Examining the Role of Cone-expressed RPE65 in Mouse Cone Function

Alexander V. Kolesnikov¹, Peter H. Tang²,³ & Vladimir J. Kefalov¹

Efficient chromophore supply is paramount for the continuous function of vertebrate cone photoreceptors. It is well established that isomerization of all-trans- to 11-cis-retinoid in the retinal pigmented epithelium by RPE65 is a key reaction in this process. Mutations in RPE65 result in a disrupted chromophore supply, retinal degeneration, and blindness. Interestingly, RPE65 has recently been found to also be expressed in cone photoreceptors in several species, including mouse and human. However, the functional role of cone-expressed RPE65 has remained unknown. Here, we used loss and gain of function approaches to investigate this issue. First, we compared the function of cones from control and RPE65-deficient mice. Although we found that deletion of RPE65 partially suppressed cone dark adaptation, the interpretation of this result was complicated by the abnormal cone structure and function caused by the chromophore deficiency in the absence of RPE65 in the pigmented epithelium. As an alternative approach, we generated transgenic mice to express human RPE65 in the cones of mice where RPE65 expression is normally restricted to the pigmented epithelium. Comparison of control (RPE65-deficient) and transgenic (RPE65-expressing) cones revealed no morphological or functional changes, with only a slight delay in dark adaptation, possibly caused by the buffering of retinoids by RPE65. Together, our results do not provide any evidence for a functional role of RPE65 in mouse cones. Future studies will have to determine whether cone-expressed RPE65 plays a role in maintaining the long-term homeostasis of retinoids in cones and their function and survival, particularly in humans.

Our nighttime and daytime visual functions are mediated by two major classes of retinal photoreceptor cells, the rods and cones, respectively. Visual perception begins with the absorption of light by visual pigments within rods (rhodopsin) and cones (cone opsins), which triggers the photosomerization of their covalently-bound chromophore (11-cis-retinal) to its all-trans conformation⁴. Activated pigments initiate a cascade of phototransduction reactions that ultimately generates a physiological response to light⁵,⁶. Bleached photoreceptors restore their photon-catching function by continuous recycling of all-trans-retinal back to 11-cis-retinal via a process called the visual (retinoid) cycle. In vertebrates, two major interrelated visual cycles have been described. The classical retinal pigmented epithelium (RPE)-based visual cycle supplies 11-cis-retinal to both rods and cones, while a more recently discovered alternative visual cycle functions through Müller glial cells of the retina to deliver chromophore specifically to cones⁷,⁸,⁹. An additional mechanism involving direct all-trans-retinal photoisomerization to 11-cis-retinal with blue light, similar to that occurring within invertebrate opsins, has also been described⁴,⁸,⁹.

A critical step in the classical visual cycle is the enzymatic isomerization of all-trans-retinol, released from the photoreceptors, to 11-cis-retinol. This reaction takes place in the RPE and is catalyzed by an abundantly expressed 65-kDa protein named RPE65¹⁰,¹¹. In addition to its crucial role in rod pigment regeneration, RPE65 is also important for the function and survival of human cone photoreceptors¹². Numerous naturally occurring mutations in the RPE65 gene result in a disrupted visual cycle and lead to a severe clinical disease called Type 2 Leber congenital amaurosis. This blinding condition is characterized by the early loss of cone-mediated vision, sensory nystagmus, and the lack of electroretinographic (ERG) signals¹³.

¹Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, Saint Louis, MO, 63110, USA. ²Department of Ophthalmology, Medical University of South Carolina, Charleston, SC, 29425, USA. ³Present address: Byers Eye Institute, Department of Ophthalmology, Stanford University School of Medicine, Palo Alto, CA, 94303, USA. Correspondence and requests for materials should be addressed to V.J.K. (email: kefalov@wustl.edu)
Surprisingly, in addition to its well-known localization within the pigmented epithelium, RPE65 protein has been also found in the cones of numerous mammalian species, including rod-dominant mouse, cow, and human 12–17 and the cone-dominant lizard 5. With the exception of lizard cones, where this protein is localized in their inner segments, in all other reported cases RPE65 is present in the cone outer segments (OS), with particular abundance in their basal region 3. Interestingly, the level of RPE65 within cones across different strains of mice appears to be inversely related to its level in the RPE 18, suggesting a possible role in the recycling of chromophore in cones.

Unlike its well-established role in the RPE, the function of cone RPE65 remains unclear. As the enzymes required for generating its all-trans-retinyl ester substrates (LRAT and ARAT) have not been identified in photoreceptors, cone RPE65 is not likely to operate as a retinoid isomerase. Instead, recent studies have hypothesized its function as a retinoid binding protein which could stabilize 11-cis-retinol delivered from Müller cells and shuttle it along the cone OS 16. In addition, based on in vitro experiments with a cone-derived 661 W cell line, another possible role of cone RPE65 could be to bind 11-cis-retinyl esters to facilitate their hydrolysis 17, or even to enhance the oxidation of 11-cis-retinol by promoting its interaction with a yet unidentified cone-specific retinol dehydrogenase 18–20. Interestingly, in contrast to mouse strains with higher levels of cone RPE65 where cone pigment is fully regenerated after dark adaptation, those with little or no cone RPE65 appear to have a mild chromophore deficiency 10. However, direct biochemical or physiological evidence for a functional role of RPE65 in cones in vivo has remained missing.

Here, we used loss and gain of function approaches to investigate the proposed physiological function of cone RPE65 in the alternative visual cycle, as well as its possible role as a structural and functional component of the cone OS that might support the viability and signaling of these photoreceptors under conditions of severe visual chromophore deficiency.

Results
Systemic administration of 9-cis-retinal partially rescues cone function and morphology in RPE65-deficient mice. In attempt to determine the potential role of RPE65 protein expressed within mouse cones, we first used the available Rpe65−/− mice 10. Expression of both M- and S-opsins, cone density, and function in this model are all severely compromised as early as two weeks after birth 21, complicating its physiological characterization. However, they can be rescued to a significant degree by systemic injections of exogenous 11-cis-retinal or 9-cis-retinal, starting before the onset of cone degeneration 21,22. We applied a previously described sustained delivery method of commercially available 9-cis-retinal to mouse photoreceptors in vivo, in which a 1:10 (by volume) mixture of the retinoid and a thermosensitive polymer Matrigel® Matrix are repeatedly injected subcutaneously. Matrigel is a growth factors-containing hydrogel which is a liquid at 4 °C and can be loaded with retinoid. Upon injection, Matrigel undergoes a phase change into its solid form at the mouse body temperature, and then it slowly releases the bound chromophore into the circulation, from where it is eventually transported to the eye and retina 23.

The efficacy of chromophore administration was first examined in the rods of Rpe65−/− mice that do not degenerate appreciably until 7 weeks of age 15. Retinoid injections were initially performed at postnatal day 10 (P10), just before mice open their eyes, and then at P14, P18, and P23. The animals were raised in constant darkness from the first injection onwards to prevent bleaching of visual pigments and to improve the effectiveness of chromophore supplementation 24. A cohort of control mice from the same litter was kept dark-reared under similar experimental conditions but without chromophore supplementation. Transretinal (ex vivo) rod ERG recordings were carried out at P28–P30 (Fig. 1). In this method, the presence of postsynaptic inhibitors blocked contributions of higher order response components (such as ON bipolar cell-driven ERG b-wave), allowing us to isolate the rod photoresponse component 25. Consistent with previously reported results 26, chromophore-deficient rods in Rpe65−/− mice exhibited ~10-fold smaller and significantly faster responses to test flashes of green light and were desensitized by approximately ~2000-fold, as compared with those from wild-type (C56BL/6j) animals (Fig. 1A,B,D). Systemic administration of Matrigel preloaded with 9-cis-retinal largely restored the sensitivity and kinetics of rod responses in this mouse model (Fig. 1A,C,D) thus confirming the efficiency of this delivery method. The remaining ~3-fold difference in rod photosensitivity between chromophore-treated Rpe65−/− mice and wild-type animals can be entirely attributed to the correspondingly lower quantum efficacy of vertebrate isorhodopsin (a pigment formed with 9-cis-retinal) compared to rhodopsin, in which opsin is bound to 11-cis-retinal 27.

We then applied the same procedure to supply the rapidly degenerating cones in Rpe65−/− mice with exogenous 9-cis-retinal (Fig. 2). To facilitate cone recordings, these animals were derived on a Gnat1−/− background (lacking rod transducin α-subunit) that eliminates the rod component of the light response without affecting cone morphology or function 28. In this study, we performed all of our physiological experiments using mid-wavelength light signal cone (M-cone) response, due to methodological limitations of using UV light and the proximity of the absorbance spectra of M-cone pigment to that of rhodopsin. This allows for a better comparison between rod and M-cone response. In contrast to rods, M-cones in untreated 4-week-old Rpe65−/−Gnat1−/− mice were essentially unresponsive to green light, indicating the rapid deterioration of their function as compared to cells from control Gnat1−/− retinas (Fig. 2A,B,D). Systemic administration of 9-cis-retinal/Matrigel mixture only partially restored cone function (Fig. 2C). Although the maximal amplitude of M-cone response recovered to ~50% of that in Gnat1−/− mice, the sensitivity of chromophore-treated cones was still ~20 times lower (indicating a 7-fold reduction, considering the use of 9-cis-retinal), and the responses revealed abnormal and substantially slower activation and inactivation (Fig. 2A,C,D). The time-to-peak of dim flash M-cone responses in Matrigel-treated animals lacking RPE65 ranged from 250 to 300 ms, as compared to only ~70 ms in control mice.

Opsin mislocalization plays a key role in the pathophysiology of cone loss in diseases involving a disrupted retinoid cycle. Previous studies in RPE65- and LRAT-deficient mice indicate that trafficking of several
phototransduction proteins (including M- and S-opsins and G-protein transducin) to the cone OS is impaired in these models, and both opsins become distributed throughout the entire length of the cell. Therefore, we characterized the localization of M-opsin in cones of 4-week-old control and 9-cis-retinal-treated Rpe65−/− animals (Fig. 3). In accordance with previous findings, prolonged supply of visual chromophore to RPE65-deficient mice largely improved targeting of M-opsin to the cone OS in the dorsal retina as compared to untreated counterparts (Fig. 3B,C). Cone numbers and density were also substantially increased in chromophore-treated Rpe65−/− animals. However, the rescue was only partial, as some M-pigment was still present in cone synaptic terminals (Fig. 3C). This was in contrast to that observed in Gnat1−/− controls, which also had a greater number of dorsal cones overall (Fig. 3A). Together with the delayed cone response kinetics evident from our ex vivo ERG recordings described above, this result indicates that even prolonged treatment with exogenous 9-cis-retinal cannot rescue fully the trafficking of phototransduction proteins to cone OS and the physiological function of M-cones in RPE65-deficient mice. Despite that, the method still allowed us to investigate how the removal of RPE65 from cones affects their dark adaptation.

Suppressed dark adaptation of M-cones in mice lacking RPE65. RPE65 has been found in cones of both rod-dominant human and cone-dominant lizard retinas, where it has been proposed to play a role as a facilitator of the cone-specific visual cycle. Therefore, we characterized the localization of M-opsin in cones of 4-week-old control and 9-cis-retinal-treated Rpe65−/− animals (Fig. 3). In accordance with previous findings, prolonged supply of visual chromophore to RPE65-deficient mice largely improved targeting of M-opsin to the cone OS in the dorsal retina as compared to untreated counterparts (Fig. 3B,C). Cone numbers and density were also substantially increased in chromophore-treated Rpe65−/− animals. However, the rescue was only partial, as some M-pigment was still present in cone synaptic terminals (Fig. 3C). This was in contrast to that observed in Gnat1−/− controls, which also had a greater number of dorsal cones overall (Fig. 3A). Together with the delayed cone response kinetics evident from our ex vivo ERG recordings described above, this result indicates that even prolonged treatment with exogenous 9-cis-retinal cannot rescue fully the trafficking of phototransduction proteins to cone OS and the physiological function of M-cones in RPE65-deficient mice. Despite that, the method still allowed us to investigate how the removal of RPE65 from cones affects their dark adaptation.

Figure 1. Treatment with 9-cis-retinal largely restores sensitivity of chromophore-deficient mouse rods. (A) Representative family of transretinal rod ERG responses from wild-type (C57BL/6J) mouse retinas. Test flashes of 505 nm light with intensities of 1.6, 4.8, 14, 33, 114, 392, 1.2 × 10^4, and 3.9 × 10^4 photons μm^−2 were delivered at time 0. (B) Representative family of transretinal rod ERG responses from Rpe65−/− mouse retinas. Test flashes of 505 nm light with intensities of 6.9 × 10^3, 1.7 × 10^4, 5.7 × 10^4, 2.0 × 10^5, 6.0 × 10^5, 2.0 × 10^6, and 5.7 × 10^6 photons μm^−2 were delivered at time 0. There were no responses if lower flash intensities were applied. (C) Representative family of transretinal rod ERG responses from retinas of Rpe65−/− animals repeatedly injected with 9-cis-retinal and Matrigel Matrix. Test flashes of 505 nm light with intensities of 14, 33, 114, 392, 1.2 × 10^4, 3.9 × 10^4, and 1.1 × 10^5 photons μm^−2 were delivered at time 0. (D) Averaged rod intensity-response functions (mean ± SEM) for wild-type (n = 3), Rpe65−/− (n = 4), and 9-cis-retinal treated Rpe65−/− (n = 7) mice. Error bars for some points are smaller than the symbol size. Hyperbolic Naka-Rushton fits yielded half-saturating intensities (I_{1/2}) of 46, 8.9 × 10^4, and 142 photons μm^−2, respectively.
control Gnat1<sup>−/−</sup> and Rpe65<sup>−/−</sup> Gnat1<sup>−/−</sup> mice (derived from breeding of the two individual knockout strains) were used. It should be noted that the original Rpe65<sup>−/−</sup> line<sup>10</sup> was generated on the C57BL/6J genetic background that has been shown to express RPE65 protein in cones<sup>16</sup>. In contrast, the original Gnat1<sup>−/−</sup> strain<sup>18</sup> was derived and maintained on the BALB/c background which lacks cone RPE65 expression<sup>16</sup>. Thus, it was important to analyze whether our composite control Gnat1<sup>−/−</sup> strain (Rpe65<sup>−/+</sup> Gnat1<sup>−/−</sup>) with a mixed C57BL/6J x BALB/c background preserved RPE65 in retinal cones. Indeed, the endogenous expression of mouse cone RPE65 protein in this line was confirmed by immunohistochemistry using PETLET anti-RPE65 antibody (Fig. 4A and its inset, green). This allowed us to compare the dark adaptation of RPE65-expressing cones in control (Gnat1<sup>−/−</sup>) retinas and RPE65-deficient cones in Rpe65<sup>−/−</sup> Gnat1<sup>−/−</sup> retinas.

We began with control recordings, first determining the dark-adapted sensitivity (S<sub>fDA</sub>) of 4-week-old control M-cones. The isolated retina was then exposed to bright light that rapidly bleached an estimated >90% of the cone visual pigment. Following this nearly complete pigment bleach, cones in control retinas were initially desensitized by almost 3 log units and then gradually recovered most of their sensitivity in the dark, with a time constant of ~1.2 ± 0.2 min (Fig. 4B). The recovery of cone sensitivity was then measured in retinas of age-matched RPE65-deficient mice (Rpe65<sup>−/−</sup> Gnat1<sup>−/−</sup>) that were raised in constant darkness and repeatedly supplemented with 9-cis-retinal/Matrigel mixture, as described in the previous section. Consistent with those results (Fig. 2D), the initial dark-adapted sensitivity of M-cones in this line was ~10 times lower. We found that the lack of RPE65 decelerated the subsequent cone dark adaptation by ~5-fold (time constant of ~6.1 ± 0.4 min) under identical (>90%) pigment bleaching conditions. Interestingly, the fractional level of cone sensitivity recovery (Sj/S<sub>fDA</sub>) by 30 min postbleach was ~1.7-fold higher in mutant retinas, as compared to controls (~60% vs. ~35%, respectively).
were replotted from Fig. 4B using the right y-axis of Fig. 4C. cis-retinal (Figs 2 and 3) could have also contributed to the suppressed dark adaptation of mutant exogenous 9-
mised when cones were lacking their RPE65 protein. However, the abnormally slow kinetics of cone responses
was necessary and sufficient to eliminate any residual cone expression of mouse RPE65 in C57BL/6J mice 16.
~0.5 cones, thus complicating the interpretation of the results obtained with this animal model.
(Fig. 5C). Surprisingly, the expected abundant expression of human RPE65 in rods (as the
antibody did not reveal the presence of endogenous mouse RPE65 in their cones; however, a normal abundance
of RPE65 was shown in the RPE layer (Fig. 5B, green signal, compare this result to the one shown in Fig. 4A).
In stark contrast, human RPE65 protein was exclusively expressed in the cone OS of transgenic animals, but
cumstance greatly facilitated the detection of transgenic RPE65 protein in mouse cones. Both M-cone density and
morphology in the retinas of control and transgenic animals were normal, as was revealed by immunolabeling
pigment in mutant retinas (Fig. 4C). Compared in this way, the difference in rates of cone dark adaptation
between control and 9-cis-retinal treated Rpe65−/−Gnat1−/− mice was even more dramatic (time constants of
~0.5 ± 0.06 min and ~6.1 ± 0.4 min, correspondingly). For better visualization, the data from mutant animals
were replotted from Fig. 4B using the right y-axis of Fig. 4C.
Together, these results may indicate that chromophore recycling through the retina visual cycle was compromised when cones were lacking their RPE65 protein. However, the abnormally slow kinetics of cone responses in RPE65-deficient mice (Fig. 2C) and incomplete rescue of cone morphology and function upon treatment with exogenous 9-cis-retinal (Figs 2 and 3) could have also contributed to the suppressed dark adaptation of mutant cones, thus complicating the interpretation of the results obtained with this animal model.

Transgenic expression of RPE65 in normal mouse M-cones. We developed an alternative approach to more directly address the possible role of cone RPE65 in dark adaptation of mammalian cone photoreceptors. To do that, we generated a novel mouse line which transgenically expresses human RPE65 protein in normal retinal cones under the control of the rhodopsin kinase promoter (see Materials and Methods). The choice of a highly homologous human RPE65 (Leu-450 isoform) was mainly dictated by the possibility to distinguish it from the endogenous mouse protein by immunohistochemistry. Again, to facilitate cone recordings, we derived this line on Gnat1−/− background. Because proven C57BL/6J mouse embryos were used for ES cells microinjection to generate a single chimeric animal, we further crossed it with original Grk1−/−− littermates with PETLET anti-RPE65 antibody did not reveal the presence of endogenous mouse RPE65 in their cones; however, a normal abundance of RPE65 was shown in the RPE layer (Fig. 5B, green signal, compare this result to the one shown in Fig. 4A).
In stark contrast, human RPE65 protein was exclusively expressed in the cone OS of transgenic animals, but not in transgene-negative controls, as was detected by immunostaining with the DALEED anti-RPE65 antibody (Fig. 5C). Surprisingly, the expected abundant expression of human RPE65 in rods (as the Grk1 promoter has to be active in all retinal photoreceptors) was not observed in our transgenic model. Although the reason for that is currently unclear (for instance, it could be caused by the instability and/or degradation of RPE65 in rods), this circumstance greatly facilitated the detection of transgenic RPE65 protein in mouse cones. Both M-cone density and morphology in the retinas of control and transgenic animals were normal, as was revealed by immunolabeling
with M-opsin antibody (Fig. 5D). As expected, transgenic human RPE65 was also expressed in ventrally-located S-cones in Gnat1−/−hRpe65+ mice that are not present in the far dorsal part of the retina, as was evidenced by immunostaining with S-opsin antibody (Fig. 5E).

To test the possible effect of transgenic expression of human RPE65 on phototransduction cascade in mouse cones, we next performed physiological experiments in perfused retinas from 6-week-old animals. Again, the analysis was limited to M-opsin expressing cones which can be selectively stimulated with visible green light. ERG recordings from isolated retinas in the presence of postsynaptic blockers revealed that under dark-adapted conditions the flash responses of M-cones from Gnat1−/−hRpe65+ mice had amplitudes, kinetics and sensitivity comparable to those of responses from control littermates (Fig. 6A–C). The comparable sensitivity of cones with and without RPE65 expression was further evident from fitting normalized averaged cone intensity-response relationships with hyperbolic functions yielding the sensitivity (S½) values of 1.7 × 10⁴ and 1.8 × 10⁴ photons μm⁻² (P > 0.05) for control (with RPE65-negative cones) and transgenic (with RPE65-positive cones) mice, respectively (Fig. 6D). Together with our immunohistochemical analysis, these results demonstrate that the transgenic expression of human RPE65 in mouse M-cones did not produce adverse effects on their viability, overall health, or phototransduction. The lack of detectable morphological or functional changes allowed us to investigate whether cone-expressed RPE65 is important for normal dark adaptation of mammalian M-cones as well as their function in bright light.

Figure 4. Delayed M-cone dark adaptation in retinas of RPE65-deficient mice. (A) Endogenous expression of RPE65 protein in cones of control Rpe65+/+Gnat1−/− mice was confirmed by immunohistochemistry using PETLET RPE65 antibody (green). Cell nuclei were stained with DAPI (blue). RPE: retinal pigmented epithelium; COS: cone outer segments; CIS: cone inner segments; ONL: outer nuclear layer. Scale bar, 15 μm. The inset shows higher magnification of the selected area. Scale bar, 10 μm. (B) Recovery of cone ERG a-wave flash sensitivity (Sf; mean ± SEM) in isolated retinas of control (n = 9) and Rpe65−/−Gnat1−/− mice injected with Matrigel loaded with 9-cis-retinal (n = 12) after bleaching >90% of cone pigment at time 0 with 505 nm LED light. Error bars for some points are smaller than the symbol size. Data were fitted with single-exponential functions that yielded time constants of ~1.2 min and 6.1 min, respectively. SfDA denotes the sensitivity of dark-adapted cones. (C) Comparison of the recovery of cone ERG a-wave flash sensitivity (Sf; mean ± SEM) in isolated retinas of control Rpe65+/+Gnat1−/− mice (n = 9, left y-axis) and Rpe65−/−Gnat1−/− littermates injected with Matrigel loaded with 9-cis-retinal (n = 12, right y-axis) under conditions of bleaching causing equal initial cone desensitization (1.5 log units). Bleaching ~40% (control Rpe65+/+Gnat1−/−) or >90% (Rpe65−/−Gnat1−/−) of cone pigment at time 0 was performed with 505 nm LED. Data from Rpe65−/−Gnat1−/− mice were replotted from (B). Data were fitted with single-exponential functions that yielded time constants of ~0.5 min and 6.1 min, respectively. The y-axis for the data from mutant mice (right) was arbitrary scaled up to match the initial dark-adapted cone sensitivity (SfDA) of the two strains.
Transgenic expression of RPE65 does not affect pigment regeneration in mouse M-cones. To address the possible role of cone-expressed RPE65 in the visual cycle, we first monitored in isolated mouse retinas which, regardless of its presence, followed a single-exponential time course with the time constant of ~1.2 ± 0.2 min (Fig. 7A). The final recovery of cone sensitivity reached ~30% in both cases.

We then performed a similar bleaching experiment in live animals where the cones were in their native environment and the combined action of the retina and RPE visual cycles contributed to their dark adaptation, as demonstrated previously32. In this case, the effect of transgenic expression of human RPE65 on the restoration of M-cone photosensitivity after >90% pigment bleach was tested by full-field ERG recordings performed in vivo. We used cone ERG b-waves to monitor postbleach cone sensitivity (Sb) changes due to very small amplitudes of photopic ERG a-waves in mice that are masked by much larger b-waves of inverse polarity representing the response of cone bipolar cells. We found that the initial phase of sensitivity recovery, driven by the retina visual cycle31, was not accelerated in transgenic Gnat1+/− hRpe65+ animals (Fig. 7B, circles). Instead, somewhat surprisingly, in the presence of cone RPE65 some data points of the recovery within first the 20 min after the bleach (marked with stars indicating statistical significance) lagged behind the control data. Linear fits to the first three postbleach recovery points (0.5–3 min) yielded rates of 0.24 min⁻¹ and 0.17 min⁻¹ for RPE65 transgene-positive and negative.
mice, respectively (Fig. 7B, black lines). Consistent with the idea that the presence of cone RPE65 does not change the turnover of visual chromophore in the RPE, the second, slow component of M-cone dark adaptation (25–65 min) and the final level of cone sensitivity recovery after the bleach were both unaltered in transgenic line. Thus, human RPE65 protein expressed in mouse M-cones lacked the ability to enhance dark adaptation of these photoreceptors, either in isolated retinas or intact eyes.

To test the possibility that transgenic RPE65 could modulate the function of cones under continuous bright light in vivo, we performed an additional ERG experiment. After recording dark-adapted M-cone b-wave flash sensitivity, green Ganzfeld light (300 cd m⁻², estimated to bleach ~0.8% M-cone pigment s⁻¹) was applied for 30 min. It induced an immediate ~2 log unit light-adaptation of cones which was followed by transient increase in sensitivity (presumably, resulting from the retina network adaptation) of comparable size for control and transgenic animals, respectively. Normalized averaged cone intensity-response relationships (mean ± SEM, no statistical significance at any light intensity) for control Gnat1⁻/⁻ (n = 6) and transgenic Gnat1⁻/⁻ hRpe65⁺ (n = 6) mice. The data were fitted with Naka-Rushton equations that yielded I₁/₂-values as in (C). Error bars for some points in (C,D) are smaller than the symbol size.

Transgenic expression of RPE65 in M-cones of RPE65-deficient mice does not improve their morphology or function. Although our results demonstrate that the transgenic expression of human RPE65 in normal mouse cones does not enhance the recycling of 11-cis-retinal through the intraretinal visual
cycle, it is still possible that this protein could play an important structural role in species whose cones express it naturally. Therefore, we further investigated if transgenic cone-specific expression of RPE65 would retard or even prevent the well-known degeneration of cones in a chromophore-deficient mouse model. To do that, we generated transgenic animals lacking endogenous RPE65 (Rpe65−/−/Gnat1−/− (n = 6) and transgenic Gnat1−/−/Rpe65−/− (n = 6)) mice after bleaching >90% of cone pigment at time 0 with 505 nm LED light. Data were fitted with single-exponential functions that yielded time constants of ~1.2 min in both cases. SDA denotes the sensitivity of dark-adapted cones. (B) Recovery of photopic ERG b-wave (Sb; mean ± SEM, *P < 0.05, ***P < 0.001) in vivo in control Gnat1−/− (n = 5) and transgenic Gnat1−/−/Rpe65−/− (n = 4) mice after bleaching >90% of cone pigment at time 0 with 520 nm LED light. Sb was normalized to the corresponding dark-adapted value (SbDA) in each case. Initial rates of Sb recovery were determined from linear fits (black lines) that yielded the slopes of 0.24 min−1 (control) and 0.17 min−1 (transgenic). Squares show the change of photopic ERG b-wave (Sb; mean ± SEM, *P < 0.05) in vivo following illumination with green 530 nm Ganzfeld background light (300 cd m−2•min−1) in control (n = 4) and transgenic (n = 4) mice. Sb was normalized to the corresponding dark-adapted value (SbDA) in each case. Error bars for some points in (A, B) are smaller than the symbol size.

**Discussion**

The role of RPE65 in recycling visual chromophore in the RPE is now firmly established. As the chromophore released from photoreceptors after bleaching their visual pigment is all-trans-retinol (Vitamin A), its RPE65-driven reisomerization back to 11-cis-retinoid is critical for maintaining continuous photoreceptor function. Similarly, RPE65 is required for the conversion of Vitamin A, taken up from the choroidal vasculature into 11-cis-retinoid for use in photoreceptors. In the absence of this enzyme, photoreceptors are deprived of chromophore, unable to detect light, and eventually degenerate. In contrast to this well-established and appreciated role of RPE65 within the RPE, its role when expressed in cone photoreceptors has remained unknown. One intriguing possibility is that cone-expressed RPE65 enables recycling of retinoids within cone photoreceptors and contributes to the rapid regeneration of cone visual pigment. However, experiments with amphibian and mouse cones have clearly shown that cones are not able to regenerate their visual pigment when isolated from the retina and RPE. Thus, an alternative hypothesis for its function is that it serves as a retinoid binding protein that facilitates the turnover of chromophore in the OS of cones, either by accelerating its release and uptake from RPE and Muller cells, or by stabilizing its 11-cis-conformation, thus protecting it from photo- and thermal decay. Thus, here we performed an extensive set of experiments to investigate the role of cone-expressed RPE65 in the pigment regeneration and dark adaptation of mouse cones.
As cone-expressed RPE65 was expected to affect specifically the turnover of chromophore in cones, we investigated how the absence or presence of RPE65 in cones affects cone dark adaptation. Slowing down chromophore removal or resupply to cones would be expected to delay their dark adaptation. Conversely, accelerating chromophore turnover or protecting the retinoid from isomerization or oxidation would be expected to accelerate or enhance cone dark adaptation. Based on these premises, we used two approaches to address the role of cone-expressed RPE65 in the function of these photoreceptors. First, we compared the function of cones in isolated retinas from control (RPE65-expressing) and Rpe65−/− (RPE65-deficient cones) mice. Second, we compared the function of cones where RPE65 was introduced transgenically and cones of mice that normally only express it in RPE cells.

Recordings from isolated retinas revealed that Rpe65−/− cones desensitize less and recover 5-fold slower than controls following exposure to bright bleaching light (Fig. 4B). The difference in dark adaptation between control cones and cones from Rpe65−/− retinas became even more pronounced when the bleach of the control cells was adjusted to produce comparable levels of initial desensitization (Fig. 4C). One potentially exciting interpretation of these results is that RPE65 in cones serves to accelerate their visual cycle and speeds up cone dark adaptation. However, this analysis was complicated by the chromophore deficiency in Rpe65−/− mice caused by the lack of a functional RPE visual cycle. This resulted in cone opsin mislocalization and degeneration, combined with largely suppressed cone function (Figs 2 and 3). While treating the mice with exogenous 9-cis-retinal and raising them in darkness ameliorated these problems, cones from Rpe65−/− mice still had abnormally slower photoresponses and were about 10-fold less sensitive than controls (Fig. 2). In addition, while improved, cone opsin mislocalization and cone loss were still present in Rpe65−/− mice even after the treatment with chromophore (Fig. 3). These issues ultimately prevented us from drawing conclusions about the role of cone-expressed RPE65 based on the loss of function experiments from Rpe65−/− animals.
To avoid issues associated with the chromophore deficiency of Rpe65<sup>−/−</sup> mice, we resorted to our second, gain of function, approach and introduced human RPE65 in the cones of mice where it normally is expressed only in the RPE. Our choice of human transgenic RPE65 was dictated by the need to distinguish it from the endogenous mouse protein by immunohistochemistry. The high level of homology (95%) between the human and mouse retinoid isomerases<sup>26</sup> and the preservation of all key structural elements in human RPE65 as compared to its mouse counterpart allowed us to introduce a very similar protein to mouse cones which was properly localized to their outer segments (Fig. 5C). The transgenic expression of RPE65 in cones did not affect their health or function in dark-adapted conditions (Figs 5 and 6), allowing us to more reliably evaluate its role in cone dark adaptation. Contrary to the results from Rpe65<sup>−/−</sup> mice, we found that introducing RPE65 in cones of a mouse strain that normally lacks cone RPE65 resulted in a slight delay in their dark adaptation <i>in vivo</i>, as well as in somewhat larger desensitization of the cones in the presence of steady bright background light (Fig. 7). Both of these results are consistent with the notion that RPE65 in mouse cones causes a minor delay in the regeneration of their visual pigment. Thus, cone RPE65 does not appear to enhance chromophore recycling or have an obvious beneficial effect for the function of these photoreceptors in mice. Consistent with this notion, expressing RPE65 in the cones of Rpe65<sup>−/−</sup> animals also did not improve cone opsin mislocalization or cone function (Fig. 8). The slight delay in cone pigment regeneration in RPE65 transgenic cones might be due to some buffering of retinoids by RPE65.

In summary, our results do not provide any evidence for a beneficial role of RPE65 in mouse cones. However, even though the visual cycle in these cells does not seem to be enhanced in the short term by the presence of RPE65, it is still possible that this protein could play a role in maintaining the long-term homeostasis of retinoids in the cones and enhance in this way the function and survival of these photoreceptors. Furthermore, although we found no effect by RPE65 in mouse cones, this protein could modulate cone function in other species, particularly in humans. A key difference between the retinas of mouse and humans in the diurnal human retina, which is absent in the nocturnal mouse. Thus, our findings do not rule out a role for cone-expressed RPE65 in modulating the function of the densely-packed foveal cones and, therefore, supporting the daytime high accuracy vision that these photoreceptors mediate in humans.

Materials and Methods

**Animals.** All experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Washington University Animal Studies Committee. Wild-type mice with a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor). Mice with a knockout of the retinal pigmented epithelium protein 65 kDa gene (Rpe65<sup>−/−</sup>) were described previously<sup>15</sup>. Rod transducin α-subunit knockout (Gnat1<sup>−/−</sup>) mice having no rod signaling<sup>28</sup> were used as controls in all cone physiological experiments. Rpe65<sup>−/−</sup> Gnat1<sup>−/−</sup> animals and their littermate Gnat1<sup>−/+</sup> controls were generated by crossing Rpe65<sup>−/+</sup> and Gnat1<sup>−/+</sup> lines. All mice with wild-type allele of Rpe65 used in this study were homozygous for its Leu450 isoform, as determined using genotyping protocol published elsewhere<sup>39</sup>.

Transgenic mice expressing human RPE65 protein in their cones (hRpe65<sup>+</sup>) were generated by the Molecular Genetics Core at Washington University, as follows. The transgene vector was constructed using standard cloning methods<sup>33</sup>. The first step was the removal of Grk1 (rhodopsin kinase) promoter from AAV-Grk1-EGFP plasmid (as an Apal–EcoRI fragment that included SV40 SD/SA sequence), followed by its insertion into hRpe65-cDNA plasmid (a gift from Dr. Jian-Xing Ma, University of Oklahoma Health Sciences Center) linearized with Apal–NotI. The final step was the retrieval of the Grk1-hRpe65 fragment from Grk1-hRpe65-cDNA plasmid with Pmel and its insertion into the pI vector (a modified pC1 vector containing SV40 late PolyA sequence, Promega) cut with EcoRV–Smal. The total size of the final Grk1(SV40 SD/SA)-hRpe65(SV40 late PolyA) sequence was ~2.4 kb.

The transgene vector was then electroporated into 129 × 1 Sw/J ES cells (SCC10 line, Siteman Cancer Center Embryonic Stem Cell Core at Washington University) and the recombinant event in selected G418-resistant clones was confirmed by Southern blotting and PCR. Positive ES cells were microinjected into C57BL/6J mouse embryos that were then implanted into surrogate C57BL/6J mothers. The resulting chimeras were bred with C57BL/6J mice to generate transgenic hRpe65<sup>+</sup> mice. These animals were further bred with Gnat1<sup>−/−</sup> mice lacking cone expression of endogenous RPE65 for five generations, to obtain Gnat1<sup>−/−</sup>−hRpe65<sup>+</sup> and control Gnat1<sup>−/+</sup> littermates. Alternatively, hRPE65<sup>+</sup> mice were mated with Rpe65<sup>−/−</sup> Gnat1<sup>−/−</sup> animals and further backcrossed to generate Rpe65<sup>−/−</sup>−Gnat1<sup>−/−</sup>−hRpe65<sup>+</sup> and control Rpe65<sup>−/−</sup>−Gnat1<sup>−/+</sup> lines. Genotyping for the presence of hRpe65 transgene in each generation, as well as that for Gnat1 and Rpe65 alleles, was performed according to protocols designed by Transnetyx, Inc.

Young adult animals of either sex (4–6-week-old) were used in all experiments. Animals were provided with standard chow (LabDiet 5053; LabDiet, Purina Mills). All mice with wild-type allele of RPE65 were maintained under a 12 h light (10–20 Lux)/12 h dark cycle and dark-adapted overnight prior to physiological recordings. Unless stated otherwise, all animals with Rpe65<sup>−/−</sup> background were dark-reared from postnatal day 10 (P10) until they reached the age of 4 weeks. For all experiments in this study, animals were euthanized by asphyxiation with rising concentration of CO<sub>2</sub>.

**Antibodies.** The PETLET polyclonal rabbit antibody to detect immunoreactive mouse RPE65 protein has been described previously<sup>35</sup> and was used at a concentration of 2 µg/ml for IHC analysis. The DALEED polyclonal rabbit antibody to detect human RPE65 protein<sup>39</sup> was used at a dilution of 2 µg/ml for IHC. The anti-M-cone opsin antibody (0.5 µg/ml) was obtained from Millipore. The anti-S-cone opsin antibody (0.4 µg/ml) was obtained from Santa Cruz Biotechnology. Alexa-488 and Alexa-594 secondary antibodies (0.1 µg/ml) were from Invitrogen.

**Immunohistochemistry.** After removal of the cornea and lens, the remaining mouse eyecup was fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at pH 7.4 for 1.3–2 h at room temperature (RT). The eyecup was then washed once in PBS for 10 min and incubated in 30% sucrose buffered...
with PBS at 4 °C overnight. Next, the eyecup was embedded in Optimal Cutting Temperature compound (Ted Pella), flash-frozen in 2-methylbutane (Sigma) on dry ice, and cut with a cryo-microtome to produce 8 μm sections in dorsal-to-ventral direction through the optic nerve head. Sections were dried for 30 min at RT, gently washed in deionized water for 10 min, dried again for 10 min at RT, and blocked for 1 h at RT with a solution containing 1% bovine serum albumin (Sigma), 1.5% goat serum, and 0.1–0.25% Triton X-100 (Sigma) in PBS. Sections were then incubated at 4 °C overnight with appropriate primary antibodies diluted in a solution containing 1% Tween-20 (Bio-Rad) and 1% Triton X-100 in PBS. Next, sections were washed once in PBS and then incubated with secondary antibodies diluted in PBS containing propidium iodide or DAPI (Invitrogen) for 2 h at RT, washed with PBS (2X, 10 min, RT), and mounted on cover slips with Fluoromount-G (Thermo Scientific) for analysis by confocal microscopy (Olympus FV1000 Confocal Microscope). Images were acquired in both directions from the central region of the retina near the optic nerve head. Immunohistochemistry experiments were typically performed on 2–3 separate animals, and representative images are shown in the respective figures.

Retinoid delivery. Retinoids were handled under dim red light. A single dose of 9-cis-retinal (Sigma) in the amount of 0.5–0.75 μg per animal was dissolved in 20 μl of absolute ethanol and combined with 180 μl of Matrigel® Matrix (Corning) at 4 °C for a final volume of 200 μl. The sample was mixed carefully and injected subcutaneously with a cooled 1 ml syringe into the dorsal torso region of mice with Rpe65−/− background at P10. From this point on, animals were placed in constant darkness for dark-rearing and injections were repeated at P14, P18, and P23. Mice were sacrificed and used at P28–P30.

Acute application of exogenous 9-cis-retinal to isolated retinas from 4-week-old dark-adapted control and transgenic mice with Rpe65−/− background (in this case, animals were raised in normal dark/light cycling conditions until P28) was performed as follows: 300 μg of dried retinoid was dissolved in 8 μl of absolute ethanol and diluted to 8 ml with L15 solution (Sigma) containing 1% lipid-free BSA. The final concentrations of 9-cis-retinal and ethanol were ~130 μM and 0.1%, respectively. Before transfer to the perfusion chamber for recordings, a whole retina mounted on filter paper was incubated in a Petri dish with 4 ml of this oxygenated solution for 1 h in the dark at RT.

Transretinal (ex vivo ERG) recordings from isolated retinas. Mice were dark-adapted overnight or dark-reared from P10 (in the case of Rpe65−/− mice), and a whole retina was removed from each mouse eyecup under infrared illumination and stored in oxygenated aqueous L15 (13.6 mg/ml, pH 7.4, Sigma) solution containing 0.1% BSA at RT. The retina was mounted on filter paper with the photoreceptor side up and placed into a perfusion chamber between two electrodes connected to a differential amplifier. The preparation was perfused with Locke's solution containing 112.5 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 10 mM HEPES, pH 7.4, 20 mM NaHCO₃, 3 mM Na succinate, 0.5 mM Na glutamate, 0.02 mM EDTA, and 10 mM glucose. This solution was supplemented with 2 mM L-glutamate and 10 μM DL-2-amino-4-phosphonobutyric acid to block postsynaptic components of the photoresponse, and with 20 μM BaCl₂ to suppress the slow glial PIII component. The perfusion solution was continuously bubbled with a 95% O₂/5% CO₂ mixture and heated to 36–37 °C.

Light stimulation was applied by 20 ms test flashes from a calibrated 505 nm LED light. The stimulating light intensity was controlled by a computer in 0.5 log unit steps. Intensity-response relationships were fitted with Naka-Rushton hyperbolic functions, as follows:

$$ R = \frac{R_{\text{max}} \cdot I^n}{I_{1/2}^n + I^n} $$

where $R$ is the transient-peak amplitude of the response, $R_{\text{max}}$ is the maximal response amplitude, $I$ is the flash intensity, $n$ is the Hill coefficient (exponent), and $I_{1/2}$ is the half-saturating light intensity. In experiments designed to monitor the post-bleach recovery of cone a-wave flash sensitivity (Sₙ, see definition below), >90% or ~40% of M-cone visual pigment was bleached with a 3 s exposure to 505 nm light. The bleached fraction was estimated from the following equation:

$$ F = 1 - e^{(-P \cdot t)} $$

where $F$ is the fraction of pigment bleached, $t$ is the duration of the light exposure (s), $I$ is the bleaching light intensity of 505 nm LED light (1.6 × 10⁶ photons μm⁻² s⁻¹), and $P$ is the photosensitivity (7.5 × 10⁻¹⁵ μm²) of mouse cones at the wavelength of peak absorbance. Photosponses were amplified by a differential amplifier (DP-311, Warner Instruments), low-pass filtered at 300 Hz (8-pole Bessel), digitized at 1 kHz and stored on a computer for further analysis. Cone a-wave flash sensitivity ($S_f$) was calculated from the linear part of the intensity-response curve, as follows:

$$ S_f = \frac{R}{R_{\text{max}}}, $$

where $R$ is the cone a-wave dim flash response amplitude, $R_{\text{max}}$ is the maximal response amplitude for that retina, and $I$ is the flash strength. Data were analyzed with Clampfit 10.4 and Origin 8.5 software.

Electroretinography (ERG). Dark-adapted control Gnat1−/− and transgenic Gnat1−/− hRpe65+ mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg).
Pups were dilated with a drop of 1% atropine sulfate. Mouse body temperature was maintained at 37 °C with a heating pad. ERG responses were measured from both eyes by contact corneal electrodes held in place by a drop of Gonak solution. Full-field ERGs were recorded with the UTAS BigShot apparatus (LKC Technologies) using Ganzfeld-derived test flashes of calibrated green 530 nm LED light (within a range from 0.24 cd·s·m⁻² to 7.45 cd·s·m⁻²). Cone b-wave flash sensitivity (Sb) calculated similarly to the a-wave (Sa) in vivo ERG recordings was first determined in the dark (from the average of up to 20 flash responses) and normalized to the maximal b-wave amplitude obtained with the brightest white light stimulus of the Xenon Flash tube (700 cd·s·m⁻²). The M-cone pigment was then bleached near completely by a 35 s exposure to bright light delivered by a 520 nm LED focused at the surface of mouse eye cornea that produced ~1.3 × 10⁵ photons·μm⁻²·s⁻¹. The bleaching fraction, Fb, was estimated by the formula defined above. After the bleach, the recovery of cone b-wave Sb was followed in darkness for up to 1 h. Mice were re-anesthetized with a smaller dose of ketamine (~1/2 of the initial dose) in the middle of that period. In a subset of experiments, bright green background Ganzfeld illumination (530 nm, 300 cd·m⁻², estimated to bleach ~0.8% M-cone pigment s⁻¹) was applied continuously for 30 min to dark-adapted animals, and the cone b-wave Sb change was monitored during that period of light exposure.

Statistics. For all experiments, data were expressed as means ± SEM. Unless stated otherwise, data were analyzed using the independent two-tailed Student’s t-test, with an accepted significance level of P < 0.05.

References
1. Ebrey, T. & Koutalos, Y.Vertebrate photoreceptors. Prog Retin Eye Res 20, 49–94 (2001).
2. Arshavsky, V. Y., Lamb, T. D. & Pugh, E. N., Jr. G proteins and phototransduction. Annual Rev of Physiology 64, 153–187 (2002).
3. Yau, K. W. Phototransduction mechanism in retinal rods and cones. The Friedenwald Lecture. Invest Ophthalmol Vis Sci 35, 9–32 (1994).
4. Kiser, P. D., Golczak, M. & Palczewski, K. Chemistry of the retinoid (visual) cycle. Chemical reviews 114, 194–232 (2014).
5. Tang, P. H., Kono, M., Koutalos, Y., Ablonczy, Z. & Crouch, R. K. New insights into retinoid metabolism and cycling within the retina. Prog Retin Eye Res 32, 48–63 (2013).
6. Wang, J. S. & Kefalov, V. J. The cone-specific visual cycle. Prog Retin Eye Res 30, 115–128 (2011).
7. Saat, J. C. Vitamin A and Vision. Sub-cellular biochemistry 81, 231–239 (2016).
8. Kolesnikov, A. V., Shukolyukov, S. A., Cornwall, M. C. & Govardovskii, V. I. Recombination reaction of rhodopsin in situ studied by photoconversion of “indicator yellow”. Vision Res 46, 1665–1675 (2006).
9. Kaylor, J. J. et al. Blue light regenerates functional visual pigments in mammals through a retinyl-phospholipid intermediate. Nature communications 8, 16 (2017).
10. Redmond, T. M. et al. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. Nature genetics 20, 344–351 (1998).
11. Jin, M., Li, S., Moghrabi, W. N., Sun, H. & Travis, G. H. Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 122, 449–459 (2005).
12. Moiseyev, G., Chen, Y., Takahashi, Y., Wu, B. X. & Ma, J. X. RPE65 is the isomerohydrolase in the retinoid visual cycle. Invest Ophthalmol Vis Sci 102, 12413–12418 (2005).
13. Jacobson, S. G., et al. Human cone photoreceptor dependence on RPE65 isomerase. Proc Natl Acad Sci USA 104, 15123–15128 (2007).
14. de Hollander, A. I., Roepman, R., Koenekoop, R. K. & Cremers, F. P. Leber congenital amaurosis: genes, proteins and disease mechanisms. Prog Retin Eye Res 27, 391–419 (2008).
15. Ziozko, S. L., Crouch, R. K., Moiseyev, G. & Ma, J. X. Identification of the RPE65 protein in mammalian cone photoreceptors. Invest Ophthalmol Vis Sci 43, 1604–1609 (2002).
16. Tang, P. H., Wheless, L. & Crouch, R. K. Regeneration of photopigment is enhanced in mouse cone photoreceptors expressing RPE65 protein. J Neurosci 31, 10403–10411 (2011).
17. Tang, P. H., Buhusi, M. C., Ma, J. X. & Crouch, R. K. RPE65 is present in human green/re cones and promotes photopigment regeneration in an in vitro cone cell model. J Neurosci 31, 18618–18626 (2011).
18. Hemati, N., et al. RPE65 surface epitopes, protein interactions, and expression in rod- and cone-dominant species. Mol Vis 11, 1151–1165 (2005).
19. Kiser, P. D., Golczak, M., Lodowski, D. T., Chance, M. R. & Palczewski, K. Crystal structure of native RPE65, the retinoid isomerase of the visual cycle. Proc Natl Acad Sci USA 106, 17325–17330 (2009).
20. Xue, Y. et al. CRALBP supports the mammalian retinal visual cycle and cone vision. J Clin Invest 125, 727–738 (2015).
21. Ziozko, S. L. et al. Downregulation of cone-specific gene expression and degeneration of cone photoreceptors in the Rpe65⁻/⁻ mouse at early ages. Invest Ophthalmol Vis Sci 46, 1473–1479 (2005).
22. Rohrer, B. et al. Cone opsin mislocalization in Rpe65⁻/⁻ mice: a defect that can be corrected by 11-cis retinal. Invest Ophthalmol Vis Sci 46, 3876–3882 (2005).
23. Tang, P. H., Fan, J., Goletz, P. W., Loomis, L. & Crouch, R. K. Effective and sustained delivery of hydrophobic retinoids to photoreceptors. Invest Ophthalmol Vis Sci 51, 5958–5964 (2010).
24. Fan, J., Crouch, R. K. & Kono, M. Light prevents exogenous 11-cis retinal from maintaining cone photoreceptors in chrophore-deficient mice. Invest Ophthalmol Vis Sci 52, 2412–2416 (2011).
25. Kolesnikov, A. V. & Kefalov, V. J. Transretinal ERG recordings from mouse retina: rod and cone responses. Journal of visualized experiments: JoVE 61 (2012).
26. Fan, J., Woodruff, M. L., Culluffo, M. C., Crouch, R. K. & Fain, G. L. Opsin activation of transduction in the rods of dark-removed Rpe65 knockout mice. J Physiol 568, 83–95 (2005).
27. Hurley, J. B., Ebrey, T. G., Honig, B. & Otolonghi, M. Temperature and wavelength effects on the photochemistry of rhodopsin, isorhodopsin, bacteriorhodopsin and their photo products. Nature 270, 540–542 (1977).
28. Calvert, P. D. et al. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha -subunit. Proc Natl Acad Sci USA 97, 13913–13918 (2000).
29. Fan, J., Rohrer, B., Frederick, J. M., Baehr, W. & Crouch, R. K. Rpe65⁻/⁻ and Lrat⁻/⁻ mice: comparable models of leber congenital amaurosis. Invest Ophthalmol Vis Sci 49, 2384–2389 (2008).
30. Zhang, H. et al. Trafficking of membrane-associated proteins to cone photoreceptor outer segments requires the chromophore 11-cis-retinal. J Neurosci 28, 4008–4014 (2008).
31. Kolesnikov, A. V., Tang, P. H., Parker, R. O., Crouch, R. K. & Kefalov, V. J. The mammalian cone visual cycle promotes rapid M/L-cone pigment regeneration independently of the interphotoreceptor retinoid-binding protein. J Neurosci 31, 7900–7909 (2011).
32. Kolesnikov, A. V. et al. Retinol dehydrogenase 8 and ATP-binding cassette transporter 4 modulate dark adaptation of M-cones in mammalian retina. J Physiol 593, 4923–4941 (2015).
33. Jones, G. J., Crouch, R. K., Wiggert, B., Cornwall, M. C. & Chader, G. J. Retinoid requirements for recovery of sensitivity after visual-pigment bleaching in isolated photoreceptors. Proc Natl Acad Sci USA 86, 9606–9610 (1989).
34. Wang, J. S., Estevez, M. E., Cornwall, M. C. & Kefalov, V. J. Intra-retinal visual cycle required for rapid and complete cone dark adaptation. Nat Neurosci 12, 295–302 (2009).
35. Sato, S., Frederiksen, R., Cornwall, M. C. & Kefalov, V. J. The retina visual cycle is driven by cis retinol oxidation in the outer segments of cones. Vis Neurosci 34, E004 (2017).
36. Boulanger, A., Liu, S., Yu, S. & Redmond, T. M. Sequence and structure of the mouse gene for RPE65. Mol Vis 7, 283–287 (2001).
37. Grimm, C. et al. Constitutive overexpression of human erythropoietin protects the mouse retina against induced but not inherited retinal degeneration. J Neurosci 24, 5651–5658 (2004).
38. Lee, E. C. et al. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics 73, 56–65 (2001).
39. Moiseyev, G., Takahashi, Y., Chen, Y., Kim, S. & Ma, J. X. RPE65 from cone-dominant chicken is a more efficient isomerohydrolase compared with that from rod-dominant species. J Biol Chem 283, 8110–8117 (2008).
40. Vinberg, F., Kolesnikov, A. V. & Kefalov, V. J. Ex vivo ERG analysis of photoreceptors using an in vivo ERG system. Vision Res 101, 108–117 (2014).
41. Sillman, A. J., Ito, H. & Tomita, T. Studies on the mass receptor potential of the isolated frog retina. I. General properties of the response. Vision Res 9, 1435–1442 (1969).
42. Nymark, S., Heikkinen, H., Haldin, C., Donner, K. & Koskelainen, A. Light responses and light adaptation in rat retinal rods at different temperatures. J Physiol 567, 923–938 (2005).
43. Nikonov, S. S., Khodolenko, R., Lem, J. & Pugh, E. N. Jr. Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings. J Gen Physiol 127, 359–374 (2006).

Acknowledgements
This work was supported by NIH grants EY019312 and EY21126 (V.J.K.), EY002687 to the Department of Ophthalmology and Visual Sciences at Washington University, and by Research to Prevent Blindness. We thank Dr. Rosalie Crouch (Medical University of South Carolina) for critical discussions of the experiments and the manuscript, as well as for financial support for P.H.T. We also thank Drs Janis Lem (Tufts University School of Medicine) for providing Gnat1−/− mice, Michael Redmond (National Eye Institute) for providing Rpe65−/− animals, Jian-Xing Ma (University of Oklahoma Health Sciences Center) for a generous gift of hRpe65-cDNA plasmid, and the members of Transgenic Core Facility at Washington University for their help with generation of hRpe65+ mice.

Author Contributions
Conception and design of the experiments: A.V.K. and V.J.K. Collection, assembly, analysis and interpretation of data: A.V.K. and P.H.T. Drafting the article or revising it critically for important intellectual content: A.V.K. and V.J.K. All authors approved the final version of the manuscript.

Additional Information
Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018