Cas9/sgRNA selective targeting of the P23H Rhodopsin mutant allele for treating retinitis pigmentosa by intravitreal AAV9.PHP.B-based delivery

Serena G. Giannelli1, Mirko Luoni1, Valerio Castoldi3, Luca Massimino1,2, Tommaso Cabassi1,2, Debora Angeloni4, Gian Carlo Demontis5, Letizia Leocani3, Massimiliano Andreazzoli6 and Vania Broccoli1,2,*

1Stem Cell and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, 20132 Milan, Italy, 2Institute of Neuroscience, National Research Council (CNR), 20129 Milan, Italy, 3Experimental Neurophysiology Unit, Institute of Experimental Neurology (INSPE), San Raffaele Scientific Institute, 20132 Milan, Italy, 4Institute of Life Sciences, Scuola Superiore Sant’Anna, 56124 Pisa, Italy, 5Department of Pharmacy, University of Pisa, 56126 Pisa, Italy and 6Department of Biology, University of Pisa, 56127 Pisa, Italy

*To whom correspondence should be addressed at: Stem Cells and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. Tel: +39 0226434616; Fax: +39 0226434621; Email: broccoli.vania@hsr.it

Abstract

P23H is the most common mutation in the RHODOPSIN (RHO) gene leading to a dominant form of retinitis pigmentosa (RP), a rod photoreceptor degeneration that invariably causes vision loss. Specific disruption of the disease P23H RHO mutant while preserving the wild-type (WT) functional allele would be an invaluable therapy for this disease. However, various technologies tested in the past failed to achieve effective changes and consequently therapeutic benefits. We validated a CRISPR/Cas9 strategy to specifically inactivate the P23H RHO mutant, while preserving the WT allele in vitro. We, then, translated this approach in vivo by delivering the CRISPR/Cas9 components in murine Rho+/P23H mutant retinae. Targeted retinae presented a high rate of cleavage in the P23H but not WT Rho allele. This gene manipulation was sufficient to slow photoreceptor degeneration and improve retinal functions. To improve the translational potential of our approach, we tested intravitreal delivery of this system by means of adeno-associated viruses (AAVs). To this purpose, the employment of the AAV9-PHP.B resulted the most effective in disrupting the P23H Rho mutant. Finally, this approach was translated successfully in human cells engineered with the homozygous P23H RHO gene mutation. Overall, this is a significant proof-of-concept that gene allele specific targeting by CRISPR/Cas9 technology is specific and efficient and represents an unprecedented tool for treating RP and more broadly dominant genetic human disorders affecting the eye, as well as other tissues.
Introduction

Retinitis pigmentosa (RP) is the most common cause of blindness and includes a large number of inherited retinal disorders (1). RP causes a primary rod photoreceptor cell death associated to night blindness with visual field restriction, followed by cone cell deterioration and loss of central vision. More than 60 genes have been identified to cause RP (2–4) and inherited in autosomal recessive (arRP) (5), dominant (adRP) (6–8) and X-linked (xRP) (9) genetic traits. Among the relatively large number of genetic loci causing adRPs, the RHODOPSIN (Rho) gene is the most prominent, accounting for 15% of retinal degenerations and 25% of adRP (10,11). RHO encodes for a rod receptor protein that, binding to retinal, can sense light and initiate the phototransduction cascade in rod photoreceptors (12). Among the 120 mutations identified in RHO, the p.Pro23Hist (P23H) is the most frequent mutation which alone accounts for ~10% of the adRP cases in North America. In animal models, this mutation causes gain-of-function pathological effects inducing RHO protein misfolding, aggregation and reduced glycosylation (13,14), thus leading to rod death and retinal impairment (15,16). In addition, the P23H mutant Rho protein was shown to destabilize rod photoreceptor disk membranes and interfere with the disc membrane reorientation process (17,18).

Currently, neither therapies are available to halt or delay RP disease progression nor photoreceptor cell death. However, given that the retina is an easily accessible, relative small, compartmentalized and immune privileged tissue, adeno-associated virus (AAV)-based methods have been particularly successful in therapeutic gene delivery. Importantly, clinical trials on severe forms of retinal dystrophies (19) have shown to be safe and rather efficacious in ameliorating patient visual acuity (20–22). Gene therapy is currently widely explored in a variety of preclinical studies but its application remains restricted to loss-of-function genetic diseases, in which gene replacement, even to a small degree, can account for functional restoration (23,24). Conversely, most of the adRP forms caused by gain-of-function mutations are excluded from these developments since silencing of the mutant pathogenic allele is required for functional rescue. In this scenario, allele-specific strategies are further complicated, given the hurdles posed by the discrimination between the wild-type (WT) and the mutated sequence, often reduced to a single nucleotide difference.

The recent introduction of the CRISPR/Cas9 system might provide an unprecedented tool for specific manipulation and disruption of the mutant alleles by selective allele gene targeting. This system employs the bacterial Cas9 endonuclease to cleave DNA sequences targeted by a short guide RNA “sgRNA” complementary to the 20 nt target sequence and providing specificity to any site of the genome proximal to a fixed protospacer adjacent motif (PAM) sequence (25). Recruited by sgRNA, Cas9 proteins cleave the DNA at a fixed distance from the PAM domain, thus producing a double strand break that triggers the endogenous DNA repair mechanisms, including the error prone non-homologous end-joining (NHEJ) pathway, that entails small insertion and/or deletion (indel), and the high-fidelity homologous directed repair (HDR), that instead leads to targeted integration of a provided DNA donor sequence (25). These two pathways can be exploited, respectively, for targeted gene disruption or repair and modification. Although these two processes are formally equivalent, taking advantage of NHEJ is easier and more efficacious since in many cellular systems is often prevalent to HDR, especially in postmitotic cells, and moreover it does not require a donor template (25). The most popular and optimized CRISPR/Cas9 system is based on Streptococcus pyogenes innate immune system (SpCas9) and relies on the NGG PAM site that is fairly abundant throughout the genome but still limited. More recently, the identification of different Cas9 orthologs and the development of engineered variants have provided a customizable variety of nucleases with different PAM sequence and, therefore, largely expanding the targeting range of this system across the genome (26,27). For instance, the D1135V/R1335Q/T1337R triple SpCas9 mutant protein (hereafter referred to as Cas9-VQR) was engineered to this purpose and recognizes the novel PAM site NGA (28).

In the past, various paradigms of gene inactivation have been taken into consideration for Rho inactivation with variable degrees of specificity and efficacy: ribozymes (29,30), short hairpin RNAs (31,32), zinc-finger nuclease (33). Thus, we decided to take advantage of the CRISPR/Cas9 expanded genome-editing capacity to achieve the specific inactivation of the P23H Rho allele to counteract the photoreceptor degeneration and disease progression. We explored this hypothesis both in vitro and in vivo, using the P23H knock-in mouse line, which represents a faithful animal model of the human disease, closely recapitulating the dynamics and pathological progression of the human retinal degeneration (34). Here, we demonstrated for the first time that the selective and efficient inactivation of the P23H Rho mutant allele in the retinal tissue upon in vivo SpCas9 variant delivery mediated either by plasmidic DNA transduction or AAVs. These results provide a strong proof-of-concept for the therapeutic exploitation of this system that aims to minimize photoreceptor degeneration and visual loss.

Results

Targeting of the P23H Rho allele based on the CRISPR/SpuCas9-VQR system

The P23H mutation is due to the pathogenic C-to-A nucleotide transversion in position 68 of the Rho coding sequence, both in human and mice. We asked whether this mutation could generate a novel PAM (neo-PAM) for one of the various Cas9 protein orthologs or variants, thus providing selectivity for the mutated allele. Intriguingly, we found a NNHRRT PAM domain that overlapped precisely with the A68 nucleotide on the antiparallel strand of both human and murine Rho gene (sgRNA-saC1) (Supplementary Material, Fig. S1). This PAM site is specific for the newly discovered variant of the Streptococcus aureus Cas9 (SaCas9-KHH) that for its smaller size is more apt for translational purposes (35,36). We mounted the SaCas9-KHH in a lentiviral vector equipped with the puromycin resistance gene (Puro) and the sgRNA-saC1 in a second vector containing the sgRNA scaffold optimized for SaCas9 and the resistance gene for blastidicin (Blast). We used WT and P23H Rho mouse embryonic fibroblasts (MEFs) to test if this guide could selectively cleave the P23H Rho mutant allele even in double Blast/Puro resistant MEFs. Unfortunately, no detectable cleavage was found either at the level of WT or P23H Rho sequence using two different methods for indel assessment, namely the T7 endonuclease I (T7EI) assay and the tracking of indels by decomposition (TIDE) analysis (37) (Supplementary Material, Fig. S2). In contrast, the same vectors equipped with a published guide targeting the EMX1 gene (26) led to a significant DNA cleavage in 293T cells even in absence of antibiotic selection, thus excluding technical issues (Supplementary Material, Fig. S2). Since it is known that sgRNA specificity is depending by its seed sequence, a stretch of 8–14 nt before the PAM domain (38,39), we focused on specific
CRISPR/Cas9 elements were mounted on the same vector to fold together with the Blast gene resistance and finally both mMUT, expressing the sgRNA in association to the proper scaf-fold together with the Blast gene resistance and finally both CRISPR/Cas9 elements were mounted on the same vector to fold together with the Blast gene resistance and finally both mMUT, expressing the sgRNA in association to the proper scaffold.

Notably, while in WT samples indel mutations were not detected (Supplementary Material, Fig. S3C), a significant amount of indel mutations in WT cells (Supplementary Material, Fig. S3G) were subjected to TIDE analysis and clone sequencing but no detectable indels were ascertained (Supplementary Material, Fig. S6). T7EI assay, TIDE and clone sequencing concordantly detected significant levels of indel on the allele complementary to the guide but none when the single nucleotide mismatch was present (Supplementary Material, Fig. S6), thus validating the feasibility of our measurements and specificity of the selected guides.

Selecting targeting of the P23H and WT Rho allele in Rho<sup>P23H</sup> murine retinas

We, then, asked whether Rho gene allele specific targeting could be achieved in vivo. To this aim, we sought to deliver the CRISPR/Cas9 system components in the retinae of P0 newborn Rho<sup>P23H</sup> mice by in vivo electroporation (42). To efficiently target the elements of the CRISPR/Cas9 system, we devised a single pCAG vector expressing the SpCas9-VQR under the strong CAG promoter and the sgRNA-mMUT under the U6 promoter. This vector was coelectroporated with a GFP reporter in the subretinal space for targeting the photoreceptor cell layer in the developing Rho<sup>P23H</sup>/<sup>-</sup> eyes (Fig. 1A). To assess in vivo efficiency and specificity of the gene allele targeting, retinae were harvested 4 weeks after electroporation and GFP<sup>+</sup> cells purified by FACsorting followed by molecular analysis (Fig. 1A). We first analyzed exclusively the P23H allele by PCR-P23H amplification, and quantified the cleavage rate in 27.5 ± 1.7% for the T7EI assays (n = 4) (Fig. 1B) but considerably higher for the TIDE analysis (Fig. 1C) and clone sequencing (77.2 ± 6.1%, n = 4; 87.8 ± 4.5, n = 3, respectively; ∼40 clones per retina) (Fig. 1D–G). This remarkable discrepancy in cleavage estimate between T7EI and the other methods can be explained by the overrepresentation of a particular Rho mutant allele with a single adenosine nucleotide insertion in the P23H Rho sequence in edited retinae (38.1 ± 3.5 by clone sequencing, n = 3) (Fig. 1E) that confers to the edited samples a minor degree of variability which would escape T7EI cleavage. This considered, we detected in the P23H Rho allele extremely high indel rate, causing frameshifts in the P23H Rho coding sequence in 100% of the cases (n = 3) (Fig. 1G), which likely leads to inactivation of the disease Rho allele. Thus, the mutant allele targeting is not only very effective but has also the potential to disrupt the P23H allele pathological functions, leading to the disease. To properly assess this issue at functional level, we cloned the four of most frequent Rho gene variants detected in our in vivo experiment, namely, the WT, P23H and the two most frequent indel mutants referred to as
IndelA and IndelB (Fig. 2A) and overexpressed them in P19 mouse cells (a cell line derived from an embryonic teratocarcinoma). Previous studies has shown that in cultured epithelial HeLa cells the WT Rho protein is targeted to the cell membrane, whereas the P23H Rho protein accumulates in the endoplasmic reticulum (ER) causing severe cellular stress (43–45). Accordingly, in transfected P19 cells the WT Rho localized on the plasma membrane while the P23H mutant protein remained trapped in the cytoplasm as shown by immunofluorescence using two different antibodies recognizing the Rho N- or the C-terminal (respectively, upstream and downstream the amino-acidic position 23 targeted in our editing approach). In contrast, the resulting translated products of the indelA and B Rho mutant alleles were undetectable using either antibody (Fig. 2B). These results are consistent with the premature degradation of their RNA products (Supplementary Material, Fig. S7A and B) caused by nonsense-mediated decay as shown to occur with other Rho mutants sharing a premature termination codon (46). The presence of a premature STOP codon in both indelA and B altered proteins was indeed predicted using ORF recognitions.

Figure 1. In vivo analysis of sgRNA-mut effect on the RhoP23H and RhoWT alleles. Experimental work-flow of the in vivo study. RhoP23H+/+ retinas were electroporated at P0 (left panel) with two pCAG expression vectors: one expressing the VQR variant of the SpCas9 together with the sgRNA-mMUT and the other an EGFP cassette. EGFP fluorescence was instrumental for the recovery upon FACS-sorting (right panel) of the transduced cells (central panel). After cell sorting, selective PCRs utilized for amplification of P23H and WT Rho alleles. Amplicons were subjected to a panel of cleavage assay (A). Analysis of indel mutations by the T7EI assay in P23H rhu (B) (n = 4) and WT alleles (C) (n = 5) in electroporated and NE retinas.TIDE analysis of representative retinal samples for P23H (D) and WT (E) Rho alleles indicating predicted indel efficiency. Clone sequencing analysis for the determination of the indel mutation on P23H (F) (n = 40) and WT (G) (n = 36) Rho alleles of electroporated retinae. The most frequent sequence found at the level of the P23H Rho allele was p.P23QfsX3, produced by the insertion of an adenine (A) nucleotide in position 68 (H), whereas the unaltered sequence was the most represented at the level of the WT allele of electroporated retinae. Quantification of the indel rate (J, L) and frameshift mutation recurrence (G, M) in P23H Rho (J, K) and WT (L, M) of electroporated retinae.
Moreover, possible pathogenicity associated to these frameshift mutations was assessed in vitro by evaluating their hypothetical ability to trigger ER stress, a significant cause underlying retinal degeneration (43). To this aim, we determined the Xbp1 spliced forms which initiates the ERAD pathway (47) using both a reporter assay and RT-PCR. Differently from the P23H mutant, the prematurely terminated forms presented basal levels of Xbp1 splicing, thus, confirming their lack of toxicity (Supplementary Material, Fig. S7C–H).

Next, we analyzed the mutation frequency of the WT Rho allele in electroporated retinae using selective amplification. In this case, T7EI analysis failed to detect significant cleavage in this allele (Fig. 1H) whereas TIDE analysis estimated it at 4.3 ± 0.9 (n = 5) (Fig. 1I). Finally, according to the clone sequencing only 2 out of 3 retinae carried indel mutations (≈10%) in the WT allele (clone sequencing: ≈40 clones per retina) (Fig. 1K–M).

Two different retinal genomic samples were tested for off-target activity at the same sites searched previously in MEFs (41). Neither TIDE analysis nor clone sequencing, revealed any detectable off-target cleavage at these sites, thus indicating a high safety profile of this treatment (Supplementary Material, Fig. S4B). Overall, these results clearly outlined that the Cas9-VQR/sgRNA-mMUT system enabled an efficient and selective targeting of the P23H, mutant, capable of a high degree of inactivation in the Rho allele in vivo.

Effect of targeting of the P23H Rho allele in Rho+/P23H mutant retinae

Given that the in vivo electroporation mediated an efficient delivery of the CRISPR/Cas9 elements in the retina, with high targeting rate of the P23H mutant Rho allele, we wondered whether
this could be sufficient to delay rod photoreceptor degeneration and especially outer segment (OS) deterioration. Outer nuclear layer (ONL) thickness (ONL) is a surrogate for photoreceptor number, thus is an important feature to assess retinal degeneration. We measured ONL thickness in electroporated eyes (only right eyes were electroporated, RE) but since electroporation could be also detrimental to retinal integrity we also assessed it in contralateral non-electroporated eyes (left eyes, LEs). Neither electroporated eyes (treated with Cas9-VQR alone or Cas9-VQR together with sgRNA-mMut) nor non-electroporated eyes presented significant differences in ONL thickness (Fig. 3A–D). Since at 1 month of age photoreceptor loss in Rho<sup>+/P23H</sup> retinae is relatively mild, thus masking possible effects of our treatment, we attempted to measure other features of photoreceptor degeneration. The OS is the photoreceptor compartment where RHO resides and vision initiate (12). This complex process requires a constant turnover of molecules, proteins and subcellular structures (48) but is also easily disrupted if one of its elements, RHO among many others, is functionally altered, thus leading to retinal degeneration (49). The first hallmark of this process is OS degeneration, characterized by initial shortening, misshaping and final complete loss. Degeneration also involves inner segment (IS), the compartment that lies between the OS and the photoreceptor soma and lodges the Golgi apparatus, ER and mitochondria. Four weeks after electroporation photoreceptor OSs were dysmorphic in shape and shorter in control, while morphology and length was evidently more preserved in treated retinae (Fig. 3E–Q). Retinae treated with both Cas9-VQR and sgRNA-mMUT (CRISPR-VQR-treated) GFP<sup>+</sup> photoreceptor displayed longer OS/IS tract (>1.50%, Fig. 3M) than in retinae treated with the Cas9-VQR only (controls). Cyclic nucleotide-gated channel alpha1 (CNGA1) and CNX (calnexin) staining were
used to identify the shape, respectively, of the OS and IS and their relative boundaries (34), thus highlighting the differences between control and CRISPR-VQR-treated retinae in the OS tract (>190%, Fig. 3M). Thus, the OS damage induced by the Rho<sup>P23H</sup> mutation was rescued by our CRISPR/Cas9-VQR treatment.

ONL degeneration progressed at 3 months of age, moreover the detrimental effect of electroporation at this stage was clearly evident as demonstrated by ONL thickness in non-electroporated retinae (Fig. 4A). Despite great variability among electroporated retinae, ONL thickness was significantly
lower in control compared with treated retinae (Fig. 4B–D, Supplementary Material, Fig. S8C and D). Also OS degeneration markedly advanced at this stage to the point that in control retinae OS loss was almost complete, whereas in CRISPR-VQR–treated retinae the OS layer was still present although strongly reduced in length (Fig. 4E–Q). Thus also at 3 months of age our treatment preserved ONL and OS length from the damage induced by the Rho<sup>+/P23H</sup> mutation, despite worsening caused by the electroporation procedure. Additional immunohistochemical characterization of the photoreceptor rescue at 1 and 3 month was performed to define more rigorously: layers appearance, RHO localization, presence of cones, microglia activation and astroglial reactivity (Supplementary Material, Fig. S8). Drastic changes were observed between 1- and 3-month-old retinae in all the stainings. No RHO delocalization was detected in any condition (Supplementary Material, Fig. S8E–H), as also expected since our mouse model does not present with it (34). Differences among control and respective treatment could be detected only in ONL thickness at 3 months (as expected, Supplementary Material, Fig. SBC and D) and in GFAP reactivity that resulted milder in treated versus control retinae at both time points although it was present in all conditions (Supplementary Material, Fig. S8Q–T).

To obtain a functional correlate of the morphological diversity between CRISPR-VQR and control–treated photoreceptors, we performed transretinal electroretinogram (ERG) recordings in 3-month-old–treated mice (Fig. 4R–T). Clearly un.injected left retinae, presented better responses to light than the right electroporated eyes, thus confirming an additional detrimental effect on the degenerative phenotype, caused by the electroporation procedure previously indicated by the histological analysis (Figs 3A–D and 4A–D). For this reason, we only compared RE recordings upon LE normalization (Fig. 4U and V, see also row data in Supplementary Material, Table S1). WT retinae produced robust a- and b-waves indicating functional photoreceptor- and bipolar-dependent currents. In contrast, waves recorded in control–treated Rho<sup>−/P23H</sup> retinae were severely blunted consistent with the observed photoreceptor cell loss. Remarkably, Rho<sup>−/P23H</sup> retinae showed a significant rescue in both a- and b-waves indicating a functional recovery of the phototransduction visual activity (Fig. 4).

**AAV-mediated delivery for targeting the P23H Rho allele in Rho<sup>−/P23H</sup> murine retinae**

AAV delivery of therapeutic gene has been proven very successful for the treatment of retinal diseases in various clinical and preclinical studies (19,20,22–24). Currently administration of viral particles for the treatment of retina or RPE has required direct injection in the subretinal space between the photoreceptor cell layer and the RPE and consequently their detachment. This procedure, although employed in retinal clinical practice is not devoid of risks and complications compared with administration into the vitreous chamber. Intravitreal (IV) injection is safer and commonly used for pharmacological treatments of retinal diseases such as age-related macular degeneration. In recent years, AAV technology has engineered a great numbers of new serotype variants with enhanced capabilities (50–52), thus making targeting of the retina using IV administration possible in the adult mice. The 7m8 serotype is an AAV2 variant generated for this purpose (51), and therefore, it was the first one we tested for delivery in Rho<sup>−/P23H</sup> eyes. With a GFP expressing AAV2–7m8 (10<sup>12</sup> vg/ml, 1 μl/eye), the overall transduction appeared limited with an evident preference for amacrine and ganglion cells (Supplementary Material, Fig. S9A–F). However, the use of the human RHO promoter (hRHO) to drive GFP expression did greatly improved photoreceptor transduction (Supplementary Material, Fig. S8). Therefore, two different AAV vectors were devised one carrying an inducible Cas9–VQR and the other carrying the sgRNAmut together with the GFP reporter and rTA transactivator under the control of the hRHO (53). Since, the TetO promoter requires the rTA transactivator and doxycycline to be activated this system allows us to restrict Cas9 expression to rod photoreceptors. To ensure continuous Cas9–VQR expression, doxycycline (2 mg/ml) was administered orally to mice since the day of IV injection and for all the duration of the experiment. Due to technical limits in performing IV injections, delivery was performed at 3 weeks of age. Two weeks after injection of the two viruses, we tested treated and untreated retinae for the presence of indel, using T7EI assay in the P23H and WT allele (Supplementary Material, Fig. S9H). Unfortunately, gene-editing molecular events were undetectable in these samples. The employment of two vectors for this strategy implies that very high level of transduction need to be achieved to have detectable gene targeting. The 7m8 could not reach this level of targeting at least with the titer employed. More recently others and we demonstrated the high transduction efficiency in neural tissues of the new synthetic capsid variant AAV9–PHP.B (50,54). This virus was developed by a targeted modification of the AAV9 capsid to potentiate its ability to cross the brain-blood barrier and infect wide areas in the neurasix (50). Given its excellent potential for therapeutic gene delivery, we tested its ability to target the retina using IV administration (Fig. 5A). Great gene delivery in the whole retina and in the RPE was detectable using an AAV9–PHP.B equipped with the CBA–GFP vector cassette (Fig. 5B–E). All retinal cellular subtypes were targeted but with different extent. Transduction was higher in rod photoreceptors (70 ± 5%) and amacrine cells (81 ± 7%) and lower in cone photoreceptor (45 ± 3%), ganglion (32 ± 2%), bipolar (15 ± 1%), horizontal (33 ± 2%) and Müller Glia cells (18 ± 2%) (Fig. 5F–M). These findings prompted us to use the AAV–PHP.B variant to treat Rho<sup>−/P23H</sup> retinae with our CRISPR/spCas9–VQR approach, by delivering the two different constructs described earlier (Fig. 6A). GFP transduction carried by the vectors was robust and diffuse in the ONL of the whole retina (Fig. 6B). Consequently, we were able to evaluate indel frequency exclusively into rod photoreceptors, after FACs-sorting for GFP<sup>+</sup> cells. The P23H Rho allele of these retinae presented a significant but moderate levels of cleavage (9.6 ± 0.4, n = 3) (Fig. 6C). On the contrary, the WT Rho allele remained completely unaltered in all the treated retinae (Fig. 6D). Unfortunately, indel quantification in these samples was hampered by the low sensitivity with both the TIDE (Fig. 6H) and the clonal analysis (data not shown). To solve this problem, we investigated the possibility of enriching for the editing events separating the cells with high and low levels of GFP transduction (GFP-high and -low, respectively) by FACs-sorting (Fig. 6E). Indeed, the GFP-high, respect to the GFP-low, population exhibited a much higher accumulation of indels (Fig. 6F), as measured by T7EI assay at the level of the P23H Rho allele. Once more, the WT Rho allele instead was not affected (Fig. 6C). Interestingly, TIDE analysis revealed an 15-fold difference in indel efficiency between the parental GFP<sup>+</sup> (Fig. 6H) and GFP-high population (Fig. 6I). This increase in indel mutation allowed for indel frequency analysis in retinal samples revealing that in viral transduced retinae as much as in electroporated retinae the most present indel by far is the insertion of a single nucleotide (Fig. 6I). Furthermore, GFP<sup>+</sup> and GFP-high samples

---
were subjected to NGS analysis to refine indel analysis and compare them with GFP+ sorted samples of electroporated retinae (Fig. 7A).

NGS analysis of edited Rho+/P23H murine retinae

For sequencing analysis, we devised a PCR that unselectively amplified both WT and P23H Rho allele, to concurrently assess their incidence and NHEJ events. Quantification of editing frequency in retinal samples was obtained using the CRISPResso toolbox (55). This analysis confirmed selectivity of our approach for the P23H Rho allele. In fact, WT Rho allele frequency in control untreated retina was comparable to treated samples, regardless of the delivery system employed or enrichment of editing events (Fig. 7B). Conversely, these two parameters strongly affected unedited P23H Rho allele frequency, that was lowest in electroporated retinae (<10%) compared with AAV9-PHP.B-injected retinae (38%). NHEJ events occurred in a complementary fashion in these samples (43 and 7%, respectively). Indel enrichment in the GFP-high compared with the GFP+ was evident as previously assessed (Fig. 7B). Regardless of indel enrichment or delivery system, retinal treatments showed very similar pattern of frameshift and in-frame indel mutation (Fig. 7C, Supplementary Material, Figs S10 and S11). Since both +1 and +2 frameshift mutation in the P23H Rho allele cause the formation of premature termination codon (Fig. 2B), the occurrence of this type of mutation is most likely to disrupt its proteic expression and its dominant-positive detrimental effect, compared with in-frame mutations. Thus, our editing strategy revealed not only high selectivity but also robust functional silencing efficiency of the disease allele.
Selective targeting of the human WT and P23H RHO gene alleles

Since efficacy of the CRISPR/spCas9-VQR system was proved in a murine model of the disease and since the sgRNA-mMUT PAM site is conserved in human genome (Supplementary Material, Fig. S1), we sought to test this approach in a convenient human cellular model. Thus, we generated two sgRNAs based on the conserved PAM site, one targeting the WT (sgRNA-hWT) and the other the P23H mutant sequence (sgRNA-hMUT) (Fig. 8A). To generate a cellular model with the P23H homozygote mutation in the RHO gene, we devised a CRISPR/Cas9-based gene-editing approach to introduce the c.68C>A nucleotide transversion into 293T cells (Supplementary Material, Fig. S12). WT or gene edited RHO/P23H/P23H 293T cells were treated with sgRNA-hWT and -hMUT and subjected to our panel of genomic cleavage assays (Fig. 8B–U). In all assays, both guides showed significantly different cleavage efficiency when targeting the complementary genome, compared with the one bearing one mismatch. In particular, T7EI assay quantified the sgRNA-hWT indel rate in WT cells in 60 ± 13% compared with none in P23H cells (n = 3), whereas sgRNA-hMUT indel rate was...
down to zero in WT but up to 58 ± 14% in mutant cells (n = 3). For the same conditions, TIDE analysis concordantly measured cleavage efficiency in 78 ± 9%, 7 ± 1%, 3 ± 1%, 76 ± 10%, respectively (n = 3). Selectivity of cleavage based on clone sequencing was even higher with 94% (in WT cells) versus 0% (in P23H cells) for the sgRNA-hWT and 89% (in p23H cells) versus 0% (in WT cells) sgRNA-hMUT. Conveniently, the fraction of indel in-frame mutation in the RHO coding sequence was very low for both human guides (similarly to sgRNAMUT) supporting the idea that this system would efficiently inactivate the pathogenic product of the P23H RHO mutant allele also in human retina.

Discussion

Here, we exploited the efficiency of the CRISPR/Cas9 technology to selectively target a single point mutation in the genome, generating the dominant gain-of-function mutant RHO allele, responsible for photoreceptor cell loss and RP. Our results provide strong evidence that this approach can lead to efficient targeting of the mutant sequence and, thus, preventing the pathogenic effects of the dominant mutation, while simultaneously preserving the WT Rho allele. Notably, we showed that targeting specificity of sgRNAs can be strongly influenced by the seed sequence up to discriminating between two sequences which differ for a single nucleotide only. This is an invaluable finding which discloses a wide use of this approach to distinguish between two or more homologous sequences.

Surprisingly, this high level of precision and efficiency in sequence-specific targeting of the designed CRISPR elements was maintained, and even increased, after local delivery in the retinal cells, indicating that this approach maintains its specificity upon in vivo delivery. Such efficient targeting of the mutant Rho allele triggered an evident delay of the degenerative retinal process and a rescue of the retina functional activity. Since the PAM genomic site is conserved between humans and mice, we were able to prove efficacy and selectivity of this gene targeting system in human cells engineered with the P23H RHO gene mutation.

Previous studies have attempted to use shRNA (30) or ribozyme-based (31,32) approaches to silence the P23H Rho mutant allele. In these and other studies, selectivity toward the P23H Rho allele is achieved using transgenic mice or rats overexpressing the mutated P23H allele from a different species (human or rodent). In this scenario, the silencing of the toxic P23H, although beneficial, cannot accurately model the biallelic composition of the human pathology, as our knock-in mice model provides. This mouse model combined with our strategy to target selectively the mutated allele, faithfully model the disease
genetic features and its potential rescue, suggesting a substantial higher therapeutic potential (30–32,56). Indeed RHO selective inactivation by the CRISPR/Cas9 system has been recently proved possible in transgenic rats carrying a murine mutant allele (57). In this study, sgRNA selectivity relied mainly on species-specific and not allelic differences. Moreover, S334X dominant Rho allele targeted in this study is not conserved in humans and, thereby, not relevant for human disease pathology. Hence, this approach provided a proof-of-principle of feasibility but is not suitable for translation into human therapy. Beyond the CRISPR/Cas9-mediated editing, other approaches have been attempted for the attenuation of dominant Rho alleles causing RP. Albeit some therapeutic benefit was achieved, the repression of the mutant allele resulted not complete and its effect was weakened with time in vivo providing relevant obstacles for any clinical development. In this regard, our approach has the strong advantage to produce a stable and definitive genetic change in the targeted cells even with a single application. An interesting therapeutic option is the suppression and replacement (31,33,53) approach based on the silencing of both WT and mutant RHO alleles, while simultaneously adding an extra functional copy of the gene resistant to silencing. Nonetheless, the drawback of this approach is that RHO ectopic expression, ensured by current gene transfer tools, cannot meet its endogenous levels, necessary for recovery.

Various mutations in the RHO gene have been identified to cause adRP. Currently, despite the Rho c.68C > A mutant allele is by far the most common mutation in North America, more than other 90 mutations have been identified in the RHO gene responsible for adRP and congenital stationary night blindness.

Figure 8. CRISPR/Cas9 selective targeting of either WT or P23H RHO allele in human cells. Schematic of sgRNA-hWT (green) and sgRNA-hMUT (orange) annealing to their target sequences (upper case), differing for a single nucleotide (underscored). Symbols indicate: *: mismatches, red line: PAM sequence, red triangle: DSB site; color blue: Pro23, purple: His23 (A). Cleavage efficiency as measured with T7EI assay. T7EI assay performed on RHO<sup>+/+</sup> (B, C) (n = 3) and RHO<sup>P23H/P23H</sup> (D, E) (n = 3) 293T cells resulted in detectable DNA cleavage only when complementarity between sgRNA and target sequence was perfect. TIDE analysis on RHO<sup>+/+</sup> cells with sgRNA-hWT (F) showed greater cleavage than sgRNA-hMUT (G) and vice versa on RHO<sup>P23H/P23H</sup> cells with sgRNA-hMUT (G) showed greater cleavage than sgRNA-hWT (F) (R<sup>2</sup> indicates the variance, a statistic value of likelihood of the TIDE prediction). Molecular cloning followed by Sanger sequencing indicated the presence of various sgRNA-hWT targeted sequences in RHO<sup>+/+</sup> cells (J, N, R) (n = 31) presenting high rate of frameshift (R) but not on RHO<sup>P23H/P23H</sup> cells (L, P, T) (n = 22) conversely when sgRNA-hMUT was applied to RHO<sup>+/+</sup> cells no indel mutations were detected (K, O, S) (n = 24) where they were present in RHO<sup>P23H/P23H</sup> cells (M, Q, U) (n = 35) resulting in a high rate of frameshift mutations (U). *P < 0.05.
Remarkably, we identified PAM domains from different Cas9 orthologs (25–27) or their variants overlapping or in close proximity (<4 bases distant) within a large set of these disease-causing mutations (Supplementary Material, Fig. S13). More in general, this methodology can have application to the dominant retinal dystrophies caused by mutations in the IMPDH1 (58), PRPF3 (6,8) and KLRH1 (59) genes.

Overall, AAVs are considered the tool-of-choice for therapeutic gene delivery in the retina. Moreover, the development of different capsid serotypes and novel variants have been instrumental for an efficient targeting of different cell populations within the retina using subretinal injections. Nonetheless, very recent studies have shown that this administration route might lead to some moderate systemic inflammation that might worsen the overall pathological state (21,22). On this regard, IV administration of AAVs has potentially a much safer profile but has often resulted in a significant reduction of retinal transduction especially for photoreceptor cells, that are the cellular target for a predominant number of retinal diseases. However, the viral vector toolbox available for retinal research has recently been remarkably expanded with the discovery of new and potent AAV variants. In this work, we demonstrated, for the first time, the great potential of the AAV-PHP.B synthetic variant for IV delivery of gene therapeutics to target the retinal photoreceptor cells. Importantly, the use of this vector allowed us to effectively deliver the CRISPR/Cas9-VQR system into the whole retina. Inactivation of the P23H Rho allele by the AAV-PHP.B delivered CRISPR/Cas9-VQR system was proven specific and effective but it was restricted to only a small fraction of photoreceptor cells (~10%). A critical aspect limiting the success of the present approach is the need of using more than one AAV to deliver all the necessary CRISPR elements. In fact, although, AAV-mediated SpCas9 genome editing in vivo has already been proved (60), its delivery cannot be performed in a single particle. This drawback was recently overcome by the identification of the new SaCas9 ortholog sufficiently small to be packaged in an AAV vector with its sgRNA (26). The use of this system enabled a strategy to edit the dystrophin mutant gene in vivo as a new therapeutic opportunity for treating Duchenne muscular dystrophy (35,36). Moreover, a new variant, SaCas9-KHH, was engineered to broaden its genome targeting range. Nonetheless, the two different sgRNAs for this Cas9, that would selectively target the P23H allele, exhibited a very poor cleavage efficiency in our experimental setting. Thus, more work is needed to broaden the effective potential, and predict the favorable targeting events for the SaCas9 and its derivatives.

Overall, we believe that, this particular approach to be therapeutically valuable necessitates of an convenient and efficient delivery of the CRISPR components to the relevant tissue and cells and that IV administration of the PHP.B variant meets these need.

Notably, this is the first time that a Cas9 variant has been explored to reach allele specific targeting. More broadly, this study represents a convincing proof-of-concept for wide application of the CRISPR system for dominant genetic human disorders, caused by mutant gain-of-function or dominant-negative gene alleles. The broad applicability of this approach depends on the identification of new bacterial Cas9 orthologs, and their engineered variants with intact nuclease activity. Recently, this pool has strongly expanded the targeting range of this system providing a significant flexibility for genome-wide engineering (27,28,38).

The employment of the gene-editing methods always raises safety concerns about off-target effects, especially when translational applications are taken into consideration. We did not observe off-target mutations at potential off-target sites in the mouse genome nor any evident long-term abnormalities in retinæ after electroporation. However, off-target mutations may occur at sites beyond those predicted in silico, hence a comprehensive and unbiased analysis, such as whole genome sequencing, would be an essential component of future efforts to definitively establish the safety of this approach (26,61–63). Various approaches have been conceived to restrict Cas9 activity in time and reduce its detrimental off-target effects (64,65).

In our system, the use of TetO promoter to induce Cas9 could be useful control its expression with doxycycline and identify a therapeutic window in which Cas9 therapeutic action is deprived of undesired effect. Moreover, this system might be implemented to clarify when during disease progression our approach is effective since as the disease progress it is less likely that gene correction can revert RP symptoms.

These results provide strong evidence that the CRISPR/Cas9 system can be tailored to target the mutant Rho allele, restoring retinal function, thus opening new strategies for human dominant genetic disorders, in particular afflict the eye. Moreover, this approach was successfully combined with IV delivery using the AAV-PHP.B variant, thus reinforcing its translational potential.

Materials and Methods

Vectors generation

The oligo (Sigma-Aldrich) pairs encoding the 20 nt guide sequences and including overhangs for ligation into the BamH1 site (Oligos List) were annealed and ligated (Clontech) into the LV-U6-filler-gRNA scaffold plasmid previously linearized with the BsmBI restriction site, thus removing the filler sequence. This vector was engineered replacing the TetO-Nfia cassette of the TetO-FUW-Nfia plasmid (66) (Addgene) with the ‘U6-promoter-filler-gRNA scaffold’ of the LentiCRISPR (38) (Addgene). To provide the LV-U6-filler-gRNA scaffold vectors with a selection, a Blast cassette under the EF1a core promoter was cloned downstream of the U6-sgRNA cassette. Moreover, this vector was further modified replacing the gRNA scaffold optimized for spCas9 with an other contained in the vector p.X601 (26) (Addgene) selective for saCas9. Replacing the Nfia cassette of the TetO-FUW-Nfia with the spCas9-p2aPuro sequence of LentiCRISPR, we obtained the TetO-spCas9-p2aPuro vector, from which the TetO-SaCas9-KHH-p2aPuro vector was derived (taking advantage of MSP2292, Addgene). The plasmid pCAG-spCas9-VQR was generated from the pCAG-sPcaS9 (cloned by replacing GFP of the pCAG-GFP, Addgene, with spCas9 of the LentiCRISPR) (42) vector by overlap-extension PCR (Phusion High-Fidelity DNA Polymerase, New England Biolabs, NEB) using appropriate primers (Oligos List) to modify codon 1135 (GAC into GTG), 1335 (AGG into CAG) and 1337 (AAC into AGA) of the native spCas9, thus producing the VQR variant. The same strategy was used for the generation the TetO-spCas9-VQR-p2a-Puro vector.

Similarly, we constructed an inducible adenovector containing the spCas9-VQR form the pX51 vector (Addgene), which was further modified replacing the MeCP2 promoter with the TetO promoter. A second adenovector was generated using an AAV2-hRHO-promoter-GFP vector (kindly donated by Dr A. Auricchio) introducing the U6-sgRNAmMut cassette upstream of the hRHOp and a GFP-t2a-rtTA cassette downstream.

WT Rho CDS sequence was amplified from whole cDNA derived from retrotranscription (iScript cDNA synthesis kit, Bio-Rad) of RNA extracted (TRIZol Reagent, Thermo Fisher Scientific) from adult retinae. The oligos employed for this purpose (Rho-EcoRI-Agel-koz-Fw and Rho-Nhel-EcoRI-R, Oligos List) allowed
for direct cloning into the pCAG-ires-GFP plasmid using EcoRI but also further subcloning in to the pCAG vector using EcoRI-NheI restriction sites. The N-terminus of the mRho WT sequence was substituted with the corresponding region of the other variants (P23H, IndelA & IndelB) using PCR amplification (oligos: Rho-EcoRI-Agel-koz-Fw and Rho-XhoI, Oligos List) and Agel and XhoI restriction site from molecular cloning.

Cell cultures

MEFs were isolated from E14.5 Rho<sup>23H</sup>/P23H, Rho<sup>23H</sup>/+ and Rho<sup>-/-</sup> mice embryos. Primary culture were derived as previously described (66) and maintained in MEF medium (Dulbecco’s Modified Eagle Medium, containing 10% fetal bovine serum, non-essential amino acids, sodium pyruvate and penicillin/streptomycin, Thermofisher Scientific). In total, 293T cells were cultured in the MEFs media, whereas P19 cell lines were maintained in α-MEM (Sigma-Aldrich) supplemented with 10% FBS. Cells were split every 3–4 days using Trypsin (Sigma-Aldrich) dissociation (0.25%) in their medium. MEFs or 293T cells were cultured every 3–4 days using Trypsin (Sigma-Aldrich) for direct cloning into the pCAG-ires-GFP plasmid using EcoRI but also further subcloning in to the pCAG vector using EcoRI-NheI restriction sites. The N-terminus of the mRho WT sequence was substituted with the corresponding region of the other variants (P23H, IndelA & IndelB) using PCR amplification (oligos: Rho-EcoRI-Agel-koz-Fw and Rho-XhoI, Oligos List) and Agel and XhoI restriction site from molecular cloning.

Viral production

Lentiviral replication-incompetent, VSVG-coated lentiviral particles were packaged in 293T cells (67). AAV replication-incompetent, recombinant viral particles were produced in 293T cells by polyethylenimine (PolyScience) cotransfection of three different plasmids: transgene-containing plasmid, packaging plasmid for rep and cap genes and pAdDeltaF6 for the three adenoviral helper genes. The cells and supernatant were harvested at 120 h. Cells were lysed in Tris buffer (50 mM Tris pH = 8, 5, 150 mM NaCl, Sigma-Aldrich) by repetitive freeze-thawing cycles (three times). To clarify, the lysate Benzonase treatment was performed (250 U/ml, 37°C for 30 min, Sigma-Aldrich) in presence of 1 mM MgCl<sub>2</sub> (Sigma-Aldrich) and cellular debris separated by centrifugation (2000g, 30 min). The viral particles in the supernatant instead were concentrated by precipitation with 8% PEG8000 (Polyethylene glycol 8000, Sigma-Aldrich), resuspended in Tris buffer and combined with corresponding cell lysates. The viral phase was isolated by iodixanol step gradient (15, 25, 40, 60% Optiprep, Sigma-Aldrich) in the 40% fraction and concentrated in phosphate buffer saline (PBS) with 100 K cutoff concentrator (Vivaspin20, Sartorius Stedim). Virus titers were determined by measuring the number of DNAse I-resistant viral particles, using qPCR with linearized genomic plasmid as a standard.

Animal models and manipulation

All procedures on animals were performed in accordance with the institutional guidelines for animal research. Homozygous P23H knock-in mice (obtained from The Jackson Laboratory, Bar Harbor, ME) were bred either with each other to maintain the homozygous line or with C57BL/6 mice (Charles River Italy, Calco, Italy) to obtain to generate P23H heterozygous embryos and pups. P0 mice were electroporated as previously described (42). Shortly, they were anesthetized by chilling on ice, and a small incision was made in the eyelid and sclera near the lens with a 33-gauge needle. In total, 0.3 μl of DNA solutions (3–6 mg/ml, 0.1% fast green) were injected into the subretinal space through the incision by means a Hamilton syringe with a 33-gauge blunt-ended needle. Upon DNA injection, five square pulses of 80 V amplitude and 50 ms duration (with 950 ms intervals) were applied to the heads of the pup using tweezer-type electrodes (model 520, 7 mm diameter, BTX, San Diego), connected to a pulse generator ECM830 (BTX). DNA was transfected only into REs. IEs were left untouched. Upon 4–12 weeks, retinae were harvested for indel analysis, histological staining or ERG recordings. For AAV IV injections, three-fourth-week-old mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg; Ketavet 100—Intervet Productions s.r.l, Aprilia, Italy) and xylazine (10 mg/kg; Rompun—Bayer s.p.a., Milano, Italy). One microliter of AAV-CBA-GFP (1 × 10<sup>12</sup> viral genomes/ml, vg/ml) or of a 1:1 mixture of AAV-Cas9-VQR (1 × 10<sup>12</sup> vg/ml) and AAV-U6sgRNA-MUT-RHOP-GFP-T2A-rTA (1 × 10<sup>12</sup> vg/ml) was intravitreally injected using a Hamilton syringe with a 33-gauge blunt-ended needle under a stereo microscope (Leica Microsystems Ltd). Doxycycline (2 mg/ml) was administered in the drinking water from the day of injection till retinal harvest. Two weeks after injection retinae were harvested for subsequent analysis.

Tissue and cell collection

Electroporated retiniae were dissected under a fluorescence dissection microscope. GFP<sup>+</sup> retiniae were selected for further analysis based on fluorescence intensity and diffusion but also retinal integrity. To perform immunohistochemical analysis dissected retiniae were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, cryoprotected in 5% sucrose (10 min) followed by 30% sucrose (10 min) and embedded in OCT compound on dry ice. Cryostat crossections (20 μm) were then cut. In case of indel analysis, retiniae were dissociated (pain 10 U/ml, Worthington, 10 min, 37°C) and FACS-sorted (MoFlo, Beckman Coulter, Inc.). Genomic DNA (gDNA) was extracted from the GFP<sup>+</sup> population thus isolated using QIAamp DNA micro kit (QIAGEN) following manufacturer instruction.

gDNA was extracted from MEFs and 293T cells using a lysis buffer (100 mM Tris–HCl pH 8, 200 mM NaCl, 5 mM EDTA, 0.5% SDS with proteinase K, Sigma-Aldrich) to incubate cells (4–24 h, 55°C). gDNA was purified using isopropanol and ethanol 70% and resuspended in water. RNA extraction was performed using Trizol Reagent according to manufacturer instruction (Trizol Reagent, Thermo Fisher Scientific). For immunohistochemical analysis, cells were fixed 4% paraformaldehyde.

Indel analysis and off-target analysis

The genomic regions that were flanking the sgRNAs target sites were amplified by PCR using 100 ng of gDNA as a template. Primers sequences for the selective or unselective amplification of both WT and P23H Rho alleles are indicated in Oligos List as well as primers for hRHO amplification. Selective Rho<sup>-</sup> and Rho<sup>P23H</sup> PCR required an annealing temperature of 66 and 68°C, respectively, while 62°C was routinely used as annealing temperature for unselective PCRs. For T7EI analysis, 200 ng of purified...
PCR products (Wizard SV gel and PCR Clean-Up System, Promega) were denatured and reannealed in NEB buffer 2 (NEB): 95°C, 5 min; 95–85°C at 2°C/s; 85–25°C at -0.1°C/s. Upon reannealing 10U of T7EI (NEB) were added and incubated at 37°C for 15 min to then be analyzed on 2% agarose gels, imaged with a Gel Doc gel imaging system (Bio-Rad). The percentage of genome modification was obtained as previously described (25). To perform TIDE assay, purified PCR products were analyzed by Sanger sequencing (GATC Biotech). The quantification of genome modification was also obtained using the TIDE software, which allowed to interpolate the chromatograms produced by direct Sanger sequencing of the previously described PCR products or which applies a deconvolution algorithm on the sequencing traces to identify the indel mutations proximal to the editing site and accurately determines their frequency in a cell population (37). Sequencing primers used for TIDE analysis are indicated in Oligos List. Amplicons obtained as earlier were also subjected to clone sequencings, which consisted in cloning of PCR products using the TOPO BLUNT cloning kit following manufacturer instruction (ThermoFisher Scientific) and then select and individually sequence clones positive for the amplicon of interest. RFLP analysis was also carried out either on amplicons (293T selection for recombined clones with BstBI) or individual clones (optimization of PCR selectivity, BanII). Briefly, 2–500 ng of DNA were incubated with 5U of BanII or BstBI (NEB) at 50°C for 15 min to then be analyzed on 2% agarose gels. Off-target were identified using COSMID online tool (41) (see Oligos List), to be tested both by TIDE analysis and clone assayed by PCR amplification (https://crispr.bme.gatech.edu). 

**List of primers**

| Oligos for overlap-extension PCR |
|----------------------------------|
| VQR-Sacl-PE | GAGGCTGTTGAAATGTAAGTGGCCGCCGACC |
| VQR-F11 | GGCGGAAGCTTCTCCGAGATTCTGCTG |
| VQR-RI1 | CAGAATAGCCAGGTTGGGTTTACAGA |
| VQR-RI2 | ACCGCGCGTTCGCGCTGCTG |
| VQR-Nhel-RE | GGCGGAGGGCCGAGTCAAGGTGACG |

**Oligos for molecular cloning**

| Oligos for molecular cloning |
|----------------------------------|
| Rh-o-EcoRI-Agel-koz-Fw | CGCGAATTCACGGTGTAGAGTCGTC |
| Rh-Nhel-EcoRI-R | CGCGAATTCACTAGATGTCGAGG |
| Rh-Xho-R | GCGCCTTCGAGATTACGCGCGAG |

**Oligos for molecular cloning**

| sgRNA saC1 | Fw: caccgGGTGTGGTACGCAGCCACTT; Rv: aaacAGCAGCCGACATACCTGG |
| sgRNA saC2 | Fw: caccgGGTGTGGTACGCAGCCACTT; Rv: aaacAGCAGCCGACATACCTGG |
| sgRNA hEMX1 | Fw: caccgGGCCTCCCAAAAAGCAGCGCG |
| sgRNA-US | Fw: caccgGTTCGCCGCCGAGTACTGCTG |
| sgRNA m MUT | Fw: caccgGGCCTCCCAAAAAGCAGCGCG |
| sgRNA REC | Fw: caccgTGACCTGCTGAGACTGCA |
| sgRNA h WT | Fw: caccgGTTCGCCGCCGAGTACTGCTG |
| sgRNA h MUT | Fw: caccgGTTCGCCGCCGAGTACTGCTG |

**Oligos for clone sequencing**

| Oligos for clone sequencing |
|----------------------------------|
| mRho seq-Fw | CTCCGAGATGCGCAATGCTG |
| hEMX1-Rv | CCATAGGGAAGGGGCCACGTG |
| hRHO-seq-Fw | CTCGCCAGATGCGCAATGCTG |

**Histological analysis and morphological measurements**

Retinal sections and fixed cells were permeabilized for 30 min in blocking solution, containing 0.2% Triton X-100 (Sigma) and 10% donkey serum (Sigma-Aldrich, 1:1000), rabbit anti-c-Na channel protein (Millipore, 1:1000), rabbit anti-CNGA (kindly provided by Dr Molday, 1:20) chicken anti-Green Fluorescent protein (ThermoFisher Scientific, 1:2000), and mouse anti-RHO (Sigma-Aldrich, 1:1000). Rabbit anti-RHO (Sigma-Aldrich, 1:1000) was used: rabbit-anti calnexin (Sigma-Aldrich, 1:500), mouse anti-CNGA (kindly provided by Dr Molday, 1:20). Primary antibodies were used: rabbit-anti calnexin (Sigma-Aldrich, 1:1000), mouse anti-CNGA (kindly provided by Dr Molday, 1:20).
(Abcam, 1:500), rabbit anti-Red-Green-Opsin (Millipore, 1:500), rabbit anti-GFAP (Dako, 1:500), rabbit Iba (Wako, 1:500). Upon wash with PBS (3×), slides were incubated for 1 h at RT in blocking solution with Hoechst and secondary antibodies mix: 488Alexa-conjugated anti-chicken coupled with either Alexa Fluor-594 and Alexa Fluor-647 anti-rabbit or anti-mouse secondary antibodies (1:1000, ThermoFisher Scientific). After PBS washes (3×), slides were mounted. Confocal images were captured with Leica TCS SP5 Laser Scanning Confocal microscope (Leica Microsystems Ltd). Confocal images were used to evaluate ONL thickness, a surrogate for photoreceptor number (43) and OS thickness (31). Quantification were made by measuring 10 electroporated positions (identified by GFP expression) evenly spaced 1 mm apart from the optic nerve (5 superior loci and 5 inferior loci) under 63 magnification. Further, digital 3D magnification was utilized for OS thickness measurements. The CNGA and CNX stainings were utilized to assess OS/IS boundary. Measurements were made using the Leica LAS AF Lite software and averaged between the different animals. Three eyes per electroporation condition were measured as well as control-lateral eyes as non-electroporated samples (NE).

RNA analysis

Upon extraction, whole RNA (TRIZol Reagent, Thermo Fisher Scientific) of transfected P19 cells was retrotranscribed (iScript cDNA synthesis kit). The absence pCAG plasmid contamination in whole cDNA was revealed by RT-PCR using following oligos (5′–3′): Fw, GGGCCGCAGCCAATCAGAG, Rv, CACAAAGG GCCCTCCGGAG and GoTaq Polymerase (Promega). mRho RNA levels were measured using quantitative real time PCR (qPCR) using Titan HotTaq EvaGreen qPCR mix (BioAtlas) by means of a C1000 Thermal Cycler (Biorad). In total, 18S mRNA levels were used as normalizer. Primers for Rho were (5′–3′): Fw, ATGAGCAACCTCCGGTTCG, Rv, ATGAGCAACCTCCGGTTCG. Primers for 18S were: Fw, GTAAACGTTGAAAACCAT, Rv, CCAT CCAATCAGGTAAGCCG. cDNA was also tested for different Xbp1 isoform by RT-PCR as already described (47). Xbp1 expression was also tested by an Xbp1 reporter plasmid (70) (kindly provided by Dr M. D’Antonio) cotransfected into p19 cells in combination with different RHO variants. In this case, XBP1 splicing was evaluated by GFP fluorescence intensity as evaluated by FACS-sorting.

ERG

Scotopic ERG responses were evaluated in WT, Cas9 VQR only and Cas9 VQR +Sg RNA mMUT mice at 3 months of age. Mice were dark-adapted for 2 (71) h before the recordings and all procedures were conducted under dim-red light. Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg; Ketavet 100—Intervet Productions s.r.l) and xylazine (10 mg/kg; Rompun—Bayer s.p.a.). Pupils were dilated with 0.5% Tropicamide (Visumidriatic—Visufarma s.p.a., Roma, Italy) and 0.04% Rho (Rompun—Bayer s.p.a.). Pupils were dilated with 0.5% Tropicamide (Visumidriatic—Visufarma s.p.a., Roma, Italy) and 0.04% Rho (Rompun—Bayer s.p.a.). Pupils were dilated with 0.5% Tropicamide (Visumidriatic—Visufarma s.p.a., Roma, Italy) and 0.04% Rho (Rompun—Bayer s.p.a.). Pupils were dilated with 0.5% Tropicamide (Visumidriatic—Visufarma s.p.a., Roma, Italy). Data were acquired at a sampling frequency of 4096 Hz, coded with 16 bits and filtered between 5 and 70 Hz. Flash stimuli, with intensity of 231 mJ and duration of 10 μs, were delivered to both eyes at a frequency of 0.5 Hz with a Flash10s photo stimulator (Micromed s.p.a.) placed 15 cm from the eyes. For each session, 6 averages of 10 trains each were used for measuring the amplitude of a-wave (baseline to negative a-wave peak) and b-wave (a-wave peak to positive b-wave peak). a-wave and b-wave amplitudes were normalized on the ERG recorded from the non-electroporated LE of the same mouse. Rho+/− mice electroporated at P0 as above were recorded at 1 month of age. Normalized ERG amplitudes were analyzed using one-way ANOVA (three mice strains at one time point) followed by LSD post hoc test.

Quantifications and statistical analysis

To the exception electrophysiological measurements (see earlier), expression or indel data are represented as the mean of biological replicates and the variation as standard standard deviation (SD). For each experiment, “n” indicates the number of independent cultures used. Statistical significance of differences between control and target data sets was determined with one-way ANOVA and post hoc analysis (Graph Pad Prism); differences with P < 0.05 were considered statistically significant.

Author Contributions

S.G.G. and V.B. conceived and planned the experiments; S.G.G. performed the experiments and analyzed data; M.L. contributed in gene cloning, sequencing and lentiviral particles production; L.M. and T.C. processed and stained isolated mouse retinae; D.A. and M.A. carried out and elaborated the Sanger sequencing data; V.C., G.D. and L.L. performed and analyzed the ERG recordings; V.B. supervised and supported the project and wrote the paper together with S.G.G.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We thank Dr A. Lombardo, K. Joung, V. Marigo, A. Recchia, E. Van Anken, M. D’Antonio and V. Ranganathan for helpful discussion and sharing of reagents. We are thankful to the FRACtal core facility for expert supervision in flow-cytometry.

Conflict of Interest statement. None declared.

Funding

This work was supported by the European Research Council (AdERC #340527) and Fondazione Roma (Call 2013, Retinitis pigmentosa). Funding to pay the Open Access publication charges for this article was provided by the European Research Council (ERC).

References

1. Hamel, C. (2006) Retinitis pigmentosa. Orphanet. J. Rare Dis., 1, 40.
2. Kennan, A., Aherne, A. and Humphries, P. (2005) Light in retinitis pigmentosa. Trends Genet., 21, 103–110.
3. Daiger, S.P., Bowne, S.J. and Sullivan, L.S. (2007) Perspective on genes and mutations causing retinitis pigmentosa. Arch. Ophthalmol., 125, 151–158.
4. Wang, D.Y., Chan, W.M., Tam, P.O.S., Baum, L., Lam, D.S.C., Chong, K.K.L., Fan, B.J. and Pang, C.P. (2005) Gene mutations
in retinitis pigmentosa and their clinical implications. Clin. Chim. Acta, 351, 5–16.

5. Ayuso, C., García-Sandoval, B., Najera, C., Valverde, D., Carballo, M. and Antinolo, G. (2008) Retinitis pigmentosa in Spain. The Spanish multicentric and multidisciplinary group for research into retinitis pigmentosa. Clin. Genet., 48, 120–122.

6. Sullivan, L.S., Bowne, S.J., Birch, D.G., Hughbanks-Wheaton, D., Heckenlively, J.R., Lewis, R.A., Garcia, C.A., Ruiz, R.S., Blanton, S.H. and Northrup, H. (2006) Prevalence of disease-causing mutations in families with autosomal dominant retinitis pigmentosa: a screen of known genes in 200 families. Investig. Ophthalmol. Vis. Sci., 47, 3052–3064.

7. Milla, E., Maseras, M., Martínez-Gimeno, M., Gamundi, M.J., Assaf, H., Esmerado, C. and Carballo, M. (2002) [Genetic and molecular characterization of 148 patients with autosomal dominant retinitis pigmentosa (ADRP)]. Arch. Soc. Esp. Oftalmol., 77, 481–484.

8. Ziviello, C., Simonelli, F., Testa, F., Anastasi, M., Marzoli, S.B., Falsini, B., Ghiglione, D., Macaluso, C., Manito, M.P., Garré, C. et al. (2005) Molecular genetics of autosomal dominant retinitis pigmentosa (ADRP): a comprehensive study of 43 Italian families. J. Med. Genet., 42, e47.

9. Tee, J.J.L., Smith, A.J., Hardcastle, A.J. and Michaelides, M. (2016) RPGR-associated retinopathy: clinical features, molecular genetics, animal models and therapeutic options. Br. J. Ophthalmol., 100, 1022–1027.

10. Iannaccone, A., Man, D., Waseem, N., Jennings, B.J., Iannaccone, A., Man, D., Waseem, N., Jennings, B.J., Dryja, T.P., McGee, T.L., Hahn, L.B., Cowley, G.S., Olsson, J.E., Falsini, B., Ghiglione, D., Macaluso, C., Manito, M.P., Garré, C. et al. (2005) Molecular genetics of autosomal dominant retinitis pigmentosa (ADRP): a comprehensive study of 43 Italian families. J. Med. Genet., 42, e47.

11. Dryja, T.P., McGee, T.L., Hahn, L.B., Cowley, G.S., Olsson, J.E., Reichel, E., Sandberg, M.A. and Berson, E.L. (1990) Mutations within the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa. N. Engl. J. Med., 323, 1302–1307.

12. Sung, C.H. and Chuang, J.Z. (2010) The cell biology of vision. Hum. Mol. Genet., 19, 953–963.

13. Tam, B.M. and Moritz, O.L. (2006) Characterization of rhodopsin P23H-induced retinal degeneration in a Xenopus laevis model of retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci., 47, 3234–3241.

14. Murray, A.R., Vuong, L., Brobst, D., Fliesler, S.J., Peachey, N.S., Gorbatyuk, M.S., Naash, M.I. and Al-Ubaidi, M.R. (2015) Glycosylation of rhodopsin is necessary for its stability and incorporation into photoreceptor outer segment discs. Hum. Mol. Genet., 24, 2709–2723.

15. Naash, M.I., Hollfield, J.G., al-Ubaidi, M.R. and Baehr, W. (1993) Simulation of human autosomal dominant retinitis pigmentosa in transgenic mice expressing a mutated murine opsin gene. Proc. Natl. Acad. Sci. U. S. A., 90, 5499–5503.

16. Orhan, E., Dalkara, D., Neuli, M., Lechauve, C., Michiels, C., Picaud, S., Leveillard, T., Sahel, J.A., Naash, M.I., LaVail, M.M. et al. (2015) Genotypic and phenotypic characterization of P23H line 1 rat model. PLoS One, 10, 1–21.

17. Haeri, M. and Knox, B.E. (2012) Rhodopsin mutant P23H destabilizes rod photoreceptor disk membranes. PLoS One, 7, e30101.

18. Sakami, S., Kolesnikov, A.V., Kefalov, V.J. and Palczewski, K. (2014) P23H opsin knock-in mice reveal a novel step in retinal rod disc morphogenesis. Hum. Mol. Genet., 23, 1723–1741.

19. den Hollander, A.I., Roepman, R., Koenenkoop, R.K. and Cremers, F.P.M. (2008) Leber congenital amaurosis: genes, proteins and disease mechanisms. Prog. Retin. Eye Res., 27, 391–419.

20. Bainbridge, J.W.B., Smith, A.J., Barker, S.S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G.E., Stockman, A., Bhattacharya, S.S. et al. (2008) Effect of gene therapy on visual function in Leber’s congenital amaurosis. N. Engl. J. Med., 358, 2231–2239.

21. Maguire, A.M., Simonelli, F., Pierce, E.A., Pugh, E.N., Mingozzi, F., Bennicelli, J., Banfi, S., Marshall, K.A., Testa, F., Surace, E.M. et al. (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. N. Engl. J. Med., 358, 2240–2248.

22. Hauswirth, W.W., Aleman, T.S., Kaushal, S., Cideciyan, A.V., Schwartz, S.B., Wang, L., Conlon, T.J., Boye, S.L., Flotte, T.R., Byrne, B.J. et al. (2008) Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. Hum. Gene Ther., 19, 979–990.

23. Georgiadis, A., Duran, Y., Ribeiro, J., Abeille-Hervas, L., Robbie, S.J., Sünkel-Laing, B., Fourali, S., Gonzalez-Cordero, A., Cristante, E., Michaelides, M. et al. (2016) Development of an optimized AAV2/5 gene therapy vector for Leber congenital amaurosis owing to defects in RPE65. Gene Ther., 23, 857–862.

24. Ali, R.R., Sarra, G.M., Stephens, C., Alwis, M.D., Bainbridge, J.W., Munro, P.M., Fauser, S., Reichel, M.B., Kinnon, C., Hunt, D.M. et al. (2000) Restoration of photoreceptor ultrastructure and function in retinal degeneration slow mice by gene therapy. Nat. Genet., 25, 306–310.

25. Ran, F.A., Hsu, P.P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat. Protoc., 8, 2281–2308.

26. Ran, F.A., Cong, L., Yan, W.X., Scott, D.A., Gootenberg, J.S., Kriz, A.I., Zetsche, B., Shalem, O., Wu, X., Makarova, K.S. et al. (2015) In vivo genome editing using Staphylococcus aureus Cas9. Nature, 520, 186–190.

27. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., Van Der Oost, J., Regev, A. et al. (2015) Cas9 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell, 163, 759–771.

28. Kleinstiver, B.P., Prew, M.S., Tsai, S.Q., Topkar, V.V., Nguyen, N.T., Zheng, Z., Gonzales, A.P., Li, Z., Peterson, R.T., Yeh, J.R. et al. (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature, 523, 481–485.

29. Lewin, A.S., Drenser, K.A., Hauswirth, W.W., Nishikawa, S., Yasumura, D., Flannery, J.G. and LaVail, M.M. (1998) Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. Nat. Med., 4, 967–971.

30. LaVail, M.M., Yasumura, D., Matthews, M.T., Drenser, K.A., Flannery, J.G., Lewin, A.S. and Hauswirth, W.W. (2000) Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy. Proc. Natl. Acad. Sci. U. S. A., 97, 11488–11493.

31. Mao, H., Gorbatyuk, M.S., Rossmillner, B., Hauswirth, W.W. and Lewin, A.S. (2012) Long-term rescue of retinal structure and function by rhodopsin RNA replacement with a single adeno-associated viral vector in P23H RHO transgenic mice. Hum. Gene Ther., 23, 356–366.

32. Tessitore, A., Parisi, F., Denti, M.A., Allocco, M., Di Vicino, U., Domenici, L., Bozzoni, I. and Auricchio, A. (2006) Preferential silencing of a common dominant rhodopsin mutation does not inhibit retinal degeneration in a transgenic model. Mol. Ther., 14, 692–699.
33. Mussolino, C., Sanges, D., Marrocco, E., Bonetti, C., Di Vicino, U., Marigo, V., Auricchio, A., Meroni, G. and Surace, E.M. (2011) Zinc-finger-based transcriptional repression of rhodopsin in a model of dominant retinitis pigmentosa. EMBO Mol. Med., 3, 118–128.

34. Sakami, S., Maeda, T., Bereta, G., Okano, K., Golczak, M., Sumaroka, A., Roman, A.J., Cideciyan, A.V., Jacobson, S.G. and Palczewski, K. (2011) Probing mechanisms of photoreceptor degeneration in a new mouse model of the common form of autosomal dominant retinitis pigmentosa due to P23H opsin mutations. J. Biol. Chem., 286, 10551–10567.

35. Nelson, C.E., Hakim, C.H., Ousterout, D.G., Thakore, P.I., Moreb, E.A., Rivera, R.M.C., Madhavan, S., Pan, X., Ran, F.A., Yan, W.X. et al. (2016) In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science, 351, 403–407.

36. Tabebordbar, M., Zhu, K., Cheng, J.K.W., Chew, W.L., Widrick, J.J., Yan, W.X., Maesner, C., Wu, E.Y., Xiao, R., Ran, F.A. et al. (2016) In vivo gene editing in dystrophic muscle and muscle stem cells. Science, 351, 403–407.

37. Brinkman, E.K., Chen, T., Amendola, M. and Van Steensel, B. (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res., 42, e168.

38. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. et al. (2013) Multiplex genome engineering using CRISPR/Cas system. Science, 339, 819–823.

39. Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O. et al. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol., 31, 827–832.

40. González, F., Zhu, Z., Shi, Z.D., Lelli, K., Verma, N., Li, Q.V. and Huangfu, D. (2014) An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. Cell Stem Cell, 15, 215–226.

41. Cradick, T.J., Qiu, P., Lee, C.M., Fine, E.J. and Bao, G. (2014) COSMID: a web-based tool for identifying and validating CRISPR/Cas off-target sites. Mol. Ther. Nucleic Acids, 3, e214.

42. Matsuda, T. and Cepko, C.L. (2004) Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc. Natl. Acad. Sci. U. S. A., 101, 16–22.

43. Chiang, W., Kroeger, H., Sakami, S., Messah, C., Yasumura, D., Matthes, M.T., Coppringer, J.A., Palczewski, K., Lavail, M.M. and Lin, J.H. (2015) Robust endoplasmic reticulum-associated degradation of rhodopsin precedes retinal degeneration. Mol. Neurobiol., 52, 679–695.

44. Illing, M.E., Rajan, R.S., Bence, N.F. and Kopito, R.R. (2002) A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system. J. Biol. Chem., 277, 34150–34160.

45. Saliba, R.S., Munro, P.M.G., Luthert, P.J. and Cheetham, M.E. (2002) The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation. J. Cell Sci., 115, 2907–2918.

46. Roman-Sanchez, R., Wensel, T.G. and Wilson, J.H. (2016) Nonsense mutations in the rhodopsin gene that give rise to mild phenotypes trigger mRNA degradation in human cells by nonsense-mediated decay. Exp. Eye Res., 145, 444–449.

47. Lin, J.H., Li, H., Yasumura, D., Cohen, H.R., Zhang, C., Panning, B., Shokat, K.M., LaVail, M.M. and Walter, P. (2007) IRE1 signaling affects cell fate during the unfolded protein response. Science, 318, 944–949.

48. Carter-Dawson, L.D. and LaVail, M.M. (1979) Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. J. Comp. Neurol., 188, 245–262.

49. Kennedy, B. and Malicki, J. (2009) What drives cell morphogenesis: a look inside the vertebrate photoreceptor. Dev. Dyn., 238, 2115–2138.

50. Deverman, B.E., Pravdo, P.L., Simpson, B.P., Kumar, S.R., Chan, K.Y., Banerjee, A., Wu, W.L., Yang, B., Huber, N. and Pasca, S.P. (2016) Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. Nat. Biotechnol., 34, 204–209.

51. Dalkara, D., Byrne, L.C., Klimczak, R.R., Visel, M., Yin, L., Merigan, W.H., Flannery, J.G. and Schaffer, D.V. (2013) In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. Sci. Transl. Med., 5, 189ra76–189ra76.

52. Körbelin, J., Doghevria, G., Michelfelder, S., Ridder, D.A., Hunger, A., Wenzel, J., Seissmann, H., Lampe, M., Bannach, J. and Asparakis, M. et al. (2016) A brain microvasculature endothelial cell-specific viral vector with the potential to neurovascular and neurological diseases. EMBO Mol. Med., 8, 609–625.

53. O’Reilly, M., Palfi, A., Chadderton, N., Millington-Ward, S., Ader, M., Cronin, T., Tuohy, T., Auricchio, A., Hildinger, M. and Rivnay, A. et al. (2007) RNA interference-mediated suppression and replacement of human rhodopsin in vivo. Am. J. Hum. Genet., 81, 127–135.

54. Morabito, C., Giannelli, S.G., Ordazzo, G., Bido, S., Castoldi, V., Indrigo, M., Cabassi, T., Cattaneo, S., Luoni, M., Cancelleri, C. et al. (2017) AAV-PHP.B-mediated global-scale expression in the mouse nervous system enables GBA1 gene therapy for wide protection from synucleinopathies. Mol. Ther., 25, 2727–2742.

55. Pinello, L., Canver, M.C., Hoban, M.D., Orkin, S.H., Kohn, D.B., Bauer, D.E. and Yuan, G.C. (2016) Analyzing CRISPR genome-editing experiments with CRISPResso. Nat. Biotechnol., 34, 695–697.

56. Latella, M.C., Di Salvo, M.T., Cocichiarella, F., Benati, D., Grisendi, G., Comitato, A., Marigo, V. and Roccia, A. (2016) In vivo editing of the human mutant rhodopsin gene by electroporation of plasmid-based CRISPR/Cas9 in the mouse retina. Mol. Ther. Nucleic Acids, 5, e389.

57. Bakondi, B., Lv, W., Lu, B., Jones, M.K., Tsi, Y., Kim, K.J., Levy, R., Akhtar, A.A., Breuning, J.J., Swendsen, C.N. et al. (2015) In vivo CRISPR/Cas9 gene editing corrects retinal dystrophy in the S334ter-3 rat model of autosomal dominant retinitis pigmentosa. Mol. Ther., 24, 556–563.

58. McGrew, D.A. and Hedstrom, L. (2012) Towards a pathologial mechanism for MPD1H-linked retinitis pigmentosa. Adv. Exp. Med. Biol., 723, 539–545.

59. Wen, Y., Locke, K.G., Klein, M., Bowne, S.J., Sullivan, L.S., Ray, J.W., Daiger, S.P., Birch, D.G. and Hughsbanks-Wheaton, D.K. (2011) Phenotypic characterization of 3 families with autosomal dominant retinitis pigmentosa due to mutations in KHHHL7. Arch. Ophthal., 129, 1475–1482.

60. Swiec, L., Heidenreich, M., Banerjee, A., Habib, N., Li, Y., Trombeta, J., Sur, M. and Zhang, F. (2015) In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat. Biotechnol., 33, 102–106.

61. Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S. and Kim, J. (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res., 24, 132–141.
62. Tsai, S.Q., Zheng, Z., Nguyen, N.T., Liebers, M., Topkar, V.V., Thapar, V., Wyvekens, N., Khayer, C., Iafrate, A.J., Le, L.P. et al. (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat. Biotechnol., 33, 187–197.

63. Wang, X., Wang, Y., Wu, X., Wang, J., Wang, Y., Qiu, Z., Chang, T., Huang, H., Lin, R. and Yee, J. (2015) Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. Nat. Biotechnol., 33, 175–178.

64. Merienne, N., Vachey, G., de Longprez, L., Meunier, C., Zimmer, V., Perriard, G., Canales, M., Mathias, A., Herrgott, L., Beltraminelli, T. et al. (2017) The self-inactivating KamiCas9 system for the editing of CNS disease genes. Cell Rep., 20, 2980–2991.

65. Shin, J., Jiang, F., Liu, J.J., Bray, N.L., Rauch, B.J., Baik, S.H., Nogales, E., Bondy-Denomy, J., Corn, J.E. and Doudna, J.A. (2017) Disabling Cas9 by an anti-CRISPR DNA mimic. Sci. Adv., 3, e1701620.

66. Caiazzo, M., Giannelli, S., Valente, P., Lignani, G., Carissimo, A., Sessa, A., Colasante, G., Bartolomeo, R., Massimino, L., Ferroni, S. et al. (2015) Direct conversion of fibroblasts into functional astrocytes by defined transcription factors. Stem Cell Reports, 4, 25–36.

67. Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Thomas, C.S. and Wernig, M. (2010) Direct conversion of fibroblasts to functional neurons. Nature, 77, 7–8.

68. Giannelli, S.G., Demontis, G.C., Pertile, G., Rama, P. and Broccoli, V. (2011) Adult human Müller glia cells are a highly efficient source of rod photoreceptors. Stem Cells, 29, 344–356.

69. Langmead, B., Trapnell, C., Pop, M. and Salzberg, S. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol., 10, R25.

70. Back, S.H., Lee, K., Vink, E. and Kaufman, R.J. (2006) Cytoplasmic IRE1alpha-mediated XBP1 mRNA splicing in the absence of nuclear processing and endoplasmic reticulum stress. J. Biol. Chem., 281, 18691–18706.

71. Pinto, L.H., Vitaterna, M.H., Siepka, S.M., Shinomura, K., Lumayag, S., Baker, M., Fenner, D., Mullins, R.F., Sheffield, V.C., Stone, E.M. et al. (2004) Results from screening over 9000 mutation-bearing mice for defects in the electroretinogram and appearance of the fundus. Vision Res., 44, 3335–3345.

72. Brandli, A. and Stone, J. (2015) Using the electroretinogram to assess function in the rodent retina and the protective effects of remote limb ischemic preconditioning. J. Vis. Exp., 9, e52658.