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In conditions of limited chromophore supply rods entrap 11-*cis*-retinal leading to loss of cone function and cell death

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RPE65 is a retinoid isomerase required for the production of 11-*cis*-retinal, the chromophore of both cone and rod visual pigments. We recently established an R91W knock-in mouse strain as homologous animal model for patients afflicted by this mutation in RPE65. These mice have impaired vision and can only synthesize minute amounts of 11-*cis*-retinal. Here, we investigated the consequences of this chromophore insufficiency on cone function and pathophysiology. We found that the R91W mutation caused cone opsin mislocalization and progressive geographic cone atrophy. Remnant visual function was mostly mediated by rods. Ablation of rod opsin corrected the localization of cone opsin and improved cone retinal function. Thus, our analyses indicate that under conditions of limited chromophore supply rods and cones compete for 11-*cis*-retinal that derives from regeneration pathway(s) which are reliant on RPE65. Due to their higher number and the instability of cone opsin, rods are privileged under this condition while cones suffer chromophore deficiency and degenerate. These findings reinforce the notion that in patients any effective gene therapy with RPE65 needs to target the cone-rich macula directly to locally restore the cones' chromophore supply outside the reach of rods.

INTRODUCTION

Vertebrate retinas have two types of photoreceptor cells mediating vision—rods and cones. Rods are exquisitely sensitive to light but cones are more critical for daytime vision, acuity and color discrimination. Despite this functional specialization, cone and rod visual pigments use the same chromophore (11-*cis*-retinal) to capture light. The retinal pigment epithelium protein of 65 kDa (RPE65) was shown to be a retinoid isomerase essential for the synthesis of 11-*cis*-retinal. RPE65 is catalyzing the conversion of all-*trans*-retinyl esters to 11-*cis*-retinol (1–3) and mutations in this enzyme cause blindness in humans (OMIM 180069) (4–6). Pathogenic mutations are found in different regions of the *RPE65* gene where they may cause RPE65-deficiency or affect protein structure, modulate docking of binding protein partners and/or influence catalytic activity (3,7).

Studies on different animal models with *RPE65* mutations indicated that the cause and timeframe of cell death is different for cones and rods. In animals with no RPE65, e.g. no

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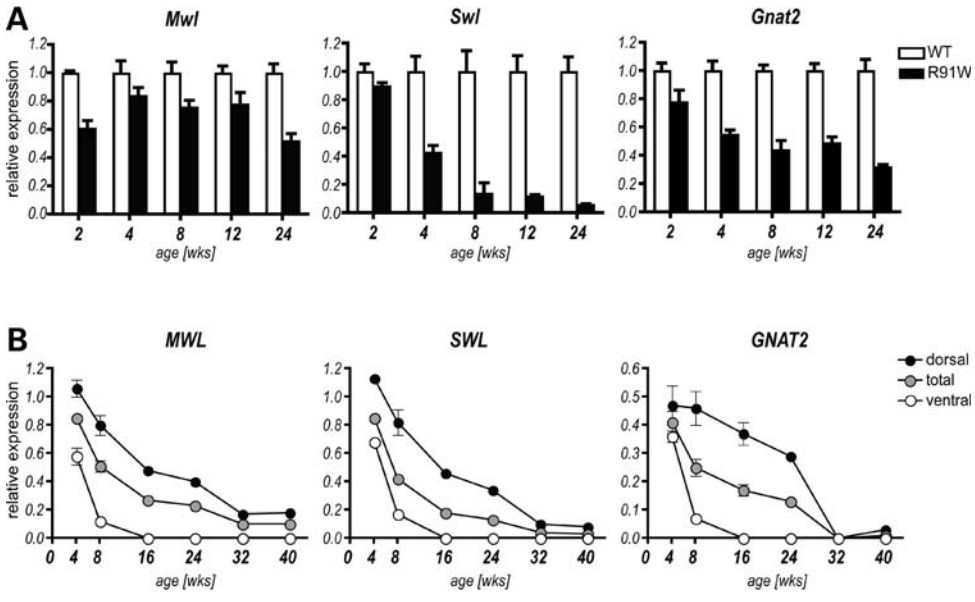

Figure 1. Geographical distribution and temporal cone loss caused by the R91W mutation. (**A**) Relative mRNA levels of cone markers as indicated. Expression was determined in retinas of wild-type and R91W mice at 4, 8, 12 and 24 weeks of age by real-time PCR. Average mRNA levels ± SD (black bars) are expressed relatively to age-matched wild-type controls, which were set to 1 (white bars). RNA isolated from three independent retinas per time-point and genotype was amplified in triplicates. (**B**) Cones were quantified by counting the number of MWL-, SWL- or GNAT2-positive outer segments in dorso-ventrally oriented retinal sections cut trough the optic nerve. Graphs represent values relative to 4-week-old R91W (set to 1) for a given cone-marker and region of the retina (mean ± SEM). An age-dependent decrease of cone marker labeling was observed resulting in an almost complete absence of cones at 32 weeks of age. GNAT2, cone-specific transducin α-subunit; MWL, middle wavelength cone opsin; SWL, short wavelength cone opsin.

11-*cis*-retinal production, rod cell death is proposed to result from constant activation of the phototransduction cascade by unliganded opsin (8). Even though this 'death signal' is present from birth, substantial degeneration occurs only late. Cones, however, appear to be more sensitive to chromophore deficiency. Patients with mutations in *RPE65* show cone photoreceptor loss early in life (9) and in Rpe65$^{-/-}$ animals, most of the cones degenerate within few weeks after birth (10).

In mouse models, early interventions, such as *Rpe65* gene replacement or retinoid supplementation therapy, rescue cone function and preserve cones against degeneration (10–13). The beneficial effect for cones has been directly attributed to 11-*cis*-retinal since it stabilizes cone opsin and promotes proper cone opsin trafficking from the inner to the outer segments (14,15). Thus, while rods are quite resistant to chromophore deficiency, cones are more vulnerable and degenerate earlier. This observation is clinically relevant and of special importance for choosing the treatment window to further improve the outcome of human trials using RPE65 gene therapy (16–19).

Studies addressing cone function and degeneration have been mainly conducted in RPE65 null animals (20–22) that lack the visual chromophore. Current literature, however, indicates that more than 50% of *RPE65* mutations in patients are missense mutations. Recently, we generated a mouse model carrying the R91W mutation in RPE65 (23), which is the most common missense mutation found in patients (24). As these patients retain useful vision at younger ages (25), it was suggested that the mutant RPE65 protein has some capability to produce 11-*cis*-retinal. In R91W mutant mice, we showed that—though strongly reduced—RPE65 protein levels enable visual function superior to that found in RPE65 null mutants. Indeed, small but detectable amounts of 11-*cis*-retinal exist in the eyes of this mouse mutant. Thus, R91W mutant mice represent a unique animal model to analyze the consequences of very limited 11-*cis*-retinal supply for retinal function and integrity.

Here, we focused on the consequences of this phenotype for cone structure and function. We analyzed the time course and characteristics of cone degeneration. Our analysis provides evidence that in R91W mutants rods and cones compete for 11-*cis*-retinal and that cone degeneration is caused by 11-*cis*-retinal insufficiency. Our data reinforce the notion that both photoreceptor types depend on RPE65 for chromophore supply.

RESULTS

Cone loss

Previous studies in Rpe65$^{-/-}$ mice have shown that cone markers are reduced very early during and after postnatal development (10). RPE65 gene delivery (12,13) or administration of 9- or 11-*cis*-retinal (10,11) prevents the downregulation of cone-specific gene expression. Recently, we have shown that 5–10% of normal 11-*cis*-retinal levels can be produced by a hypomorphic RPE65 protein carrying the R91W amino acid substitution (23). Here, we sought to analyze the influence of these limited amounts of 11-*cis*-retinal on the structural and functional integrity of cones.

To approach this question, we examined the mRNA levels of cone-specific markers in 2-, 4-, 8-, 12- and 24-week-old R91W and corresponding wild-type animals (Fig. 1A). All of the tested cone markers {cone opsins [middle wavelength

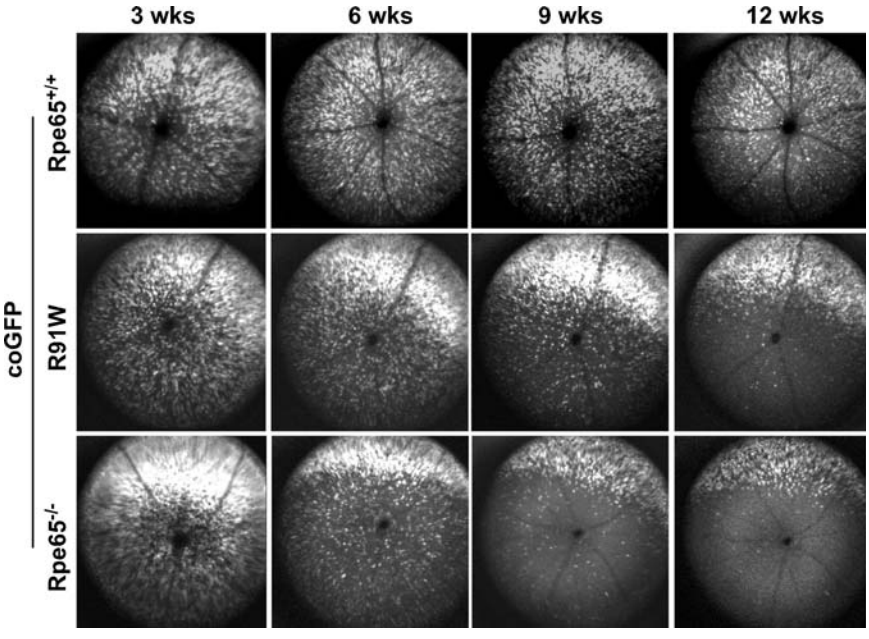

Figure 2. Cone loss in different Rpe65-related genetic backgrounds monitored *in vivo*. Fundus autofluorescence in transgenic mouse lines expressing GFP under the control of a cone opsin promotor (coGFP) monitored by scanning laser ophthalmoscope. Mice were on different *Rpe65* genetic backgrounds (R91W, $Rpe65^{-/-}$ and $Rpe65^{+/+}$) as indicated and fluorescence was monitored every 3 weeks until 12 weeks of age in individual animals. Shown is the temporal cone loss of one representative mouse per genotype. coGFP transgenic mice on a wild-type *Rpe65* background served as controls ($Rpe65^{+/+}$). Note the accelerated loss of GFP-positive cells from the ventral retina in $Rpe65^{-/-}$ animals.

(Mwl), short wavelength (Swl)] and cone-specific transducin α-subunit (Gnat2)} were transcriptionally downregulated as early as at 2 weeks of age (Fig. 1A). At 4 weeks, a time-point when the mouse retina can be considered mature, Mwl expression was reduced to 84% and by 24 weeks only 52% of wild-type levels were expressed (Fig. 1A). The Swl marker was even more suppressed: to 43% in 4 weeks and to only 6% in 24-week-old animals (Fig. 1A). Expression of Gnat2 was 55–32% in the period between 4 and 24 weeks of age (Fig. 1A).

We additionally analyzed these cone markers by immunostaining in 4-, 8-, 16-, 24-, 32- and 40-week-old animals and quantified the number of cells positive for MWL, SWL and GNAT2-cone opsin (Fig. 1B). A dorso-ventral gradient of expression for MWL and SWL cones has been described in the mouse retina (26). In order to analyze the spatial localization of cones, we quantified their dorso-ventral distribution in R91W (see Materials and Methods) at different ages. We compared it with 4-week-old wild-type animals, levels of them were set to 1 for dorsal, ventral or total expression, respectively (Fig. 1B). By 4 weeks of age, the most prominent cone loss occurred in the ventral retina of R91W mice. The MWL and SWL opsins were reduced to 58% and 68%, respectively, when compared with age-matched wild-type controls (Fig. 1B). Degeneration continued gradually and by 16 weeks of age the ventral retina of R91W mice was virtually cone-free. The dorsal retina was better preserved at all time-points tested (Fig. 1B). At 4 weeks of age, the amount of MWL- and SWL-positive cells in the dorsal retina was comparable to the respective wild-type control (Fig. 1B). However, at the same time-point, GNAT2 levels were already reduced to 47%. After 32 weeks of age, GNAT2-positive cells were no longer detectable, but some MWL- and SWL-positive cells remained in the retina of R91W mice (Fig. 1B).

The SWL cones seemed to be the most strongly affected cone type. Between 4 and 8 weeks of age, more than 50% of SWL-positive cells were lost in R91W animals. In the same period, MWL dropped only by 40% (Fig. 1B and data not shown). By 24 weeks, only 16% of SWL cones remained in the upper central retina, whereas 27% of the MWL marker was still detectable.

To explore the morphological consequences of *Rpe65* mutations on cone degeneration *in vivo*, we crossed R91W and $Rpe65^{-/-}$ strains to a transgenic mouse line that expresses GFP under the control of a cone opsin promoter (coGFP). Using scanning laser ophthalmoscopy (SLO) to detect GFP-positive cells, we followed cone degeneration in individual animals in the period between 3 and 12 weeks of age (Fig. 2). The loss in GFP signal, which is representative for cone loss, was detectable already at 6 weeks of age in both *Rpe65* mutant mice strains (Fig. 2). The signal started disappearing in the ventral region of both $Rpe65^{-/-}$;coGFP and R91W;coGFP retinas. However, while in $Rpe65^{-/-}$;coGFP mice this polarization was strongly established already at 6 weeks of age, degeneration in R91W;coGFP mice proceeded more gradually (Fig. 2). These data are in line with our immunostaining data showing that the ventral retina in R91W animals was more prone to cone degeneration.

Cone opsin mislocalization

Lack of 11-*cis*-retinal has been implicated in improper cone opsin trafficking, which results in cone degeneration (14,15).

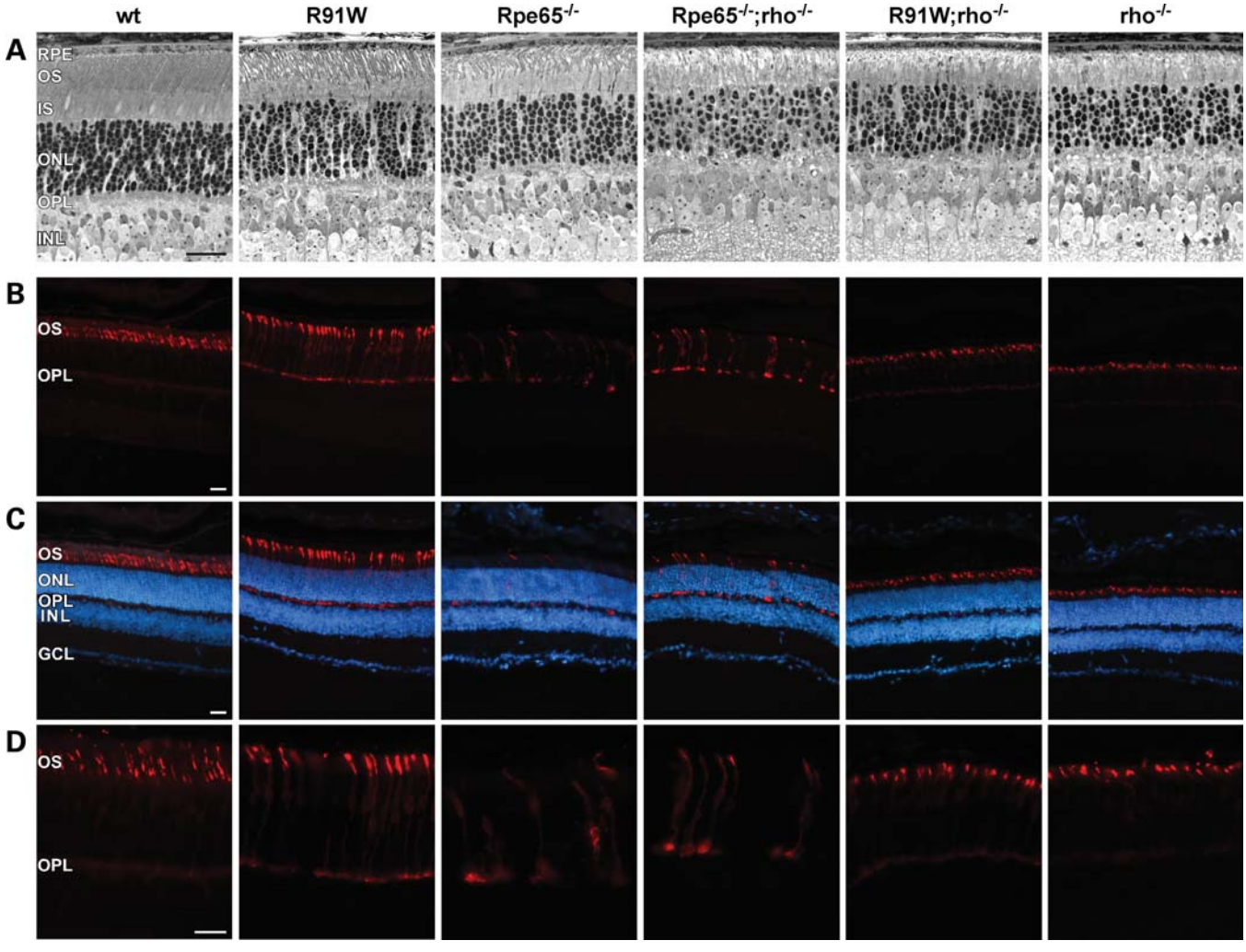

Figure 3. Cone opsin mislocalization in R91W can be corrected by ablation of rod outer segments. Retinas of age-matched 4-week-old mice of indicated genotypes were analyzed. (**A**) Representative light microscopy photographs of retinal morphology. Note the slightly better structural preservation of OS in R91W mice when compared with Rpe65$^{-/-}$ single mutant animals. No gross morphological differences were detected among the three rho$^{-/-}$ mutant mice (Rpe65$^{-/-}$;rho$^{-/-}$, R91W;rho$^{-/-}$ and rho$^{-/-}$, respectively). (**B**–**D**) Immunolocalization of short wavelength (SWL) cone opsin at low (B and C) or higher magnification (D). Nuclei were contrasted with DAPI (C). SWL localized to cone outer segments in all animals. However, in R91W, Rpe65$^{-/-}$ and Rpe65$^{-/-}$;rho$^{-/-}$ mutant mice, SWL mislocalized to synaptic termini in the OPL, to perinuclear regions in the ONL and to cellular protrusions. Ablation of rod OS almost fully corrected cone opsin mislocalization in R91W;rho$^{-/-}$ but not in Rpe65$^{-/-}$;rho$^{-/-}$ mice. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 25 μm.

Here, we analyzed the impact of limited amounts of 11-*cis*-retinal in R91W mice on cone structural integrity.

We compared retinal morphology of six different mouse strains (wt, R91W, Rpe65$^{-/-}$, Rpe65$^{-/-}$;rho$^{-/-}$, R91W;rho$^{-/-}$ and rho$^{-/-}$) at 4 weeks of age by light microscopy (Fig. 3A) and by immunostaining of SWL cone opsin (Fig. 3B–D). As previously reported (23), the first signs of degeneration were already present in 4-week-old R91W and Rpe65$^{-/-}$ animals, visible as shorter and less densely packed photoreceptor outer segments (Fig. 3A). We did not observe major differences in retinal morphology between animals on a rho$^{-/-}$ background (Fig. 3A: Rpe65$^{-/-}$;rho$^{-/-}$, R91W;rho$^{-/-}$ and rho$^{-/-}$). Immunostaining of SWL cone opsin in wild-type mice predominantly stained cone outer segments (Fig. 3B: wt). In R91W animals, however, a strong cone opsin specific signal was detected in synaptic termini of photoreceptors and a weak perinuclear staining was observed as well (Fig. 3B: R91W). In Rpe65$^{-/-}$ animals, the staining pattern was qualitatively similar, but signal intensity was generally lower and a reduced number of cones was observed (Fig. 3B: Rpe65$^{-/-}$).

Using rho$^{-/-}$ animals, we analyzed if structural ablation of rod opsin in R91W would rescue cone opsin mislocalization. This experiment was based on the assumption that 11-*cis*-retinal in R91W;rho$^{-/-}$ mice should be exclusively available to cones. As control we used Rpe65$^{-/-}$;rho$^{-/-}$ animals. Ablation of rod opsin in Rpe65$^{-/-}$ mice had no effect on cone opsin mislocalization (Rpe65$^{-/-}$;rho$^{-/-}$, Fig. 3B). In contrast, in R91W;rho$^{-/-}$ mice cone opsin localization was corrected. It was absent from synaptic termini and was found predominantly in outer segments (Fig. 3B: R91W;rho$^{-/-}$). Immunostaining was almost indistinguishable

to single rho$^{-/-}$ mutants (Fig. 3B: rho$^{-/-}$). To ensure that the rescue effect was indeed mediated by 11-*cis*-retinal, we measured the retinoid content in the eyes by HPLC. The 11-*cis*-retinal and 11-*cis*-retinol content in eyes of 4-week-old R91W;rho$^{-/-}$ animals was 1.3 ± 0.1 pmol and 2.0 ± 0.2 per eye, respectively. No 11-*cis*-retinoids were detected in Rpe65$^{-/-}$;rho$^{-/-}$ double mutants, as reported before (27).

These data show that the rescue of cone opsin misrouting observed in R91W;rho$^{-/-}$ can be attributed to the availability of 11-*cis*-retinal for cones and suggest that cone and rod opsins compete for the chromophore.

Photoreceptor competition for the visual chromophore

Ablation of RPE65 eliminates cone function and remnant minimal visual responses can be attributed to rod function due to the presence of 9-*cis*-retinal forming the visual pigment isorhodopsin (28,29). R91W mice contain similar amounts of 9-*cis*-retinal, but, in contrast to Rpe65$^{-/-}$ mice, they also contain small amounts of 11-*cis*-retinal (23). Accordingly, R91W mice are about 1 log unit more sensitive to light than Rpe65$^{-/-}$ animals under scotopic conditions. The previous functional analysis indicated that electroretinogram (ERG) responses under photopic conditions might originate from both the rod and the cone system (23).

To specifically analyze the source of vision in R91W mutant mice, we abolished rod function by intercrossing R91W knock-in mice with rho$^{-/-}$ and Gnat1a$^{-/-}$ knock-out animals. Rho$^{-/-}$ mice do not develop rod outer segments but initially have a functional cone system (30). Similar to the rho$^{-/-}$ animals, Gnat1a$^{-/-}$ mice have no rod function, but, in contrast, develop normal rod outer segments with regular rod opsin content (31). Functionally, retinas of both mouse strains are considered as pure-cone retinas.

We generated R91W;Gnat1a$^{-/-}$, R91W;rho$^{-/-}$ double mutant and R91W;Gnat1a$^{-/-}$;rho$^{-/-}$ triple mutant mice and compared their function with the previously described wild-type, R91W, rho$^{-/-}$ and Rpe65$^{-/-}$;rho$^{-/-}$ mutant mice (Fig. 4A and B). By the time of analysis all mice were 4-weeks-old.

Dark-adapted ERG intensity series revealed severely depressed responses in R91W mice when compared with wild-type animals (Fig. 4A). As expected, minimal or no responses were detected under the same conditions in rho$^{-/-}$ and all double- or triple mutant animals due to the functional rod ablation (Fig. 4A).

To analyze light-adapted responses, photopic flash intensity series across a 6-log-unit intensity range was recorded in all genotypes (Fig. 4B). Under this condition, R91W and rho$^{-/-}$ revealed very similar threshold sensitivities when compared with wild-type animals (Fig. 4B). Ablation of rod opsin in R91W (R91W;rho$^{-/-}$) caused an additional reduction in sensitivity by 1–1.5 log units (Fig. 4B). However, photoreceptors in R91W;Gnat1a$^{-/-}$ animals lacking rod function but not rod outer segments were much more desensitized than those in R91W;rho$^{-/-}$ animals (Fig. 4B). The severely desensitized response in R91W;Gnat1a$^{-/-}$ was not due to the absence of 11-*cis*-retinal. HPLC analysis of the retinoid content showed that R91W;Gnat1a$^{-/-}$ contained 23.7 ± 1.2 pmol, an amount similar to single mutant R91W animals (23). The mice with the better ERG response (R91W;rho$^{-/-}$) had with 1.3 ± 0.1 pmol per eye an even lower content of 11-*cis*-retinal.

The strongly reduced cone function in R91W;Gnat1a$^{-/-}$ mice thus suggested that in the presence of limited amounts of 11-*cis*-retinal, a substantial cone response can only be generated when rod opsin is absent, as in R91W;rho$^{-/-}$. To prove this principle and to exclude a possible non-specific effect of the Gnat1a$^{-/-}$ knock-out, we generated R91W;rho$^{-/-}$;Gnat1a$^{-/-}$ triple mutant animals. We found no difference in ERG responses between R91W;rho$^{-/-}$ double mutants and R91W;Gnat1a$^{-/-}$;rho$^{-/-}$ triple mutants (Fig. 4B). This suggests that 11-*cis*-retinal in the triple mutants (captured by rod opsin in R91W;Gnat1a$^{-/-}$ animals) was now available for cone opsins, supporting our hypothesis of chromophore competition between rod and cone opsins.

DISCUSSION

We investigated the cone pathophysiology resulting from 11-*cis*-retinal chromophore insufficiency. We show that (i) the R91W mutation causes an early progressive geographic cone atrophy characterized by a ventro-dorsal gradient of degeneration; (ii) cone opsin mislocalization in R91W can be rescued by the ablation of rod opsin; and (iii) ablation of rod opsin increases cone function in R91W mice.

Collectively, our data suggest that rod opsin and cone opsins compete for the available 11-*cis*-retinal. In a situation where only 5–10% of normal 11-*cis*-retinal levels are present (as in R91W knock-in mice), rod opsins seem to entrap almost all available chromophore. Rods may outcompete cones for the following reasons: (i) rods massively outnumber cones; (ii) cones have much higher spontaneous rate of chromophore dissociation (32); and (iii) rods have a larger interface with the RPE—the major chromophore source.

The genetic ablation of rod opsin increases the availability of 11-*cis*-retinal and improves cone structure and function. The functional ablation of rods (Gnat1a$^{-/-}$), however, is not sufficient, which indicates that rod opsin works as a 'chromophore trap' preventing cone function. Our data also suggest that mice do not have an isolated pathway of chromophore delivery specifically reserved for cones, as supply of the chromophore for both rods and cones depends on RPE65.

Cone degeneration

We have shown by RT–PCR, immunofluorescence and by SLO *in vivo* analysis that cone markers were very early reduced in R91W mice. The ventral retina was most affected and was devoid of cones already at 16 weeks of age. It has been shown that the mouse ventral retina is enriched with SWL opsin (26) and SWL cones are reduced particularly early in Rpe65$^{-/-}$ mice (10). Thus, the geographic cone atrophy was qualitatively similar in Rpe65 knock-out and R91W knock-in mice, and in both models, SWL cones were more severely affected than MWL cones. However, in Rpe65 knock-outs, SWL cone opsin loss is more pronounced: Swl RNA is reduced by more than 50% by 2 weeks of age as

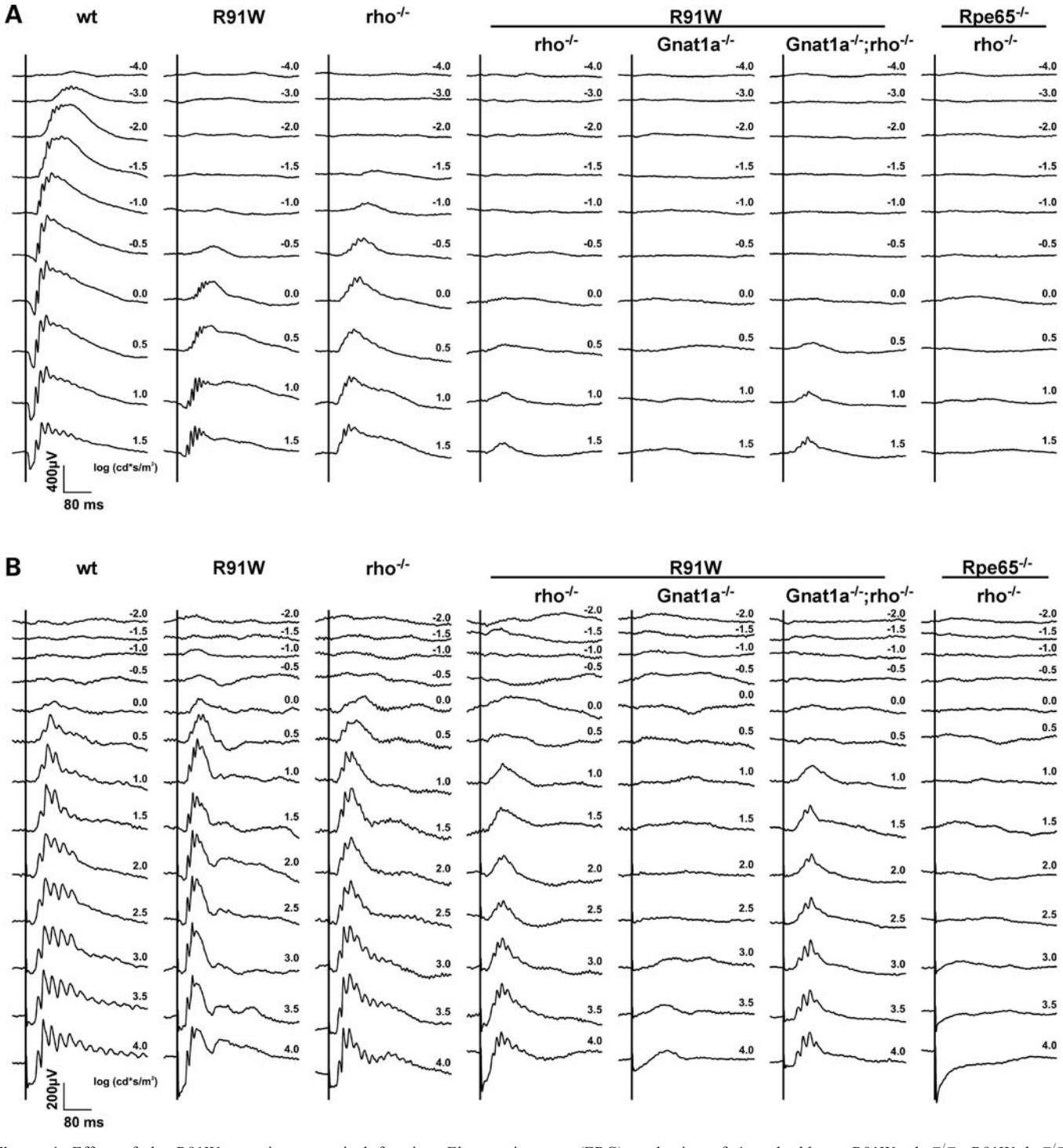

Figure 4. Effect of the R91W mutation on retinal function. Electroretinogram (ERG) evaluation of 4-week-old wt, R91W, rho$^{-/-}$, R91W;rho$^{-/-}$, R91W;Gnat1a$^{-/-}$, R91W;Gnat1a$^{-/-}$; rho$^{-/-}$ and Rpe65$^{-/-}$;rho$^{-/-}$ mice. Single flash ERGs were recorded under dark-adapted (**A,** scotopic) and light-adapted (**B,** photopic) conditions. Additional deletion of rho in R91W and Rpe65$^{-/-}$ enabled the examination of pure cone function in the absence of the rod system. The vertical lines indicate the timing of the light flash. Note that an initial negative deflection in the higher intensity range (above 1.5 log cd*s/m^2) is no retinal electrical response but a flash artifact.

opposed to a reduction of only 10% in R91W knock-in mice (data not shown). We also qualitatively followed cone cell loss in *Rpe65* mutant animals *in vivo* by using coGFP reporter animals monitored by SLO. This method clearly showed that the cone degeneration in R91W animals proceeded more slowly than in Rpe65 knock-out mice. It has to be noted that in coGFP mice the interference with opsin production or the presence of GFP is toxic for cones leading to their death by

about 6 months of age, with some individual variability (data not shown). Thus, the true cone status can probably only be assessed during the first 3–4 months of life in coGFP line and the respective double mutant mice.

By immunofluorescence, we noted a certain discrepancy in the number of GNAT2-positive cells when compared with MWL- and SWL-positive cells. At 4 weeks of age, fewer GNAT2-positive cells were detected than MWL or SWL cone opsin-labeled cells. This discrepancy cannot be explained by lower transcriptional activity since mRNA expression of all three markers was comparably reduced. This may suggest that in degenerating cone cells, GNAT2 is less stable than cone opsins. A similar conclusion has been made in young Lrat$^{-/-}$ and Rpe65$^{-/-}$ mice where posttranslational degradation was accounted as reason for decreased GNAT2 levels (14).

Cone preservation and chromophore competition

An optimal delivery of 11-*cis*-retinal to cones has not only a functional relevance but it is also important for structural maintenance. Recently, Rohrer *et al*. (11) detected cone opsin mislocalization in Rpe65$^{-/-}$ mice, which was corrected upon administration of 11-*cis*-retinal or 9-*cis*-retinal (10). Based on this observation, the authors hypothesized that cones degenerate because of opsin mislocalization as a direct result of 11-*cis*-retinal starvation. Later studies showed, accordingly, that viral *Rpe65* gene delivery to Rpe65$^{-/-}$ mice restores cones (12,13). Finally, 11-*cis*-retinal has been shown to be necessary for proper trafficking of membrane-associated phototransduction components from their synthesis place within the cone inner segment trough the connecting cilium to the outer segment (14,15). Retinas of R91W single mutant mice receive only small [5–10% of wild-type (23)] amounts of 11-*cis*-retinal. Immunostaining data show that cone opsin in R91W animals is mislocalized. This suggests that most of the available 11-*cis*-retinal in R91W animals ends up in rods for reasons explained below. This further suggests that chromophore starvation is the cause for cone degeneration in R91W animals—as for 11-*cis*-retinal deficient animals (Rpe65$^{-/-}$ and Lrat$^{-/-}$). As R91W mice show delayed cone cell death compared with retinas without measurable chromophore, our results suggest that the progression of cone degeneration can be directly attributed to the degree of impairment in the 11-*cis*-retinal supply.

In situations of limited 11-*cis*-retinal delivery to retinas where the vast majority of photoreceptors are rods, the chromophore uptake is presumably based on a 'tyranny of the majority' rule, which favors rods over cones. Additionally, the thermodynamic stability of the rod opsin–11-*cis*-retinal holocomplex and the proximity to the RPE as chromophore source are also favorable for rods. A competition between rods and cones for limited amounts of chromophore has been previously described *in vitro* (33). In our experiments, a competitive chromophore uptake is supported by the fact that even though comparable amounts of 11-*cis*-retinal were found in age-matched R91W single- and R91W;Gnat1a$^{-/-}$ double mutant animals (~25 pmol/retina), the later had only minor ERG responses under photopic conditions. This argues that most 11-*cis*-retinal in R91W is captured by rods and that the retinal function recorded in R91W under photopic conditions is almost completely mediated by the rod system. However, once rod opsin is taken away from the retina (R91W;rho$^{-/-}$), the chromophore becomes available for cones resulting in a corrected opsin targeting and improved cone function.

Biochemical and genetic evidence for a cone-specific visual cycle involving Müller cells and a novel type of all-*trans* to 11-*cis*-retinol isomerase different from RPE65 has been provided by studies in lower vertebrates (34–39). Our findings, however, argue against separate pathways for the regeneration of cone and rod-specific visual pigments in mice. We clearly show that chromophore delivery to both rods and cones strictly depends on functional RPE65. In R91W animals, no other enzyme can compensate for the reduced activity of the mutant RPE65 protein and only the physical ablation of the rod opsin chromophore trap enables cones to incorporate sufficient chromophore to correctly localize opsin to their outer segments. Furthermore, a putative RPE65-independent cone visual cycle involving Müller cells would be expected to be only marginally affected by the presence or absence of rods. The close physical proximity of cones and Müller cells would strongly reduce the ability of rods to compete for the chromophore regenerated and released by Müller cells specifically for cones. Indirect evidence from the current literature further supports the importance of a strong RPE65 activity for proper cone function: the RPE65 protein from the cone-dominated chicken retina is a more efficient enzyme than RPE65 from rod dominated species (40) and the central RPE layer of primates, which localizes to the cone-rich area of the retina, has a higher RPE65 protein expression and activity than the RPE in the peripheral, rod-rich retina (9). The resulting wealthier local chromophore pool in the macular region of the primate retina and the physical absence of competing rods in this area may less strongly affect chromophore delivery to cones in the fovea allowing initial useful central vision by RPE65 patients. Since blue cones are reported to be completely absent from the central fovea (41,42) and instead localize to rod-dominated regions of the human retina, blue cones may be in strong competition with rods for the available chromophore. Except for the macular region, the human retina is comparable to the mouse retina. Our data thus predict that blue cones in RPE65 patients may be more affected than other cone types and may not receive sufficient chromophore for proper function and survival, as proposed recently (43). Indeed, most patients with RPE65 mutations report early difficulties with blue color vision (43,44) supporting our conclusions based on the R91W mouse model.

To our knowledge, this study demonstrates for first time an *in vivo* competition between rods and cones for limited amounts of chromophore and its functional and pathological consequences. Our data suggest that a successful RPE65 gene therapy requires delivery of the functional gene directly to the macular region to supply cones and avoid chromophore 'theft' by rods. The importance of 11-*cis*-retinal supplementation for the cone structural integrity should also be considered in therapies aiming at the inhibition/slowing of the visual cycle and thus at the reduction of available chromophore in the retina. It seems important that the therapeutic value of visual cycle inhibitor compounds should be carefully

Table 1. PCR primers used for genotyping

Genotype	Forward (5′–3′)	Reverse (5′–3′)	Size (bp)
coGFP	CAATTAAGAGATCAGGTAGTGT	AGTTCACCTTGATGCCGTTCTT	641
R91W[a]	GCTGGTCTTGCCTGTATCA	GTCAGAGACAGTGCTGTGTT	998
Rpe65$^{+/+}$	GATGTGGGCCAGGGCTCTTTGAAG	CCCAATAGTCTAGTAATCACAGATG	546
Rpe65$^{-/-}$	GATGTGGGCCAGGGCTCTTTGAAG	GGGAACTTCCTGACTAGGGGAGG	459
Rho$^{+/+}$	TCTCTCATGAGCCTAAAGCT	ATGCCTGGAACCAATCCGAG	470
Rho$^{-/-}$	TCTCTCATGAGCCTAAAGCT	TTCAAGCCCAAGCTTTCGCG	310
Gnat1a$^{+/+}$	TATCCACCAGGACGGGTATTC	GCGGAGTCATTGAGCTGGTAT	387
Gnat1a$^{-/-}$	TATCCACCAGGACGGGTATTC	GGGAACTTCCTGACTAGGGGAGG	273

[a]PCR product was additionally cut with *Taq*I restriction enzyme. Expected fragments: 998 bp for knock-in and 619 bp + 379 bp for wild-type.

evaluated with respect to their specific impact on the cone visual system.

MATERIALS AND METHODS

Mice

All procedures concerning animals were in accordance with the regulations of the Veterinary Authority of Zurich and with the statement of 'The Association for Research in Vision and Ophthalmology' for the use of animals in research. All animals were raised in cyclic light (12:12 h; 60 lux at cage level).

The knock-in mice with an amino acid exchange of Arg by Trp at position 91 in *Rpe65* (R91W) on a 129S6 genetic background are described elsewhere (23). Wild-type (129S6), Rpe65$^{-/-}$ (20), rho$^{-/-}$ (30) and Gnat1a$^{-/-}$ (31) mice were maintained at the animal facility of the University Hospital Zurich. Transgenic coGFP mice, in which GFP expression is driven by 5′ regulatory sequences of the human red pigment (45), were provided by Thomas Hughes, Yale University School of Medicine. The R91W;rho$^{-/-}$, Rpe65$^{-/-}$;rho$^{-/-}$, R91W;Gnat1a$^{-/-}$, R91W;rho$^{-/-}$;Gnat1a$^{-/-}$, R91W;coGFP, Rpe65$^{-/-}$;coGFP double- or triple transgenic mice were generated by classical breeding schemes. Genotyping was performed by PCR on tail genomic DNA using primer pairs indicated in Table 1.

Histology

Animals were sacrificed and the nasal part of the eye was marked for orientation. The enucleated eyes were fixed overnight in 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer and processed as described previously (46). Semi-thin sections (0.5 μm) of Epon-embedded tissue were prepared from the central retina, counterstained with methylene blue and analyzed using a light microscope (Axiovision, Zeiss, Jena, Germany).

Immunofluorescence and cone quantification

Animals were sacrificed, their eyes were marked nasally, enucleated and perforated at the cornea to allow better penetration of the fixative. After fixation for 1 h in 4% PFA (in PBS), the eyes were cryoprotected in 25% sucrose solution. The eyes were embedded in albumin from hen egg white (Fluka, Buchs, Switzerland) and cut dorsal to ventral through the optic nerve head. Sections (14 μm) were blocked for at least 1 h at room temperature in PBS containing 0.2% Triton X-100 and 10% normal goat serum (Dako, Zug, Switzerland) or 10% normal horse serum (Jackson Immunoresearch, Westgrove, PA, USA). Primary antibodies used were: rabbit anti-GNAT2 (Santa Cruz, CA, USA), goat anti-SWL-opsin (Santa Cruz) and rabbit anti-MWL (Chemicon, Temecula, CA, USA). The immunostaining procedure was as described previously (13).

The most transversal sections, cut through the optic nerve, were chosen to quantify the number of cells positive for GNAT2, MWL and SWL-cone opsins, respectively. The immunoreactive outer segments were counted from periphery to periphery using a BX60 microscope equipped for epifluorescence (Olympus Suisse SA, Aigle, Switzerland) and coupled to the analySIS® 3.0 software (Soft Imaging System). At least four eyes per condition and genotype were analyzed.

RNA isolation and RT–PCR

RNA isolation from retinal tissue and reverse transcription were performed as described previously (46). cDNA quantification was carried out by real-time PCR using the LightCycler 480 Sybr Green I Master kit and a LightCycler 480 instrument (Roche, Mannheim, Germany). cDNAs were amplified with primers for Gnat2, Mwl and Swl (10) and normalized to actin (47). The normalization was done using the comparative threshold cycle method. Relative values were calculated using a suitable calibrator sample. At least three retinas from three different animals were analyzed in triplicates.

Electroretinography

Electroretinograms were recorded binocularly according to previously described procedures (28,48) in anesthetized mice with dilated pupils using a Ganzfeld bowl, DC amplifier, and a PC-based control and recording unit (Multiliner Vision; VIASYS Healthcare GmbH, Hoechberg, Germany). Recordings were obtained in both scotopic (dark-adapted overnight) and photopic (light-adapted 10 min at 30 cd/m^2) conditions.

Single white-flash stimulus intensity ranged from −4 to 1.5 log cd*s/m^2 under scotopic and from −2 to 4 log cd*s/m^2 under photopic conditions, divided into 10 and 13 steps, respectively. For the intensities from 2 to 4 log cd*s/m^2, a

Mecablitz 60CT4 flash gun (Metz, Germany) added to the Ganzfeld bowl was used. Ten responses per intensity were averaged with an inter-stimulus interval of either 5 or 17 s (>1 cd*s/m^2). At least three animals per genotype were analyzed at 4 weeks of age.

In vivo monitoring by SLO

Scanning laser ophthalmoscopy was obtained in dark-adapted GFP-positive mice according to procedures reported previously (49). Mice were anesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg) and the pupils were dilated with tropicamide eye drops (Mydriaticum Stulln, Pharma Stulln, Stulln, Germany). SLO was performed with a Heidelberg Retina Angiograph (Heidelberg Engineering), a confocal scanning-laser ophthalmoscope. Laser wavelength used for fundus autofluorescence visualization was 488 nm.

HPLC determination of retinoids

Mice were dark-adapted overnight. All the following steps were carried out under dim red light. Animals were sacrificed, and lens and vitreous were removed from the eye through a slit in the cornea. The rest of the tissue including the retina and eyecup was snap frozen in liquid nitrogen until further analysis. Retinoid extraction and HPLC analysis was performed as described previously (50). Three to six animals per genotype were analyzed.

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Conflict of Interest statement. None declared.

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