivating the cone function in the foveal region of RCD human patients with the short GIRK2/opsin phototransduction cascade. The activation of the remaining cone opsin by a light stimulus will trigger the short phototransduction cascade and lead to vision restoration in RCD patients, even at an intermediate or advanced stage of the disease.

This new approach has thus the potential to maintain and/or restore, high acuity and color vision requiring only low light intensities in human patients.

A clear advantage of microbial opsins is their robustness and millisecond scale kinetics (Packer et al., 2013) [26]. For systems using other opsins, it should be considered that in order to respond to another light stimulus, the cascade has to be deactivated to recover light sensitivity. In absence of this, cones may stay hyperpolarized after GIRK2 channel activation limiting their ability to modulate synaptic transmission at a movie rate compatible with motion vision. In the present approach, depolarization of the cones was made possible thanks to the arrestin that is still maintained at very late stages of the disease in both RCD models. This was noticeable in the flicker ERG traces showing responses of the retina during repetitive light stimuli and also by the improved optokinetic reflex of treated mice.

Lastly, the fact that incorporation of GIRK2 enhances existing light responses in cones even prior to complete outer segment loss offers the possibility of implementing this gene therapy in mid stages of the disease. Retinal degeneration in mice is much faster than in humans, thus a few days of therapeutic efficiency in mice is equal to several years in humans. Nonetheless, even when endogenous light responses disappear, retinas expressing GIRK2 are still able to respond to light, generating response amplitudes that correspond to remaining cone numbers. This suggests that patients with remaining foveal cones can benefit from this treatment even if they have no detectable light perception at the beginning of treatment (Figure 10). Thus this approach can be used as long as cone cells remain. Indeed a decrease in the response of treated cones to light stimuli was recorded, which was consistent with decrease in cone numbers and the fact that we did not transduce all cones due to subretinal injection further limited the beneficial effect. AAV vectors showing better lateral spread can be used to

increase transduced cone numbers beyond the bleb (Khabou et al., 2018; International patent application WO 2018134168) [27, 28]. In order to increase the therapeutic window, neurotrophic factors can be implemented alongside the approach of the present invention. Indeed, AAV-mediated secretion of neurotrophic factors such as the rod-derived cone viability factors (RdCVF), have been shown to delay cone cell death and may be combined with GIRK2 mediated sensitization (Byrne et al., 2015) [29].

It has now been found that a mutated form of GIRK1 channel, GIRK1 F137S, induces significantly more ion efflux than GIRK2 in the context of a short GIRK/opsin phototransduction cascade. This result suggests that incorporation of GIRK1 F137S in cones will provide for an improved gene therapy over GIRK2 gene therapy of RCD.

An object of the present invention is therefore a vector comprising a nucleotide sequence encoding subunit 1 of G-protein-gated inwardly rectifying potassium channel (GIRK1) F137S.

The nucleotide sequence encoding GIRK1 F137S can be under the control of a cone-specific promoter such as pR1.7 or a functional variant thereof, or minimal M-opsin promoter, in particular in a pMNTC expression cassette, or a GRK promoter or truncated version thereof (G Protein-Coupled Receptor Kinase, GRK1 in particular).

The vector of the present invention can further comprise a nucleotide sequence encoding a mammalian cone opsin. For example, the mammalian cone opsin is a short wavelength cone opsin (SWO), e.g. from *mus musculus* or human cone opsin. Where present in the same vector, the nucleotide sequence encoding GIRK1 F137S, and the nucleotide sequence encoding a mammalian cone opsin are preferably under the control of a same promoter, in particular a cone-specific promoter such as pR1.7 or a functional variant thereof, or minimal M-opsin promoter, in particular in a pMNTC expression cassette, or a GRK promoter or truncated version thereof (G Protein-Coupled Receptor Kinase, GRK1 in particular).

For the purposes of the present invention, "GIRK1 F137S" means a nucleotide sequence encoding a mutated form of wild-type human GIRK1 (SEQ ID NO: 1) or mouse GIRK1 (SEQ ID NO: 4) comprising a substitution of Phe137 by Ser and which retain the ability to respond to light when co-expressed with an opsin. The GIRK1 F137S may differ from wild-type GIRK1 by the substitution of Phe₁₃₇ by Ser, only (e.g. as in SEQ ID NO: 2), or by a limited number of mutation(s), e.g. substitution and/or deletion and/or insertion of at most 1, 2, 3, 4, or 5 of an amino acid(s), in addition to the substitution of Phe₁₃₇ by Ser. For example, a nucleotide sequence encoding GIRK1 F137S comprises or consists of a nucleotide sequence encoding the polypeptide of sequence SEQ ID NO:2, or comprises or consists of the nucleotide

sequence SEQ ID NO: 3, or comprises or consists of a nucleotide sequence encoding the polypeptide of sequence SEQ ID NO: 5.

Human wild-type GIRK1	
Amino acid sequence (501Aa)	MSALRRKFDDYQVVTSSSGSLQPQGPQDPQQQLVPKKKRQRFVDK NGRCNVQHGNLGSSESRYSDFLFTTLVDLKWRWNLFILTYTVAWLFMAS MWWVIAYTRGDLNKAHVGNYPVANVYNFPSAFLFF ₁₃₇ LETEATIGYGYRY ITDKCPEGIILFLFQSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMR DGKLTLMFRVGNLRNSHMVSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFS TGADQLFLVSPLTICHVIDAKSPFYDLSQRSMQTEQFEIVVILEGIVETTGMT CQARTSYTEDEVWGHRRFPVISLEEGFFKVDYSQFHATFEVPTPPYSVKE QEEMLLMSSPLIAPAITNSKERHNSVECLDGLDDITTKLPSKLQKITGREDFP KKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNSEKLVSKTTKMLSDPMS QSVADLPPKLQKMAGGAARMEGNLPAKLRKMNSDRFT (SEQ ID NO: 1)
Human GIRK1 F137S	
Nucleotide sequence	ATGAGCGCCCTGAGAAGAAAGTTTCGGCGACGACTACCAGGTGGTCACC ACAAGCAGCTCTGGCTCTGGACTGCAGCCTCAAGGACCTGGACAGGAT CCTCAGCAGCAGCTGGTGCCCAAGAAACGGCAGAGATTCTGTGGAC AAGAACGGCCGGTGCAATGTGCAGCACGGCAATCTGGGCAGCGAGACA AGCAGATACCTGAGCGACCTGTTCAACCACTGGTGGACCTGAAGTGG CGGTGGAACCTGTTTCATCTTCATCCTGACCTACACCGTGGCCTGGCTGT TCATGGCCAGCATGTGGTGGGTTATCGCCTACACCAGAGCGACCTGA ACAAGGCCCATGTGGGCAACTACACCCCTTGCCTGGCCAACTGTGTACA ACTTCCCTAGCGCCTTCTGTTCAGCATCGAGACAGAGGCCACAATCGG CTACGGCTACCGGTACATCACCGACAAGTGCCCCGAGGGAATTATCCTG TTCCTGTTCCAGTCCATCCTGGGCTCTATCGTGGATGCCTTCTGATCG GCTGCATGTTTCATCAAGATGAGCCAGCCTAAGAAGCGGGCCGAGACAC TGATGTTTAGCGAGCACGCCGTGATCAGCATGCGCGACGGAAACTGA CCCTGATGTTTCAGAGTGGGCAACCTGCGGAACAGCCACATGGTGTGAG CCCAGATCAGATGCAAGCTGCTGAAGTCCAGACAGACCCCTGAGGGCG AGTTCCTGCCTCTGGATCAGCTGGAAGTGGACGTGGGCTTTAGCACAG GCGCCGATCAGCTGTTTCTGGTGTCCCTCTGACCATCTGCCACGTGAT CGACGCTAAGAGCCCCTTCTACGATCTGAGCCAGCGGAGCATGCAGAC CGAGCAGTTTGAGATCGTGGTTCATCCTGGAAGGCATTGTGGAACACC GGCATGACCTGCCAGGCCAGAACAGCTACACAGAGGATGAGGTGCTGTG TGGGGCCACAGATTCTTCCCGTGATCTCTCTGGAAGAGGGCTTCTTCA AGGTGGACTACAGCCAGTTCACGCCACCTTTGAGGTGCCACACCTC CTTACAGCGTGAAAGAACAAGAGGAAATGCTGCTGATGAGCAGCCCTCT GATCGCCCCTGCCATCACCAACAGCAAAGAACGGCACAACAGCGTGGA ATGCCTGGACGGCCTGGATGACATCACCAACAGCTGCCTTCCAAGCT GCAGAAGATCACCGGCAGAGAGGACTTCCCCAAGAACTGCTGAGAAT GAGCAGCACCACCAGCGAGAAGGCCTACTCTCTGGGCGACCTGCCTAT GAAGCTGCAGCGGATCTCTAGCGTGCCCGGCAACTCTGAGGAAAAGCT GGTTTCCAAGACCACCAAGATGCTGAGCGATCCCATGAGCCAGTCCGT GGCTGATCTGCCACCTAACTGCAGAAAATGGCTGGCGGAGCCGCCAG AATGGAAGGCAATCTGCCTGCCAACTGCGCAAGATGAAGTCCGACCG GTTACC (SEQ ID NO : 3)
Amino acid sequence (501Aa)	MSALRRKFDDYQVVTSSSGSLQPQGPQDPQQQLVPKKKRQRFVDK NGRCNVQHGNLGSSESRYSDFLFTTLVDLKWRWNLFILTYTVAWLFMAS MWWVIAYTRGDLNKAHVGNYPVANVYNFPSAFLFS ₁₃₇ LETEATIGYGYRY ITDKCPEGIILFLFQSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMR DGKLTLMFRVGNLRNSHMVSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFS

	TGADQLFLVSPLTICHVIDAKSPFYDLSQRSMQTEQFEIVVILEGIVETTGMT CQARTSYTEDEVWGHRRFFPVISLEEGFFKVDYSQFHATFEVPTPPYSVKE QEEMLLMSSPLIAPAITNSKERHNSVECLDGLDDITTKLPSKLQKITGREDFP KKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNSEEKLVSKTTKMLSDPMS QSVADLPPKLQKMAGGAARMENLPAKLRKMNSDRFT (SEQ ID NO : 2)
Mouse wild-type GIRK1	
Amino acid sequence (501Aa)	MSALRRKFGDDYQVVTSSSGSGLQPQGPQGQPQQQLVPKKKRQRFVDK NGRCNVQHGNLGSSETSRYLSDLFTTLVDLKWRWNLFIFILTYTVAWLFMAS MWWVIAYTRGDLNKAHVGNYPVCVANVYNFPSAFLFF ₁₃₇ LETEATIGYGYRY ITDKCEGIIILFLFQSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMR DGKLTLMFRVGNLNRNSHMSVSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFS TGADQLFLVSPLTICHVIDAKSPFYDLSQRSMQTEQFEVVVILEGIVETTGMT CQARTSYTEDEVWGHRRFFPVISLEEGFFKVDYSQFHATFEVPTPPYSVKE QEEMLLMSSPLIAPAITNSKERHNSVECLDGLDDISTKLPSKLQKITGREDFP KKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNSEEKLVSKTTKMLSDPMS QSVADLPPKLQKMAGGPTRMEGNLPAKLRKMNSDRFT (SEQ ID NO : 4)
Mouse GIRK1 F137S	
Amino acid sequence (501Aa)	MSALRRKFGDDYQVVTSSSGSGLQPQGPQGQPQQQLVPKKKRQRFVDK NGRCNVQHGNLGSSETSRYLSDLFTTLVDLKWRWNLFIFILTYTVAWLFMAS MWWVIAYTRGDLNKAHVGNYPVCVANVYNFPSAFLFS ₁₃₇ LETEATIGYGYRY ITDKCEGIIILFLFQSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMR DGKLTLMFRVGNLNRNSHMSVSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFS TGADQLFLVSPLTICHVIDAKSPFYDLSQRSMQTEQFEVVVILEGIVETTGMT CQARTSYTEDEVWGHRRFFPVISLEEGFFKVDYSQFHATFEVPTPPYSVKE QEEMLLMSSPLIAPAITNSKERHNSVECLDGLDDISTKLPSKLQKITGREDFP KKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNSEEKLVSKTTKMLSDPMS QSVADLPPKLQKMAGGPTRMEGNLPAKLRKMNSDRFT (SEQ ID NO : 5)

Another object of the present invention is a pharmaceutically acceptable carrier including a vector of the present invention.

- According to a particular embodiment of the present invention, the pharmaceutically acceptable carrier can include a vector comprising a nucleotide sequence encoding GIRK1 F137S as described above and a vector comprising a nucleotide sequence encoding a mammalian cone opsin. For example, the mammalian cone opsin is a short wavelength cone opsin (SWO), e.g. from *mus musculus* or human long wavelength-sensitive (OPN1LW), medium wavelength-sensitive (OPN1MW), short wavelength-sensitive (OPN1SW). According to an embodiment, the mammalian cone opsin is human Long-wave-sensitive opsin 1 (SEQ ID NO:6).

Long-wave-sensitive opsin 1 (OPN1LW) homo sapiens	
	MVLKAEHTRSPSATLPSNVPSCRSLSSSEDGPGSSSLADGGLAHNLQDSVRHRILYLSE QLRVEKASRDGNTVSYLKLVSADRHQVPHIQQAFKVNQRASATIAQIEHRLHQCHQQL QELEEGCRPEGLLLMAESDPANCEPPSEKALLSEPPEPGGEDGPVNLPHASRPFILESRF QSLQQGTCLLETEDVAQQQNLLLQKVKAELAAKRFHISLQESYHSLKERSLTDLQLLLESL QEEKCRQALMEEQVNGRLQGQLNEIYNLKHNLACSEERMAYLSYERAKEIWEITETFKSR ISKLEMLQQVTQLEAAEHLQSRPPQMLFKFLSPRLSLATVLLVFVSTLCACPSSLISSRLCT

CTMLMLIGLVLAWQRWRAIPATDWQEWVPSRCRLYSKDSGPPADGP (SEQ ID NO:6)

According to a particular embodiment of the present invention, the pharmaceutically acceptable carrier is for example chosen from solid-lipid nanoparticles, chitosan nanoparticles, liposome, lipoplex or cationic polymer.

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According to a particular embodiment of the present invention, the vector of the present invention is a virus, chosen from an adeno-associated virus (AAV), an adenovirus, a lentivirus, an SV40 viral vector. According to a particular embodiment of the present invention, the present invention is equal to or less than 30 nm in size. For example it is an adeno-associated virus (AAV), preferably an AAV8, or an AAV2-7m8 or AAV9-7m8 capsid variant as described in the international patent application WO 2012/145601.

An AAV2-7m8 or AAV9-7m8 capsid variant is an AAV2 or AAV9 virus comprising a 7 to 11 amino acid long insertion peptide in the GH loop of the VP1 capsid protein, wherein the insertion peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7).

The genomic and polypeptide sequences of various serotypes of AAV, as well as the sequences of the native inverted terminal repeats (ITRs), Rep proteins, and capsid subunits including VP1 protein are known in the art. Such sequences may be found in the literature or in public databases such as GenBank or Protein Data Bank (PDB). See, e.g., GenBank and PDB AF043303 and 1LP3 (AAV2), AY530579 and 3UX1 (AAV9 (isolate hu.14)), the disclosures of which are incorporated by reference herein for teaching AAV nucleic acid and amino acid sequences. Exemplary amino acid sequence of wild-type VP1 for AAV9 and AAV2 are shown in SEQ ID NO: 8 and SEQ ID NO:9, respectively.

20

wild-type AAV9 VP1 capsid protein	
MAADGYLPDWLEDNLSEGIREWALKPGAPQPKANQQHQDNARGLVLPGYKYLGPNG GLDKGEPVNAADAALEHDKAYDQQLKAGDNPYLKYNHADAQERLKEDTSFGGNLG RAVFQAKKRLLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAAKKRLNFG QTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCD SQWLGDRTVTTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYGYSTPWGYFDFNRFHC HFSPRDWQRLINNNWGFPRKRLNFKLFNIQVKEVTDNNGVKTIANNLSTVQVFTDSDYQ LPYVLGSAHEGCLPPFPADVFMIPQYGYLTLDGSGQAVGRSSFYCLEYFPSQMLRTGNN FQFSYEFENVPFHSSYHSQSLDRLMNPLIDQYLYLSKTINGSGQNQQTLKFSVAGPSN MAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASH KEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITNEEEIKTTNPVATESYQQVATNHQSA Q ₅₈₈ Q ₅₈₉ AQTGWVQNQGILPGMVWQDRDVLQGPWAKIPHTDGNFHPSPLMGGFGMKH PPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTS NYYSNNVEFAVNTEGVYSEPRPIGTRYLTR (SEQ ID NO: 8)	
wild-type AAV2 VP1 capsid protein	

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MAADGYLPDWLEDTLSEGIQWWKLKPGPPPKPAERHKDDSRGLVLPGYKYLGPFGN
LDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADADEFQERLKEDTSFGGNLGR
AVFQAKKRVLEPLGLVEEPVKTAGKGRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQ
TGDADSVDPDQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCD
STWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHC
HFSPRDWQRLINNNWGFPRKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQ
LPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGN
NFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYLSRTNTPSGTTTQSRLQFSQAGA
SDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVPNGPAMA
SHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQR
GN587R588QAATADVNTQGVLPGMVWQDRDVYLQGPWAKIPHTDGHFHPSPMLGGFGLK
HPPQILIKNTVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENS KRWNPEIQYT
SNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL
(SEQ ID NO: 9)

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Preferably, the insertion site of the insertion peptide in the GH loop of the VP1 capsid protein is between amino acids 587 and 588 of AAV2 wild-type VP1 capsid protein, between amino acids 588 and 589 of AAV9 wild-type VP1 capsid protein.

- 5 According to some embodiments, the insertion peptide has a length of 7 amino acids, 8 amino acids, 9 amino acids, 10 amino acids, or 11 amino acids.

The insertion peptide may comprise one or more spacer amino acids at the N- and/or C-terminus of amino acid sequence LGETTRP (SEQ ID NO: 7). Preferably, the spacer amino acids are selected from the group consisting of Ala, Leu, Gly, Ser, and Thr, more preferably from the group consisting of Ala, Leu, and Gly.

10 According to an embodiment, the insertion peptide comprises or consists of sequence AALGETTRPA (SEQ ID NO: 10), LALGETTRPA (SEQ ID NO: 11), or GLGETTRPA (SEQ ID NO: 12), preferably comprises or consists of sequence AALGETTRPA (SEQ ID NO: 10) or LALGETTRPA (SEQ ID NO: 11).

15 According to a particular embodiment, the viral vector, in particular AAV, AAV8, AAV2-7m8 or AAV9-7m8, comprises the polynucleotide of interest (nucleotide sequence encoding GIRK1 F137S or a functional derivative thereof, and/or nucleotide sequence encoding mammalian cone opsin) under the control of a cone-specific promoter, preferably a pR1.7 or a functional variant thereof, or a minimal M-opsin promoter, in particular in a pMNTC expression cassette. In said AAV, the polynucleotide of interest which is operatively linked to the cone-specific promoter, e.g. promoter pR1.7, minimal M-opsin promoter or pMNTC, is preferably flanked by two adeno-associated virus inverted terminal repeats (AAV ITRs), preferably AAV2 ITRs.

25 pR1.7 is a 1.7 kilobases synthetic promoter based on the human red opsin promoter sequence described in Hum Gene Ther. 2016 Jan;27(1):72-82. As used herein, "pR1.7" denotes the promoter of sequence SEQ ID NO:13 and functional variants thereof. "Functional

variants" of the pR7.1 promoter typically have one or more nucleotide mutations (such as a nucleotide deletion, addition, and/or substitution) relative to the native pR7.1 promoter (SEQ ID NO: 13), which do not significantly alter the transcription of the polynucleotide of interest. In the context of the present invention, said functional variants retain the capacity to drive a strong expression, in cone photoreceptors, of the polynucleotide of interest. Such capacity can be tested as described by Ye et al. (Hum. Gene Ther. 2016;27(1):72–82) and Khabou et al. (JCI Insight. 2018, 3(2): e96029).

Another example of cone-specific promoter which may be used is a minimal M-opsin promoter region such as disclosed in WO2015142941, in particular in SEQ ID NO:55 or SEQ ID NO: 93 as disclosed in WO2015142941. Instant sequence SEQ ID NO:14 is identical to SEQ ID NO: 93 of WO2015142941.

In an embodiment, the polynucleotide of interest which is placed under the control the minimal M-opsin promoter region, is inserted in a pMNTC expression cassette comprising an optimized enhancer, optimized promoter, optimized 5'UTR, optimized intron, optimized kozak and optimized polyA region (SEQ ID NO:95 of WO2015142941).

pR1.7 promoter	
ggaggctgaggggtggggaaagggcatgggtgttcatgaggacagagctccgttcatgcaatgaaaagagtgttgagac ggatgggtggtgactggactatacactacacacggtagcgatggtacacttgtattatgtatatttaccagatcttttaagtg caaaggcaaatggccaaatggctcctgtcctatagctgtagcagccatcggtgttagtgacaaagccccctgagtcgaagt acagcagcccccataactcctaactcggtctcccgcgtggagtcatttaggagtagtcgcattagagacaagtccaacatcta atctccacccctggccagggccccagctggcagcgaggggtgggagactccgggcagagcagagggcgctgacattggg ccggcctggtggtgccctctggccttccccaggggcccttcttctgggcttcttggccgacctgctcccgtcctctc ccccatcccacccctcaccctcgttctcctatcctctctagtgctccctccacttccatccacccctctgcaagagtgtgg accacaaatgagtttccactggcctggggacacacgctgccccacaggtgctgagtgacttctaggacagtaactctgcttag gctaaaatgggactgtacttctgttagccctaatacatcaattagcagagccgggtgaagggtgcagaacctaccgcttccaggc ctcctcccactctgccacctccactctcctctgggatgtggggcgtggcacacgtgtggccagggcattggtgggattgca ctgagctgggtcattagcgtaactctggacaagggcagacagggcgagcggaggccagctccggggctcagggaaggc tgggggcttccccagacacccactcctcctctgctggacccccacttcatagggcacttctgttctcaaagggttccaaat agcatggtggccttgatgccaggaagcctcagagtgcttatctccctctagacagaagggaatctcggtcaagagg agaggtcgccctgttaaggccaccagccagctcatggcggaatgggacaaggctggccagccatcccaccctcagaa gggaccgggtggggcaggtgatctcagaggaggctcacttctgggtctcacattcttgatccgggtccaggcctcgccctaa atagctccctgggcttcaagagaaccacatgagaaaggaggattcgggctctgagcagttcaccaccacccccagct gcaaatcctgaccggtgggtccacctgccccaaaggcgagcagggacagtagaagggaacagagaacacataaacac agagagggccacagcggtcccacagtcaccgccaccttctggcggggatgggtggggcgtctgagttgttccagca aatccctctgagccgcttgcgggctcgctcaggagcaggggagcaagaggtgggaggaggaggtctaagtccaggc ccaattaagagatcaggtagtgtagggttggagctttaaggtaagaggccgggctgatcccacaggccagataaag cgccgtgaccctcaggtgatgcccagggccggctgccgtcggggacagggcttccatagcc (SEQ ID NO:13)	
minimal M-opsin promoter region	
Ccagcaaatccctctgagccgccccggggctcgccctcaggagcaaggaagcaaggggtgggaggaggaggtctaag tcccaggcccaattaagagatcagatggtgtaggatttgggagctttaaaggtaagaggcccggtgatccactggccgg tataaagcaccgtgaccctcaggtgacgcaccagggccggctgccgtcggggacagggcttccatagccag (SEQ ID NO:14)	

pMNTC
cctacagcagccaggggtgagattatgaggtgagctgagaatatcaagactgtaccgagtagggggccttggcaagtgtg agagcccggcagctggggcagagggcgagtagcgggtgctgttacggacctctcaaacgaggtaggaaggtcagaagt caaaaaggggaacaaatgatgttaaccacacaaaaatgaaaatccaatggttgatccattccaaatacacaaggcaa cggataagtgtacggggccaggcacagaaggccatgcaccgtaggattgcactcagagctcccaaatgcataggaatag aaggggtgggtgcaggaggtgaggggtgggaaagggcatgggtgttcatgaggacagagctccgtttcatgcaatgaa aagagttggagacggatgggtgggtgactggactatacacttacacacggtagcgatgggtacactttgtatgtatatttaccac gatcttttaaggtgtcaaaaggcaaatggccaaatggttcctgtcctatagctgtagcagccatcggtgtgtatgacaaagccc ctgagtcaagatgacagcagccccataactcctaactcggtctcccgcgtggagtcatttaggagtagtcgcattagagaca agtccaacatctaatttccaccctggccagggccccagctggcagcgaggggtgggagactccgggcagagcagagggc gctgacattggggcccggtggcttgggtccctctggccttccccaggggcccttcttctggggcttcttgggccgactg ctccgctcctctcccccatccacccctcaccctcgttctcctatcctctctagtgctccctccacttccacccctctg caagagtgtgggaccacaaatgagtttccctggcctggggacacacgtgccccacaggtgctgagtgacttctaggaca gtaatctgctttaggtctaaaatgggacttgaatctgttagccctaatcatcaattagcagagccggtgaaggtgcagaacctac cgcttctccagggcctcctccacctctgccacctccactctccttctgggatgtgggggctggcacacgtgtggcccagggcat tggtgggattgactgagctgggtcattagcgaatcctggacaagggcagacagggcgagcgaggggccagctccgggg ctcaggcaaggtgggggcttccccagacacccactcctcctctgctggacccccacttcatagggcacttcgtgttctcaa agggcttccaaatagcatggtggccttggatgccaggggaagcctcagagttgcttatcctctagacagaagggaatct cggtaagaggggagaggtgcctgttcaaggccaccagccagctcatggtcggtaatgggacaagggtggccagccatc ccaccctcagaagggaagggcgggtggggcaggtgactcagaggaggtcacttctgggtctcacattctccagcaaatccctt gagccgccccgggggctcgccctcaggagcaaggaagcaaggggtgggaggaggaggtctaagtcccaggcccaatta agagatcagatgggttaggatttggagctttaaaggtgaagaggcccggtgatccactggccgggtataaagcacctg accctcaggtgacgcaccagggccggctgccgtcggggacagggcttccatagcccaggttaagtcaaggttacaagac aggttaaggagaccaatagaaactgggctgtcgagacagagaagactcttgcgttctgataggcacctattgttactga catccacttgccttctctccacaggcccagagaggagacagggccgcccacc (SEQ ID NO:15)

The promoter and the polynucleotide of interest are operatively linked. As used herein, the term “operably linked” refers to two or more nucleic acid or amino acid sequence elements that are physically linked in such a way that they are in a functional relationship with each other. For instance, a promoter is operably linked to a coding sequence if the promoter is able to initiate or otherwise control/regulate the transcription and/or expression of a coding sequence, in which case the coding sequence should be understood as being “under the control of” the promoter. Generally, when two nucleic acid sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They will usually also be essentially contiguous, although this may not be required.

According to an embodiment, the vector is an AAV9 (AAV9-7m8-pR1.7) or an AAV2 (AAV2-7m8-pR1.7) comprising:

- a VP1 capsid protein in which a 7 to 11 amino acid long insertion peptide is inserted in the GH loop of said VP1 capsid protein relative to wild-type AAV9 VP1 capsid protein, at a position localized between amino acids 588 and 589 of wild-type AAV9 VP1 capsid protein, wherein said peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7); and
- the polynucleotide of interest (nucleotide sequence encoding GIRK1 F137S and/or nucleotide sequence encoding mammalian cone opsin) under the control of a pR1.7 promoter.

In said AAV9-7m8 or AAV2-7m8, the insertion peptide has a length of 7 amino acids, 8 amino acids, 9 amino acids, 10 amino acids, or 11 amino acids. Preferably, the insertion peptide comprises one or more spacer amino acids at the N- and/or C-terminus of amino acid sequence LGETTRP (SEQ ID NO: 7). Preferably, the spacer amino acids are selected from the group consisting of Ala, Leu, Gly, Ser, and Thr, more preferably from the group consisting of Ala, Leu, and Gly. According to an embodiment, the insertion peptide comprises or consists of sequence AALGETTRPA (SEQ ID NO: 10), LALGETTRPA (SEQ ID NO: 11), or GLGETTRPA (SEQ ID NO: 12); preferably comprises or consists of sequence AALGETTRPA (SEQ ID NO: 10) or LALGETTRPA (SEQ ID NO: 11).

The vectors of the invention are produced using methods known in the art. In short, the methods generally involve (a) the introduction of the AAV vector into a host cell, (b) the introduction of an AAV helper construct into the host cell, wherein the helper construct comprises the viral functions missing from the AAV vector and (c) introducing a helper virus into the host cell. All functions for AAV virion replication and packaging need to be present, to achieve replication and packaging of the AAV vector into AAV virions. The introduction into the host cell can be carried out using standard virology techniques simultaneously or sequentially. Finally, the host cells are cultured to produce AAV virions and are purified using standard techniques such as iodixanol or CsCl gradients or other purification methods. The purified AAV virion is then ready for use.

Another object of the present invention is a nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S as described above, for use as a medicament. In particular, said nucleic acid is for use in treating rod-cone dystrophy (RCD). In other words, another object of the present invention is a method of treating a RCD in a mammal in need thereof, the method comprising administering to the mammal a nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S as described above.

In an embodiment, the nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S comprises or consists of a nucleotide sequence encoding the polypeptide of sequence SEQ ID NO: 2, or comprises or consists of the nucleotide sequence SEQ ID NO: 3.

The nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S may be in a vector selected from the group consisting of an adeno-associated virus (AAV), an adenovirus, a lentivirus, and SV40 viral vector.

According to an embodiment, the nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S is under the control of the pR1.7 promoter or of a functional variant of said promoter.

According to an embodiment, the nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S is in a vector which is an AAV2 or AVV9 virus comprising a 7 to 11 amino acid long insertion peptide in the GH loop of the VP1 capsid protein, wherein the insertion peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7).

5 Preferably, the vector is a recombinant AAV2 or AAV9 vector comprising:

- a VP1 capsid protein in a 7 to 11 amino acid long insertion peptide is inserted in the GH loop of said VP1 capsid protein relative to wild-type AAV9 VP1 capsid protein, at a position localized between amino acids 588 and 589 of wild-type AAV9 VP1 capsid protein, wherein said peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7); and

10 - the nucleotide sequence encoding the mammalian cone opsin under the control of a pR1.7 promoter.

According to an embodiment, said insertion peptide comprises or consists of amino acid sequence AALGETTRPA (SEQ ID NO: 8), LALGETTRPA (SEQ ID NO: 9), or GLGETTRPA (SEQ ID NO: 10).

15 According to an embodiment, the nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S further comprises a sequence encoding a mammalian cone opsin, as described above.

According to another embodiment, the nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S is for use in treating RCD in combination with another nucleic acid
20 encoding a mammalian cone opsin, as described above. In other words, another object of the present invention is a method of treating a RCD in a mammal in need thereof, the method comprising administering to the mammal a nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S and another nucleic acid encoding a mammalian cone opsin. In said method, the nucleic acids are therefore in separate forms and may be formulated in a single
25 pharmaceutical composition or in separate pharmaceutical compositions.

In one embodiment, the nucleic acid comprising a nucleotide sequence encoding GIRK1 F137S comprises the expression cassette from plasmid pAAV2-5'ITR-pR1.7-hGIRK-F137S-BGHpA-AAV2-3'ITR (having the sequence as set forth in SEQ ID NO:16), pAAV2-5'ITR-pR1.7-hGIRK-F137S-WPREmut6-BGHpA-AAV2-3'ITR (having the sequence as set forth in SEQ ID NO:17) or pAAV2-5'ITR-pR1.7-hGIRK-F137S-WPREmut6deltaATG-BGHpA-AAV2-3'ITR (having the sequence as set forth in SEQ ID NO:18).

pAAV2-5'ITR-pR1.7-hGIRK-F137S-BGHpA-AAV2-3'ITR
cctgcaggcagctgcgcgctcgctcactgagggccgcccgggcaaagcccggtcggtggcgaccttggcgcccg gcctcagtgagcgagcgagcgagcgagagggagtgccaactccatcactaggggtcctctagacaacttgtatag aaaagttgggaggctgaggggtgggaaaggcatgggtgttcatgaggacagagctccgtttcatgcaatgaaaga gtttggagacggatggtggtgactggactatacacttacacacggtagcgatggtacacttgtattatgtatatttac

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(SEQ ID NO:16)

pAAV2-5'ITR-pR1.7-hGIRK-F137S-WPREmut6-BGHpA-AAV2-3'ITR

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[illegible]

Another object of the present invention is a pharmaceutical composition comprising the vector or the pharmaceutically acceptable carrier of the present invention, with a pharmaceutically acceptable carrier, diluent or excipient.

Another object of the present invention is a vector, a carrier or a pharmaceutical composition of the present invention, for use in treating rod-cone dystrophy (RCD).

Rod-cone dystrophy (RCD) is a heterogeneous group of diseases such as Retinitis Pigmentosa (RP), in particular non-syndromic X-linked Retinitis Pigmentosa (XLRP), autosomal recessive RP, autosomal dominant RP. The most common syndromic forms of RCD include Usher syndrome, Bardet-Biedl syndrome, Refsum disease, Bassen-Kornzweig syndrome and Batten disease.

The RCD subject to be treated is a mammal, in particular a non-human or human primate, preferably a human. The RCD in the mammal may be at an early, intermediate or advanced stage of the disease. In RCD subjects at intermediate or advanced stage of the disease, transduction of the subjects' cones with a nucleotide sequence encoding GIRK1 F137S would be sufficient to achieve vision restoration provided cone opsin and cone arrestin are still expressed in the patients' cone cell bodies. In RCD subjects whose cone cell bodies

no longer express cone opsin, transduction of the subjects' cones with a nucleotide sequence encoding GIRK1 F137S and a mammalian cone opsin would be required.

Accordingly, treatment of RCD may be implemented by administering the vector(s), carrier or pharmaceutical composition of the present invention to the mammal, so as to achieve
5 transduction of cones with the GIRK1 F137S transgene, or GIRK1 F137S and mammalian cone opsin transgenes.

In other words, another object of the present invention is a method of treating a RCD in a mammal in need thereof, the method comprising administering to the mammal an effective amount of the vector or the carrier of the pharmaceutical composition of the present invention.

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Accordingly, in a first embodiment, the vector comprising a nucleotide sequence encoding GIRK1 F137S, carrier including said vector, or a pharmaceutical composition comprising the vector or carrier is for use in treating rod-cone dystrophy in a RCD mammalian subject whose cone cells still express endogenous cone opsin. According to an embodiment,
15 the vector further comprises a nucleotide sequence encoding a mammalian cone opsin. According to another embodiment, the vector does not comprise a nucleotide sequence encoding a mammalian cone opsin. According to an embodiment, the carrier further includes a vector comprising a nucleotide sequence encoding a mammalian cone opsin. According to another embodiment, the carrier does not include a vector comprising a nucleotide sequence
20 encoding a mammalian cone opsin.

In a second embodiment, the vector comprising a nucleotide sequence encoding GIRK1 F137S, carrier including said vector, or a pharmaceutical composition comprising the vector or carrier is for use in treating rod-cone dystrophy in a RCD mammalian subject whose cone cells no longer express endogenous cone opsin. According to this embodiment, the
25 vector further comprises a nucleotide sequence encoding a mammalian cone opsin, or the carrier further includes a vector comprising a nucleotide sequence encoding a mammalian cone opsin.

Treatment of RCD may also be implemented by transducing a mammalian cone precursor cell with vector(s), carrier or pharmaceutical composition of the present invention,
30 and administering the transduced mammalian cone precursor cell to the retina, in particular to the fovea region, of the RCD mammal.

In other words, another object of the present invention is a method of treating a RCD in a mammal in need thereof, the method comprising administering to the mammal an effective
35 amount mammalian cone precursor cell transduced with the vector or the carrier of the pharmaceutical composition of the present invention.

The invention also relates to a cone precursor cell comprising a heterologous nucleic acid encoding GIRK1 F137S, or encoding GIRK1 F137S and a mammalian cone opsin, for use in a method of treating a RCD. Accordingly, it is also provided a method a method of treating a RCD in a mammal in need thereof, the method comprising administering to the

5 mammal a cone precursor cell comprising a heterologous nucleic acid encoding GIRK1 F137S, or encoding GIRK1 F137S and a mammalian cone opsin. As used herein, the term « heterologous nucleic acid » refers to a gene, polynucleotide or nucleic acid sequence that is not in its natural environment.

10 Cone precursor cells are not-fully differentiated, non-dividing cells committed to differentiate into cone cells.

In an embodiment, cone precursor cells are obtained from retina of donor (e.g. cadaver eye donor) or from the RCD subject to be treated, preferably from the RCD subject to be treated. In another embodiment, cone precursor cells are obtained from stem cells, in particular embryonic stem cells, induced pluripotent stem (iPS cells), adult stem cells or fetal stem cells.

15 In another embodiment, cone precursor cells are obtained from differentiated embryonic stem cells. According to one embodiment, embryonic stem cells are non-human embryonic stem cells. According to another embodiment, human embryonic stem cells may be used with the proviso that the method itself or any related acts do not include destruction of human embryos. Preferably cone precursor cells are obtained by differentiation of stem cells, preferably from

20 differentiation of adult stem cells or induced pluripotent stem cells, more preferably from differentiation of induced pluripotent stem cells obtained from somatic cells, e.g. fibroblasts, of the RCD subject to be treated.

Embryonic stem cells are able to maintain an undifferentiated state or can be directed to mature along lineages deriving from all three germ layers, ectoderm, endoderm and

25 mesoderm. Embryonic stem cells can be reprogrammed towards cone photoreceptors by manipulation of key developmental signaling pathways as described in the international patent application WO2018055131. For example, it may be used antagonists of the nodal and wnt pathway in addition to activin-A and serum (Watanabe K et al. Nat Neurosci. 2005 Mar;8(3):288-96.), or inhibition of the Notch signaling pathway can be implemented (Osakada

30 F et al. Nat Protoc. 200;4(6):811-24). Cone precursor cells can be obtained from embryonic stem cells using any protocol known by the skilled person (Osakada F et al. Nat Biotechnol. 2008 Feb;26(2):215-24; Amirpour N et al. Stem Cells Dev. 2012 Jan;21(1):42-53; Nakano T et al. Cell Stem Cell. 2012 Jun 14;10(6):771-85; Zhu Y et al. Plos One. 25 2013;8(1):e54552; Yanai A et al. Tissue Eng Part C Methods. 2013 Oct;19(10):755-64; Kuwahara A et al. Nat

35 Commun. 2015 Feb 19;6:6286; Mellough CB et al. Stem Cells. 2015 Aug;33(8):2416-30; Singh K et al. Stem Cells Dev. 2015 Dec 1;24(23):2778-95).

Preferably, cone precursor cells are obtained from iPS cells or adult stem cells, more preferably from iPS cells. Induced pluripotent stem (iPS) cells are derived from a non-pluripotent cell, typically an adult somatic cell, by a process known as reprogramming, where the introduction of only a few specific genes are necessary to render the cells pluripotent (e.g. OCT4, SOX2, KLF4 and C-MYC in human cells). One benefit of use of iPS cells is the avoidance of the use of embryonic cells altogether and hence any ethical questions thereof. Photoreceptor precursor cells can be obtained from iPS cells using any differentiation method known by the skilled person.

In particular, photoreceptor precursor cells can be obtained from human iPS cells by a method as disclosed in Garita-Hernandez et al., 2019, Nat Commun 10, 4524. Human iPS are expanded to confluence in iPS medium (e.g. Essential 8™ medium, GIBCO, Life Technologies). After 80% confluence, the medium was switched to a proneural medium (e.g. Essential 6™ medium supplemented with 1 % N2 supplement (100X); GIBCO, Life Technologies). The medium was changed every 2–3 days. After 4 weeks of differentiation, neural retina-like structures grew out of the cultures and were mechanically isolated. Pigmented parts, giving rise to RPE were carefully removed. The extended 3D culture in Maturation medium (DMEM/F-12 medium supplemented with 2% B-27™ Supplement (50X), serum free, and 1% MEM Non-Essential Amino Acids Solution (100X); GIBCO, Life Technologies) allowed the formation of retinal organoids. Addition of 10 ng/ml Fibroblast growth factor 2 (FGF2, Preprotech) at this point favoured the growth of retinal organoids and the commitment towards retinal neurons instead of RPE lineage. In order to promote the commitment of retinal progenitors towards photoreceptors, Notch signalling was specifically blocked for a week starting at day 42 of differentiation using the gamma secretase inhibitor DAPT (10 µM, Selleckchem). Floating organoids were cultured in 6 well-plates (10 organoids per well) and medium was changed every 2 days.

Photoreceptor precursor cells can also be obtained from human iPS cells using any other protocol known by the skilled person (Lamba, Osakada and colleagues (Lamba et al. Proc Natl Acad Sci USA. 2006 Aug 22;103(34):12769-74; Lamba et al. Plos one. 2010 Jan 20;5(1):e8763; Osakada et al. Nat. Protoc. 2009;4(6):811-24; Meyer JS et al. Proc Natl Acad Sci USA. 2009 Sep 29; 106(39):16698-703 ; Meyer JS et al. Stem Cells. 2011 Aug ;29(8) :1206-18; Mellough CB et al. Stem Cells. 2012 Apr;30(4):673-86; Boucherie C et al. Stem Cells. 2013 Feb ;31(2) :408-14 ; Sridhar A et al. Stem Cells Transl Med. 2013 ;2(4) :255-64 ; Tucker BA et al. Elife. 2013 Aug 27;2:e00824 ; Tucker BA et al. Stem Cells Transl Med. 2013 Jan ;2(1) :16-24 ; eichman S et al. Proc Natl Acad Sci USA. 2014 Jun 10 ;111(23) :8518-23 ; Zhong X et al. Nat Commun. 2014 Jun 10 ;5 :4047 ; Wang X et al. Biomaterials. 2015 Jun ;53 :40-9).

The cone precursor comprise a heterologous nucleic acid encoding i) GIRK1 F137S, or ii) encoding GIRK1 F137S and a mammalian cone opsin. Where the cone precursor cells comprise a heterologous nucleic acid encoding GIRK1 F137S, or a functional derivative thereof, and a mammalian cone opsin, the cone precursor cells either comprise i) a
5 heterologous nucleic acid encoding both GIRK1 F137S and a mammalian cone opsin, or ii) a heterologous nucleic acid encoding GIRK1, and another heterologous nucleic acid encoding a mammalian cone opsin.

Said cone precursor cells may be prepared by introducing into said cone precursor cells said heterologous nucleic acid(s), or an expression cassette or vector comprising said nucleic
10 acid(s), by any method known to the skilled person. According to an embodiment, a cone precursor cell comprising a heterologous nucleic acid encoding GIRK1 F137Sf, or encoding GIRK1 F137S and a mammalian cone opsin, is prepared by infecting the cone precursor cell with a viral vector as described above, in particular with an AAV vector, preferably the AAV8, AAV2-7m8 or AAV9-7m8.

15 In another aspect, the invention therefore further refers to a method of preparing a cone precursor cell comprising a heterologous nucleic acid encoding GIRK1 F137S, or encoding GIRK1 F137S and a mammalian cone opsin, said method comprising infecting cone precursor cells with a viral vector or carrier according to the invention, and recovering infected cone precursor cells.

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The vector, carrier, or pharmaceutical composition, or cone precursor cells may be administered by any suitable route known to the skilled person in particular by intravitreal or subretinal or suprachoroidal administration.

In an embodiment, the viral vector, carrier or pharmaceutical composition is injected at
25 a dose comprised between 10^7 to 10^{15} vg/eye, preferably between 10^{11} and 10^{15} vg/eye, even more preferably 10^{11} and 10^{13} vg/eye.

In an embodiment, the viral vector, carrier or pharmaceutical composition is administered by intravitreal injection.

In one embodiment, the AAV vector is an AAV2 or AAV9 or modified versions thereof
30 such as AAV2-7m8 or AAV9-7m8 and said vector is administered by intravitreal injection.

In an embodiment, the viral vector, carrier or pharmaceutical composition is administered by subretinal injection.

The fovea is a small region in the central retina of primates of approximately equal to or less than 0.5 mm in diameter that contains only cone photoreceptor cells, and highest
35 density of cones in the whole retina. The fovea dominates the visual perception of primates by providing high-acuity color vision. The highest density of cones is found at the center of the

fovea (<0.3 mm from the foveal center), devoid of rod photoreceptors. Cone density decreases by up to 100-fold with distance from the fovea.

Cone cells in the fovea are the primary targets of gene therapies aiming to treat inherited retinal diseases like retinitis pigmentosa. Usually, viral vectors encoding therapeutic proteins are injected “subretinally”, i.e. into the subretinal space between the photoreceptors and the retinal pigment epithelium (RPE) cells in order to provide gene delivery to cones.

The subretinal delivery leads to the formation of a “bleb”, which refers to a fluid-filled pocket within the subretinal space of the injected eye. In this approach, gene delivery is limited to cells that contact the local bleb of injected fluid. Retinal detachment, and in particular foveal detachment, that occurs during subretinal injections is a concern in eyes with retinal degeneration.

Advantageously, when the vector is an AAV9-7m8 vector (in particular AAV9-7m8-pR1.7 vector), the vector (or carrier of pharmaceutical composition comprising said vector) can be administered by a distal subretinal injection, or in the periphery of the fovea, and then spread laterally to reach the foveal region. According to an embodiment the bleb is formed greater than or equal to 0.5 millimeters away from the center of the fovea, without detaching the foveal region. In an embodiment, said AAV9-7m8 viral vector is formulated in a solution and 50 to 100 μ L of solution are injected continuously in 20 to 30 seconds. In an embodiment, said AAV9-7m8 viral vector is formulated in a solution at a concentration of 1×10^{10} to 1×10^{12} vg/mL (viral genome/mL), preferably of 0.5×10^{11} to 5×10^{11} vg/mL, still preferably of 1×10^{11} vg/mL. Preferably, the cone precursor cells are administered by intraocular injection, preferably by subretinal space injection, more preferably by injection between the neural retina and the overlying PE. The amount of cone precursor cells to be administered may be determined by standard procedure well known by those of ordinary skill in the art. Physiological data of the patient (e.g. age, size, and weight) and type and severity of the disease being treated have to be taken into account to determine the appropriate dosage. The cone precursor cells may be administered as a single dose or in multiple doses. In particular, each unit dosage may contain, from 100,000 to 300,000 cone precursor cells per μ L, preferably from 200,000 to 300,000 cone precursor cells per μ L.

Brief description of the figures

Figure 1 represents phototransduction cascade (A) normal phototransduction cascade (B) short phototransduction cascade with an animal opsin and GIRK2 channel. PDE: phosphodiesterase. CNG: cyclic-nucleotide gated channels. cGMP: cyclic guanosine monophosphate.

Figure 2 represents plasmids (A) CMV-GIRK2-GFP and (B) CMV-SWO-mCherry.

Figure 3 represents what remained in the phototransduction cascade in rd10 mice using immunohistochemistry (A-D) retinal cross-section of a control WT mouse stained with (A) opsin, (B) transducin, (C) PDE and (D) cone arrestin. (E-H) retinal cross-section of a rd10 mouse at P14 stained with (E) opsin, (F) transducin, (G) PDE and (H) cone arrestin. (I-L) Retinal cross-section of a rd10 mouse at P150 stained with (I) opsin, (J) transducin, (K) PDE and (L) cone arrestin. ONL: outer nuclear layer. INL: inner nuclear layer. GC: ganglion cells. Scale bar is 50µm. Inset scale bar is 25µm.

Figure 4 represents preliminary data. (A) Eye fundus of GIRK2-GFP expression in rd10 mouse one week post-injection (**site of injection*) (B) Photopic ERG amplitude in rd10 mice at P33, injected with AAV-SWO-tdTomato and AAV-GIRK2-GFP. Control mice are injected with AAV-GFP (n=12). $P=0,0002$. (C) Representative flickers ERG at P33. (D) Measure of the visual acuity by optokinetic test in rd10 mice, injected with AAV-SWO-tdTomato and AAV-GIRK2-GFP. Control mice are injected with AAV-GFP. Control mice were injected with AAV-GFP (n=8).

Figure 5 represents GIRK2-mediated vision. (A) Photopic ERG amplitude in rd10 mice at P41, injected with AAV-SWO-tdTomato and/or AAV-GIRK2-GFP. Control mice are injected with AAV-GFP (n=12). $P_{SWO+GIRK2}=0,0381$ and $P_{GIRK2}=0,0021$. (B) Measure of the visual acuity by optokinetic test in rd10 mice, injected with AAV-SWO-tdTomato and/or AAV-GIRK2-GFP. Control mice are injected with AAV-GFP. Control mice were injected with AAV-GFP (n=7). (C) Representative flickers ERG at P41.

Figure 6 represents long term efficiency. (A) Photopic ERG amplitude in rd10 mice, injected with AAV-GIRK2-GFP. Control mice are injected with PBS (n=6). (B) Measure of the visual acuity by optokinetic test in rd10 mice, injected with AAV-GIRK2-GFP. Control mice are injected with PBS (n=6). (C) Number of cones of wild-type mice and non-injected rd10 mice over time (n=6). $Pvalue_{(P50-P365)}=0,0022$. (D) Linear regression correlation between the ERG amplitudes and the number of cones in rd10 mice (n=6). $Pvalue_{non-injected}=0,0482$. $Pvalue_{AAV-GIRK2-GFP}=0,0007$. $Pvalue_{PBS}=0,0104$.

Figure 7 represents what remained in the phototransduction cascade in huP347S^{+/-} mice using immunohistochemistry. (A-D) Retinal cross-section of a control WT mouse stained with (A) opsin, (B) transducin, (C) PDE and (D) cone arrestin. (E-H) retinal cross-section of a huP347S^{+/-} mouse at P14 stained with (E) opsin, (F) transducin, (G) PDE and (H) cone arrestin. (I-L) retinal cross-section of a huP347S^{+/-} mouse at P150 stained with (I) opsin, (J) transducin, (K) PDE and (L) cone arrestin. ONL: outer nuclear layer. INL: inner nuclear layer. GC: ganglion cells. Scale bar is 50µm. Inset scale bar is 25µm.

Figure 8 represents universality of the approach. (A) Photopic ERG amplitude in huP347S^{+/-} mice, injected with AAV-GIRK2-GFP. Control mice are injected with PBS (n=6). (B) Measure of the visual acuity by optokinetic test in huP347S^{+/-} mice, injected with AAV-GIRK2-GFP. Control mice are injected with PBS (n=6). (C) Number of cones of wild-type mice and non-injected huP347S^{+/-} mice over time (n=6). *Pvalue* (P50-P365) = 0,0022. (D) Linear regression correlation between the ERG amplitudes and the number of cones in huP347S^{+/-} mice (n=5). *Pvalue* non-injected = 0,0313. *Pvalue* AAV-GIRK2-GFP = 0,0146. *Pvalue* PBS = 0,0497.

Figure 9 represents the efficiency of the mouse GIRK2 in HEK cells transfected with two plasmids: CMV-SWO-mCherry and CMV-GIRK2-GFP.

Figure 10 represents phenotyping of a normal volunteer and retinitis pigmentosa patients for eligible patient population. Upper panel (A) shows the fundus and OCT images of the back of the eye in a normal individual along with adaptive optics images of cone dominated regions of the retina. Middle panel (B) shows a pie-chart distribution of advanced RCD patients. Lower panels represent OCT and AOSLO images of different patients. AOSLO confocal (up) and AOSLO split detection (low) in vivo retinal images of a patient with retinitis pigmentosa (age 77, male). Acquired fields are located at the transition between regions of presumed dormant cones (i.e. morphologically intact - as indicated by the IS/OS line visible in OCT, clear inner segment mosaic visible in AOSLO split detection, and clear cone mosaic indicating intact inner and outer segments in AOSLO confocal - though with reduced function according to the patient's visual acuity; yellow bars) and damaged or absent cones (indicated by the absent IS/OS line in OCT, and the blurred inner segment and cone mosaics in AOSLO split detection and confocal respectively; red bars). Yellow arrows indicate cones that appear to be degenerating, with absent OS in confocal but present IS in split detection. Scale bars, 200µm.

Figure 11 represents immunohistochemistry labeling cone phototransduction cascade proteins in normal and RP human retina. (A) Retinal cross-section of a 86 years old control human retina (20x). (B) Retinal cross-section of a 75 years old human retina affected by retinitis pigmentosa (RP) and having night blindness and loss of peripheral vision (40x). (A-B) stained with Opn1mw, (bright) and nuclear stain DAPI (dark). ONL: outer nuclear layer. INL: inner nuclear layer. GC: ganglion cells. Scale bar is 50µm. Inset scale bar is 25µm.

Figure 12 shows characterization of GIRK1 F137S currents elicited by activation of mOpn4L in HEK293 cells. A) When activated with blue light (471 nm, exposure from 10 to 20 s on the axis of abscissae) mOpn4L induces a strong GIRK1 F137S-mediated current that concludes upon stimulation with lime light (560 nm, exposure from 20 to 60 s on the axis of abscissae) and results in a statistically significant difference in B) the flux of ions through HEK293 membranes and C) the electrical current density per capacitance.

Figure 13 shows characterization of GIRK1 or GIRK2 currents elicited by activation of mOpn4L in HEK293 cells when activated with blue light (471 nm, exposure from 10 to 20 s on the axis of abscissae) and terminated upon stimulation with lime light (560 nm, exposure from 20 to 60 s on the axis of abscissae).

5 Figure 14: shows the current density per capacitance of HEK293 cells expressing A1) hGIRK1 F137S, B1) hGIRK2 or C1) truncated rGIRK2. The current response, elicited by the activation of mOpn4, is shown in comparison to constructs with c-terminal eGFP fusion and untransfected cells. The current density of individual cells is illustrated by individual data points. Significant differences between conditions are marked with * (Kruskal-Wallis One Way
10 Analysis of Variance on Ranks with all pairwise multiple comparison procedures (Dunn's Method) $P < 0.05$). Significant differences between conditions are marked with * (Mann-Whitney Rank Sum Test; $P = 0.002$). When activated with blue light mOpn4 induces a GIRK-mediated current that concludes upon stimulation with lime light as visible in exemplary traces of A2) hGIRK1 F137S, B2) hGIRK2 and C2) truncated rGIRK2 T constructs.

15 Figure 15 A) Stimulation of HEK293 cells expressing mOpn4 and either hGIRK1 F137S, hGIRK1 F137S – eGFP, or hGIRK2 produces a reliably observable current in whole-cell patch clamp recordings, while currents of hGIRK2-eGFP, rGIRK2 or rGIRK2-eGFP after mOpn4 activation are less likely to be observable. B) Current density per capacitance of HEK293 cells expressing the different GIRK constructs (top) or their eGFP tagged versions
20 (bottom). The current density of individual cells is illustrated by individual data points. Significant differences between conditions are marked with * (Kruskal-Wallis One Way Analysis of Variance on Ranks with all pairwise multiple comparison procedures (Dunn's Method) $P < 0.05$).

Figure 16 represents the visual acuity in rd10 mice after bilateral subretinal injection at
25 P15 of AAV8-pR1.7-hGIRK1F137S at 5E7 or 5E8 vg/eye compared to vehicle-injected rd10 mice or naïve (not injected) rd10 mice.

Figure 17 represents the plasmid pAAV2-5'ITR-pR1.7-hGIRK-F137S-BGHpA-AAV2-3'ITR.

Figure 18 represents the plasmid pAAV2-5'ITR-pR1.7-hGIRK-F137S-WPREmut6-
30 BGHpA-AAV2-3'ITR.

Figure 19 represents the plasmid pAAV2-5'ITR-pR1.7-hGIRK-F137S-WPREmut6deltaATG-BGHpA-AAV2-3'ITR.

35 **EXAMPLES**

EXAMPLE 1 : MATERIAL AND METHODS

1. Animals

C57BL/6j^{rd10/rd10} (rd10) mice were used in these experiments. They have a mutation on the rod PDE gene leading to a dysfunctional phototransduction cascade and a rod-cone dystrophy. The second model used is the huRhoP347S^{+/-} mouse. The homozygous strand of this mouse present a KO of mouse rhodopsin (mRho) gene and a KI of human rhodopsin (huRho) with a mutation (P347S) (Millington-Ward et al., 2011) [30]. The homozygous males were crossed C57BL/6j (wild-type) females to obtain heterozygous mice. These mice have a similar phenotype as the rd10 mice but the degeneration rate is lower.

2. AAV injections

Mice were first anesthetised with intraperitoneal injections of 0.2 ml/20g ketamine (Ketamine 500, Vibrac France) and xylazine (Xylazine 2%, Rompun) diluted in 0.9% NaCl. Eyes were dilated with 8% Neosynephrine (Neosynephrine Faure 10%, Europhtha) and 42% Mydriaticum (Mydriaticum 0.5%, Thea) diluted in 0.9% NaCl.

A total volume of 1 µl of vector solution was injected subretinally. Fradexam, an ophthalmic ointment, was applied after injection. The list of injected viral vectors is presented below:

Injection Table				
Mice	Eyes	viral vector injected	viral vector titration	volume injected
rd10	both	AAV8-mCAR-GFP	10 ¹¹ rAAV particles	1 µl for all conditions
rd10	both	AAV8-mCAR-GIRK2-GFP+ AAV8-mCAR-SWO-tdTomato		
rd10	both	AAV8-mCAR-GIRK2-GFP		
rd10	right	PBS		
rd10	left	AAV8-PR1.7-GIRK2-GFP	5.10 ⁸ rAAV particles	
huRhoP347S ^{+/-}	right	PBS		
huRhoP347S ^{+/-}	left	AAV8-PR1.7-GIRK2-GFP	5.10 ⁸ rAAV particles	

3. Eye fundus examination

One week after subretinal injection, mice were anesthetised by isofluorane inhalation. Eyes were dilated and then protected with Lubrithal eye gel (VetXX). Fundus imaging was

performed with a fundus camera (Micron III; Phoenix research Lab) equipped with specific filters to monitor GFP or tdTomato expression in live anesthetised mice.

4. Electrophoretography (ERG) recordings

5 To evaluate retinal function, electrophoretography recordings (ERG) were recorded (espion E2 ERG system; Diagnosys). Several tests were performed at different time points after injections of the viral vectors. Mice were anesthetised with intraperitoneal injections of 0.2 ml/20g ketamine (Ketamine 500, Vibrac France) and xylazine (Xylazine 2%, Rompun) diluted in 0.9% NaCl. Mice were then placed on a heated pad at 37°C. Eyes were dilated with
10 Neosynephrine (Neosynephrine Faure 10%, Europhtha) and Mydriaticum (Mydriaticum 0.5%, Thea) diluted in 0.9% NaCl. Eyes were protected with Lubrithal eye gel before putting electrodes on the corneal surface of each eye. The reference electrode was inserted under the skin into the forehead and a ground electrode under the skin in the back.

ERG recordings were done under two conditions: (i) photopic condition, which reflects
15 con-driven light responses – 6ms light flashes were applied every second during 60 seconds at increasing light intensities (0.1/1/10/50cd s/m) after an adaptation of 5 minutes at 20cd s/m – and (ii) flicker condition, which are rapid frequency light stimuli that reflect cone function (70 flashes at 10Hz et 1cd s/m).

Graph and statistical analysis were performed using GraphPad.

20

5. Optokinetic test

Visual acuity was measured using an optokinetic test scoring the head turning movement of a mouse placed in front of moving bars. Testing was performed using a computer-based machine consisting of four computer monitors arranged in a square to form
25 an optokinetic chamber. A computer program was designated to generate the optokinetic stimuli, consisting of moving alternate black and white stripes. The spatial frequency is ranging from 0.03 to 0.6 cyc/deg. The program enabled modulation of stripe width and direction of bar movement.

30 6. Immunohistochemistry and confocal imaging

Animals were sacrificed by CO₂ inhalation, and the eyes were enucleated and fixed in 4% paraformaldehyde-PBS for 1h at room temperature. The eyes were dissected either as eyecups for immunohistochemistry or prepared as flat mounts for cell counting. The eyecups were then cryoprotected with a gradient of PBS-Sucrose 10% for 1h and then in PBS-Sucrose
35 30% overnight. The eyecups were embedded in OCT and 12 µm thick cryostat sections (ThermoFisher) were cut and mounted on glass slides. The sections were washed in PBS

(3x5 mins) and stained against different antibodies (see table below) and DAPI (1:2000). The sections were finally washed in PBS, mounted in Fluoromount Vactashield (Vector Laboratories) and coverslipped for imaging using laser-confocal microscopy (Olympus IX81). For flat-mount retina stainings, the protocol is the same except that the tissue was not cryoprotected. Images were analysed using FIJI software.

Antibody table		
Target	Host	Clonality/Conjugated
PRIMARY ANTIBODIES		
Red/Green opsin (M/L opsin)	Rabbit	Polyclonal
Mouse Cone arrestin	Rabbit	Polyclonal
PDE6C	Rabbit	Polyclonal
GNAT2	Rabbit	Polyclonal
PNA-Lectin	Conjugated with FITC	
SECONDARY ANTIBODIES		
Anti-rabbit	Donkey	AF 546

7. Cell counts

Flat mount retinas of rd10 and huRhoP347S^{+/−} mice were stained using antibodies against mouse cone arrestin – mCAR (1:10000) and DAPI (1:2000). The double stained cells counted at different ages. Retinas from 5 animals (n=10) were used for each age and were oriented dorso-ventrally and naso-temporally. Serial optical sections were obtained to cover the thickness of the entire outer nuclear layer (ONL). Two scanning areas of 211.97 x 211.97 μm were made in each of the four regions in all retinas. Counts of cone cells were performed manually using the FIJI software by the reconstruction of the images (z stack) covering the entire thickness of the ONL. Average density values of each retina were calculated to obtain the number of cone cells per mm^2 at different ages.

8. *In vitro* test of the efficiency of mouse GIRK2

HEK cells were transfected with two plasmids: CMV-SWO-mCherry and CMV-GIRK2-GFP (Figure 2) according to a well-known procedure in the art. HEK293 cells were cultured and recorded in dark room conditions after transfection. Cells were placed in the recording chamber of a microscope equipped with a 25x water immersion objective (XLPlanN-25 x -W-MP/NA1.05, Olympus) at 36 °C in oxygenated (95% O₂/5% CO₂) Ames medium (Sigma-Aldrich) enriched with an addition of 1 mM9-cis-retinal. KGluconate was added to the external

solution in order to get a high extracellular potassium concentration leading to a cell potassium reversal potential of -40mV.

For Whole-cell recordings, the Axon Multiclamp 700B amplifier (Molecular Device Cellular Neurosciences) was used, GIRK-mediated K⁺-currents were recorded in voltage-clamp configuration at -80 mV, using borosilicate glass pipettes (BF100-50-10, Sutter Instrument) pulled to 5MΩ and filled with 115 mM K Gluconate, 10 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 1.5 mM EGTA, 10 mM HEPES, and 4 mM ATP-Na₂ (pH 7.2).

During experiments, a CCD camera (Hamamatsu Corp.) was used to visualize cells using a trans-illuminated infrared-light. A monochromatic light source (Polychrome V, TILL photonics) was used to stimulate cells during electrophysiological experiments with light flashes at 400 nm.

9. Patient eye fundus imaging

Adaptive optics scanning laser ophthalmoscopy (AOSLO) (Roorda et al Opt Exp 2002) was used to image cone photoreceptor mosaic at cell resolution. The AOSLO device used (MAORI, PSI, Andover, MA, USA) allows simultaneous imaging over a 2-degree field of view of intact cones with both inner and outer segments (IS, OS) from light scattered along the optical axis (confocal mode) and inner segments (IS) from multiply scattered light scattered off axis (split detection mode). This allows us to evaluate cone presence and health, with differential imaging of IS versus IS+OS for each cone.

EXAMPLE 2: RESULTS

1. The changes in the phototransduction cascade in degenerating cones

The phototransduction cascade was first analysed in the rd10 mouse model by studying its components using immunohistochemistry, at different time points during retinal degeneration. Immunofluorescence staining was performed against cone opsin, transducing, phosphodiesterase and cone arrestin proteins of the phototransduction cascade that interact directly with cone opsin.

Figure 3 shows that only the cone opsin and arrestin were still expressed and localized around the cone cell body at late stage of the disease.

2. Cone opsin and GIRK2-mediated vision restoration

Based on immunohistochemistry and previous findings with cone opsins expressed in neurons, it was first studied why delivering a mouse short wavelength cone opsin (SWO) fused with tdTomato and rat truncated GIRK2 fused with GFP using two AAV vectors mixed in equimolar ratios would enhance cone cell's response to light. Thus two AAVs were injected

subretinally to degenerating rd10 mouse retinas at p15 (Figure 4A). This led to a significant increase in photopic ERG amplitudes in treated eyes compared to controls (Figure 4B). Flicker ERGs confirmed that the recovery mechanism was still active in these cone cells expressing GIRK2 allowing them to follow a fast stimulus (Figure 4C). The rd10 animals treated with
5 GIRK2 showed also an improved optokinetic reflex compared to controls (Figure 4D).

Next it was studied if the endogenous cone opsin, still present in degenerating cones, was functional and sufficient to activate GIRK2 channel in this mouse model. For this, a single AAV8 vector encoding GIRK2 in fusion with GFP was delivered. This led to similar increases in photopic ERG amplitudes and optokinetic reflex in treated eyes compared to controls
10 confirming that GIRK2 alone was sufficient to increase light sensitivity via G protein coupled signalling involving cone opsin (Figure 5A-B). Flicker ERGs were also robustly amplified with this approach (Figure 5C).

3. GIRK2-mediated vision restoration: long-term efficacy

15 Photopic ERG recordings were performed to monitor the cone response to light stimuli at different time points after treatment with GIRK2 and in absence of treatment. These ERGs were done under two conditions: (i) photopic with light flashes applied every second during 60 seconds at increasing light intensities and (ii) flicker stimulation with repetitive flashes during 60 seconds. Data were collected on a weekly basis until p50 and then every 10 to 13 days
20 until 11 weeks of age and showed a gradual decline in ERG amplitudes for both controls and treated eyes (Figure 6A). Moreover, these results are consistent with the optokinetic test, both controls and treated eyes with GIRK2 show a decreased optokinetic reflex over time (Figure 6B). This decline was to be expected as cone numbers also decreased over time in the rd10 mice (Figure 6C). The number of cone photoreceptors remaining in rd10 retinas were counted
25 to correlate decreases in cone numbers with decrease in ERG amplitudes. Indeed, the decrease in light responses was proportional to number of remaining photoreceptors (Figure 6D). It was thus concluded that GIRK2 increased light responses in remaining cones so long as cones remained alive but, as expected, it did not slow down the loss of cone cells.

30 4. GIRK2-mediated vision restoration in an RCD model caused by mutant rhodopsin

Having in mind the goal of creating a mutation-independent therapy, the approach was tested in another mouse model – with a different causal mutation. To this aim experiments were done in a heterozygous mouse model called mRho^{-/-}huRhoP347S^{+/-} carrying a knock in for P347S mutant human rhodopsin. Mutant human rhodopsin and absence of mouse
35 rhodopsin led to a rod-cone dystrophy in this complementary model. Here, the same set of experiments was repeated as that was done in the rd10 mouse model. First, the

phototransduction cascade proteins interacting with cone opsin at different time points was analysed (Figure 7). It was noticed that: (i) the degeneration rate was slower compared to rd10 and (ii) similar to the rd10 model only the opsin and the arrestin persisted in cone cell bodies at P150.

5 Next, mice were injected at P15 with the same AAV vectors encoding for GIRK2 fused with GFP and recorded ERGs to monitor cone response to light stimuli at various time points (Figure 8A). The response amplitudes of treated eyes were significantly higher than that of control eyes until P100. Moreover, flicker ERG responses were also similarly improved in this mouse model. Similarly to rd10 mice, this mouse model also shows an improved optokinetic
10 reflex that decreases over time in both control and treated conditions (Figure 8B). This decline is to be expected as cone numbers also decreases over time in this RCD mouse model (Figure 8C). The decrease in time in ERG amplitudes also correlated with a decrease in cone numbers in this model (Figure 8D). This was again consistent with the fact that the approach did not stop the degeneration but allowed for enhanced light sensitivity via GIRK2.

15 **5. Efficiency of the mouse GIRK2 in an *in vitro* test**

Light stimulations (400nm, 5 seconds, fullfield) activated GIRK currents in HEK cells expressing both GIRK and SWO (Short Wavelength Opsin) (Figure 9). GIRK channels are modulated in a membrane-delimited, fast manner via the Gi/o pathway and the expression of
20 the mouse GIRK channel was membrane bound. The amplitudes and kinetics of light-induced activation and deactivation of GIRK channels with SWO, induce large GIRK current amplitudes during a 5 s light pulse.

EXAMPLE 3: GIRK1 F137S elicits stronger current than GIRK2 upon stimulation with blue light (471 nm)

25 Human embryonic kidney 293 (HEK293) stably expressing mouse Opn4L-mCherry are maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM), 4.5 g/l D-glucose, supplemented with 10 % fetal bovine serum (Gibco) and penicillin/streptomycin in a humidified incubator under 5% CO₂. HEK293 cells are transfected with FuGENE® HD (Promega)
30 according to the manufacturer's protocol and incubated for 18–24 h before recordings. Retinaldehyde are added to a final medium concentration of 1 µM.

For GIRK channel recordings GIRK constructs are expressed in HEK293 cells stably expressing Opn4L-mCherry. Cells are cultured and recorded in dark room conditions after transfection. GIRK-mediated K⁺-currents are measured and analyzed as described below. The
35 external solution is as follows: 20 mM NaCl, 120 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-KOH, pH 7.3 (KOH). Patch pipettes (2–5 MΩs) are filled with internal solution: 100

mM potassium aspartate, 40 mM KCl, 5 mM MgATP, 10 mM HEPES-KOH, 5 mM NaCl, 2 mM EGTA, 2 mM MgCl₂, 0.01 mM GTP, pH 7.3 (KOH). Cells are recorded in external solution containing 1 μ M 9-cis retinal (Sigma). Cells are visualized using a trans-illuminated red light (590 nm) or green light filter (480 nm) during experimental manipulations. Whole-cell patch clamp recordings of HEK293 cells are performed with an EPC10 amplifier (HEKA). Currents are digitized and filtered with the internal 10-kHz three-pole Bessel filter (filter 1) in series with a 2.9-kHz 4-pole Bessel filter (filter 2) of the EPC10 amplifier. Series resistances are partially compensated between 70 and 90%.

For analyzing and comparing GIRK currents HEK293 cells are voltage clamped at -60 mV. For determination of the baseline current a 500 ms long voltage ramp from -100 to +50 mV is applied before light application. Then a 10 sec light pulse of 471 nm is applied at -60 mV. The size of the GIRK currents is related to the conductance of the cell before and after light activation.

For analyzing possible differences in the efficiency of G protein modulation and desensitization between the different GIRK currents we apply light pulses in the range between 0.1 – 60 sec and vary the intensity of light as described in our publications (Eickelbeck, D. *et al. Commun. Biol.* 2, (2019); Spoida, K. *et al. Melanopsin Curr. Biol.* 26, 1206–1212 (2016); Masseck, O. a. O. A. *et al. Neuron* 81, 1263–1273 (2014)).

As visible from the comparison of Figures 12 and 13, GIRK1 F137S induces significantly more ion efflux than truncated rat GIRK2 (about 17-fold higher) in the context of a short GIRK/opsin phototransduction cascade in HEK cells, while wild-type GIRK1 is ineffective at inducing ion efflux. This result suggests that incorporation of GIRK1 F137S in cones will provide for an improved gene therapy over GIRK2 gene therapy of RCD.

Figures 14 and 15 also confirm that GIRK1 F137S (with or without GFP tag) induces significant ion efflux, as compared to either human or rat GIRK2 (with or without GFP tag).

EXAMPLE 4: GIRK1 F137S vision restoration in an RCD model caused by mutant rhodopsin

The same experimental model as in Example 2.2 was used in order to demonstrate that AAV-mediated expression of human GIRK1 F137S in the eyes of rd10 mice led to greater visual acuity at P37, as determined by an optokinetic test. The results are shown in Figure 16. P15 rd10/rd10 mice received a subretinal injection of AAV8-PR1.7-hGIRK1 F137S at a dose of 5e8vg/eye or 5e7vg/eye. At P30 and P37, OKT measurements were performed to assess visual function. Significant visual improvements were observed 3 weeks after administration with 5e8vg/eye, at P37.

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CLAIMS

1. A vector comprising a nucleotide sequence encoding subunit 1 of G-protein-gated
5 inwardly rectifying potassium channel (GIRK1) F137S.
2. A vector according to claim 1 wherein said vector is a virus, chosen from an adeno-
associated virus (AAV), an adenovirus, a lentivirus, an SV40 viral vector.
- 10 3. A vector according to claim 1 or 2, wherein the vector is an AAV2 or AAV9 virus
comprising a 7 to 11 amino acid long insertion peptide in the GH loop of the VP1 capsid protein,
wherein the insertion peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7).
4. A vector according to any one of claims 1 to 3, wherein the nucleotide sequence
15 encoding subunit 1 of G-protein-gated inwardly rectifying potassium channel (GIRK1) F137S
is under the control of a cone-specific promoter.
5. A vector according to claim 4, wherein the cone-specific promoter is pR1.7 or a
functional variant thereof, or minimal M-opsin promoter, in particular in a pMNTEC expression
20 cassette, or a GRK promoter or truncated version thereof.
6. The vector according to any one of claims 1 to 4, wherein the nucleotide sequence
encoding GIRK1 F137S comprises the sequence SEQ ID NO: 3.
- 25 7. The vector according to any of the above claims, wherein the vector is a recombinant
AAV9 vector comprising:
 - a VP1 capsid protein in a 7 to 11 amino acid long insertion peptide is inserted in the GH
loop of said VP1 capsid protein relative to wild-type AAV9 VP1 capsid protein, at a position
localized between amino acids 588 and 589 of wild-type AAV9 VP1 capsid protein, wherein
30 said peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 5); and
 - the nucleotide sequence encoding GIRK1 F137S under the control of a pR1.7 promoter.
8. The vector according to any of the above claims, wherein said insertion peptide
comprises or consists of amino acid sequence AALGETTRPA (SEQ ID NO: 10),
35 LALGETTRPA (SEQ ID NO: 11), or GLGETTRPA (SEQ ID NO: 12).

9. The vector according to any of the above claims, further comprising a nucleotide sequence encoding a mammalian cone opsin.
10. A pharmaceutically acceptable carrier comprising the vector defined in any one of claims 1 to 9.
11. The pharmaceutically acceptable carrier according to claim 10, which further comprises a vector comprising a nucleotide sequence encoding a mammalian cone opsin.
12. The pharmaceutically acceptable carrier according to claim 10 wherein the vector comprising a nucleotide sequence encoding a mammalian cone opsin:
- a) is selected from the group consisting of an adeno-associated virus (AAV), an adenovirus, a lentivirus, and SV40 viral vector; or
 - b) is an AAV2 or AAV9 virus comprising a 7 to 11 amino acid long insertion peptide in the GH loop of the VP1 capsid protein, wherein the insertion peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7); or
 - c) is a recombinant AAV9 vector comprising:
 - a VP1 capsid protein in a 7 to 11 amino acid long insertion peptide is inserted in the GH loop of said VP1 capsid protein relative to wild-type AAV9 VP1 capsid protein, at a position localized between amino acids 588 and 589 of wild-type AAV9 VP1 capsid protein, wherein said peptide comprises amino acid sequence LGETTRP (SEQ ID NO: 7); and
 - the nucleotide sequence encoding the mammalian cone opsin under the control of a pR1.7 promoter.
13. The pharmaceutically acceptable carrier according to any one of claims 10 to 12 wherein the carrier is chosen from the group consisting of solid-lipid nanoparticles, chitosan nanoparticles, liposome, lipoplex and cationic polymer.
14. The vector according to any one of claims 1 to 9 or pharmaceutically acceptable carrier according to any one of claims 10 to 13, wherein the mammalian cone opsin is a short wavelength cone opsin (SWO).
15. A pharmaceutical composition comprising the vector according to any one of claims 1 to 9 and 14, or the pharmaceutically acceptable carrier according to any one of claims 10 to 14, with a pharmaceutically acceptable carrier, diluent or excipient.

16. The pharmaceutical composition according to claim 15, further comprising a vector comprising a nucleotide sequence encoding a mammalian cone opsin.
17. The vector according to any one of claims 1 to 9 and 14, the pharmaceutically acceptable carrier according to any one of claims 10 to 14, or the pharmaceutical composition according to claim 15 or 16, for use in treating rod-cone dystrophy (RCD).
18. The vector, pharmaceutically acceptable carrier or pharmaceutical composition for the use according to claim 17, wherein the vector, pharmaceutically acceptable carrier or pharmaceutical composition is administered by subretinal injection at distance of the fovea.
19. The vector, pharmaceutically acceptable carrier or pharmaceutical composition for the use according to any of claims 17 or 18 wherein the vector, pharmaceutically acceptable carrier or pharmaceutical composition is administered by subretinal injection a) in a region adjacent to the superior or inferior temporal branch of retinal artery; b) at a distance of 2–3 optic disk diameter away from the center of the fovea; and c) at a position localized in the geometric shape delineated by the branches of temporal retinal artery and temporal retinal vein.
20. The vector, pharmaceutically acceptable carrier or pharmaceutical composition for the use according to claim 17, wherein the vector, pharmaceutically acceptable carrier or pharmaceutical composition is administered by intravitreal injection.
21. A nucleic acid comprising a sequence encoding subunit 1 of G-protein-gated inwardly rectifying potassium channel (GIRK1) F137S for use in treating rod-cone dystrophy (RCD).
22. A cone precursor cell comprising a heterologous nucleic acid encoding subunit 1 of G-protein-gated inwardly rectifying potassium channel (GIRK1) F137S for use in treating rod-cone dystrophy (RCD).
23. The cone precursor cell for the use according to claim 22, wherein the cone precursor cell is obtained from the RCD subject to be treated.
24. The cone precursor cell for the use according to claim 22 or 23, wherein the cone precursor cell is obtained by differentiation induced pluripotent stem cells obtained from somatic cells of the RCD subject to be treated.
25. The cone precursor cell for the use according to any one of claims 22 to 24, wherein the cone precursor cell is administered by subretinal space injection.

26. A method of preparing a cone precursor cell comprising a heterologous nucleic acid encoding GIRK1 F137S or a functional derivative thereof, said method comprising infecting a cone precursor cell with a viral vector according to any one of claims 1 to 9 and 14, or a
5 pharmaceutically acceptable carrier according to any one of claims 10 to 14.