shRNA knockdown of *guanylate cyclase 2e* or *cyclic nucleotide gated channel alpha 1* increases photoreceptor survival in a cGMP *phosphodiesterase* mouse model of retinitis pigmentosa

Joaquin Tosi [a, b], Richard J. Davis [a, b], Nan-Kai Wang [a, b, c, d], Matthew Naumann [a, b], Chyuan-Sheng Lin [a], Stephen H. Tsang [a, b, *]

[a] *Bernard & Shirlee Brown Glaucoma Laboratory, Department of Pathology & Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY, USA*
[b] *Edward S. Harkness Eye Institute, Columbia University, New York, NY, USA*
[c] *Department of Ophthalmology, Chang Gung Memorial Hospital at Linkou, Taipei, Taiwan*
[d] *Chang Gung University College of Medicine, Taoyuan, Taiwan*

Abstract

In vertebrate rods, dark and light conditions produce changes in guanosine $3',5'$-cyclic monophosphate (cGMP) and calcium (Ca^{2+}) levels, which are regulated by the opposing function of several proteins. During the recovery of a bright flash, guanylate cyclase (GUCY) helps raise cGMP to levels that open cGMP-gated calcium sodium channels (CNG) to increase Na^+ and Ca^{2+} influx in the outer segment. In contrast, light activates cGMP phosphodiesterase 6 (PDE6) causing rapid hydrolysis of cGMP, CNG closure, and reduced Na^+ and Ca^{2+} levels. In *Pde6b* mouse models of retinitis pigmentosa (RP), photoreceptor death is preceded by abnormally high cGMP and Ca^{2+} levels, likely because of continued synthesis of cGMP by guanylate cyclases and unregulated influx of Ca^{2+} to toxic levels through CNG channels. To reverse the effects of *Pde6b* loss of function, we employed an shRNA knockdown approach to reduce the expression of *Gucy2e* or *Cnga1* in *Pde6b*^{H620Q} photoreceptors prior to degeneration. *Gucy2e*- or *Cnga1*-shRNA lentiviral-mediated knockdown GUCY2E and CNGA1 expression increase visual function and photoreceptor survival in *Pde6b*^{H620Q} mice. We demonstrated that effective knockdown of GUCY2E and CNGA1 expression to counteract loss of PDE6 function may develop into a valuable approach for treating some patients with RP.

Keywords: PDE, cGMP-phosphodiesterase ● cGMP, guanosine $3',5'$-cyclic monophosphate ● GNAT1, guanine nucleotide-binding protein G(t) subunit alpha-1 ● *Pde6b*^{H620Q}, a mouse line carrying a missense mutation in *Pde6b* ● (GUCY2e), (GUCY2f) guanylate cyclases 2e and 2f ● CNGA1, cGMP-gated cation channels ● shRNA, short hairpin RNA ● lentivirus ● ERG, electroretinogram ● ROS, rod outer segments ● gene therapy

Introduction

Photoreceptor cell death is the dominant pathological feature in many retinal diseases, including retinitis pigmentosa (RP) and age-related macular degeneration (AMD) [1–3]. About 36,000 cases worldwide of simplex and familial RP are caused by loss of function mutations in cGMP phosphodiesterase (PDE6) [4–7]. Although significant advances in our understanding of RP have been made, the exact interplay between defective PDE6 and the onset of RP pathogenesis remains poorly understood.

Several *Pde6b* mouse models of RP, including *Pde6b*^{rd1}, *Pde6b*^{rd10} and *Pde6b*^{H620Q}, have been used to study the mechanisms involved in the disease [8–10]. Loss of PDE6 enzyme activity has been shown to result in increased levels of cGMP in *Pde6b*^{rd1} mice

*Correspondence to: Stephen H. TSANG, M.D., Ph.D.,
Edward S. Harkness Eye Institute,
160 Fort Washington Avenue, Research Annex,
Room 513, New York, NY 10032, USA.
Tel.: +1-212-342-1189
Fax: +1-212-305-4987
E-mail: sht2@columbia.edu

[11–15] and high Ca^{2+} in $Pde6b^{H620Q}$ mice [8]. It has been hypothesized that $Pde6b$ loss of function leads to high cGMP and consequently excessive Ca^{2+} influx, which is toxic to photoreceptors [8, 15–17].

During rod phototransduction, cGMP and Ca^{2+} are regulated by PDE6, guanylate cyclase (GUCY2), and cGMP-gated Na^+/Ca^{2+} (CNG) channels. The level of cGMP is controlled by opposing activities of PDE6 and GUCY2 and, at sufficiently high levels, CNG channels open to allow increased Ca^{2+} influx into the rod outer segment (ROS). Thus, during photoexcitation and recovery, changes in cGMP and Ca^{2+} levels occur in parallel. Light stimulates PDE6 activation, rapid reduction in cGMP, closure of CNG channels and reduced Ca^{2+} influx. After a light flash, PDE6 is inhibited, and cGMP and Ca^{2+} are restored to nominal levels by GUCY2 activity and opening of CNG channels, respectively [18–36].

Knowledge gained from the $Pde6b$ models has been used for testing different drug and gene therapies [8, 37–43]. Attempts to prevent or block photoreceptor death performed with Ca^{2+} channel blockers has showed limited efficacy. d-*cis*-Diltiazem was tested for the ability to reduce Ca^{2+} concentrations without affecting cGMP and was reported to prevent the loss of both rods and cones in $Pde6^{rd1}$ mice [44, 45]. However, the extent of protection originally reported has been controversial [46–49]. The specificity of d-*cis*-diltiazem for rod CNG channels has been demonstrated to be lower than l-*cis*-diltiazem, suggesting this isoform might have a greater effect. However, significant rescue of $Pde6b$ mutant photoreceptors performed with l-*cis*-diltiazem is uncertain [44–50].

Gene therapy approaches to increase photoreceptor survival has also been employed. The common denominator in these studies is the use of a vector designed to express PDE6β in $Pde6b$ mutant photoreceptors. Different vectors based on adenovirus, adeno-associated virus, lentivirus, simian immunodeficiency virus and herpes simplex virus have been tested for their ability to delay degeneration. Although significant morphological and functional restoration has been reported, incomplete long-term rescue was achieved in all cases [8, 38–41, 43].

In this report, we took an alternative approach to remedy the condition of excess cGMP and Ca^{2+} in $Pde6b$ mutant mice. We employed lentivirus shRNA technology to knockdown the expression of $Gucy2e$ and $Cnga1$ to rescue retinal degeneration. We injected viral particles sub-retinally in newborn (P5) $Pde6b^{H620Q}$ mutant and C57BL/6J mice, measured changes in gene expression, and photoreceptor function and survival. Our results demonstrate this approach is a viable alternative to previously tested pharmacological and gene therapy methods.

Materials and methods

Mouse lines and husbandry

Mice were used in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology, as well as the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience. $Pde6b^{H620Q}$ mice used in this experiment were bred from a colony of mice that has been previously reported [8, 51]. All $Pde6b^{H620Q}$ mice analysed in this study are homozygotes and will be referred to as $Pde6b^{H620Q}$ mutants or $Pde6b^{H620Q}$ mice. $Pde6b^{H620Q}$ and $Pde6b^{rd1}$ strains are in the C3H background and age-matched C57BL/6J (B6) mice were used as controls (The Jackson Laboratory, Bar Harbor, ME, USA).

Histochemical analyses

Mice were sacrificed and haematoxylin and eosin retinal sections were obtained as described [19, 20, 52, 53]. The number and morphology of photoreceptors of lentiviral shRNA injected eyes were compared to control eyes. Briefly, quantification of photoreceptor nuclei was conducted on several sections containing the optic nerve as follows: the distance between the optic nerve and the ciliary body was divided into four quadrants and three rows of nuclei were counted within each single quadrant. Averages and standard deviations were calculated from 10-30 animals for each time-point. The corneal scar at the 6 o'clock position allowed the identification of the injected inferior retinal half of the sample analysed. Sectioning proceeded along the long axis of the segment so that each section contained upper and lower retina as well as posterior pole.

Immunoblot analysis

Retinas were homogenized in 10% sodium dodecyl sulphate (SDS) by brief sonication and denatured at 100°C for 5 min. Following centrifugation, total protein content per sample was measured by the DC Protein Assay method (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated by SDS polyacrylamide gel electrophoresis. Samples were then transferred to nitrocellulose membranes, which were blocked in 3% bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 150 mmol/l NaCl, 100 mmol/l Tris (pH 7.4) and 0.5% Tween-20 (BSA-TTBS). Membranes were incubated with either rabbit antibody to the GUCY2E (1:500, kindly provided by Alexander Dizhoor), CNGA1 (1:12, kindly provided by Robert Molday), mouse monoclonal IgG_1 to rhodopsin (1:10,000, 1D4, sc-57432; Santa Cruz Biotechnology) or mouse monoclonal IgG_{2b} to α Tubulin (1:500, 6-11B-1, sc-23950; Santa Cruz Biotechnology) antibodies in BSA-TTBS. After washing in TTBS, filters were incubated with either goat anti-rabbit conjugated horseradish peroxidase secondary antibodies (1:10,000, sc-2004; Santa Cruz Biotechnology) or goat anti-mouse IgG- horseradish peroxidase secondary antibodies (1:10,000, sc-2005; Santa Cruz Biotechnology). After washing, antibody complexes were visualized by chemilluminescence detection (Immobilon Western, Millipore Corporation, Billerica, MA, USA) and Kodak BioMax film (Kodak, Rochester, NY, USA).

Transduction of lentiviral vectors

To knockdown $Gucy2e$ or $Cnga1$ expression, we injected shRNA lentivirus (1.5 μl, ~2 × 10^7 ml transducing units (TU) per ml in DMEM with 10% heat-inactivated fatal bovine serum and penicillin-streptomycin) sub-retinally into the right eye of $Pde6b^{H620Q}$ mice at P5 ($n = 75$ per gene) and in age-matched control C57BL/6J ($n = 75$). Virus particles were injected at the 6 o'clock position and at 1.5 mm from the limbus, producing a

Table 1 Table shows the shRNA lentiviral plasmids clone sequence, clone ID and titre used to suppress the expression of guanylate cyclase 2e (*Gucy2e*) (**A**) and cyclic nucleotide gated channel alpha 1 (Cnga1) (**B**)

A		
TRC shRNA target set clone sequence	**Clone ID**	**Titre**
CCGGGCCTAGAGTTAGAGTAGTGATCTCGAGATCACTACTCTAACTCTAGGCTTTTT	NM_008192.1-717s1c1	1.7×10^7 TU/ml
CCGGCGGTGCCCATGATGTCTATAACTCGAGTTATAGACATCATGGGCACCGTTTTT	NM_008192.1-2757s1c1	1.8×10^7 TU/ml
CCGGCCTGTTCAAGAGCATCAACAACTCGAGTTGTTGATGCTCTTGAACAGGTTTTT	NM_008192.1-2418s1c1	2.3×10^7 TU/ml
CCGGGCCTGAATCGTGACTTTGGTTCTCGAGAACCAAAGTCACGATTCAGGCTTTTT	NM_008192.1-257s1c1	2.6×10^7 TU/ml
B		
TRC shRNA target set clone sequence	**Clone ID**	**Titre**
CCGGCCTGACAAACTAAGGGCAGAACTCGAGTTCTGCCCTTAGTTTGTCAGGTTTTTG	NM_007723.1-1382s1c1	2.0×10^7 TU/ml
CCGGCCGATATGTTTGTACGAACAACTCGAGTTGTTCGTACAAACATATCGGTTTTTG	NM_007723.1-648s1c1	2.6×10^7 TU/ml
CCGGGCTATCAAACAGTACATGAATCTCGAGATTCATGTACTGTTTGATAGCTTTTTG	NM_007723.1-1256s1c1	2.9×10^7 TU/ml
CCGGGCCTGTATCTTGGCTGAATATCTCGAGATATTCAGCCAAGATACAGGCTTTTTG	NM_007723.1-1934s1c1	3.4×10^7 TU/ml
CCGGGCTGTTAAGGATCTCTCGAATCTCGAGATTCGAGAGATCCTTAACAGCTTTTTG	NM_007723.1-832s1c1	3.5×10^7 TU/ml

TRC: the RNAi consortium.

sub-retinal bubble in mid-periphery retina. The left eye was injected with *CMV::EGFP* (1.5 μl, 2×10^7 TU/ml) lentivirus or saline sub-retinally and used as control. Anaesthesia and surgery were performed as described [8].

shRNA vectors deliver a short 21-nucleotide stem hairpin RNA duplexes, designed to decrease the expression of retinal *Gucy2e* and *Cnga1*. The lentiviral transduction particles were made from sequence-verified shRNA lentiviral plasmid vectors for mouse genes (SHVRS, Mission® Lentiviral Transduction Particles; Sigma-Aldrich, St. Louis, MO, USA). Clone sets injected consist of four *Gucy2e* and five *Cnga1* individual clones targeting different regions of the mRNA for each gene. Clone sets used for this analysis are shown in Table 1. Employing different clones allow us to screen for the most effective shRNA for gene knockdown. We expect at least a knockdown efficiency of >70% with one construct from each gene targeted [54].

Electroretinograms (ERGs)

Visual function was evaluated as described [55–60]. ERGs were performed weekly from P35 to P90 to assess global retina function in injected and control eyes. We measured ERG b-wave enhancement of rod, maximal and cone responses from shRNA-lentivirus transduced C57BL/6J and *Pde6b*[H620Q] mice. Enhancement is defined as the difference in maximum ERG responses of transduced and control fellow eyes, in μV.

Results

The efficiency of shRNA knockdown to ameliorate degeneration was assessed by biochemical, histological and physiological measurements. Initially, four *Gucy2e* and five *Cnga1* shRNA

clones were pre-screened for the ability to rescue degeneration. Each clone was injected into the sub-retinas of a litter of pups. At P56, injected and control retinas were compared histologically and the ability of the clone to rescue degeneration was determined. The most efficient shRNA-*Gucy2e* and shRNA-*Cnga1* clone was selected for further study (Table 1).

Biochemical assessment: effective and specific knockdown of GUCY2E and CNGA1 expression

The ability of shRNA-*Gucy2e* and shRNA-*Cnga1* viral clones to knockdown protein expression was tested in C57BL/6J mice. Protein levels in retinal lysates were tested by immunoblotting 15 and 60 days after injection. A significant difference in GUCY2E and CNGA1 expression levels was observed between control and shRNA-*Gucy2e* and shRNA-*Cnga1* retinas, respectively (Fig. 1A and B). In contrast, expression of the rod-specific protein, GNAT1, was not significantly different between experimental and control retinas. shRNA-*Cnga1* did not significantly reduce GUCY2E expression.

Histological assessment: C57BL/6J photoreceptors are not detectably affected by shRNA-*Gucy2e* and shRNA-*Cnga1* transduction

To determine if reduction of GUCY2E and CNGA1 expression results in changes in photoreceptor numbers or morphology,
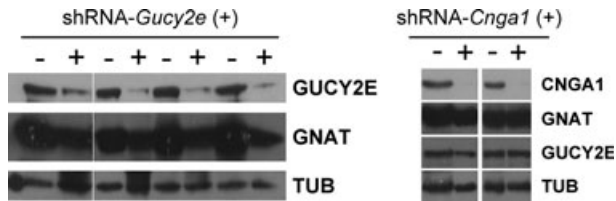
Fig. 1 Immunoblotting of retinal lysates from P60 wild-type C57BL/6J mice transduced with shRNA-*Gucy2e* (**A**, +), shRNA-*Cnga1* (**B**, +) or shRNA-*GFP* control lentivirus (**A** and **B**, −). The antibodies used were anti-GUCY2E, anti-GNAT1 and anti-CNGA1. GNAT1 is a rod-specific protein, which serves as a control for photoreceptor protein content. Bottom panel is α-tubulin (TUB), which controls for protein loading (25 μg per lane).

sections from shRNA-*Gucy2e* and shRNA-*Cnga1* injected and control C57BL/6J retinas were stained by haematoxylin and eosin and compared. Quantification of photoreceptor nuclei was conducted on several sections; retinae transduced by shRNAs have nine rows of photoreceptor nuclei lining areas from the optic nerve to ora serrata, similar to control injected eyes. Ten retinae were analysed for each viral vector. C57BL/6J shRNA transduced retinae showed no statistically significant differences in retinal structure compared to controls (Table 2). Moreover, neither noticeable morphological changes nor abnormal staining patterns were observed between injected or control retinas (Fig. 2A–D).

Morphological rescue after transduction of *Pde6b^H620Q* photoreceptors after shRNA-mediated knockdown of GUCY2E or CNGA1

Because shRNA knockdown of GUCY2E expression is not associated with gross photoreceptor degeneration in P60 control mice, we tested the ability of the shRNA-*Gucy2e* vector to rescue *Pde6b* mutant photoreceptors from degeneration. Lack of outer segments along with a single row of photoreceptor nuclei is typically observed at eight weeks in *Pde6b^H620Q* mutants because of the natural retinal degeneration process. In contrast, *Pde6b^H620Q* retinae transduced by shRNA-*Gucy2e* showed the number of photoreceptor nuclei was visibly higher than controls (Fig. 3A and B). *Pde6b^H620Q* retinae transduced by shRNA-*Gucy2e* have on average four rows of photoreceptor nuclei lining areas, whereas *Pde6b^H620Q* retinae transduced by shRNA-*GFP* or saline exhibited a single row of photoreceptor nuclei without outer segments. Calculated t-tests for total averages in *Gucy2e* retinas *versus* controls gave *P*-values of 0.0211.

To evaluate whether reducing *Cnga1* expression rescued degeneration, we compared histological sections from injected and control *Pde6b^H620Q* eyes at P56. shRNA-*Cnga1* injected eyes showed regions containing OS and an average of five rows of photoreceptor nuclei, whereas control retinas showed a single row of photoreceptor nuclei with scant OS at P56 (Fig. 3C and D). Differences were quantified in both individual quadrant and total

averages (Table 2D). Calculated total averages of *Cnga1* transduced *versus* control retinas gave very highly statistically significant *P*-values of 0.0001. Together, these results show photoreceptor survival in *Pde6b^H620Q* mutants is improved for 2 months after shRNA-*Gucy2e* or shRNA-*Cnga1* transduction.

Functional assessment: effect of *Gucy2e* and *Cnga1* knockdown on photoreceptor activity in C57BL/6J controls and *Pde6b^H620Q* mice

We measured ERG responses in C57BL/6J mice to assess the effect of shRNA-*Gucy2e* or shRNA-*Cnga1* transduction on global retinal function between 1 and 3 months (Figs 4 and 6). In general, ERG function was depressed in eyes transduced with either shRNA-*Gucy2e* or shRNA-*Cnga1*, compared to control eyes transduced with shRNA-*GFP* or injected with saline. This reduction was observed in comparisons of response traces between injected and control eyes (Fig. 4) and from comparisons of averaged b-wave amplitudes for isolated rod, maximal cone-rod and isolated cone responses (Fig. 6). Although reductions in responses were noted, there was not a complete extinction of signals from the shRNA-transduced retinas up to P90.

Next, we measured global ERG responses in *Pde6b^H620Q* mice after shRNA-*Cnga1* or shRNA-*Gucy2e* transduction between 1 and 3 months (Figs 5 and 6). In contrast to the effect on C57BL/6J retinas, knockdown of both genes produced enhancement of *Pde6b^H620Q* retinal function, compared to fellow control mutant eyes. In particular, enhancement from shRNA-*Cnga1* transduction was detected from P35 to P90, whereas improved photoreceptor function from shRNA-*Gucy2e* was observed only at P35 (Fig. 6).

For shRNA-*Gucy2e*, the largest difference between experimental and control eyes were in maximal mixed rod-cone responses at P35: 105 μV *versus* 15 μV, respectively (Fig. 5). A modest improvement in b-wave peaks was also detected in isolated rod and isolated cone responses (Fig. 6). However, mice tested at P60 and P90 showed no enhancement (Figs 5 and 6).

Improved retinal function was observed from P35 to P90 in shRNA-*Cnga1* transduced *Pde6b^H620Q* mutant retinas. There was preservation of maximal ERG a-wave responses up to 60 days (Fig. 5) and isolated rod, maximal rod-cone and cone responses ERG b-wave responses up to 90 days (Fig. 6). To confirm that this effect was not because of non-specific expression of shRNAs or surgical injury response, we also tested mice injected with shRNA-*GFP* and saline. The ERGs from these mice did not reveal any statistical significant difference between injected eyes and non-injected eyes (data not shown).

Our interpretation of preserved rod-specific ERGs is that *Gucy2e* and *Cnga1* knockdown compensates for sub-normal PDE activity to prevent rods from dying because of cGMP/Ca^{2+} toxicity. Based on our studies of the *Pde6b^H620Q* mouse, mutant retinas express PDE6 with lower than normal specific activity and show diminished isolated rod-specific ERG responses, before significant degeneration [8]. Lowering the activity of GUCY2 in rods,

Table 2 Quantitative analysis of photoreceptor nuclear rows

A	Total AVG	Total S.D.	B		Total AVG	Total S.D.
shRNA-*Gucy2e*	9.31	0.255	shRNA-*Cnga1*		8.91	0.265
CONTROL	9.23	0.391	CONTROL		8.09	0.201
No. of C57BL/6J Eyes	10		*No. of C57BL/6J Eyes*		10	
P-value and statistical significance			*P*-value and statistical significance			
The two-tailed *P*-value = 0.3614			The two-tailed *P*-value = 0.2419			
No statistically significant difference			No statistically significant difference			
C	**Total AVG**	**Total S.D.**	**D**		**Total AVG**	**Total S.D.**
shRNA-*Gucy2e*	4	1.8	shRNA-*Cnga1*		4.4	0.9
CONTROL	1.1	0.3	CONTROL		1.2	0.3
No. of Pde6b^{H620Q} Eyes	30		*No. of Pde6b^{H620Q} Eyes*		30	
P-value and statistical significance			*P*-value and statistical significance			
The two-tailed *P*-value equals 0.0211			The two-tailed *P*-value is less than 0.0001			
Statistically significant			Very highly statistically significant			

The average (AVG) number of photoreceptor nuclei in each transduced eye was counted. The results are shown as means and standard deviation (S.D.).
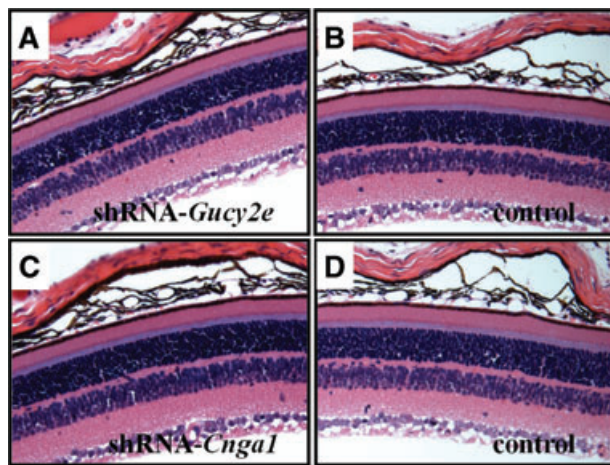

Fig. 2 shRNA knockdown has no detectable gross effects on photoreceptor survival or structure. Lentiviral shRNA targeting of *Gucy2e* (**A**) and *Cnga1* (**C**) in C57BL/6J mice exhibit identical retinal structure as control retinas (**B**, **D**) injected with saline. Eight to ten rows of photoreceptor nuclei were seen in shRNA transduced and saline injected retinae.

Fig. 3 Lentiviral shRNA targeting of *Gucy2e* (**A**) and *Cnga1* (**C**) in *Pde6b^{H620Q}* mice. At P56, the control retinae (**B**, **D**) show a single row of photoreceptors with scattered outer segments, as is typically observed in *Pde6b^{H620Q}* mutants [8] (yellow arrows). In contrast, surrounding the injection site, there are rod outer segments and eight rows of photoreceptor nuclei after shRNA-*Cnga1* and five rows of nuclei after shRNA-*Gucy2e* transduction (yellow brackets).

through knockdown of *Gucy2e,* may reduce the built-up of cGMP to a level that can be managed by the endogenous *Pde6b^{H620Q}* mutant enzyme to rescue cells from dying. Lowering the activity of CNG in *Pde6b^{H620Q}* rods, through knockdown of *Cnga1* may limit the level Ca^{2+} in *Pde6b^{H620Q}* to a level that permit longer rod survival and function.
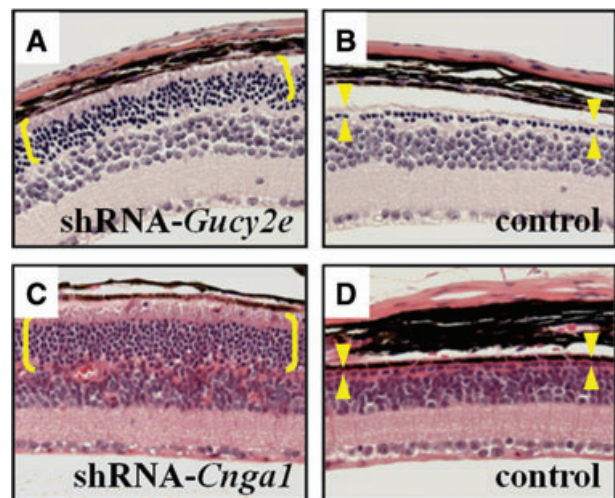
Discussion

We show delivery of shRNAs by lentiviral sub-retinal transduction results in reduction of GUCY2E and CNGA1 expression and
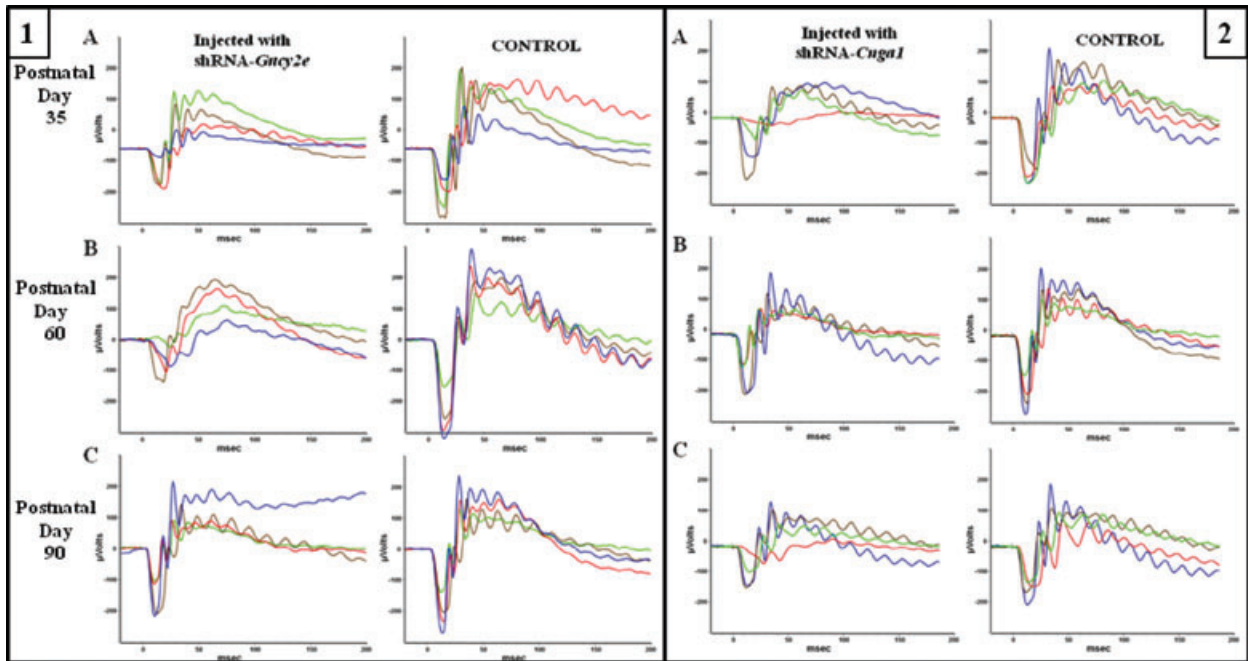
Fig. 4 Maximal dark-adapted ERG traces of C57BL/6J (wild-type) mice transduced with shRNA-*Gucy2e* (**1**) and shRNA-*Cnga1* (**2**), and corresponding control eyes at postnatal days 35, 60 and 90. ERGs were performed on both eyes simultaneously; each colour trace represented recordings from an individual subject and corresponding control.
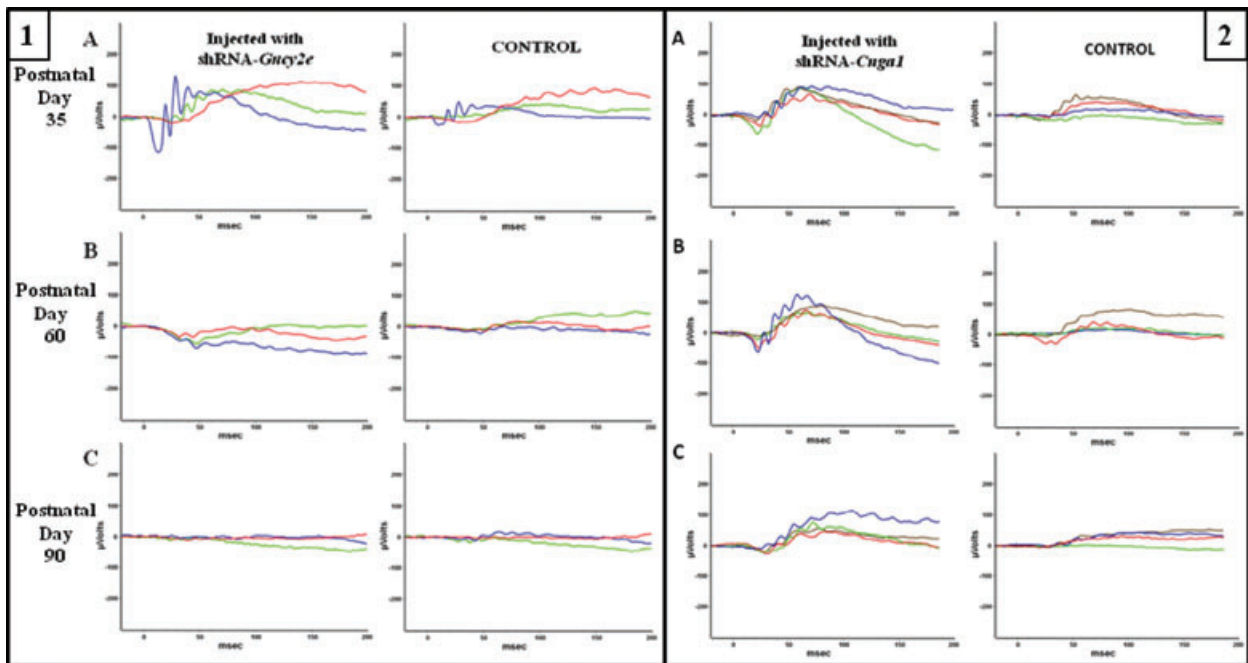

Fig. 5 Functional rescue of neuronal signaling in *Pde6b*^{H620Q} retinas transduced with shRNA- *Gucy2e* (**1**) and shRNA-*Cnga1* (**2**). Each mutant received a sub-retinal injection of shRNA-*Gucy2e* (**1**) and shRNA-*Cnga1* (**2**) lentivirus in the right eye and saline in the left eye (control) at P5. Maximal dark-adapted ERGs were then performed simultaneously on both eyes of *Pde6b*^{H620Q} mice at postnatal days 35, 60 and 90. Significant a-wave improvement are seen in shRNA transduced eyes at day 35. Each colour trace depicted recordings from an individual subject and corresponding control.
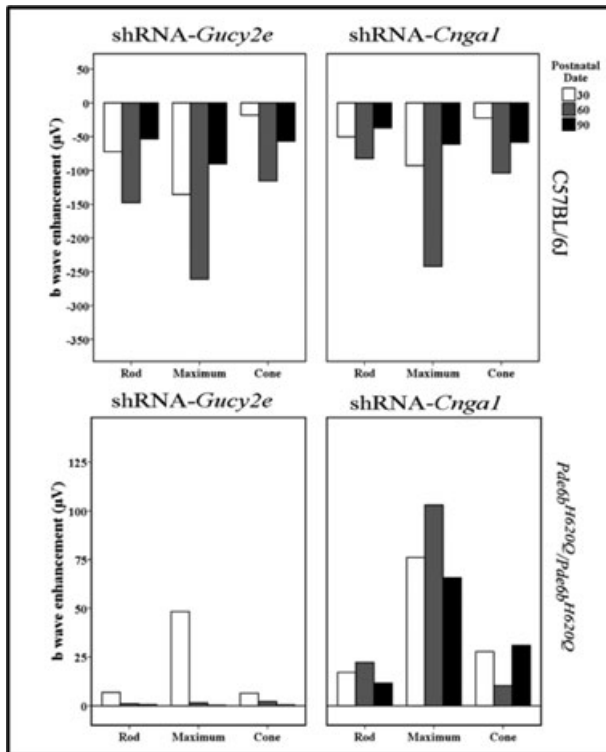
Fig. 6 ERG b-wave enhancement of rod-specific, maximal and cone responses from shRNA-lentivirus transduced C57BL/6J and *Pde6b*[H620Q] mice. The upper panels display rod, maximal and cone ERG b-wave enhancement from C57BL/6J mice aged 1–3 months after transduction with shRNA-*Gucy2e* (left) or shRNA-*Cnga1* (right). Lower panels show rod-specific, maximal and cone ERG b-wave enhancement from *Pde6b*[H620Q] mice aged 1–3 months after transduction with shRNA-*Gucy2e* (left) or shRNA-*Cnga1* (right) (White = 30 days postnatal; light shaded = 60 days postnatal; Black solid = 90 days postnatal).

significant morphological and functional rescue of *Pde6b*[H620Q] photoreceptors. These data support the model that high levels of intracellular cGMP and/or Ca^{2+} plays a role in the PDE6-associated RP.

In normal photoreceptors, the concentration of cGMP is controlled by the balance between its synthesis by GUCY2 and its hydrolysis by PDE6 [37–43]. GUCY2 is encoded by *Gucy2e* and *Gucy2f*, which function together to control cGMP production in photoreceptors [61]. These genes exhibit functional redundancy, as the *Gucy2e/Gucy2f* double mutant exhibits more severe phenotypes than either single mutant alone. For example, although full field ERGs are reduced in *Gucy2e* homozygotes and normal in *Gucy2f* mutants, no ERG responses were detected in *Gucy2e/Gucy2f* double homozygotes. Similarly, ONL thickness is significantly reduced by 6 months in *Gucy2e/Gucy2f* double homozygotes, in contrast to ONL thickness of *Gucy2e* mutants [61–63]. Our observation of *Gucy2e* knockdown in control mice associated with reduced ERG responses, in the absence of signif-

icant rod degeneration during our assay period, is consistent with the previously described *Gucy2e* phenotype [61].

CNGB1 and CNGA1 constitute CNG in rods. Because rods in *Cngb1* mutants show reduced sensitivity to light [64], we expected to see reduced ERG responses in the shRNA-*Cgna1* transduced control mice. Loss of the CNG complex could, by itself, lead to slow degeneration of rods over a period of four months [65]. However, because shRNA knockdown generally phenocopies a hypomorphic allele, we did not expect shRNA-*Cnga1* to induce significant degeneration in the 2-month period of our experiments.

High intracellular cGMP levels are toxic to photoreceptors [3, 43, 66]. In *Pde6b*[rd1] mice, PDE6 activity is undetectable and cGMP is significantly elevated above controls [43]. In this model, cGMP accumulates to high levels because GUCY2 continue to synthesize cGMP at a basal rate in the absence of PDE6 activity. In *Pde6b*[H620Q] mice, PDE6 specific activity is sub-normal suggesting that cGMP metabolism is abnormal in these mice as well [8]. Because PDE6 and GUCY2 directly catalyse reactions involving cGMP, we expected suppression of GUCY2E expression would result in increased *Pde6b*[H620Q] photoreceptor survival. Our results showing increased photoreceptor survival after *Gucy2e* knockdown support cGMP as an initiator of the retinal degeneration process in *Pde6b* mutant mice.

However necessary elevated cGMP may be in initiating cell death in these mouse models, other downstream changes are likely to be involved and may be more directly responsible for provoking degeneration. Light-sensitive conductance in photoreceptors is controlled by the effect of cGMP on Ca^{2+} influx through regulation of CNG channels [67–70]. Because abnormal Ca^{2+} levels have been implicated in causing photoreceptor cell death [15], toxicity associated with cGMP may be secondary to abnormal regulation of CNG channels [8, 16]. Indeed, our data show down-regulation of CNG channels by shRNA is associated with increased photoreceptor survival.

There are several possible reasons that may explain why the shRNA-*Cnga1* approach rescued photoreceptors, compared to published reports performed with Ca^{2+} channel blockers that did not produce rescue [46–49]. First, the mouse line *Pde6b*[H620Q] is a partial loss of function mutant exhibiting a less severe rate of degeneration. In contrast, the *Pde6b*[rd1] allele, which is a total loss of function mutation, was used for the calcium blocker studies. This may not be a sufficient reason, as we tested l-*cis*-diltiazem in *Pde6b*[H620Q] mice at three different doses (54, 108 and 216 mg/kg/day) over the course of 2 weeks and did not detect changes in retinal degeneration relative to that in controls (unpublished results). A second possibility is that there is lower bioavailability Ca^{2+} channel blockers after systemic delivery compared to local sub-retinal transduction of shRNA-*Cnga1*. Finally, the mechanisms of action of the two approaches are different: drug binding to Ca^{2+} channel blocker *versus* RNAi-mediated reduced Ca^{2+} channel expression might also account for the difference in apparent effectiveness.

To date, success of viral gene therapies to rescue vision has been limited to non-photoreceptor specific diseases such as *Rs1*,

RPE65-related early onset-retinal dystrophy, or photoreceptor diseases involving minimal cell death [71–75]. Previous attempts to rescue *Pde6b* mutant photoreceptors performed with viral PDE6 expression vectors has had limited success [37–41, 43]. Although future work is necessary, this approach may not provide enough long-term wild-type PDE6 activity to block excess Na^+/Ca^{2+} entry [38–43]. Our work suggests that downstream interventions may augment this approach. Furthermore, because cGMP and Ca^{2+} may be a common alteration leading to cell death in other signal-dependent neurodegenerative diseases, future manipulation of cGMP or cation levels with shRNA may allow for the conversion of a progressive degeneration into a stationary disease.

Both RP and the atrophic (dry) form of AMD are characterized by an initial loss of rod photoreceptors. AMD manifests clinically by abnormal kinetics in dark adaptation and decreased rod-mediated visual function, followed by the death of cones and the retinal pigment epithelium. Rods are also affected earlier than cones in normal aging. Because reduced PDE6 function is a common denominator in many cases of photoreceptor degenerations, it may be possible to develop treatments based on decreasing cGMP production by GUCY2 and influx of Ca^{2+}/Na^+ through CNGA1.

Ultimately, the results from these studies will open the possibility of genetic modification in Irish Setters with PDE6B-related degeneration and humans with photoreceptor diseases.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Dryja TP, Berson EL.** Retinitis pigmentosa and allied diseases: implications of genetic heterogeneity. *Invest Opthal Vis Sci*. 1995; 36: 1197–200.

2. **Berson EL.** Retinitis Pigmentosa: the Friedenwald Lecture. *Invest Opthal Vis Sci*. 1993; 34: 1655–76.

3. **Tsang SH, Gouras P, Yamashita CK, et al.** Retinal degeneration in mice lacking the gamma subunit of the rod cGMP phosphodiesterase. *Science (New York, NY)*. 1996; 272: 1026–9.

4. **McLaughlin ME, Ehrhart TL, Berson EL, et al.** Mutation spectrum of the gene encoding the b subunit of rod phosphodiesterase among patients with autosomal recessive retinitis pigmentosa. *Proc Nat Acad Sci*. 1995; 92: 3249–53.

5. **Bird AC.** Retinal photoreceptor dystrophies: the LI. Edward Jackson Memorial Lecture. *Am J Opthalmol*. 1995; 119: 543–62.

6. **Hartong DT, Berson EL, Dryja TP.** Retinitis pigmentosa. *Lancet*. 2006; 368: 1795–809.

7. **Daiger SP, Bowne SJ, Sullivan LS.** Perspective on genes and mutations causing retinitis pigmentosa. *Arch Ophthalmol*. 2007; 125: 151–8.

8. **Davis R, Tosi J, Janisch K, et al.** Functional rescue of degenerating photoreceptors in mice homozygous for a hypomorphic cGMP phosphodiesterase 6 allele (Pde6bH620Q). *Invest Ophthalmol Vis Sci*. 2008; 49: 5067–76.

9. **Barhoum R, Martinez-Navarrete G, Corrochano S, et al.** Functional and structural modifications during retinal degeneration in the rd10 mouse. *Neuroscience*. 2008; 155: 698–713.

10. **Gargini C, Terzibasi E, Mazzoni F, et al.** Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: a morphological and ERG study. *J Comp Neurol*. 2007; 500: 222–38.

11. **Chang B, Hawes NL, Hurd RE, et al.** Retinal degeneration mutants in the mouse. *Vision Res*. 2002; 42: 517–25.

12. **Chang B, Hawes NL, Pardue MT, et al.** Two mouse retinal degenerations caused by missense mutations in the beta-subunit of rod cGMP phosphodiesterase gene. *Vision Res*. 2007; 47: 624–33.

13. **Punzo C, Cepko C.** Cellular responses to photoreceptor death in the rd1 mouse model of retinal degeneration. *Invest Ophthalmol Vis Sci*. 2007; 48: 849–57.

14. **Bowes C, Li T, Danciger M, et al.** Retinal degeneration in the rd mouse is caused by a defect in the b subunit of rod cGMP phosphodiesterase. *Nature (Lond.)*. 1990; 347: 677–80.

15. **Doonan F, Donovan M, Cotter TG.** Activation of multiple pathways during photoreceptor apoptosis in the rd mouse. *Invest Ophthalmol Vis Sci*. 2005; 46: 3530–8.

16. **Fain GL, Lisman JE.** Light, Ca^{2+}, and photoreceptor death: new evidence for the equivalent-light hypothesis from arrestin knockout mice [In Process Citation]. *Invest Ophthalmol Vis Sci*. 1999; 40: 2770–2.

17. **Lisman J, Fain G.** Support for the equivalent light hypothesis for RP. *Nat Med*. 1995; 1: 1254–5.

18. **Tsang SH, Gouras P.** Photoreceptors and photoreceptor dysfunctions. In: Adelman G, Smith B, editors. Encyclopedia of neuroscience. Amsterdam: Elsevier Science, 2004. pp. 1633–44.

19. **Tsang SH, Burns ME, Calvert PD, et al.** Role of the target enzyme in deactivation of photoreceptor g protein *in vivo*. *Science*. 1998; 282: 117–21.

20. **Tsang SH, Gouras P, Yamashita CK, et al.** Retinal degeneration in mice lacking the g subunit of rod cGMP phosphodiesterase. *Science*. 1996; 272: 1026–9.

21. **Tsang SH, Woodruff ML, Chen CK, et al.** GAP-independent termination of photoreceptor light response by excess gamma subunit of the cGMP-phosphodiesterase. *J Neurosci*. 2006; 26: 4472–80.

22. **Tsang SH, Woodruff ML, Janisch KM, et al.** Removal of phosphorylation sites of

{gamma} subunit of phosphodiesterase 6 alters rod light response. *J Physiol*. 2007; 579: 303–12.

23. **Tsang SH, Yamashita CK, Doi K, *et al.*** *In vivo* studies of the gamma subunit of retinal cGMP-phophodiesterase with a substitution of tyrosine-84. *Biochem J*. 2001; 353: 467–74.

24. **Tsang SH, Yamashita CK, Lee WH, *et al.*** The positive role of the carboxyl terminus of the gamma subunit of retinal cGMP-phosphodiesterase in maintaining phosphodiesterase activity *in vivo*. *Vision Res*. 2002; 42: 439–45.

25. **Fain GL, Matthews HR, Cornwall MC, *et al.*** Adaptation in vertebrate photoreceptors. *Physiol Rev*. 2001; 81: 117–51.

26. **Stryer L.** Visual excitation and recovery. *J Biol Chem*. 1991; 266: 10711–4.

27. **Yarfitz S, Hurley JB.** Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J Biol Chem*. 1994; 269: 14329–32.

28. **Burns ME, Baylor DA.** Activation, deactivation, and adaptation in vertebrate photoreceptor cells. *Annu Rev Neurosci*. 2001; 24: 779–805.

29. **Burns ME, Arshavsky VY.** Beyond counting photons: trials and trends in vertebrate visual transduction. *Neuron*. 2005; 48: 387–401.

30. **Fung BK-K, Hurley JB, Stryer L.** Flow of information in the light-triggered cyclic nucleotide cascade of vision. *Pro Natl Acad Sci, USA*. 1981; 78: 152–6.

31. **Arshavsky VY, Lamb TD, Pugh EN Jr.** G proteins and phototransduction. *Annu Rev Physiol*. 2002; 64: 153–87.

32. **Zhang X, Cote RH.** cGMP signaling in vertebrate retinal photoreceptor cells. *Front Biosci*. 2005; 10: 1191–204.

33. **Baehr W, Devlin MJ, Applebury ML.** Isolation of bovine ROS phosphiesterase. *J Biol Chem*. 1979; 254: 11669–77.

34. **Fung BKK, Young JH, Yamane HK, *et al.*** Subunit stoichiometry of retinal rod cGMP phosphodiesterase. *Biochemistry*. 1990; 29: 2657–64.

35. **Guo LW, Grant JE, Hajipour AR, *et al.*** Asymmetric interaction between rod cyclic GMP phosphodiesterase gamma subunits and alpha/beta subunits. *J Biol Chem*. 2005; 280: 12585–92.

36. **Guo LW, Muradov H, Hajipour AR, *et al.*** The inhibitory gamma subunit of the rod cGMP phosphodiesterase binds the catalytic subunits in an extended linear structure. *J Biol Chem*. 2006; 281: 15412–22.

37. **Cella W, Greenstein VC, Zernant-Rajang J, *et al.*** G1961E mutant allele in the Stargardt disease gene ABCA4 causes bull's eye maculopathy. *Exp Eye Res*. 2009; 89: 16–24.

38. **Kumar-Singh R, Farber DB.** Encapsidated adenovirus mini-chromosome-mediated delivery of genes to the retina: application to the rescue of photoreceptor degeneration. *Hum Mol Genet*. 1998; 7: 1893–900.

39. **Bennett J, Tanabe T, Sun D, *et al.*** Photoreceptor cell rescue in retinal degeneration (rd) mice by *in vivo* gene therapy. *Nat. Med*. 1996; 2: 649–54.

40. **Jomary C, Vincent KA, Grist J, *et al.*** Rescue of photoreceptor function by AAV-mediated gene transfer in a mouse model of inherited retinal degeneration. *Gene Ther*. 1997; 4: 683–90.

41. **Takahashi M, Miyoshi H, Verma IM, *et al.*** Rescue from photoreceptor degeneration in the rd mouse by human immunodeficiency virus vector-mediated gene transfer. *J Virol*. 1999; 73: 7812–6.

42. **Lem J, Flannery JG, Li T, *et al.*** Retinal degeneration is rescued in transgenic rd mice by expression of the cGMP phosphodiesterase b subunit. *Proc Natl Acad Sci USA*. 1992; 89: 4422–6.

43. **Farber DB, Flannery JG, Bowes-Rickman C.** The rd mouse story: seventy years of research on an animal nodel of inherited retinal degeneration. *Prog Retinal Res*. 1994; 13: 31–64.

44. **Frasson M, Sahel JA, Fabre M, *et al.*** Retinitis pigmentosa: rod photoreceptor rescue by a calcium-channel blocker in the rd mouse. *Nat Med*. 1999; 5: 1183–7.

45. **Vallazza-Deschamps G, Cia D, Gong J, *et al.*** Excessive activation of cyclic nucleotide-gated channels contributes to neuronal degeneration of photoreceptors. *Eur J Neurosci*. 2005; 22: 1013–22.

46. **Takano Y, Ohguro H, Dezawa M, *et al.*** Study of drug effects of calcium channel blockers on retinal degeneration of rd mouse. *Biochem Biophys Res Commun*. 2004; 313: 1015–22.

47. **Pearce-Kelling SE, Aleman TS, Nickle A, *et al.*** Calcium channel blocker D-cis-diltiazem does not slow retinal degeneration in the PDE6B mutant rcd1 canine model of retinitis pigmentosa. *Mol Vis*. 2001; 7: 42–7.

48. **Pawlyk BS, Li T, Scimeca MS, *et al.*** Absence of photoreceptor rescue with D-cis-diltiazem in the rd mouse. *Invest Ophthalmol Vis Sci*. 2002; 43: 1912–5.

49. **Bush RA, Kononen L, Machida S, *et al.*** The effect of calcium channel blocker diltiazem on photoreceptor degeneration in the rhodopsin Pro213His rat. *Invest Ophthalmol Vis Sci*. 2000; 41: 2697–701.

50. **Barabas P, Cutler Peck C, Krizaj D.** Do Calcium Channel Blockers Rescue Dying Photoreceptors in the Pde6b (rd1) Mouse? *Adv Exp Med Biol*. 664: 491–9.

51. **Hart AW, McKie L, Morgan JE, *et al.*** Genotype-phenotype correlation of mouse pde6b mutations. *Invest Ophthalmol Vis Sci*. 2005; 46: 3443–50.

52. **Tsang SH, Chen J, Kjeldbye H, *et al.*** Retarding photoreceptor degeneration in *Pdeg^{tm1}/Pdeg^{tm1}* mice by an apoptosis suppressor gene. *Invest Opthal Vis Sci*. 1997; 38: 943–50.

53. **Ortega S, Ittmann M, Tsang SH, *et al.*** Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci USA*. 1998; 95: 5672–7.

54. **Stewart SA, Dykxhoorn DM, Palliser D, *et al.*** Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA*. 2003; 9: 493–501.

55. **Tsang SH, Woodruff ML, Jun L, *et al.*** Transgenic mice carrying the H258N mutation in the gene encoding the beta-subunit of phosphodiesterase-6 (PDE6B) provide a model for human congenital stationary night blindness. *Human Mutat*. 2007; 28: 243–54.

56. **Hood DG, Birch DG.** Rod phototransduction in retinitis pigmentosa: estimation and interpretation of parameters derived from the rod a-wave. *Invest Opthal Vis Sci*. 1994; 35: 2948–61.

57. **Hood DC, Birch DG.** A computational model of the amplitude and implicit time of the b-wave of the human ERG. *Vis Neurosci*. 1992; 8: 107–26.

58. **Birch DG, Hood DC, Nusinowitz S, *et al.*** Abnormal activation and inactivation mechanisms or rod transduction in patients with autosomal dominant retinitis pigmentosa and the Pro-23-His Mutation. *Invest Ophthalmol Vis Sci*. 1995; 36: 1603–14.

59. **Lyubarsky AL, Pugh ENJ.** The recovery phase of the murine rod response from electroretinographic recordings. *J Neurosci*. 1996; 16: 563–71.

60. **Goto Y, Peachey NS, Ripps H, *et al.*** Functional abnormalities in transgenic mice expressing a mutant rhodopsin gene. *Invest Opthal Vis Sci*. 1995; 36: 62–71.

61. **Baehr W, Karan S, Maeda T, *et al.*** The function of guanylate cyclase 1 and guanylate cyclase 2 in rod and cone photoreceptors. *J Biol Chem*. 2007; 282: 8837–47.

62. **Coleman JE, Semple-Rowland SL.** GC1 deletion prevents light-dependent arrestin translocation in mouse cone photoreceptor cells. *Invest Ophthalmol Vis Sci.* 2005; 46: 12–6.

63. **Blom N, Gammeltoft S, Brunak S.** Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol.* 1999; 294: 1351–62.

64. **Zhang Y, Molday LL, Molday RS, *et al.*** Knockout of GARPs and the beta-subunit of the rod cGMP-gated channel disrupts disk morphogenesis and rod outer segment structural integrity. *J Cell Sci.* 2009; 122: 1192–200.

65. **Huttl S, Michalakis S, Seeliger M, *et al.*** Impaired channel targeting and retinal degeneration in mice lacking the cyclic nucleotide-gated channel subunit CNGB1. *J Neurosci.* 2005; 25: 130–8.

66. **Lolley RN, Farber DB, Rayborn ME, *et al.*** Cyclic GMP accumulation causes degeneration of photoreceptor cells: simulation of an inherited disease. *Science (New York, NY).* 1977; 196: 664–6.

67. **Nakatani K, Yau KW.** Sodium-dependent calcium extrusion and sensitivity regulation in retinal cones of the salamander. *J Physiol.* 1989; 409: 525–48.

68. **Torre V, Matthews HR, Lamb TD.** Role of calcium in regulating the cyclic GMP cascade of phototransduction in retinal rods. *Proc Natl Acad Sci.* 1986; 83: 7109–13.

69. **Hodgkin AL, Nunn BJ.** Control of light-sensitive current in salamander rods. *J Physiol.* 1988; 403: 439–71.

70. **Wensel TG, Stryer L.** Reciprocal control of retinal rod cyclic GMP phosphodiesterase by its g subunit and transducin. *Proteins: Struct Funct Genet.* 1986; 1: 90–9.

71. **Min SH, Molday LL, Seeliger MW, *et al.*** Prolonged recovery of retinal structure/function after gene therapy in an Rs1h-deficient mouse model of x-linked juvenile retinoschisis. *Mol Ther.* 2005; 12: 644–51.

72. **Zeng Y, Takada Y, Kjellstrom S, *et al.*** RS-1 gene delivery to an adult Rs1h knockout mouse model restores ERG b-wave with reversal of the electronegative waveform of X-linked retinoschisis. *Invest Ophthalmol Vis Sci.* 2004; 45: 3279–85.

73. **Bainbridge JW, Smith AJ, Barker SS, *et al.*** Effect of gene therapy on visual function in Leber's congenital amaurosis. *N Engl J Med.* 2008; 358: 2231–9.

74. **Maguire AM, Simonelli F, Pierce EA, *et al.*** Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med.* 2008; 358: 2240–8.

75. **Hauswirth WW, Aleman TS, Kaushal S, *et al.*** 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.* 2008; 19: 979–90.