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Gene therapy with a promoter targeting both rods and cones rescues retinal degeneration caused by AIPL1 mutations

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Aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) is required for the biosynthesis of photoreceptor phosphodiesterase (PDE). Gene defects in AIPL1 cause a heterogeneous set of conditions ranging from Leber's congenital amaurosis (LCA), the severest form of early-onset retinal degeneration, to milder forms such as retinitis pigmentosa (RP) and cone-rod dystrophy. In mice, null and hypomorphic alleles cause retinal degeneration similar to human LCA and RP, respectively. Thus these mouse models represent two ends of the disease spectrum associated with AIPL1 *gene defects in humans. We evaluated whether adeno-associated virus (AAV)-mediated gene replacement therapy in these models could restore PDE biosynthesis in rods and cones and thereby improve photoreceptor survival. We validated the efficacy of human* AIPL1 *(isoform 1) replacement gene controlled by a promoter derived from the human rhodopsin kinase (RK) gene, which is active in both rods and cones. We found substantial and long-term rescue of the disease phenotype as a result of transgene expression. This is the first gene therapy study in which both rods and cones were targeted successfully with a single photoreceptor-specific promoter. We propose that the vector and construct design used in this study could serve as a prototype for a human clinical trial.*

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Introduction

Inherited retinal degenerations are a group of conditions that may result from mutations in well over 130 different genes (http://www. sph.uth.tmc.edu/Retnet/).[1–8] These highly heterogeneous conditions affect the function and viability of rod and cone photoreceptor cells, ultimately leading to photoreceptor loss and blindness. Retinitis pigmentosa (RP) is the most common form of inherited retinal degenerations. It generally begins with night blindness and reduced and delayed electroretinograms (ERG) early in life.[9] Visual deficits usually become marked at middle age. In most instances, rod photoreceptors are primarily affected in RP, and cones die as a secondary outcome to rod loss. Leber's congenital amaurosis (LCA) refers to a more severe, early-onset form of disease involving both rods and cones with loss of vision in childhood.[10–14] It is caused by mutations in at least 13 genes, one of which codes for aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1).[1]

AIPL1 mutations have been estimated to cause ~7% of recessive LCA[4] and have also been associated with cone–rod dystrophy and RP.[1,4,5] At least 20 disease-causing mutations in *AIPL1* have been reported (Human Gene Mutation Database (HGMD); http://www.hgmd.org). The variability in phenotype may be explained by the nature of the mutations. Some of these mutations lead to truncation of the reading frame and are not expected to produce a functional protein. Others are missense mutations, which may not abolish protein function completely.[15–17] In the retina, AIPL1 protein is found exclusively in photoreceptors.[18] Three mouse models of *AIPL1* deficiency have been produced and analyzed. Two of these models reproduce the *Aipl1 null* mutation (the *Aipl1*$^{-/-}$ mouse), which carry targeted disruption in the *Aipl1* gene and produces no AIPL1.[2,6] Retinal degeneration in the *Aipl1 null* mice is rapid; all photoreceptors are lost by 3 weeks of age. The other model represents an *Aipl1* hypomorphic mutation (*Aipl1*$^{h/h}$). This line produces a lower AIPL1 expression at ~20% of the wild type (WT) level,[3] and shows slower photoreceptor loss. Retinal morphology appears normal until after 3 months of age, and thereafter degeneration ensues at a slow rate

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such that by 10 months of age half of the photoreceptors would be lost. Analyses of the *null* and hypomorphic mutant mice have shown that AIPL1-linked retinopathy is due to a perturbation in the biosynthesis/stability of photoreceptor cGMP phosphodiesterase (PDE6). Very little PDE is found to accumulate in the *null* mutant.[2] In the hypomorphic mutant, there is a decline in PDE level proportional to the reduced level of AIPL.[13] This effect is highly specific for PDE, as analysis of a large number of photoreceptor proteins found no change in their expression levels in the hypomorphic mutant.[3] These data, in conjunction with the homology of AIPL1 to members of the FK506 binding protein (FKBP) family of chaperone proteins,[19–21] suggest that AIPL1 is a specialized chaperone evolved to assist in photoreceptor PDE biosynthesis.

Although established literature has only defined the role for AIPL1 in rod photoreceptor PDE synthesis, our recent study showed that AIPL1 is also required for the normal accumulation of cone PDE[22] (and our unpublished data). The photoreceptor disease due to *AIPL1* gene mutations can therefore be attributed to an insufficiency of rod and cone PDEs. Thus, an effective therapy for this condition should aim to restore rod and cone PDE biosynthesis, through reconstituting AIPL1 function, to a level sufficient to sustain photoreceptor function and survival. As cones are primarily responsible for useful daytime vision in humans, rescuing cone photoreceptors is an essential component for a successful treatment. In preclinical animal studies, an easily quantifiable, and thus very useful, outcome measure predictive of successful treatment would be an increase in PDE levels in rods and cones of recipient animals.

To drive expression of a therapeutic transgene in both rods and cones, we would ideally adopt a promoter that is active in both types of photoreceptors but not in any other retinal cells including the RPE. The recently characterized human RK promoter appears to meet the major criteria for this purpose.[7] It is relatively small in size and is selectively active in rods and cones, but not in RPE or any other retinal neurons. This study was carried out to test a clinically relevant gene therapy paradigm for AIPL1 deficiency incorporating this RK promoter as the key transcription regulatory element. Our primary objective was to validate a promoter design that drives specific expression of the transgene in both cones and rods at a level that effects substantial rescue of the disease phenotype. Given the variable severity of disease seen in patients with AIPL1 defects, we assessed the efficacy of gene replacement therapy in treating both *Aipl1* null ($Aipl1^{-/-}$) and *Aipl1* hypomorphic ($Aipl1^{hypo}$) mutant mice that simulate different rates of human retinal degeneration. We examined the effect of gene transfer and confirmed a strong and stable rescue of the disease phenotype using our vector design.

Results

The human RK promoter drives AIPL1 transgene expression and raises PDE levels in rods and cones of Aipl1^{hypo} *mice*

Human retinal disease caused by AIPL1 insufficiency involves both rods and cones. Thus, the design of a gene-based therapy would need to target both rods and cones in order to fully restore retinal function. We have previously shown that an RK promoter derived from the human rhodopsin kinase gene is highly efficient in driving reporter gene expression in both rods and cones.[7] This promoter has the added advantage of being short (below 300 bases). Hence, it can be readily accommodated into adeno-associated virus (AAV) vectors that have limited packaging capacity. This promoter was incorporated into the design of all of our replacement gene constructs for this study (Figure 1a). The human (isoform 1) and murine *Aipl1* cDNAs were PCR amplified from human and murine retinal cDNA and cloned into an AAV plasmid backbone between the RK promoter and an SV40 polyadenylation site. As an added tag for detecting the expression of the transgenes, a zsGreen reporter sequence was linked to the mouse or human *AIPL1* cDNA through an internal ribosomal entry site (Figure 1a). These two replacement gene constructs were packaged into AAV5 vectors.

We crossed our original line of hypomorphic mutant ($Aipl1^{h/h}$) and the $Aipl1^{-/-}$ mouse to produce the F1 generation of hypomorphic mice designated $Aipl1^{hypo}$. The $Aipl1^{hypo}$ mice would in theory produce half of the PDE level than the original hypomorphic line, and hence a faster disease course so that the study duration could be shortened. Immunoblotting experiments confirmed that the $Aipl1^{hypo}$ mice produced a lower level of AIPL1 protein than did $Aipl1^{h/h}$ mice, and phenotype analyses showed that the onset of retinal degeneration was earlier in the $Aipl1^{hypo}$ mice than in the $Aipl1^{h/h}$ mice (data not shown). The $Aipl1^{hypo}$ mice were therefore used as recipient animals for the gene therapy experiments. Adult $Aipl1^{hypo}$ mice (at $\sim$5 months of age) were given a single subretinal injection of the AAV vector carrying either the mouse or human AIPL1 transgene in one eye and a control vector expressing a green fluorescent protein reporter in the fellow eye. Cohorts of mice were analyzed for gene expression and for phenotype rescue at different time points after the treatment. At 6 weeks after the injection, a group of mice were killed. Their retinas were dissected and subjected to western blotting analysis. Treated $Aipl1^{hypo}$ mice showed increased AIPL1 protein levels approaching that of the WT, whereas control retinas had much lower expression (Figure 1b). To see whether PDE synthesis was also increased as a result of elevated AIPL1, we probed western blots with rod and cone PDE-specific antibodies (Figure 1b). The results showed that rod PDE was markedly elevated approaching that of the WT level. Cone PDE was also increased in the treated eyes, although it was still somewhat lower than that in the WT retinas (Figure 1b). We also carried out immunofluorescence staining for AIPL1 and both rod and cone PDEs at 4–6 weeks after treatment. The results showed increased specific expression of these three proteins in photoreceptors after treatment (data not shown), consistent with the western blotting data. These data clearly showed that (1) the RK promoter is suitable for driving AIPL1 gene expression specifically in both cone and rod photoreceptor cells; and (2) increased AIPL1 expression leads to a corresponding increase in PDE synthesis.

At least three splice variants from the human *AIPL1* gene could be found in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA), suggesting the possibility that multiple isoforms of AIPL1 protein may exist and be functionally

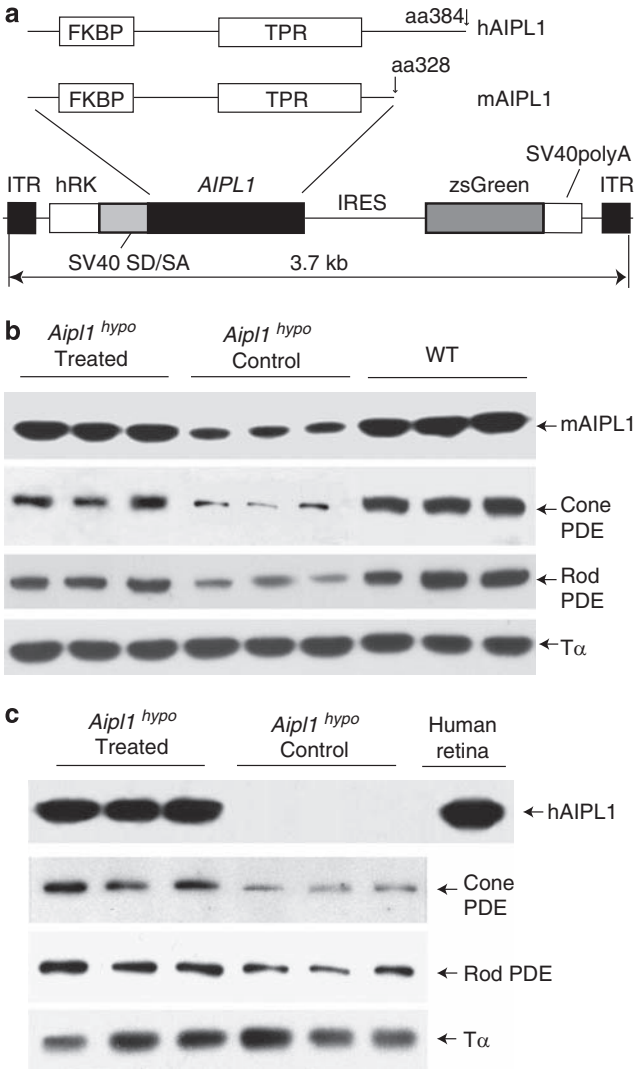


Figure 1 Targeted expression of murine and human AIPL1 in the hypomorphic mutant retinas. (**a**) Construction of AAV vectors expressing the AIPL1 expression cassette. Murine and human AIPL1 sequences and recognizable motifs are shown schematically at the top. Note that human AIPL1 has a different C-terminal region from murine AIPL1 and is longer by 60 amino acid residues. AAV vector construct design is shown at the lower portion of the figure. In this construct, *AIPL1* transcription is driven by a human RK promoter (−112 to +183 of the proximal region of human RK promoter). The transcript from the vector is expected to be bicistronic, with AIPL1 transcript being linked at its 3′ end to a zsGreen reporter. SV40SD/SA, splice donor/acceptor site from SV40 virus; IRES, internal ribosomal entry site; SV40polyA, polyadenylation site from SV40 virus; ITR, inverted terminal repeats from AAV2. (**b**) Western blotting for mAIPL1 at 6 weeks after subretinal injection of AAV5-RK-mAIPL1. Hypomorphic retinas had much reduced AIPL1 levels. Treatment led to a marked increase of AIPL1, although they remained somewhat below those of the WT. Rod and cone PDE levels were also increased. (**c**) Western blotting for hAIPL1 at 6 weeks after subretinal injection of AAV5-RK-hAIPL1. Using a hAIPL1-specific antibody, treated retinas showed a strong band of AIPL1 co-migrating with that from human retina. Rod and cone PDE levels were also increased, although the extent of increases appeared somewhat lesser than those treated with the mAIPL1 vector. For both panels b and c, three independent treated retinas were analyzed. Transducin α-subunit is shown as a loading control.

significant. To address which isoform is the predominant variant in the human retina, we performed immunoblotting experiments with an antibody that was generated against an antigen spanning the full-length human AIPL1 sequence. On immunoblots we found a single strong AIPL1 protein band from human retinal extract that matched precisely the size of AIPL1 isoform 1 that was cloned into the AAV expression vectors (Figure 1c). The other two variants were not detected in our hands by immunoblotting. These data show that, of the three putative variants of AIPL1, isoform 1 is by far the predominant form in the human retina and that only this isoform needs to be considered for future clinical gene therapy trials.

Elevated AIPL1 and PDE levels improve rod and cone photoreceptor function in the Aipl1^{hypo} *mice*

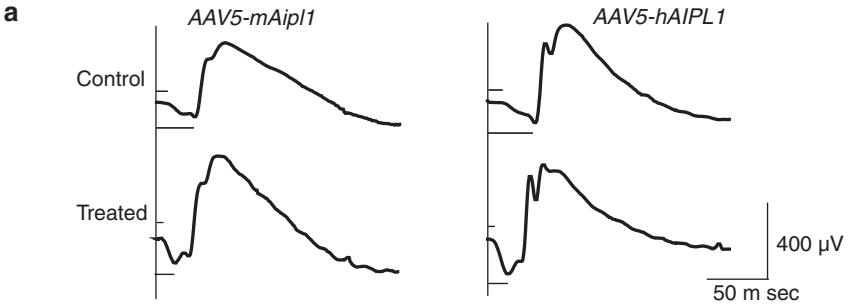
Elevated PDE synthesis would be ameliorative to the cause of disease at the molecular level in this disease model and therefore would be expected to alleviate the disease phenotype. To assess photoreceptor function, mice were tested by ERG at 6 and 23 months after injection.

Dark- and light-adapted ERGs were recorded to evaluate responses from rod and cone photoreceptors, respectively. Simultaneous bilateral recordings were performed to optimize comparison of the treated (OS) and control (OD) eyes. Previously, we have shown that the hypomorphic *Aipl1* mutant has a characteristic delayed onset of rod photoresponse at an early stage of disease.[3] This is manifested as a delayed *A*-wave latency and a prolonged *A*-wave implicit time in the rod ERG. This distinct phenotype can be explained by a delay in diffusion-limited encounter of activated transducin with PDE[23] because of the lower concentration of the latter in the mutant photoreceptors. Consistent with the previous study, we found that rod *A*-wave latencies and implicit times were markedly prolonged in control eyes compared with WT values (Figure 2a, table). AAV-mediated AIPL1 gene delivery, however, significantly accelerated the response kinetics in treated eyes (Figure 2a). Both the murine ($n=12$; $P<0.001$) and human AIPL1 ($n=5$; $P<0.01$) transgenes were effective in restoring the onset of photoresponses (Figure 2a).

Treatment with the AAV vector carrying the mouse *Aipl1* transgene led to substantial preservation of photoreceptor function as measured by ERG *A*- and *B*-wave amplitudes. At 6 months after treatment, the geometric mean ERG *A*-wave amplitude in the treated eyes (117 μV) was significantly higher than that in the control eyes (73 μV; $P<0.001$). Similarly, the geometric mean ERG *B*-wave amplitude in the treated eyes (507 μV) was also significantly higher than that in the control eyes (355 μV; $P<0.002$). ERG amplitudes favored the treated eye in all but one mouse tested (Figure 2b). Mice receiving the human transgene showed a possible treatment effect that did not reach statistical significance, given the small size of the cohort ($n=5$), and were not characterized further. Instead, we switched efforts to delivering the human *AIPL1* transgene to the null mutant (see section below).

The treatment effect with the mouse *AIPL1* transgene was long lasting. At 23 months after injection (28 months old), the treatment effect remained quite pronounced

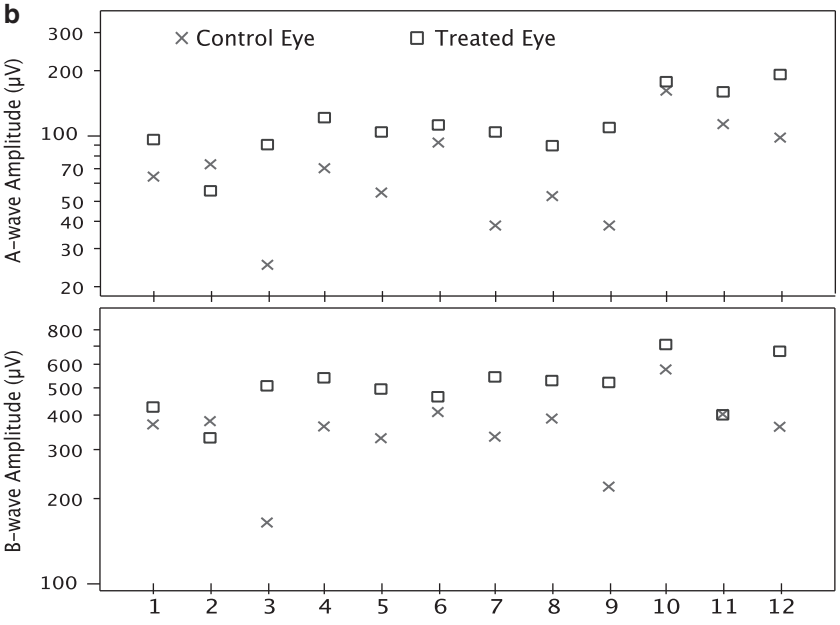
based on both rod and cone ERG amplitudes (Figure 2c). Four of five control eyes had no recordable rod ERG and one showed a very small rod ERG (*A*-wave = 6 μV, *B*-wave = 25 μV). In contrast, substantial rod ERGs were recorded from the treated eyes (geometric mean, 79 μV for *A*-wave; 336 μV for *B*-wave; $P = 0.0002$ and

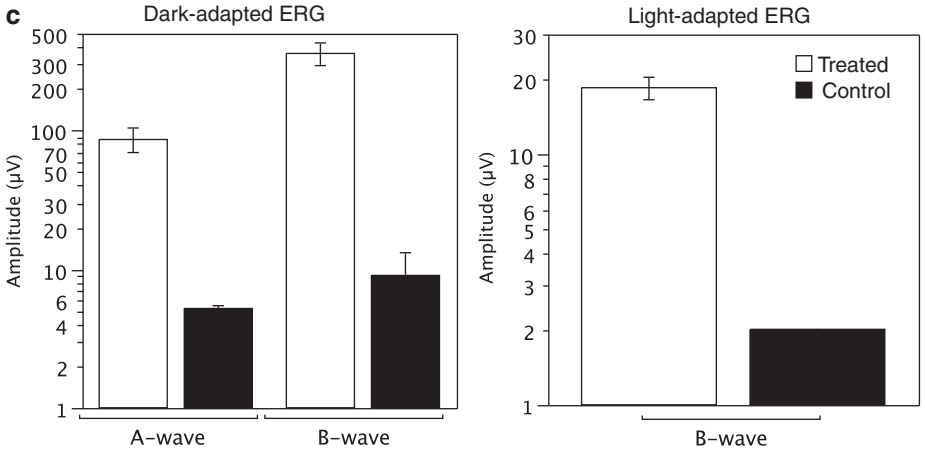


Rod A-wave Mean latencies and Implicit Times in *Aipl1* Hypomorphic Mice

Eye	N	Latency†	*P*-value (vs. Control)	*P*-value (vs. WT)	Implicit Time†	*P*-value (vs. Control)	*P*-value (vs. WT)
Control	17	8.4 ± 0.5	---------	<0.0001	27.1 ± 6.0	----------	0.0002
Treated	17*	5.0 ± 1.2	<0.0001	0.50	17.6 ± 2.9	<0.0001	0.97
WT	5	4.2 ± 0.4	<0.0001	----------	17.2 ± 1.8	<0.0002	--------

*Merging of eyes treated with mouse and human AIPL1 vectors.
†Mean ± standard error (msec)





$P = 0.0003$, respectively). The cone ERG was not detectable in any of the control eyes, whereas all of the treated eyes displayed detectable cone ERG amplitudes (geometric mean, 18 μV; $n = 5$; $P = 0.0001$).

Elevated AIPL1 and PDE levels promote rod and cone photoreceptor survival in the Aipl1^{hypo} *mice*

To evaluate the expression patterns of AIPL1 and PDE and other markers of photoreceptor survival, groups of mice were killed for immunofluorescence staining analysis following ERG tests. Figure 3 shows frozen sections from representative treated and control eyes at 6 months after injection that were labeled for AIPL1, rod and cone PDEs, and rod and cone opsins. As seen from these images, the treated retina had a much thicker outer nuclear (photoreceptor) layer as well as longer inner/outer segments. AIPL1 was expressed primarily from the transgene, as the endogenous AIPL1 was barely visible in the control retina. It localized predominantly to the inner segments (Figure 3a), consistent with the normal distribution pattern for this protein in WT retinas.[3] Rod and cone PDEs were strongly labeled in the treated retinas (Figures 3b and c), and they were localized normally to the outer segments. Rod and cone opsin labeling illustrated well-organized outer segments (Figures 3d and e). There were substantially more photoreceptors remaining in the treated retinas than in the control retinas.

Transgene expression was strictly limited to photoreceptors. This was shown by both AIPL1 immunofluorescence and the linked zsGreen reporter, seen as dotted green signals in the photoreceptor layer of all treated retinas (left panels). The zsGreen reporter was translated from a bi-cistronic mRNA that also carried the coding sequences for AIPL1. Thus, it would replicate the expression pattern of the AIPL1 transgene. In all retinas examined, zsGreen was completely absent from RPE or inner retinal neurons (although some of the injected material would unavoidably leak into the intravitreal space). These observations served to confirm that the RK promoter is highly specific for photoreceptor cells. In most treated retinas, zsGreen reporter was seen over the entire length of the section, consistent with pan retinal expression of the AIPL1 transgene and rescue.

Figure 4 shows retinal histology of treated and control eyes at 6 months after injection. Low-magnification views of control and treated retinas (left panels) show a thicker photoreceptor layer throughout the retina in the treated eye, indicating a pan-retinal rescue from the treatment. This was to be expected from our observation that a single subretinal injection of AAV vectors usually leads to transduction of nearly the entire photoreceptor population[7] (also see Figure 7f). Higher magnification images (right panels) show that the control retina retained about 2–3 rows of photoreceptor nuclei with shortened inner and outer segments in the best area. In contrast, the treated retinas had at least six rows of photoreceptor cells throughout, with longer inner and outer segments. Electron microscopy of the treated eyes showed well-organized outer segment disc structures (data not shown). At 23 months after injection (mice at 28 months of age), treated eyes retained 4–5 rows of photoreceptors with well-organized inner and outer segments that were near WT length, whereas the control eyes had none (Figure 4b). Labeling for rhodopsin showed elongated outer segments and correct localization in the outer segments (data not shown). To examine whether cone photoreceptors are also preserved in the treated retinas, we stained retinal sections with cone photoreceptor markers (cone arrestin and cone opsin; Figure 4c). The results showed well-organized cone outer segments in the treated retinas. There was no staining in the control retinas indicating degeneration of cones (data not shown). The well-preserved cone photoreceptor morphology is consistent with the larger cone ERG responses in the treated eyes. These data suggest that mouse *AIPL1* gene therapy preserved cone as well as rod photoreceptors in *Aipl1^{hypo}* mice and that the effect was long lasting.

Human AIPL1 *gene delivery by the fast onset AAV8 vector restores PDE synthesis in rod and cone photoreceptors of* Aipl1$^{-/-}$ *mutant mice*

The *Aipl1$^{-/-}$* mice manifest a rapid course of degeneration so that by age postnatal (P) day 20 essentially all photoreceptors are lost. Initially, we attempted to rescue the null mutant with AAV5-mAIPL1 and AAV5-hAIPL1 vectors that were designed for use in the hypomorphic mutant. At the time we initiated this project, AAV5 was considered the most efficient vector for introducing genes into photoreceptors. The attempt to rescue the null mutant with AAV5 vectors proved unsuccessful, even when mice were injected as early as P10. This result was not surprising, given that an AAV5 vector typically requires 4–6 weeks to reach maximal expression levels, and thus it would not be able to express the transgene fast enough to effect a rescue. Subsequently, it became known that AAV8 vectors could mediate transgene expression in the retina with much faster kinetics than AAV5.[24,25] In our own laboratory using AAV8 vector carrying a green fluorescent protein reporter gene, we usually found expression at 3 days after injection and maximal expression at 1–2 weeks. Therefore, we subsequently packaged the same AAV-hAIPL1 construct into an AAV8 vector and performed subretinal injections in the null mutant mice. In our pilot studies, we injected the

Figure 2 Functional rescue shown by ERG analysis after subretinal injection of AAV5 vectors in the *Aipl1^{hypo}* mice. (**a**) Representative ERG tracings from treated and control eyes at 6 months after injection. Both *mAIPL1*- and *hAIPL1*-treated retinas displayed faster response kinetics in terms of rod *A*-wave latencies (upper shorter lines) and implicit times (lower longer lines). Data from eyes treated with mouse and human *AIPL1* transgenes were merged in the table. *A*-Wave latencies from the treated eyes (mean ± s.e.m., 5.0 ± 1.2 ms, $n = 17$) were significantly shorter than those of the control eyes (8.4 ± 0.5 ms; $n = 17$; $P < 0.0001$). Similarly, *A*-wave implicit times for treated eyes (17.6 ± 2.9 ms; $n = 17$) showed faster response kinetics than controls (27.1 ± 6.0 ms; $n = 17$; $P < 0.0001$). (**b**) Dark-adapted ERG *A*- and *B*-wave amplitudes for treated and fellow control eyes of *Aipl1^{hypo}* mice ($n = 12$) at 6 months after injection (age 11 months old). On average, both *A*- and *B*-wave amplitudes were significantly higher for the treated eyes than for the control eyes ($P < 0.002$; see Results section). (**c**) Geometric mean dark- and light-adapted ERG amplitudes from treated and control eyes at 23 months after injection (28 months old). Although reduced relative to WT values, all treated eyes showed readily recordable ERGs, whereas ERGs from controls eyes were undetectable except for one animal. Error bars, ± s.e.m.; $n = 5$.

AAV8-hAIPL1 vector into the mutant mice at P9–13. We found that the best treatment effect was in mice injected at P9; hence, all subsequent mice analyzed in the current study were injected at P9.

To test whether the AAV8-hAIPL1 vector directed expression of the correct AIPL1 gene product, we first analyzed a group of mice at 4 weeks after injection by western blotting. As shown in Figure 5a, treatment with the AAV8-hAIPL1 vector led to a single band of protein signal on western blot co-migrated with the hAIPL1 protein from human retinal homogenate. This band was absent in control retinas. Treatment with a mAIPL1 vector produced a smaller protein, which co-migrated with the endogenous mouse AIPL1 in WT mouse retinas. An issue of some importance is the relative expression level of *AIPL1* transgene from the AAV vector. Although this question was easily addressed for a mouse *Aipl1* transgene by directly comparing it with the WT retina, estimation of human *AIPL1* transgene expression in the mouse retina would require an antibody that recognizes an identical epitope so as to bind to mouse and human proteins with equal affinity. To approximate such an antibody, we affinity-purified an antiserum generated against a full-length human AIPL1 protein using mouse AIPL1 as the affinity ligand. Western blotting (Figure 5a) and immunofluorescence staining (data not shown) using this antibody indicated that the treated retinas expressed hAIPL1 at a higher level than that of endogenous mAIPL1 in WT retina.

As AIPL1 is required for the biosynthesis/stability of PDE, no PDE accumulates in the photoreceptors of *Aipl1*$^{-/-}$ mice. We therefore examined whether treatment with the AAV8-hAIPL1 vector led to substantial accumulation of rod and cone PDEs in the null mutant retinas. Using antibodies that were specific for rod and cone PDEs we showed that treatment with the human AIPL1 vector indeed led to a substantial accumulation of PDE in the retina, although the levels were still lower than those of the WT (Figure 5b).

Human AIPL1 *transgene restores rod and cone function and slows photoreceptor degeneration in the* Aipl1$^{-/-}$ *mice*

At 4 weeks after subretinal injections, rod and cone ERGs were recorded from both eyes of all recipient mice. As shown in Figure 6a, control eyes had no detectable ERG responses consistent with previous studies.[6] Treatment restored photo-responses as shown by the substantial ERG amplitudes from both rod and cone photoreceptors (Figures 6a and b). The geometric mean values for both the *A*-wave (42 μV) and the *B*-wave (302 μV) of treated eyes were significantly higher than those of control eye values ($n = 55$; $P < 0.0001$ in both cases). The geometric mean value for cone ERG *B*-wave amplitude (29 μV) of treated eyes was also significantly higher than that of the control eyes ($n = 20$; $P < 0.0001$). Although the response

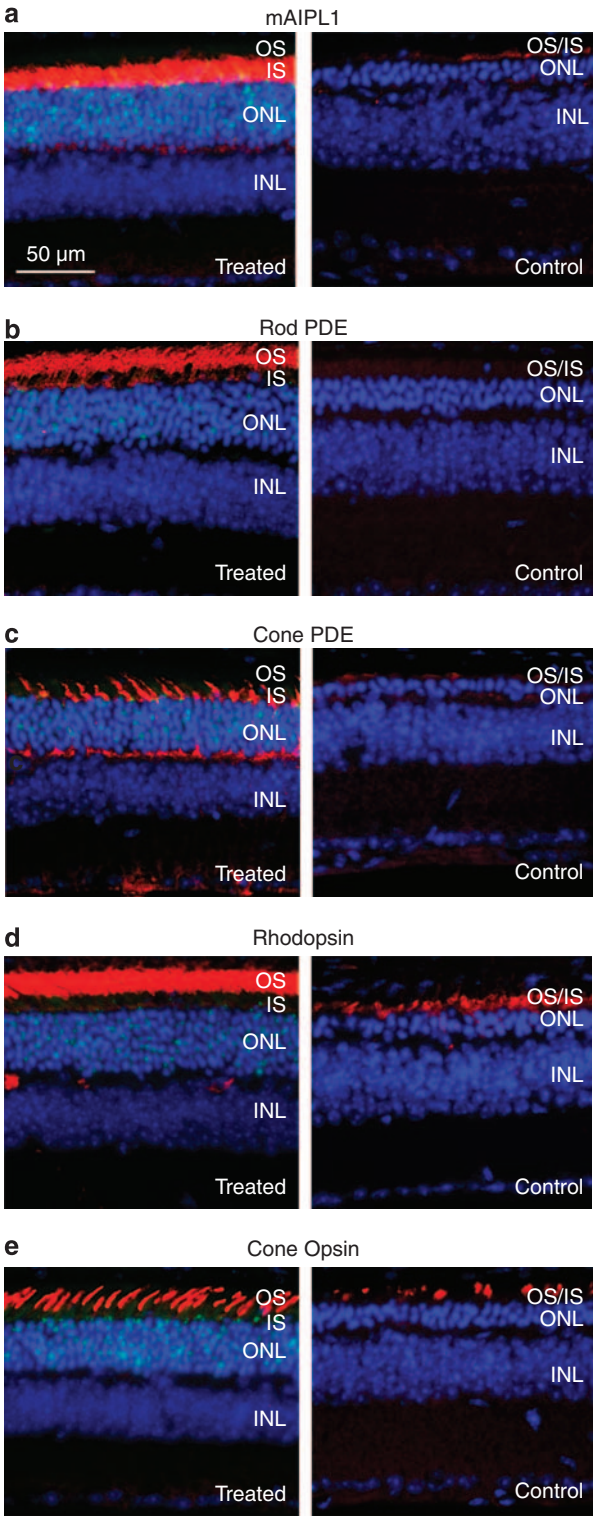


Figure 3 Immunofluorescence (red) analysis of treated and control *Aipl1*^{hypo} retinas at 6 months after injection with AAV5-RK-mAIPL1. AIPL1 (**a**) is expressed abundantly in the inner segments of the treated retina but is barely visible in the control retina. Rod and cone PDE (**b** and **c**) levels are also much higher in the treated retinas. Rhodopsin (**d**) and cone opsin (**e**) staining highlights well-maintained rod and cone photoreceptor outer segments in the treated retinas. The subcellular distribution patterns for each of those proteins in the treated retinas are indistinguishable from those of WT (data not shown). Note the reduction in outer nuclear layer thickness and the degenerated outer segments in the control retinas. Cell nuclei were counterstained with Hoechst dye 33342 (deep blue). Co-expression of the linked zsGreen reporter is restricted to the photoreceptor layer (punctate green signals in single channel; appearing as light blue in the merged image). OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer.

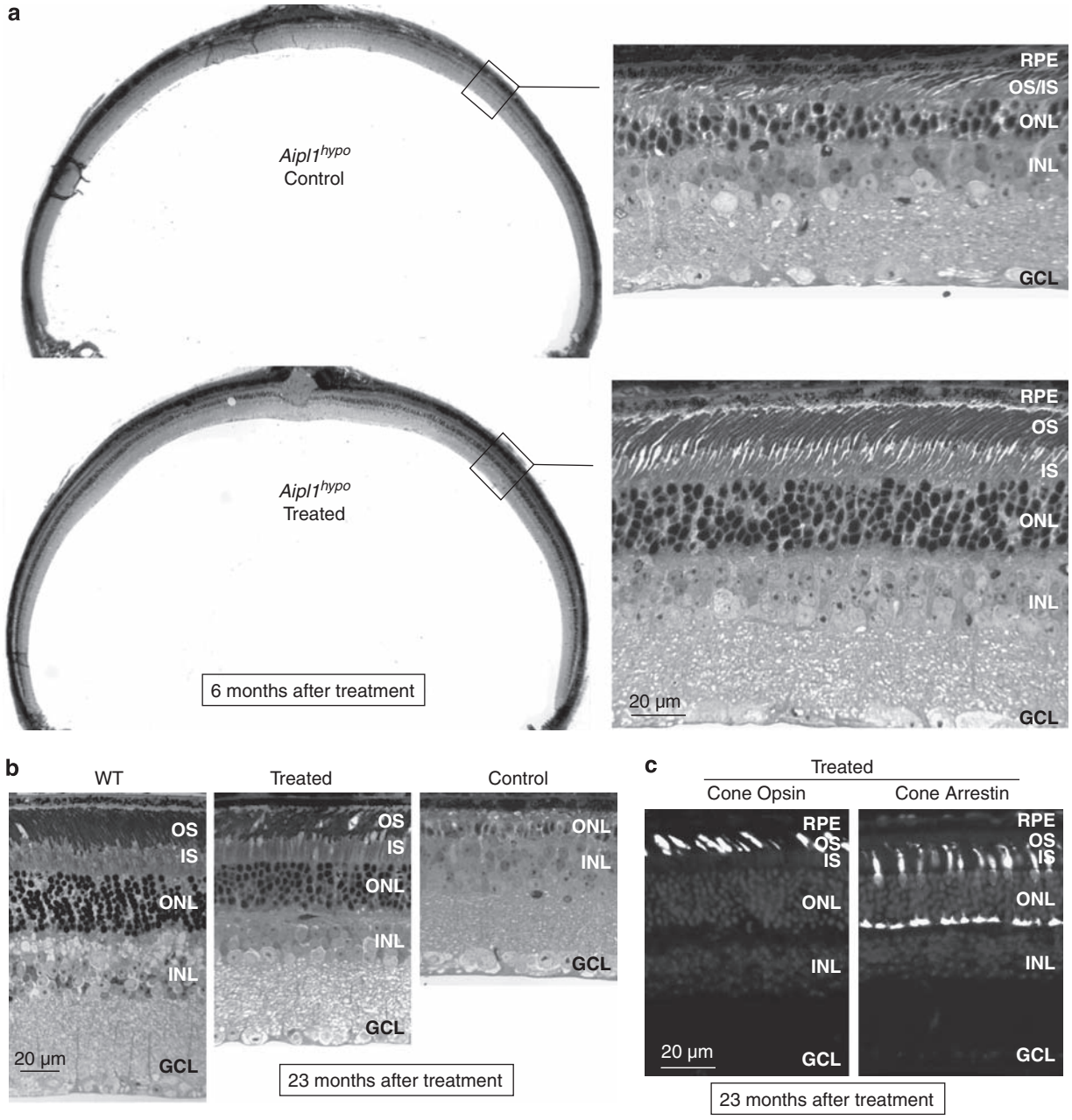


Figure 4 Morphologic analysis of *Aipl1*^{hypo} retinas after treatment with the AAV5-RK-mAIPL1 vector. (**a**) Semithin (1 μm) light micrographs of treated and control *Aipl1*^{hypo} retinas at 6 months after injection. Lower magnification images (left panel) show a thicker photoreceptor layer throughout the treated retina. Higher magnification images (right panel) show nearly normal appearing inner and outer segments in the treated retina, as well as a much thicker nuclear layer in the treated retina compared with the control. (**b**) Treated and control *Aipl1*^{hypo} retinas at 23 months after injection (28 months of age). The treated retina (left panel) maintained 5–6 rows of photoreceptor nuclei and shortened but well-organized inner and outer segments. In contrast, the control retina (right panel) had a single row of photoreceptor remaining, with no discernable inner or outer segments. (**c**) Immunofluorescence analysis for cone photoreceptor markers in the treated retinas at 23 months after injection. Both cone opsin (left) and cone arrestin (right) staining show near normal density of cone cells and well-maintained cone outer segments. There was no signal for cone arrestin or cone opsin in the control retinas. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer.

amplitudes in treated eyes remained at only 50% of WT values, this is nevertheless consistent with a strong rescue when the likelihood of modest tissue damage from the injections is taken into account.

Visual evoked potentials (VEPs), which reflect activity in the visual cortex in response to light stimulation of the retina, provide a gauge of the health of the inner retina's ability to transmit responses to visual cortex. Although loss of AIPL1 is not expected to impact the inner retina directly, we wished to determine whether the absence of photoreceptor function from birth in this mutant might have affected the inner retina owing to deafferentation. As shown in Figure 6c, VEPs were undetectable in mice that only received control

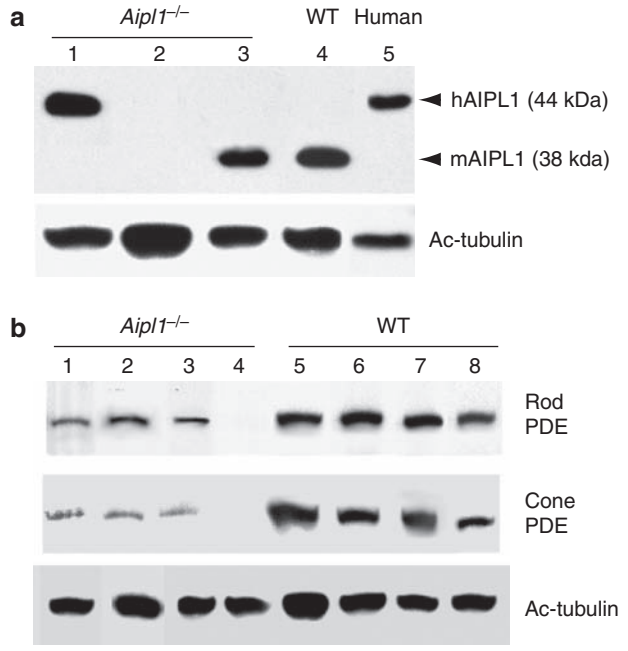


Figure 5 AAV8-mediated expression of human AIPL1 in the null mutant (*Aipl1*$^{-/-}$) retinas. (**a**) Western blotting for AIPL1 in treated and control retinas with an antibody that recognizes both murine and human AIPL1. Retinal homogenates from treated and control retinas at 4 weeks after injection were analyzed. Treated retina (lane 1) showed a 44-kDa protein that co-migrated with the AIPL1 protein from human retina (lane 5). A control retina (lane 2) had no AIPL1. A retina treated with AAV carrying the murine AIPL1 construct (lane 3) expressed a smaller protein that co-migrated with AIPL1 from WT mouse retinas (lane 4) (**b**) Western blotting for rod and cone PDEs at 4 weeks after injection of the AAV8-RK-hAIPL1 vector. Treated retinas from three different mice (lanes 1, 2 and 3) showed a partial restoration of rod and cone PDE synthesis, whereas a control retina (lane 4) had no PDE at all. The expression levels of both rod and cone PDEs remained lower than those of endogenous WT retinas (lanes 5–7). For comparison, a WT retina having received subretinal injection of a control vector (AAV-RK-EGFP; lane 8) was also included. For both panels a and b, acetylated α-tubulin (Ac-tubulin) was probed for as a loading control.

injections. In contrast, mice treated with the AAV8-hAIPL1 vector displayed a VEP, which averaged more than 50% of WT values. This not only shows functionality of the retina in subserving vision but also shows that inner retinal function did not suffer downstream losses at this time point.

Subretinal injection of the AAV8 vector carrying the human AIPL1 transgene mediated a high level of expression of hAIPL1 in the mutant mouse retinas. The transgenic human AIPL1 was distributed to the normal subcellular locations, that is, the inner segments and, to a lesser extent, the perinuclear cytoplasm and the synaptic terminals (Figure 7a). Rod and cone PDE synthesis was also restored and both proteins exhibited a normal distribution pattern (Figures 7b and c). Staining with rhodopsin and cone opsin also showed normal distribution for these two proteins. Furthermore, rod and cone cells appeared normal, with elongated outer segments (Figures 7d and e). The rescuing effect from a single subretinal injection appeared pan-retinal, as indicated by the restored PDE synthesis spanning the entire retina (Figure 7f).

To examine the extent of photoreceptor cell rescue in treated eyes compared with control eyes at a higher resolution, we embedded three pairs of eye samples in Epon and analyzed semithin sections (Figure 8a). The control retinas all showed no recognizable photoreceptor cells anywhere in the span of the retina, whereas the treated retinas all showed 5–6 rows of photoreceptor nuclei and well-organized inner and outer segments. Electron microscopy showed well-stacked disc membranes in the outer segments of the treated retinas (Figure 8b). Histological examination of treated and control eyes at 5 months after injection is shown in Figure 8c. The rescue effect was seen to be persistent in the treated *Aipl1*$^{-/-}$ mice.

Discussion

The past decade witnessed major advances in the technology to delivery genes stably to the retina. At present, the most effective gene delivery vectors are those derived from AAV.[26–31] Gene therapy studies have been performed in a number of animal models and have met with some degree of success.[32–39] We recently conducted a proof-of-concept gene therapy study in *Aipl1* mutant mice using a non-tissue-specific promoter (CMV) and obtained substantial and long-term rescue of the rod photoreceptor disease phenotype.[40] The first clinical trials for human LCA due to a defect in RPE65 targeted the RPE cells and produced encouraging results.[41–43] These studies suggest that replacement gene therapy mediated by AAV vectors is the most promising potential therapy for retinal degenerations, especially for those severe forms such as LCA that are unlikely to respond to more conservative forms of treatment.

This study presents the first successful example of targeting a transgene construct to both rods and cones simultaneously with a single tissue-specific promoter. In preparation for gene therapy in humans, we have designed the transgene expression constructs so that they are driven by the RK promoter previously shown to drive specific expression of a reporter gene in rods and cones in the context of AAV-mediated gene delivery. With this design, we showed increased production of AIPL1 in photoreceptor cells and increased levels of rod and cone PDE. As a result, retinal degeneration was significantly slowed. We observed improved photoreceptor cell survival, preservation of outer segment morphology and stabilization of retinal function. Importantly, the RK promoter used in the vector constructs to drive *AIPL1* transgene expression proved effective in rescuing both rod and cone photoreceptors.

Our data suggest that the RK promoter is well suited for driving *AIPL1* transgene expression in photoreceptors for gene therapy purposes. Both rhodopsin kinase and AIPL1 are considered relatively abundant in photoreceptors. In this study, the short RK promoter was able to restore mAIPL1 expression to near normal levels in the hypomorphic mutant. In the case of human AIPL1 expression in the null mutant, the data appear to indicate a somewhat higher expression level of the AIPL1 transgene relative to the endogenous expression level, assuming that the antibody used for that experiment indeed detects mouse and human AIPL1 equally. Regardless of this, the RK promoter, existing as an

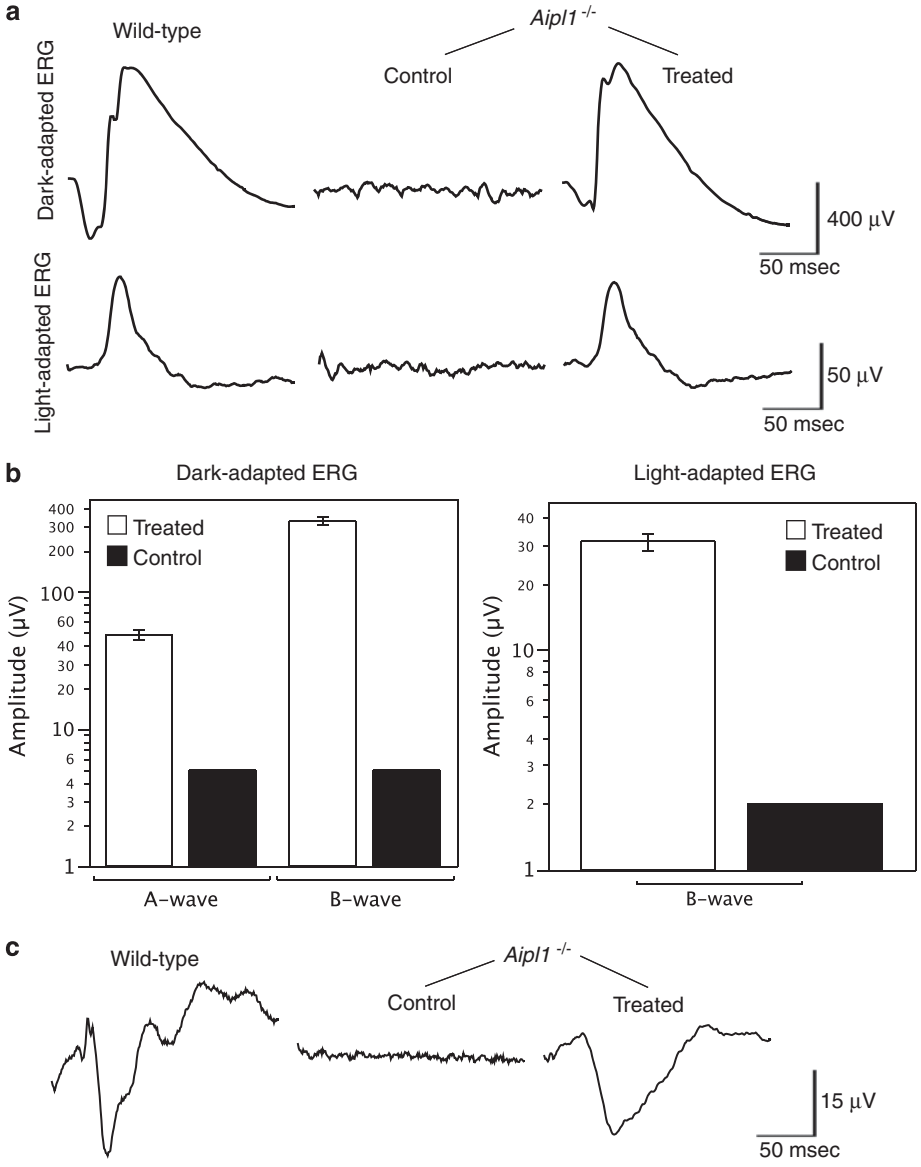


Figure 6 Restoration of retinal function in the *Aipl1*$^{-/-}$ retinas after treatment with AAV8-RK-hAIPL1. (**a**) Representative ERG traces from treated and control eyes. For both dark-adapted and light-adapted ERGs, treated retinas showed substantial light responses, whereas control eyes (middle) had undetectable responses. ERG amplitudes from treated eyes remained lower than those of WT eyes (left). (**b**) Bar graphs showing geometric mean ERG amplitudes of treated and control eyes at 4 weeks after injection. Left panel: *A*- and *B*-wave amplitudes of dark-adapted ERGs ($n=55$). The geometric mean values for both the *A*-wave (42 μV) and the *B*-wave (302 μV) of the treated eyes were significantly different from control eye values ($P<0.0001$ in both cases). Right: light-adapted (cone) *B*-wave amplitudes for the treated eyes ($n=20$). The geometric mean value (29 μV) for the treated eyes was significantly different from that of the control eyes ($P<0.0001$). Bar, ± s.e.m. (**c**) Visual evoked potentials recorded from the treated and control mice ($n=3$ for WT and treated animals; $n=5$ for controls). All treated mice exhibited a substantial VEP, whereas control mice had no detectable VEP. The geometric mean VEP amplitude from treated mice (16 μV) was smaller than that of WT mice (24 μV), but was significantly improved over the controls.

episomal element in the transgene DNA, drives AIPL1 expression to a level that is close to the endogenous AIPL1 expression level. With regard to specificity of expression, previous studies using a green fluorescent protein reporter have shown the RK promoter to be highly specific;[7,44,45] the reporter expression was found in rods and cones but not in RPE or inner retinal neurons. In this study, a linked zsGreen reporter translated from the bicistronic mRNA was also strictly limited to the photoreceptor layer, with no expression at all in RPE or inner retinal neurons. The fact that the RK promoter drove AIPL1 expression in cones in this study is evidenced by the increase and/or restoration of cone PDE synthesis as well as by functional and morphological rescue of cone photoreceptors in both the hypomorphic and null mutants. The RK promoter used in this study may be useful for driving other therapeutic transgenes that are normally expressed in rods and cones. Several known LCA genes, including *TULP1*, *RPGRIP1*, *GUCY2D*, as well as the X-linked

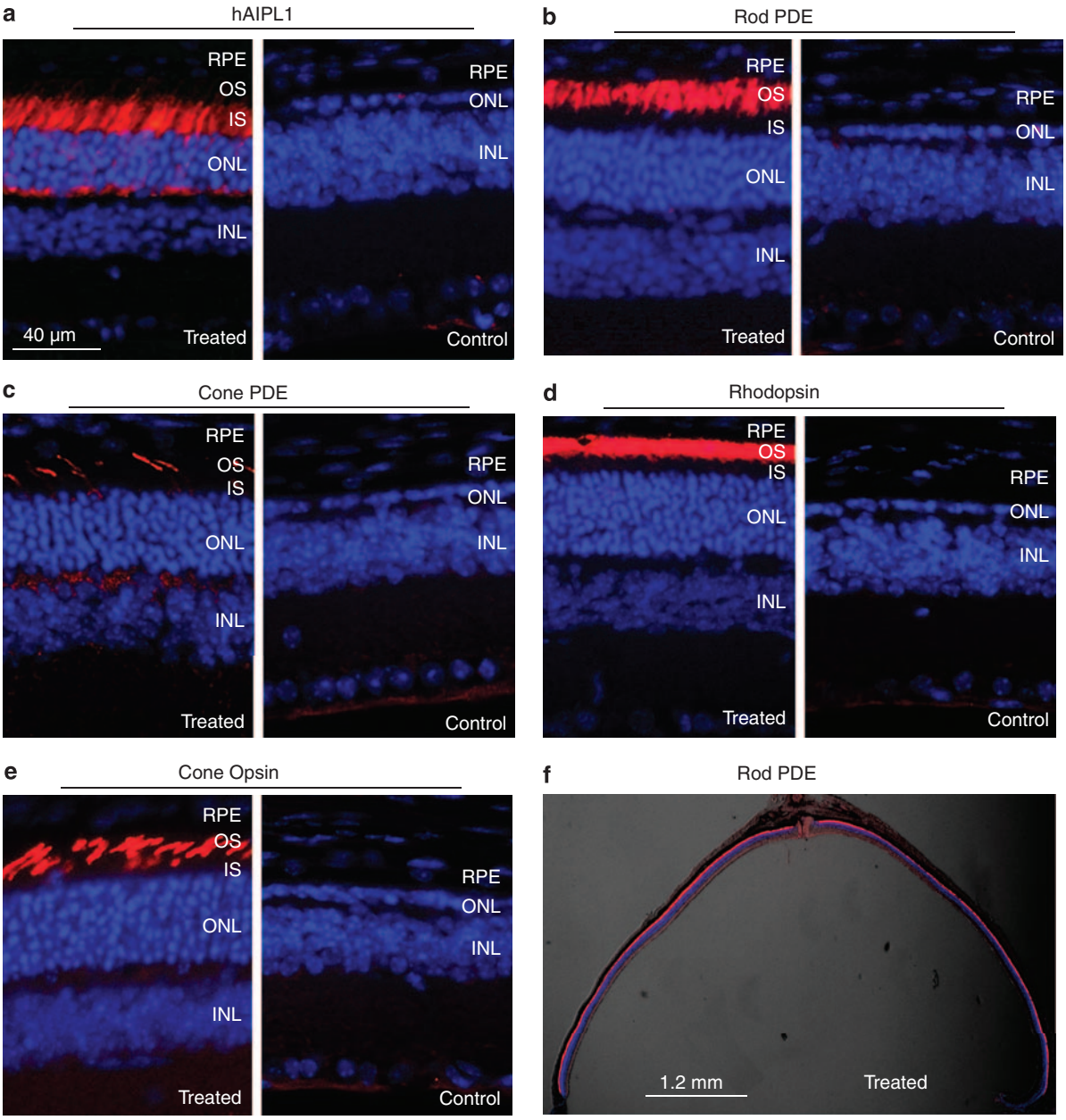


Figure 7 Immunofluorescence (red) analyses of treated and control *Aipl1*$^{-/-}$ retinas at 4 weeks after injection with AAV8-RK-hAIPL1. AIPL1 (**a**) is expressed abundantly in the inner segments of the treated retina but is absent in the control retina. Rod and cone PDE (**b** and **c**) levels are also much higher in the treated retinas. Rhodopsin (**d**) and cone opsin (**e**) staining illustrates well-maintained rod and cone photoreceptor outer segments in the treated retinas. The subcellular distribution patterns for each of those proteins in the treated retinas are indistinguishable from those of WT (data not shown). At this age (P30), only a single row of photoreceptors remained in the control retinas. A lower magnification image of a treated retina stained with rod PDE is shown in panel (**f**) to illustrate expression of the protein throughout the retina, indicating pan-retinal transduction by the injected AAV vector. Cell nuclei were counterstained with Hoechst dye 33342 (deep blue). RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer.

RPGR gene, are normally expressed in adult rods and cones.

This study largely corroborated our previous study[40] showing that mutant models with very different rates of disease progression respond favorably to AIPL1 replacement gene therapy. Similar to the previous study, two different *AIPL1* models were tested in this study. One was a hypomorphic mutant with a much reduced, but still detectable, expression of AIPL1 protein in photoreceptors. We have previously shown that in the hypomorphic mutant, PDE in rods and cones is present at much reduced levels[3,22] (and our unpublished data). Although photoreceptors initially develop and function nearly normally, they eventually degenerate. The relatively slow disease course of this model probably replicates the disease course of some of the patients who

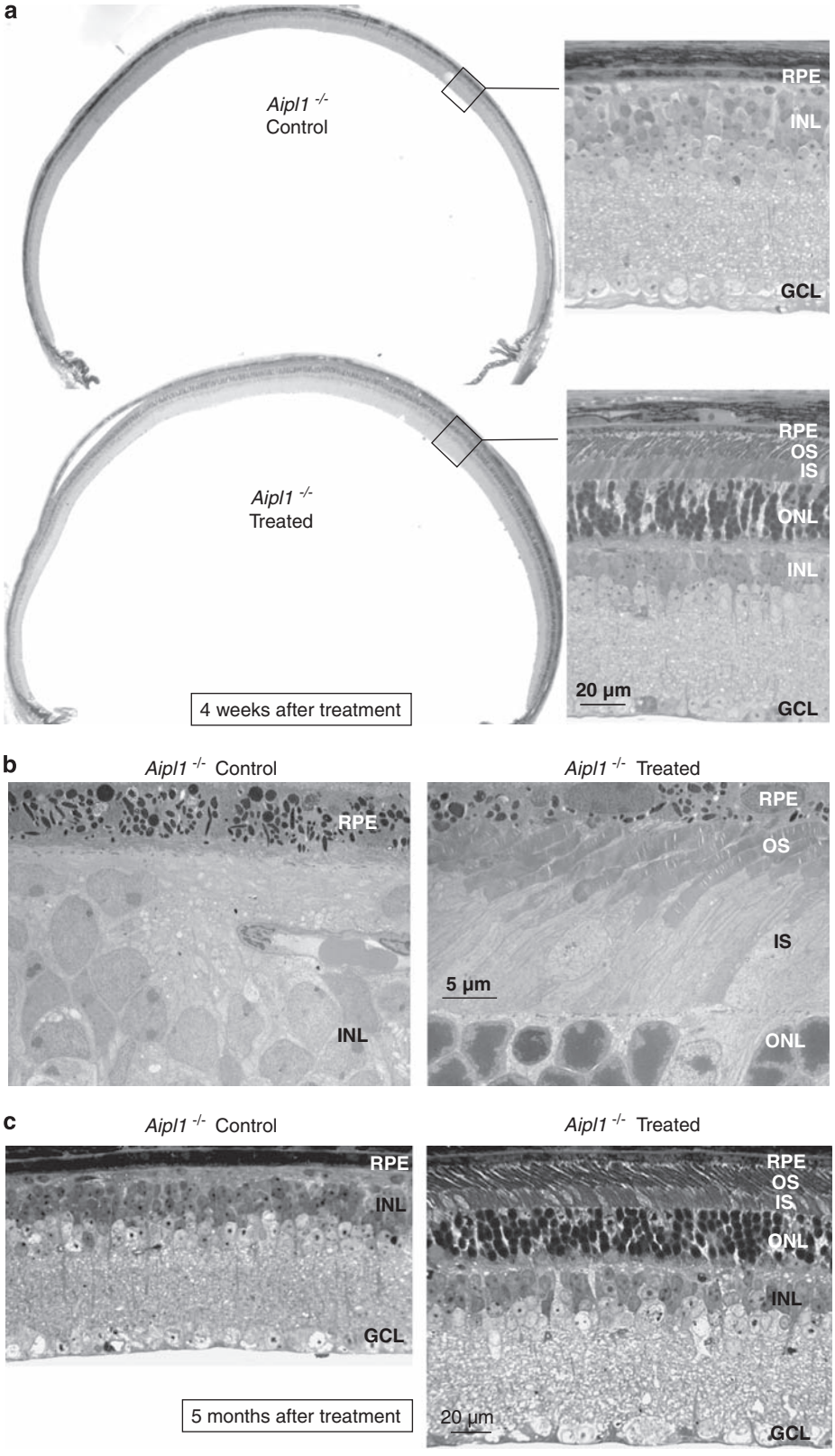


Figure 8 Morphological analysis of treated and control *Aipl1*$^{-/-}$ retinas at 4 weeks after injection. (**a**) Semithin (1 µm) light micrographs of treated and control *Aipl1*$^{-/-}$ retinas. Lower magnification images (left) show a thicker photoreceptor layer throughout the treated retina. Higher magnification images (right) show well-preserved inner and outer segments in the treated retina, and 6–7 rows of photoreceptor nuclei in the treated retinas. In contrast, there is a sparse row photoreceptor nuclei remaining in the control retinas, with no inner or outer segments. (**b**) Electron microscopy analysis of treated and control retinas. The treated retina (right) retained well-organized outer segment disc structures. (**c**) Light microscopic sections of control (left) and treated (right) retinas at 5 months after injection. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer.

carry missense mutations characterized by a course of degeneration more consistent with RP rather than with LCA. A second mouse model used in this study was the null mutant. In this model, there is no AIPL1 and, as a result, little, if any, PDE could accumulate. Photoreceptors degenerate very rapidly so that by 20 days of age, the degeneration is virtually complete.[2,6] Both models responded well to AAV vector-mediated gene therapy. However, AAV5 vectors worked well in the hypomorphic model but not in the null model. This is apparently related to the very fast onset of disease in the null mutant and the relatively slow onset of expression from the AAV5 vectors. Switching from AAV5 to AAV8 with the same expression construct profoundly altered the outcome, giving a strong therapeutic rescue in the null mutant. AAV-mediated transgene expression in the *Aipl1* mutant models appears stable and the efficacy of rescue is long lasting. In the hypomorphic mutant, AAV5-mediated gene expression remained in the retina at 23 months after injection and the photoreceptors were well maintained at that age. Similarly, AAV8-mediated delivery of the human *AIPL1* transgene in the null mutant appears stable for at least 5 months after injection, the latest time point we examined. We have observed no apparent increase in mortality in mice that have received vector injections.

With a view toward a future clinical trial in humans, the main objective of this study was to test and validate a human AIPL1 replacement gene driven by a photoreceptor-specific promoter of human origin. This has proven largely successful, as the AAV8-RK-hAIPL1 vector clearly forced expression of human AIPL1 in the recipient *Aipl1* null mouse retinas, restored rod and cone PDE synthesis, restored photoreceptor function as illustrated by ERGs and rescued photoreceptors from degeneration. We propose that the basic construct design as illustrated in this study, after inactivation of the internal ribosomal entry site and linked zsGreen reporter, meets the expectation for an effective gene therapy vector for *AIPL1* gene mutations. As such it can be moved toward a clinical trial in humans with retinal degeneration caused by these mutations.

Materials and methods

Animals

Hypomorphic Aipl1 mutant mice ($Aipl1^{hypo}$) used in this study were offspring from a cross between Aipl1 hypomorphic mice ($Aipl1^{h/h}$) that we previously published[3] and a line of *Aipl1 null* ($Aipl1^{-/-}$) mice generated by targeted disruption.[6] The $Aipl1^{h/h}$ line of mice was estimated to have AIPL1 at ~20% of WT level, and the null mice do not express AIPL1. The F1 generation ($Aipl1^{hypo}$) from this cross is expected to have an even lower level of AIPL1 relative to $Aipl1^{h/h}$ mice. The $Aipl1^{hypo}$ mice were chosen for this study because they underwent a slightly faster rate of retinal degeneration than the $Aipl1^{h/h}$ line of mice, so as to shorten the duration of the study. The $Aipl1^{hypo}$ and $Aipl1^{-/-}$ mice were used as recipients for gene therapy in this study. WT mice used in the study were C57BL from Charles River Laboratory (Wilmington, MA, USA). Mice were maintained in an animal facility under 12-h light/12-h dark lighting cycle.

Plasmid constructions and production of recombinant AAV5 and AAV8

The murine and human *AIPL1* cDNA were PCR amplified from murine or human retinal cDNA using primers that were designed to encompass the entire coding region. The cDNAs thus obtained were sequenced to verify fidelity. Human AIPL1 isoform 1 was chosen for this study. To construct the AAV vectors, AIPL1 cDNAs were inserted into the multiple cloning site of the parental pAAV-RK-zsGreen vector. The resulting pAAV-RK-hAIPL1 and pAAV-RK-mAIPL1 vectors were packaged into AAV. AAV2/5 and AAV2/8 pseudotyped vectors were generated by tripartite transfection (AAV vector plasmid encoding the gene of interest, AAV helper plasmid pLT-RC03 encoding AAV Rep proteins from serotype 2 and Cap proteins from serotype 5 or 8 and adenovirus helper mini-plasmid pHGTI-Adeno1) into 293A cells. The transfection was performed using a protocol developed by Xiao *et al*.[46] At 2 days after transfection, cells were lysed by repeated freeze and thaw cycles. After initial clearing of cell debris, the nucleic acid component of the virus producer cells was removed by Benzonase treatment. The recombinant AAV vector particles were purified by iodixanol density gradient. The purified vector particles were dialyzed extensively against phosphate buffered saline (PBS) and titrated by dot blot hybridization.

Subretinal injection

Mice were placed under general anesthesia with an intraperitoneal injection of ketamine (90 mg kg^{-1})/xylazine (9 mg kg^{-1}). A 0.5% proparacaine solution was applied to the cornea as a topical anesthetic. Pupils were dilated with topical application of cyclopentolate and phenylephrine hydrochloride. Under an ophthalmic surgical microscope, a small incision was made through the cornea adjacent to the limbus using an 18-gauge needle. A 33-gauge blunt needle fitted to a Hamilton syringe was inserted through the incision while avoiding the lens and pushed through the retina. All injections were made subretinally in a location within the nasal quadrant of the retina. Each animal received 0.5–1 μl of AAV at 1×10^{12} particles per ml. Treatment vectors were typically given in the left eye and control vectors/vehicle were given in the fellow eye (referred throughout this text as 'treated' or 'control', respectively). Visualization during injection was aided by addition of fluorescein (100 mg ml^{-1} AK-FLUOR, Alcon, Fort Worth, TX, USA) to the vector suspensions at 0.1% by volume. Fundus examination during the injection found the entire retina detached, confirming successful subretinal delivery.

Histology and immunofluorescence

For both light microscopy and transmission electron microscopy, enucleated eyes were fixed for 10 min in 1% formaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5). After removal of the anterior segments and lens, the eye cups were left in the same fixative at 4 °C overnight. Eye cups were washed with buffer, post-fixed in osmium tetroxide, dehydrated through a graded

alcohol series and embedded in Epon. Semi-thin sections (1 μm) were cut for light microscopy observations. For electron microscopy, ultrathin sections were stained in uranyl acetate and lead citrate before viewing on a JEOL 100CX electron microscope (JEOL USA, Inc., Peabody, MA, USA).

For immunofluorescence, eyes were enucleated, placed in fixative and their anterior segments and lens were removed. The fixative was 2% formaldehyde, 0.25% glutaraldehyde/PBS. Duration of fixation was typically 20 min. The fixed tissues were soaked in 30% sucrose/PBS for at least 2 h, shock frozen and sectioned at 10-ím thickness in a cryostat. Sections were collected into PBS buffer and remained free floating for the duration of the immunostaining process. The sections were viewed and photographed on a laser scanning confocal microscope (model TCS SP2; Leica Microsystems Inc., Bannockburn, IL, USA).

Two antibodies for AIPL1 were used in this study. One was generated against the full-length mouse AIPL1 protein as described.[3] This antibody detects mouse AIPL1 and cross-reacts weakly with human AIPL1. The second antibody was generated against the full-length human AIPL1 protein[2] and affinity-purified against the mouse AIPL1 antigen. This antibody was assumed to recognize mouse and human AIPL1 proteins equally. Antibody for cone PDE protein was generated against the first 200 amino acid residues of mouse cone PDE. Any cross-reactivity to rod PDE was removed by absorbing against mouse rod PDE α- and β-subunit. Antibody for mouse cone opsin was generated by immunizing chickens with synthetic peptides corresponding to a region near the C terminus of blue cone opsin (CRKPMADESDVSGSQKT) and to the N terminus of green cone opsin (MAQRLTGEQTLDHYEDSTHAS), respectively. Chicken antisera were affinity-purified. Other antibodies used in the study include monoclonal antibody for rhodopsin (rho 1D4; gift of Robert Molday)[47] and rod PDE (rod PDE β-subunit; Affinity BioReagent, Golden, CO, USA).

ERG and VEP recording

For ERG recording, mice were dark-adapted overnight and anesthetized with sodium pentobarbital (i.p.) before testing; both pupils of each animal were topically dilated with phenylephrine hydrochloride and cyclopentolate hydrochloride, and mice were then placed on a heated platform. Rod dominated responses were elicited in the dark with 10-μs flashes of white light (1.37×10^5 cd m^{-2}) presented at intervals of 1 min in a Ganzfeld dome. Light-adapted, cone responses were elicited in the presence of a 41-cd m^{-2} rod-desensitizing white background with the same flashes (1.37×10^5 cd m^{-2}) presented at intervals of 1 Hz. ERGs were monitored simultaneously from both eyes with a silver wire loop electrode in contact with each cornea topically anesthetized with proparacaine hydrochloride and wetted with Goniosol. A saline saturated cotton wick was placed in the mouth as the reference. An electrically shielded chamber served as ground.

For VEP recording, mice were dark-adapted, their pupils dilated, and anesthetized with ketamine/xylazine (i.p.). Responses were elicited with flashes (1.37×10^5 cd m^{-2}) of light presented at 1 Hz to one eye while the fellow eye remained occluded. Eyes were patched with Coverlet Adhesive eye occlusor (Beiersdorf Jobst, Charlotte, NC, USA), cut to fit the small size of the mouse eye and sealed around the edges with black plastic tape. VEPs were monitored with subdermal electrodes in the scalp over the visual cortex as the positive electrode and over the frontal cortex as the reference.

All responses were differentially amplified at a gain of 1000 (−3 db at 2 and 300 Hz; AM502, Tektronix Instruments, Beaverton, OR, USA), digitized at 16-bit resolution with an adjustable peak-to-peak input amplitude (PCI-6251, National Instruments, Austin, TX, USA) and displayed on a personal computer using custom software (Labview, version 8.2, National Instruments). Independently for each eye, cone ERGs were conditioned by a 60-Hz notch filter and an adjustable artifact-reject window, summed ($n = 4$–20), and then fitted to a cubic spline function with variable stiffness to improve signal–noise without affecting their temporal characteristics; in this way, we could resolve cone *B*-wave responses as small as 2 μV. For VEP recordings, consecutive waveforms were averaged ($n = 50$) after suppressing the heart-beat artifact with an adjustable low-pass digital lter (cutoff at 50 Hz) and rejecting waveforms containing movement artifacts by an adjustable voltage window.

Statistical analysis

JMP, version 6 (SAS Institute, Cary, NC, USA) was used to compare outcomes in treated versus untreated eyes by the one-tailed paired *t*-test. For these interocular comparisons, nondetectable dark-adapted (single flash) amplitudes were coded as 5 μV and nondetectable light-adapted (averaged) amplitudes were coded as 2 μV.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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