Rpe65$^{-/-}$ and *Lrat*$^{-/-}$ Mice: Comparable Models of Leber Congenital Amaurosis

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Purpose. The *Rpe65*$^{-/-}$ mouse, used as a model for Leber congenital amaurosis, has slow rod degeneration and rapid cone loss, presumably because of the mistrafficking of cone opsins. This animal does not generate 11-*cis* retinal, and both cone loss and rod response are restored by 11-*cis* retinal administration. Similarly, the *Lrat*$^{-/-}$ mouse does not produce 11-*cis* retinal. The authors sought to determine whether the same effects on rod and cone opsins in the *Rpe65*$^{-/-}$ mouse are also present in the *Lrat*$^{-/-}$ mouse, thereby establishing that these changes can be attributed to the lack of 11-*cis* retinal rather than to some unknown function of RPE65.

Methods. Rod and cone opsins were localized by immunohistochemical methods. Functional opsin levels were determined by regeneration with 11-*cis* retinal. Isorhodopsin levels were determined from pigment extraction. Opsin phosphorylation was determined by mass spectrometry.

Results. Rods in both models degenerated slowly. Regenerable rod opsin levels were similar over the 6-month time course investigated, rod opsin was phosphorylated at a low level (approximately 10%), and minimal 9-*cis* retinal was generated by a nonphotic process, giving a trace light response. In both models, S-opsin and M/L-opsin failed to traffic to the cone outer segments appropriately, and rapid cone degeneration occurred. Cone opsin mistrafficking in both models was arrested on 11-*cis* retinal administration.

Conclusions. These data show that the *Lrat*$^{-/-}$ and *Rpe65*$^{-/-}$ mice are comparable models for studies of Leber congenital amaurosis and that the destructive cone opsin mistrafficking is caused by the lack of 11-*cis* retinal. (*Invest Ophthalmol Vis Sci.* 2008;49:2384–2389) DOI:10.1167/iovs.08-1727

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Vision is initiated by the absorption of a photon by photosensitive proteins located in rod and cone photoreceptors. These proteins (opsins) are G-coupled protein receptors and have as their ligand 11-*cis* retinal, which isomerizes to the all-*trans* form with light, initiating the visual transduction cascade. The rod photoreceptors and their opsin rhodopsin have been extensively studied (for a recent review see Ridge and Palczewski[1]), but it is the cones that are critical for normal human daylight vision.

The generation of 11-*cis* retinal is critical for visual pigment regeneration and maintenance of normal visual function. The 11-*cis* retinal is generated in the retinal pigment epithelium (RPE) from all-*trans* retinyl esters and is then transported to the photoreceptors for the regeneration of the functional pigment.[2] The process by which 11-*cis* retinal is generated (the retinoid cycle) has been the subject of study of many laboratories. The protein RPE65, a major protein of the RPE, has been shown to be critical for the production of 11-*cis* retinal[3] and recently has been identified as the long-sought isomerohydrolase,[4–6] using the all-*trans* retinyl esters[7] as the substrate. The possibility of a separate process for the generation of 11-*cis* retinal for cones has been raised,[8–10] but this pathway does not appear to be a major pathway in rod-dominant retinas.

The Rpe65 knockout mouse (*Rpe65*$^{-/-}$) has been extensively studied.[3] This animal has been shown to have no 11-*cis* retinal, but low levels of 9-*cis* retinal are generated, possibly by thermal isomerization, which leads to the formation of minimal levels of isorhodopsin (the photosensitive rod opsin/9-*cis* retinal pigment) and thus a minuscule visual response as measured by the electroretinogram (ERG).[11] The level of pigmentation of the animal affects the quantities of this isomer; less is formed in the pigmented mouse.[12] Large amounts of the all-*trans* esters accumulate as the conversion of the esters to the 11-*cis* retinal has been arrested.[3] Rod photoreceptor degeneration is slow, and the opsin itself is found to be phosphorylated.[13,14] Several groups have shown that the administration of 9- or 11-*cis* retinal can restore rod function, even in fully adult 18-month-old mice.[13,15]

However, unlike rods, cone photoreceptors are found to degenerate rapidly in the *Rpe65*$^{-/-}$ mouse.[16] Cone opsins (both the S-opsin and the M/L-opsin) are found to be mislocalized in *Rpe65*$^{-/-}$ mice, but the repeated administration of 11-*cis* retinal at an early age (approximately postnatal day [P] 10) for several days results in opsin moving to the cone outer segment (COS), which prevents rapid cone degeneration.[17] Recently, with the use of adenovirus-expressing *Rpe65* to efficiently deliver the *Rpe65* gene to the RPE of *Rpe65*$^{-/-}$ mice, it has been shown that rod function can be restored, as expected, but also that cone opsin mislocalization can be corrected.[18]

However, it has been demonstrated that RPE65 is expressed at very low levels in cone outer segments in several different species[19,20] (WB, JMF, preliminary unpublished observations, 2007). The function of RPE65 in cones is unknown. To confirm that the mislocalization of cone opsin was attributed to the lack of 11-*cis* retinal rather than some unknown function of RPE65,

it was necessary to examine another model in which the synthesis of 11-*cis* retinal is blocked.

Lecithin-retinol acyltransferase (LRAT) catalyzes the esterification of all-*trans* retinol to all-*trans* retinyl esters[21] and is found in several tissues, including the RPE.[22] In the RPE, LRAT has a key role in the retinoid cycle[23] because it is critical for the formation of the esters, which is the essential substrate for the retinoid isomerase. The *Lrat*$^{-/-}$ mouse generated only trace levels of all-*trans* retinyl esters and no 11-*cis* retinal was found, indicating the retinoid visual cycle was blocked at the esterification step. Cone and rod visual functions were found to be attenuated,[24] but the responsible chromophore for the residual visual function was not identified.

In this study, we have compared rod and cone degeneration in *Rpe65*$^{-/-}$ and *Lrat*$^{-/-}$ mouse models in which the retinoid cycle is disrupted and no 11-*cis* retinal is synthesized. We conclude that the disruption of the retinoid cycle in these two models results in similar patterns of retinal degeneration: rapid cone degeneration with cone opsin mislocalization to the inner segment and relatively slow rod degeneration with correct rod opsin localization.

Methods

Animals

Lrat$^{-/-}$ mice were genotyped as described.[24] *Rpe65*$^{-/-}$ mice[3] were provided by T. Michael Redmond (Laboratory of Retinal Cell and Molecular Biology, National Eye Institute). Unless otherwise noted, all experiments were conducted on cyclic light-reared, age-matched animals. For isorhodopsin accumulation measurements, animals 2 months of age were dark-reared for 8 weeks. Animal experiments were performed in accordance with the policy for the Use of Animals in Neuroscience research and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The procedures were approved by the Medical University of South Carolina Animal Care and Use Committee and the Institutional Animal Care and Use Committee at the University of Utah. *Lrat*$^{-/-}$ mice were housed in the dark starting at P10. Experimental animals were injected intraperitoneally with 11-*cis* retinal (0.5 mg/dose) in 100 μL vehicle (10% ethanol, 10% bovine serum albumin, 0.9% NaCl) on days P10, P13, and P17. Animals were killed at age P20 for the cone opsin localization experiments. Littermate control mice were injected on the same schedule with vehicle alone.

Pigment Measurements

Tissue isolation and all subsequent steps were conducted under dim red light (GBX-2; Eastman Kodak, Rochester, NY). The retina was dissected and homogenized in 1% dodecylmaltoside (buffered with 100 mM sodium phosphate buffer, pH 7.4). The sample was shaken at 4°C for 2 hours, centrifuged (88,000*g* for 10 minutes), and measured in a Cary 300 spectrophotometer (Varian, Walnut Creek, CA). Difference spectra were determined from measurements before and after bleaching with white light in the presence of freshly prepared 20 mM hydroxylamine, pH 7.0. The isorhodopsin concentration was calculated based on absorption at λ_{max} = 487 nm using the extinction coefficient: ε (isorhodopsin) = 43,000 $M^{-1}cm^{-1}$.[25] To determine the regenerable opsin present, homogenized retinas of age-matched animals were supplemented with 11-*cis* retinal (80 μM) for 2 hours before obtaining difference spectra.[14] Samples were washed extensively (eight times) with 100 mM phosphate buffer after the incubation with retinal, which resulted in lower levels of pigment than reported from direct extraction techniques. Rhodopsin concentrations were calculated using the extinction coefficient of 40,000 $M^{-1}cm^{-1}$.[26]

Opsin Phosphorylation Measurements

Opsin phosphorylation was determined using mass spectrometry, as described.[14,27] In brief, retinas were homogenized in 8 M urea and digested overnight with Asp-N (5 ng/200 μL; Sigma, St. Louis, MO) in 10 mM Tris buffer, pH 7.6, at 37°C. Supernatants were collected by centrifugation (120,000*g*) and analyzed online with an ion-trap mass spectrometer (Finnigan LTQ; Thermo-Finnigan Instrument Systems, Inc., San Jose, CA). The values are slightly different from those previously published[14] because of improved instrumentation.

Immunohistochemistry

Age-matched mouse eyes were immersion-fixed for 2 hours using freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cryoprotected. Eyes were embedded, frozen, and sectioned at 12- to 14-μm thickness before incubation for immunocytochemistry (described in Rohrer et al.[17] and Baehr et al.[28]). Affinity-purified primary antibodies were applied to each group of two to four sections in a humidified chamber overnight at 4°C. Propidium iodide (1:3000 dilution; Invitrogen, Carlsbad, CA) was added to the solution containing FITC- or Alexa-488 (Molecular Probes, Eugene, OR)–conjugated secondary antibody. Sections were viewed using either an inverted laser scan confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) with a 40×-1.3 NA oil objective lens and optical slit setting of less than 0.9 μm or a fluorescence microscope (Carl Zeiss). The following antibodies were used in this study: rhodopsin (1:1000; generously provided by Robert Molday, University of British Columbia), anti-S-opsin and anti-M/L-opsin (1:500; Chemicon/Millipore, Temecula, CA), and anti-S-opsin and anti-M/L-opsin (1:500; generously provided by Jeannie Chen, University of Southern California).

Peanut Agglutinin Lectin Labeling

The RPE–choroid layer was separated from the retina–lens complex and fixed in 4% formaldehyde in phosphate-buffered saline for 4 hours at 4°C. After three washes with buffer (30 minutes each at 4°C), retinas were incubated with the 0.2 mg/mL peanut agglutinin (PNA) lectin FITC-conjugated (lectin *Arachi hypogaea*; Sigma, St. Louis, MO) overnight at 4°C. Retinas were washed three times (20 minutes each at 4°C), mounted on a slide, and coverslipped after application of an antifade solution (Prolong; Molecular Probes).[20] Samples were viewed with a fluorescence microscope (Axioplan II; Carl Zeiss) using a 100-W mercury light source and FITC filters. Cones were counted from the ventral field (n = 6 eyes).

Results

Rod Degeneration Slow in *Rpe65*$^{-/-}$ and *Lrat*$^{-/-}$ Mice

Rod photoreceptors in both the *Rpe65*$^{-/-}$ and the *Lrat*$^{-/-}$ mouse are reported to degenerate slowly.[3,24] A comparison of the two models with the wild-type (*wt*) mouse at P30 (Fig. 1A) shows the *Lrat*$^{-/-}$ mouse and the *Rpe65*$^{-/-}$ mouse to be comparable with rod outer segments decreased in length and abundance of rod nuclei when compared with *wt* mice. Rod opsin is present in the rod outer segments with no evidence of mislocalization. To determine that the opsin in the two models can form rhodopsin if the ligand 11-*cis* retinal is available, in vitro experiments were conducted, adding 11-*cis* retinal to retina homogenates and assaying for the formation of rhodopsin. Measurements at several ages demonstrate that the levels are similar (Fig. 1B). Although regenerated rhodopsin levels were certainly decreased from those of the *wt* animals, there was no significant variation between these two models and no significant decrease between 1 and 6 months of age in either model.

Isorhodopsin Formed in *Lrat*$^{-/-}$ Mouse

In both the *Rpe65*$^{-/-}$ and the *Lrat*$^{-/-}$ mouse, a small light response is generated as measured by the ERG.[3,24] However, no 11-*cis* retinal has been detected in either animal. In the

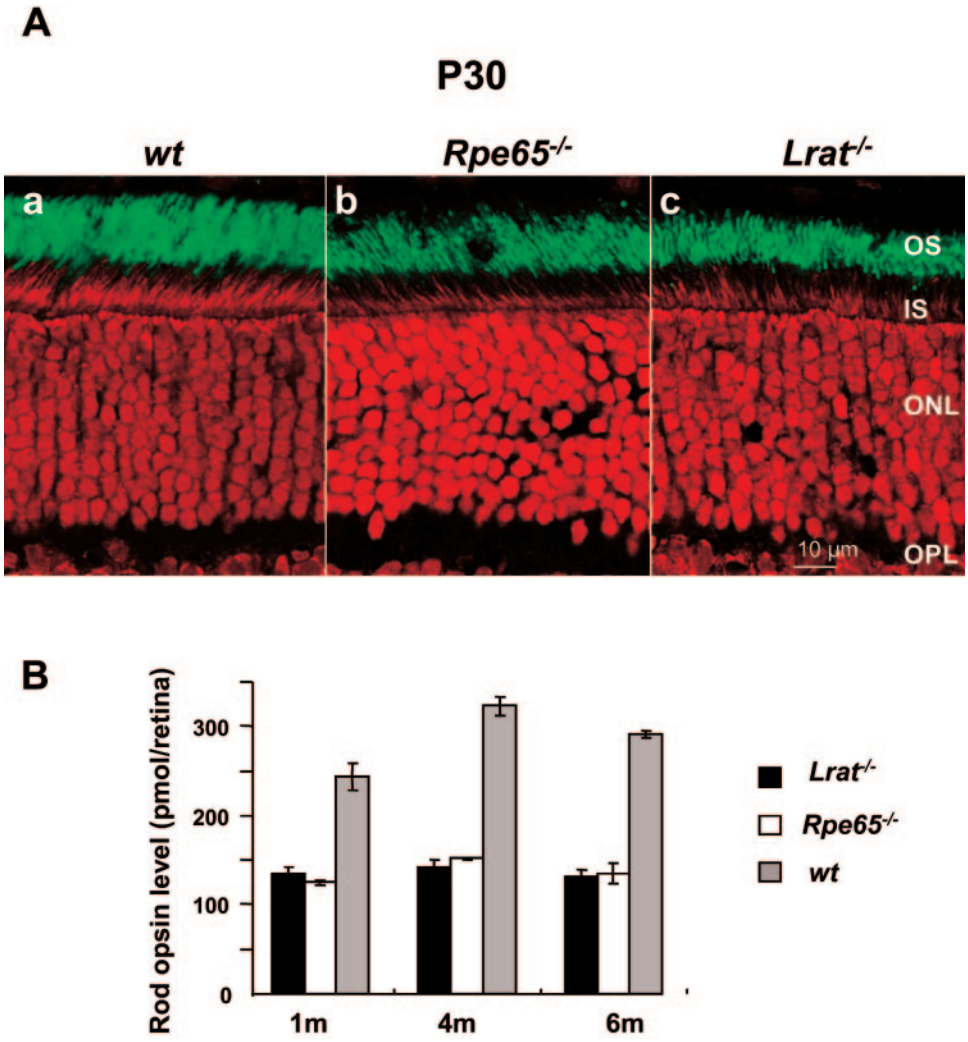

FIGURE 1. Comparison of rod opsin in 1-month-old *wt,* $Rpe65^{-/-}$, and $Lrat^{-/-}$ retinas. (**A**) Immunocytochemistry. Sections were probed using antibody directed against rhodopsin (*green*). Nuclei were counterstained with propidium iodide (*red*). Scale bar, 10 μm. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer. (**B**) Relative regenerable opsin levels in $Lrat^{-/-}$ and $Rpe65^{-/-}$ retinas. Levels of available opsin were determined by the incubation of homogenized retinas of age-matched 1-month-old, 4-month-old, and 6-month-old animals reared in cyclic light with 11-*cis* retinal (80 μM) for 2 hours. Difference spectra were determined from measurements before and after bleaching in the presence of hydroxylamine (20 mM). Available opsin levels were calculated from regenerated rhodopsin concentrations using ε = 40,000 $M^{-1}cm^{-1}$ at λ_{max} = 500 nm. Data are presented as mean ± SEM; n = 3. *White bars*: $Rpe65^{-/-}$ mice. *Black bars*: $Lrat^{-/-}$ mice. *Gray bars*: *wt* mice.

$Rpe65^{-/-}$ mouse, it has been found that a small amount of 9-*cis* retinal is generated, leading to the formation of the rod pigment isorhodopsin. On dark-rearing over a number of weeks, this pigment accumulates to a significant level, indicating that the generation of the 9-*cis* retinal is by a nonphotic process.[11] Because it had been shown that 11-*cis* retinal is also lacking in the $Lrat^{-/-}$ mouse,[24] we investigated whether these animals also generated 9-*cis* retinal. Care was taken that the animals had similar coat pigmentation because this has been shown to have an effect on the levels of 9-*cis* retinal.[12] After 8 weeks of dark-rearing, isorhodopsin levels were found to be 12.6 ± 0.8 pmol in the $Lrat^{-/-}$ mouse retina compared with 27.3 ± 0.6 pmol/retina in the age-matched $Rpe65^{-/-}$ animals dark-reared for the same period (Fig. 2). Therefore, we attributed the small light-induced ERG response in the $Lrat^{-/-}$ mouse to this 9-*cis* retinal. The finding that less 9-*cis* retinal was generated in the $Lrat^{-/-}$ animals than the $Rpe65^{-/-}$ mice suggests that the 9-*cis* retinal might have arisen from the ester form, which was elevated in the $Rpe65^{-/-}$ animals, but the exact source of this isomer remains unknown.

Rod Opsin Phosphorylated in the $Lrat^{-/-}$ Mouse

The rod opsin in the $Rpe65^{-/-}$ mouse is known to be phosphorylated.[13,14] To determine whether the opsin in the $Lrat^{-/-}$ mouse is also phosphorylated, analysis of the opsin from the animals was performed by mass spectrometry using known methodology.[14] As shown for the $Rpe65^{-/-}$ mouse, $Lrat^{-/-}$ opsin was phosphorylated, independent of light exposure. The opsin phosphorylation level in the light-adapted $Lrat^{-/-}$ mouse (13.1% ± 0.7%) was slightly increased over that

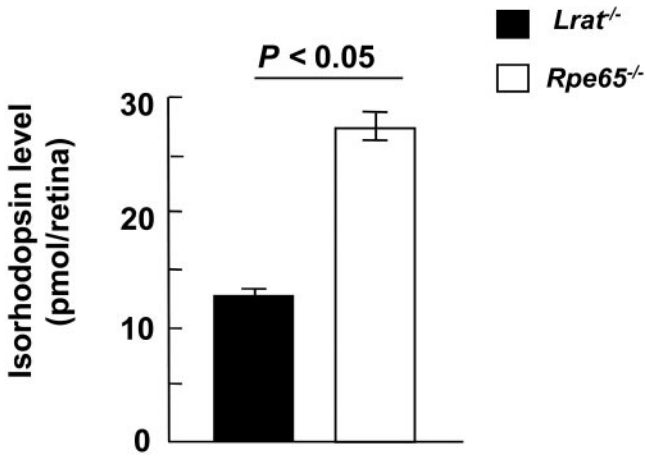

FIGURE 2. Relative isorhodopsin levels in $Lrat^{-/-}$ and $Rpe65^{-/-}$ retinas. Animals 2 months old were dark-reared for 8 weeks. Pigment levels were determined from difference absorption spectra. Data are presented as mean ± SEM (n = 3) and analyzed by two-tailed Student's t-test, accepting a significant value of $P < 0.05$. *White bars*: $Rpe65^{-/-}$ mice. *Black bars*: $Lrat^{-/-}$ mice.

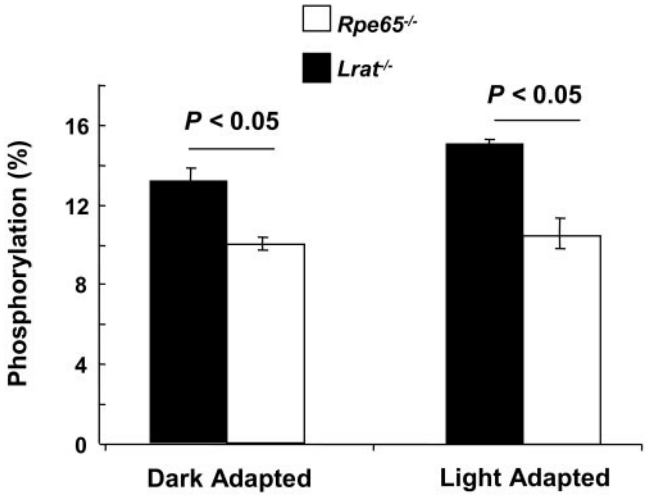

FIGURE 3. Opsin phosphorylation levels in $Lrat^{-/-}$ and $Rpe65^{-/-}$ mice. Retinas of 2-month-old $Rpe65^{-/-}$ and $Lrat^{-/-}$ mice were homogenized in 8 M urea, and the pellet was digested with Asp-N to cleave the opsin C terminus. The peptides were analyzed by HPLC/MS/MS using an LTQ mass spectrometer. Data are presented as percentages of phosphorylated peptides (mean ± SEM; $n = 3$) and analyzed by the two-tailed Student's *t*-test, accepting a significant value of $P < 0.05$. *White bars*: $Rpe65^{-/-}$ mice. *Black bars*: $Lrat^{-/-}$ mice.

found in the light-adapted $Rpe65^{-/-}$ mouse (10.4% ± 0.7%; $P < 0.05$). As expected, there was no difference in phosphorylation levels between dark- and light-adapted animals in either model because there was only a minimal light response (Fig. 3). These results support the hypothesis that this low level of constitutive opsin phosphorylation may have a role in slowing rod degeneration.

Cone Photoreceptors Rapidly Degenerate

The cones of the $Rpe65^{-/-}$ mice are known to degenerate rapidly.[16] In the $Lrat^{-/-}$ mouse, the cones also degenerate very quickly. Figure 4 demonstrates that the M/L-opsin is almost totally absent at P28 for both models and that S-opsin is totally gone at P42, demonstrating that the cones are rapidly lost in both models and that the two phenotypes appear to be comparable. When cones are counted in flat mount PNA lectin preparations from P30 animals (ventral sections, as shown in Fig. 4), the cones were found to be less than 5% of *wt* cones for the $Rpe65^{-/-}$ and the $Lrat^{-/-}$ mice ($Lrat^{-/-}$, 2.1% ± 0.7%; $Rpe65^{-/-}$, 4.6% ± 2.7%; $P = 0.02$).

Cone Opsin Mislocalization

Mislocalization of S-opsin and M/L-opsin has been shown to occur in the $Rpe65^{-/-}$ mouse. When studied in a mouse model lacking the rod opsin pool (the $Rpe65^{-/-}$ $Rho^{-/-}$ mouse), both the S-opsin and the M/L-opsin were observed to be distributed throughout the cone inner segment, cone cell body, axon, and synaptic pedicle while essentially absent in the COS.[17] At P28, COS disintegration appeared to be far advanced in the $Lrat^{-/-}$ mouse retina, and M/L-opsin mislocalized to the inner segment, perinuclear region, axon, and synaptic pedicle (Figs. 4B, 5B, 5E, arrows).

Cone Opsin Mislocalization Corrected with Administration of 11-*cis* Retinal

On repeated administration of 11-*cis* retinal to very young $Rpe65^{-/-}$ $Rho^{-/-}$ animals, both opsins were targeted to the cone outer segment.[17] To study the effect of 11-*cis* retinal treatments in the $Lrat^{-/-}$ retinas, littermates were randomly assigned to the experimental or the control groups and were kept in the dark starting at P10. The animals were injected intraperitoneally with 11-*cis* retinal (0.5 mg/dose in 100 μL vehicle [10% ethanol, 10% bovine serum albumin, 0.9% NaCl]) or vehicle alone on days P10, P13, and P17. The animals were killed at age P20. As shown in Figure 5, the mislocalization of cone opsins (both S-opsin and M/L-opsin) was partially corrected after the administration of 11-*cis* retinal. Compared with untreated retinas, the synaptic pedicles are less intensely stained, whereas more cone outer segments stained heavily for the cone opsins, indicating more cone opsin migrated properly into the outer segment after 11-*cis* retinal injections. Results in the $Lrat^{-/-}$ mouse are less dramatic than in the $Rpe65^{-/-}Rho^{-/-}$ mouse because of the large pool of rod opsin taking up the available 11-*cis* retinal. These results confirmed that the 11-*cis* retinal was critical to normal cone opsin trafficking.

Discussion

The purpose of this study was to determine whether the two mouse models lacking 11-*cis* retinal have similar patterns of rod and cone degeneration and are, therefore, both appropriate models for Leber congenital amaurosis. Even though both the *Lrat* and the *Rpe65* genes are candidate genes for Leber congenital amaurosis (e.g., see Thompson et al.[29] and Marlhens et al.[30]), the rationale for this comparison was the concern that the unexpected cone opsin mistrafficking observed in the $Rpe65^{-/-}$ mouse might be related to some unknown function of RPE65, possibly within the cone photoreceptors. We therefore examined the $Lrat^{-/-}$ mouse, which also does not produce 11-*cis* retinal but has normal RPE65 production and localization. The rod opsin levels in these two models are essentially identical, with some opsin phosphorylation that is not observed in *wt* animals. Both models show a minuscule level of 9-*cis* retinal production. The rods in both these models degenerate slowly and show no evidence of opsin mislocalization.

The cones of the $Lrat^{-/-}$ and the $Rpe65^{-/-}$ mice degenerate rapidly, with eventual complete loss of cone function. Immunohistochemistry indicates that the cone opsins in both models are not trafficking to the outer segments appropriately and the remaining outer segments appear stunted. The degeneration and cone opsin mistrafficking can be arrested with administration of 11-*cis* retinal at an early age. We have confirmed functional cone recovery in the $Rpe65^{-/-}$ $Rho^{-/-}$ mouse, but were unable to confirm recovery of cone function in the $Lrat^{-/-}$ mouse because of the interference of rods. We proposed that the lack of trafficking to the outer segments was the cause of cone degeneration. The cone opsin localization patterns looked similar in these two models lacking 11-*cis* retinal, with opsin found throughout the inner segment down to the pedicle. This mistrafficking appears to be a post-Golgi targeting disorder because the opsin is exported from the endoplasmic reticulum but cannot be transported to the outer segment.

In general, rod pigments are more stable than cone pigments, such as in their lability to hydroxylamine.[31] Another distinction between the rod and cone opsins is that cone opsins are known to be more highly phosphorylated than rod opsins on light activation because they have more serines and threonines in the C terminus.[32] In addition, the two mouse cone opsins lack the posttranslational modification of cysteine palmitylation, proposed to form the eighth "helix" on rod opsins.[33] Our data may suggest that the 11-*cis* retinal induces a conformational change necessary for recognition by a chaperone or a transporting complex. Without this conformational change, the cone opsins cannot be transported to the outer segment, where it is required for disc (and therefore outer segment) stability, similar to the requirement for rhodopsin in rod outer segment formation.[34,35]

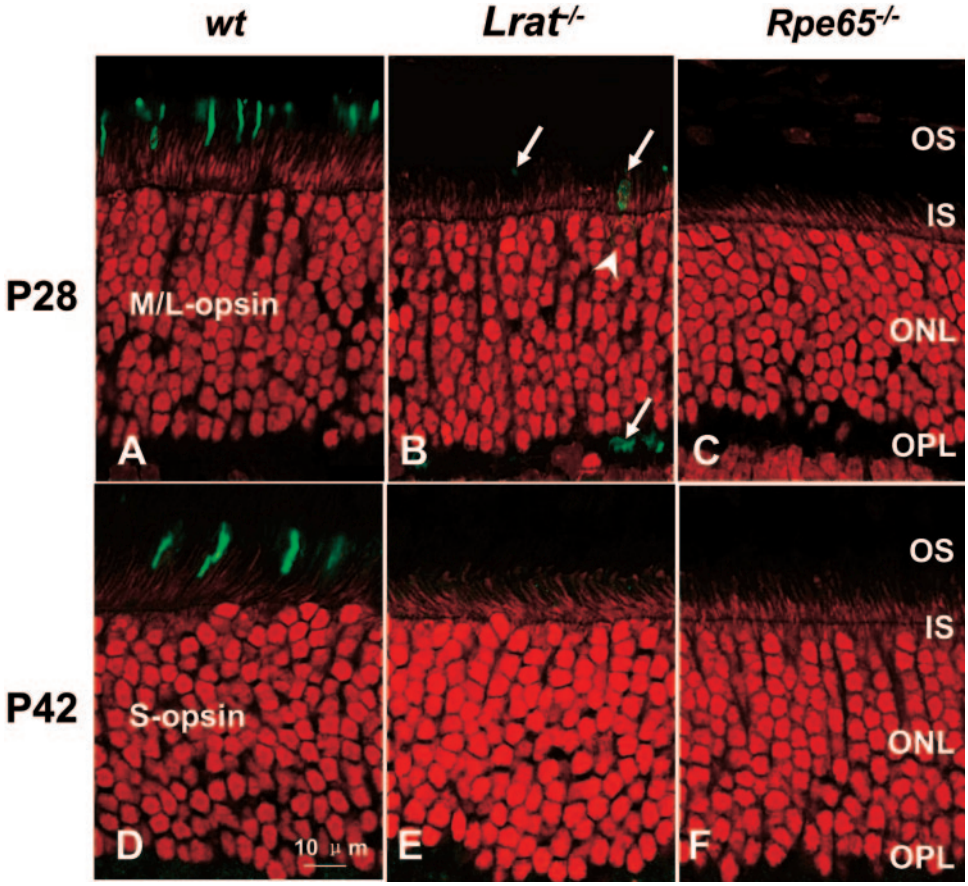

FIGURE 4. Confocal immunolocalization of cone opsins in *wt*, $Lrat^{-/-}$, and $Rpe65^{-/-}$ retinas. (**A–C**) P28 *wt* and mutant retinas are stained for M/L-opsin. The M/L-opsin is nearly absent at this stage. A perinuclear ring (**B**, *arrowhead*) represents the endoplasmic reticulum region where M/L-opsin (*arrow*) is synthesized. (**D–F**) P42 *wt* and mutant retinas are stained for S-opsin. The S-opsin is completely missing in the $Lrat^{-/-}$ and $Rpe65^{-/-}$ retinas at this stage, whereas the *wt* cones appear healthy with the opsin in the outer segments. Nuclei are contrasted with propidium iodide (*red*). Scale bar, 10 μm. All retinal sections passed through the optic nerve, and photoreceptors were imaged ventral (inferior) to the nerve where the degeneration was most advanced. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer.

The trafficking of rod opsin has been studied in several species (for a recent review, see Deretic[36]). Our results indicate that rod opsin traffics normally in the absence of 11-*cis* retinal. There is some in vitro evidence that 11-*cis* retinal improves protein stability of certain rhodopsin mutants and movement from the endoplasmic reticulum to the cell membrane,[37] but there are no reports of 11-*cis* retinal availability affecting normal rhodopsin trafficking in vivo. The C-terminal

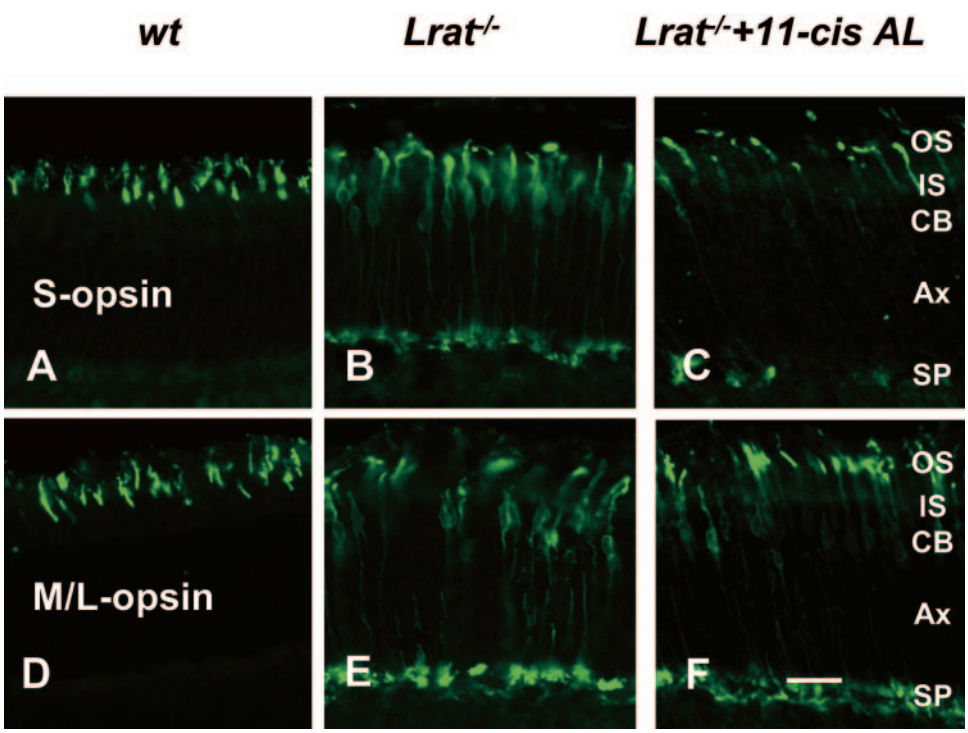

FIGURE 5. Effect of 11-*cis* retinal on cone opsin distribution. Retinal sections (14-μm thick, dorsal of the optic nerve) of P20 mice were stained with antibodies against S-opsin (**A–C**) and M/L-opsin (**D–F**) with and without 11-*cis* retinal injections. (**A**, **D**) C57BL/6 *wt* mice. (**B**, **E**) $Lrat^{-/-}$ mice. (**C**, **F**) $Lrat^{-/-}$ mice with11-*cis* injections. For the untreated knockout mice, the opsin was distributed throughout the entire cell (cone inner and outer segment, cell body, axon, and synaptic pedicle). On 11-*cis* retinal administration, less cone opsin was localized inappropriately to the synaptic pedicle, whereas more cone opsin was appropriately localized to the outer segment. OS, outer segment; IS, inner segment; CB, cell body; Ax, axon; SP, synaptic pedicle. Scale bar, 25 μm.

amino acids of rhodopsin have been implicated as essential for proper targeting,[38] and a general VXPX motif for targeting has been postulated[36] to be involved in sorting into post-Golgi carriers through binding with ARF-4.[39] Sorting mechanisms for cone pigments are unknown, but each cone pigment primary sequence carries a C-terminal sequence VSPA (M/L-opsin) or VGPH (S-opsin), each of which can qualify as a targeting sequence. Lack of correct targeting of cone pigments in the absence of 11-*cis* retinal could be explained by masking of the C-terminal targeting sequence or other targeting signals in the C-terminal region (or elsewhere in the cytoplasmic domain of cone pigments). It is conceivable that the presence of 11-*cis* retinal enables cone pigments to assume a conformation in which the targeting signal(s) are exposed.

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