Cellular/Molecular

Deletion of GRK1 Causes Retina Degeneration through a Transducin-Independent Mechanism

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***Rpe65*$^{-/-}$ mice are unable to produce 11-*cis*-retinal, the chromophore of visual pigments. Consequently, the pigment is present as the apoprotein opsin with a minute level of pigment containing 9-*cis*-retinal as chromophore. Notably, a 10–20% fraction of this opsin is mono-phosphorylated independently of light conditions. To determine the role of rhodopsin kinase (GRK1) in phosphorylating this opsin and to test whether eliminating this phosphorylation would accelerate photoreceptor degeneration, we generated the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mouse. The retinae of *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice had negligible opsin phosphorylation, extensive degeneration with decreased opsin levels, and diminished light-evoked rod responses relative to *Rpe65*$^{-/-}$ mice. These data show that opsin phosphorylation in the *Rpe65*$^{-/-}$ mouse is due to the action of GRK1 and is neuroprotective. However, despite the higher activity of unphosphorylated opsin, the severe loss of opsin in the rapidly degenerating *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice resulted in lower overall opsin activity and in higher rod sensitivity compared with *Rpe65*$^{-/-}$ mice. In *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice where transduction activation was blocked, degeneration was only partially prevented. Therefore, increased opsin activity in the absence of phosphorylation was not the only mechanism for the accelerated retinal degeneration. Finally, the deletion of GRK1 triggered retinal degeneration in *Grk1*$^{-/-}$ mice after 1 month, even in the absence of apo-opsin. This degeneration was independent of light conditions and occurred even in the absence of transducin in *Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice. Taken together, our results demonstrate a light-independent mechanism for retinal degeneration in the absence of GRK1, suggesting a second, not previously recognized role for that kinase.**

Introduction

Vision is initiated when light is absorbed by visual pigments, triggering the isomerization of 11-*cis*-retinal to the all-*trans* isomer, resulting in activation of the visual cascade. The lifetime of the activated pigment is limited by a two-step process: active rhodopsin is first phosphorylated by rhodopsin kinase (GRK1) and then becomes completely inactivated when visual arrestin binds (Arshavsky, 2002). Phosphorylation is a vital first step in the timely termination of phototransduction.

In vitro, rhodopsin phosphorylation has been shown to be catalyzed by both GRK1 (Ohguro et al., 1996; Chen et al., 1999) and protein kinase C (PKC) (Newton and Williams, 1993; Williams et al., 1997). *In vivo*, the role of GRK1 in terminating phototransduction has been clearly demonstrated by studies on the *Grk1*$^{-/-}$ mouse (Chen et al., 1999) and by deleting the rhodopsin phosphorylation sites (Chen et al., 1995; Mendez et al., 2000). Phosphorylation of rhodopsin is the only reported role for GRK1 in the retina. PKC is also expressed in rod outer segments (Kelleher and Johnson, 1986; Williams et al., 1997) and has been proposed to have a role in rhodopsin phosphorylation. However, no effect on rod phototransduction was observed on the use of PKC activators or inhibitors (Xiong et al., 1997) and Adams et al. (2003) concurred that GRK1 is the main enzyme for rhodopsin phosphorylation. The phosphorylation target of PKC in photoreceptors is still unclear.

The *Rpe65*$^{-/-}$ mouse does not form 11-*cis*-retinal in the retinal pigment epithelium (Redmond et al., 1998). Consequently, the majority of visual pigment in *Rpe65*$^{-/-}$ rods is in the form of the apoprotein, opsin, together with minute amounts ($<$0.1%) of isorhodopsin (Fan et al., 2003). The rods in *Rpe65*$^{-/-}$ mice degenerate slowly (Fan et al., 2005), and this degeneration can be blocked by ablating the α-transducin gene, *Gnat1* (Woodruff et

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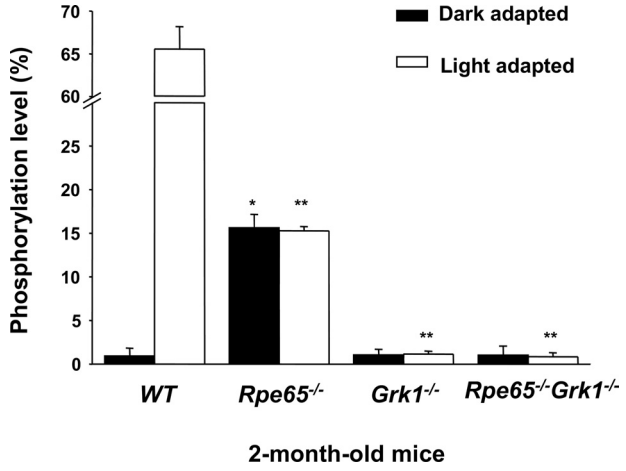

Figure 1. Effect of deletion of *Rpe65* and *Grk1* on opsin phosphorylation. Retinae of cyclic-light-reared, 2-month-old WT, *Rpe65* $^{-/-}$, *Grk1* $^{-/-}$, and *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice were homogenized in 8 M urea and digested with endoproteinase Asp-N in 10 mM Tris buffer at pH 7.6 to cleave the opsin C terminus, which was analyzed online with an LCQ mass spectrometer. In the *Grk1* $^{-/-}$ and *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice, no significant opsin phosphorylation was observed. White bars, Animals exposed to room light for 6 h; black bars, animals dark-adapted for 12 h. Data are shown as the percentage of rhodopsin C terminus containing phosphorylation, independent of the multiplicity of phosphorylation, and presented as mean ± SEM; $n = 3$.

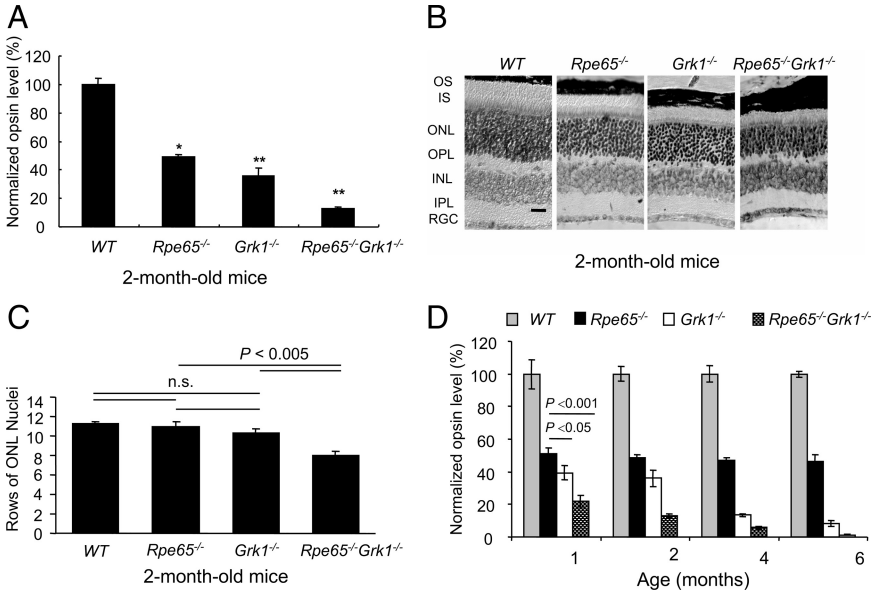

Figure 2. Effect of deletion of *Rpe65* and *Grk1*on retinal opsin levels and morphology. ***A***, Opsin levels were calculated from rhodopsin that formed upon the addition of 11-*cis*-retinal. Data were generated from 2-month-old cyclic-light-reared mice. The relative opsin levels in each strain were normalized to the average of wild-type opsin concentration and shown as a fraction of the wild type. ***B***, Retinal morphology of 2-month-old WT, *Rpe65* $^{-/-}$, *Grk1* $^{-/-}$, and *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice. Light micrographs of paraffin-embedded retinae sections from the superior central region of the retina. While morphology of the inner retina was unaffected by the different genotypes at that resolution, the outer retina showed shortening of the inner and outer segments progressing in severity from the *Rpe65* $^{-/-}$ < *Grk1* $^{-/-}$ ≪ *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ (scale bar, 30 μm). INL, Inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; OPL, outer plexiform layer; OS, outer segments; RGC, retinal ganglion cells; n.s., not significant. ***C***, Bar graph representing rows of photoreceptor cell nuclei counted in the superior central region of the retinae of 2-month-old mice from the 4 strains; mean ± SEM; $n = 5$. The cell loss was significant for the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice. ***D***, Opsin levels with age, assayed as for ***A***. The relative opsin levels in each strain were normalized to the average of wild-type opsin concentration at each age, and shown as the fraction of the wild type. Gray bar, WT mice; black bar, *Rpe65* $^{-/-}$ mice; white bar, *Grk1* $^{-/-}$ mice; hatched bar, *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice.

al., 2003), indicating that degeneration occurs due to the constitutive activation of the rod signal transduction cascade by the opsin. Notably, the rod opsin in the *Rpe65* $^{-/-}$ mouse as well as in the *Lrat* $^{-/-}$ mouse, which also lacks 11-*cis*-retinal (Batten et al., 2004), is mono-phosphorylated (10–20%) independent of light history (Ablonczy et al., 2002; Fan et al., 2008). It is unknown which kinase is responsible for this opsin mono-phosphorylation.

In this study, we generated the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$, *Rpe65* $^{-/-}$*Grk1* $^{-/-}$*Gnat1* $^{-/-}$, and *Grk1* $^{-/-}$*Gnat1* $^{-/-}$ mice to address the following questions: (1) is GRK1 the kinase that phosphorylates opsin in *Rpe65* $^{-/-}$ mice? (2) Does removal of phosphorylation affect the extent of rod degeneration? And (3) is the photoreceptor degeneration due to activation of the rod signaling transduction cascade? Based on opsin phosphorylation, opsin levels, retinal morphology, and transcorneal and isolated retinal electroretinograms (ERGs), we conclude that GRK1 is the kinase that phosphorylates opsin. Surprisingly, the retinae of *Rpe65* $^{-/-}$*Grk1* $^{-/-}$*Gnat1* $^{-/-}$ and *Grk1* $^{-/-}$*Gnat1* $^{-/-}$ mice show progressive degeneration independent of light exposure even though the classic visual transduction pathway is blocked by removal of rod α-transducin. This degeneration is particularly evident beyond 2 months of age. Thus, our results demonstrate a previously undescribed light- and transduction cascade-independent mechanism for retinal degeneration driven by the lack of GRK1.

Materials and Methods

Animals. Rpe65 $^{-/-}$ (B6;129-Rpe65 ^{tm1Tmr}) mice were the generous gift of Dr. Michael Redmond (National Eye Institute, National Institutes of Health) and genotyped as described previously (Redmond et al., 1998; Redmond and Hamel, 2000). *Grk1* $^{-/-}$ mice (B6;129-Grk1 ^{tm1Mis}) were generated as described previously (Chen et al., 1999). *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice (B6;129-Rpe65 ^{tm1Tmr} Grk1 $^{tm1Mis/Kwy}$) were generated by cross-breeding the *Rpe65* $^{-/-}$ mice with *Grk1* $^{-/-}$ mice. Western blot analysis confirmed that GRK1 and RPE65 were not detectable in homogenates of eyecups from *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice. *Gnat1* $^{-/-}$ mice (B6;129-Gnat1 ^{tm1Jl}) were the generous gift of Dr. Janis Lem (New England Medical Center and Tufts University School of Medicine, Boston, MA). *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ *Gnat1* $^{-/-}$ mice were generated by cross-breeding the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice and *Gnat1* $^{-/-}$ mice and genotyped as described previously (Calvert et al., 2000). Western blot analysis confirmed the genotype results. Age-matched C57BL/6 (WT) mice were purchased from Harlan. Animals were reared under cyclic light (12 h light/12 h dark) with the ambient light intensity at the eye level being 85 ± 18 lux until experimentation. For some experiments, mice were raised under constant darkness from birth with animal husbandry performed under very dim red light (0.3 lux). All experiments were performed in accordance with the policy on the Use of Animals in Neuroscience Research and were approved by the Medical University of South Carolina Animal Care and Use Committee and the Washington University Animal Studies Committee.

Opsin quantification. Retinae from dark-adapted mice (12 h) were collected under infrared light. A pair of retinae was homogenized with a glass syringe in 500 μl of 10 mM Tris-HCl containing 1 mM EDTA (pH 7.5), AEBSF [1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydro-chloride; Roche Molecular Biochemicals], protease inhibitor mixture tablet (1 tablet/10 ml;

Complete Mini; Roche Molecular Biochemicals), and 10 μg of DNase I (Sigma). Samples were centrifuged (27,000 × *g*, 15 min), and the supernatant was discarded. The pellets were suspended in 100 μl of 0.1 M sodium phosphate buffer (pH 7.4) and incubated with 11-*cis*-retinal (final concentration, 80 μM) at 4°C on a rotator for 12 h. Samples were centrifuged, and the resultant pellets were resuspended in 100 μl of 1% *n*-dodecyl-β-D-maltoside (ULTROL grade; Calbiochem) in 0.1 M sodium phosphate buffer (pH 7.4) for solubilization (2 h, 4°C on a rotator). Unsolubilized material was removed by centrifugation (100,000 × *g* for 15 min), and the supernatant was analyzed by spectrophotometry (Cary 300, Varian). Samples were exposed to white light (Fiber Optic Illuminator, Model 190, 50 W, 60 Hz; Dolan-Jenner Industries) for 10 min in the presence of hydroxylamine hydrochloride (pH 7.0, 20 mM final concentration). Pigment levels were determined by subtracting the postbleach from the prebleach spectra. Rhodopsin concentrations were calculated using the extinction coefficient of 40,000 M^{-1} cm^{-1} (Wald and Brown, 1958; Dartnall, 1968).

Transcorneal ERG recordings. Overnight dark-adapted mice were anesthetized using xylazine (20 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.). Pupils were dilated with phenylephrine hydrochloride (2.5%) and atropine sulfate (1%). Contact lens electrodes (Bayer et al., 2001) were placed on both eyes accompanied by 2.5% Gonak hypromellose ophthalmic demulcent solution. Full-field ERGs were recorded as described previously (Gresh et al., 2003), using the universal testing and electrophysiologic system 2000 (UTAS E-2000; LKC Technologies). Single flashes of 10 μs duration and various intensities (2.48 × 10^{-2}, 2.48 × 10^{-1}, 1.56 and 2.48 cd*s/m^2) were used for stimulation under scotopic conditions.

Transretinal ERG recordings. WT, *Rpe65*$^{-/-}$, and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice were reared in 12/12 h light/dark cycle and were dark-adapted overnight before experiments. To slow down retinal degeneration, *Grk1*$^{-/-}$ mice and a subpopulation of *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice were reared in constant darkness from birth. Recordings were made in mice approximately 1 month old. Following killing, the eyes were removed under dim red light. All subsequent manipulations were done under infrared light. The eye was hemisected, and the retina was isolated and stored in Locke solution at 4°C. One-quarter of the isolated retina was mounted on filter paper with the photoreceptor side up and placed on the recording chamber with an electrode connected to the bottom. A second electrode was placed above the retina. The perfusing Locke solution (112 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM HEPES, 20 mM $NaHCO_3$, 3 mM Na_2-succinate, 0.5 mM Na-glutamate, 10 mM glucose) was equilibrated with 95% O_2/5% CO_2, heated to 34–37°C, and contained, in addition, 2 mM L-glutamic acid to block higher order components of the photoresponse (Sillman et al., 1969). The electrode solution (140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM HEPES, 10 mM glucose, pH 7.4) under the retina contained, in addition, 10 mM $BaCl_2$ to suppress the glial component of the photoresponse (Bolnick et al., 1979; Nymark et al., 2005). Responses were amplified by a differential amplifier (DP-311; Warner Instruments). For application of 11-*cis*-retinal, an isolated retina was incubated in electrode solution containing 100 μM 11-*cis*-retinal (dissolved in 0.1% ethanol) and 1% bovine serum albumin at room temperature for 1.5 h before recording. Due to the low light sensitivity of *Rpe65*$^{-/-}$ *Grk1*$^{-/-}$ rods and to be able to compare sensitivity between genotypes with a wide range of sensitivity, white light rather than 510 nm monochromatic light was used for photostimulation. White test flashes were delivered from a calibrated light source via computer-controlled shutters. For attenuated light, the flash intensity was set by a combination of neutral-density filters. To expand the stimulation range for unattenuated light, the flash duration was increased sequentially from the standard 10 ms to 20, 40, and 80 ms.

Intensity-response data were fitted by Equation 1, as follows:

$$\frac{R}{R_{max}} = \frac{I^n}{I^n + I_o^n}$$

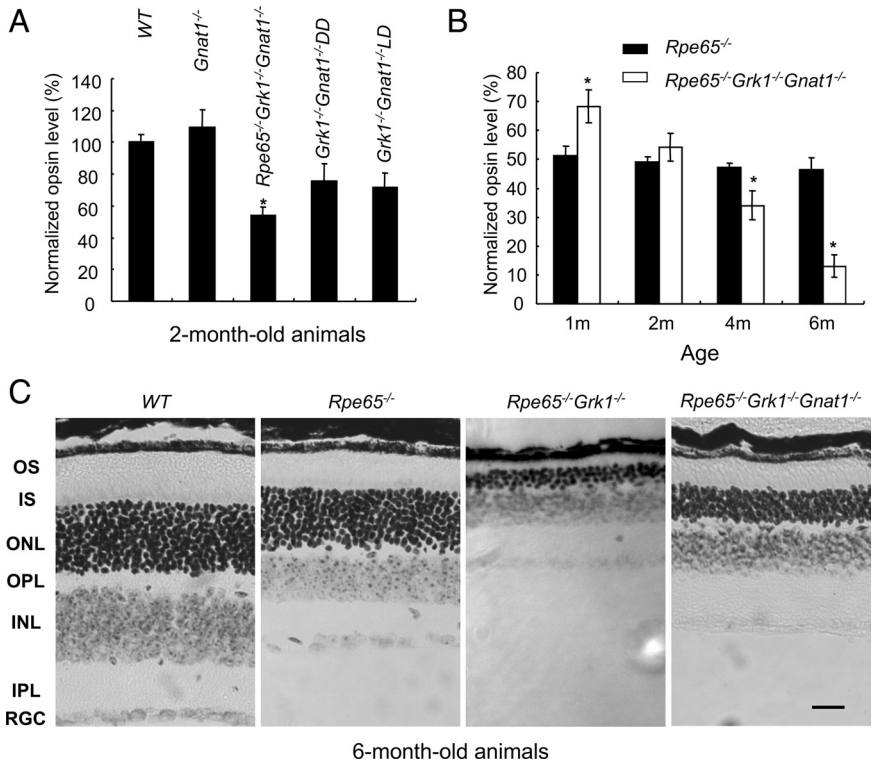

Figure 3. Effect of deletion of *Gnat1* on photoreceptor degeneration in the absence of *Grk1*. ***A***, Opsin levels were calculated as for Figure 2*A*. Data are for 2-month-old mice. The relative opsin levels were normalized to the average of wild-type opsin concentration and shown as the fraction of wild type. ***B***, Opsin levels from *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice with age, assayed as for ***A***. ***C***, Retinal morphology in 6-month-old WT, *Rpe65*$^{-/-}$, *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice. Light micrographs of paraffin-embedded retinae of 6-month-old cyclic-light-reared animals were taken from the superior central region of the eye to compare the thicknesses of the different layers. Note the extreme degeneration in the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ retina. Scale bar, 20 μm. INL, Inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; OPL, outer plexiform layer; OS, outer segments; RGC, retinal ganglion cells.

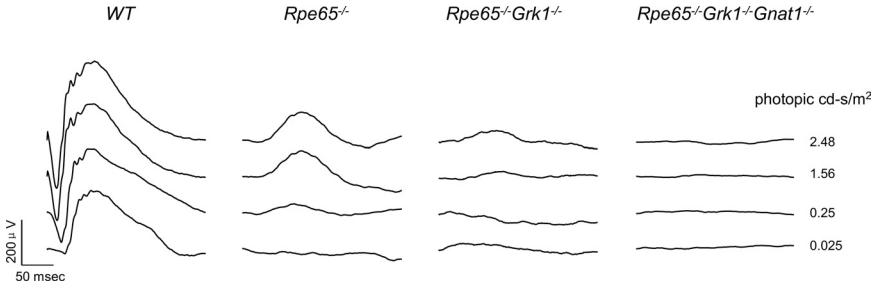

Figure 4. Scotopic transcorneal ERG responses. Families of single-flash ERGs were recorded from 2-month-old WT, *Rpe65*$^{-/-}$, and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice in response to increasing light intensities 2.48 × 10^{-2}, 2.48 × 10^{-1}, 1.56 and 2.48 cd*s/m^2.

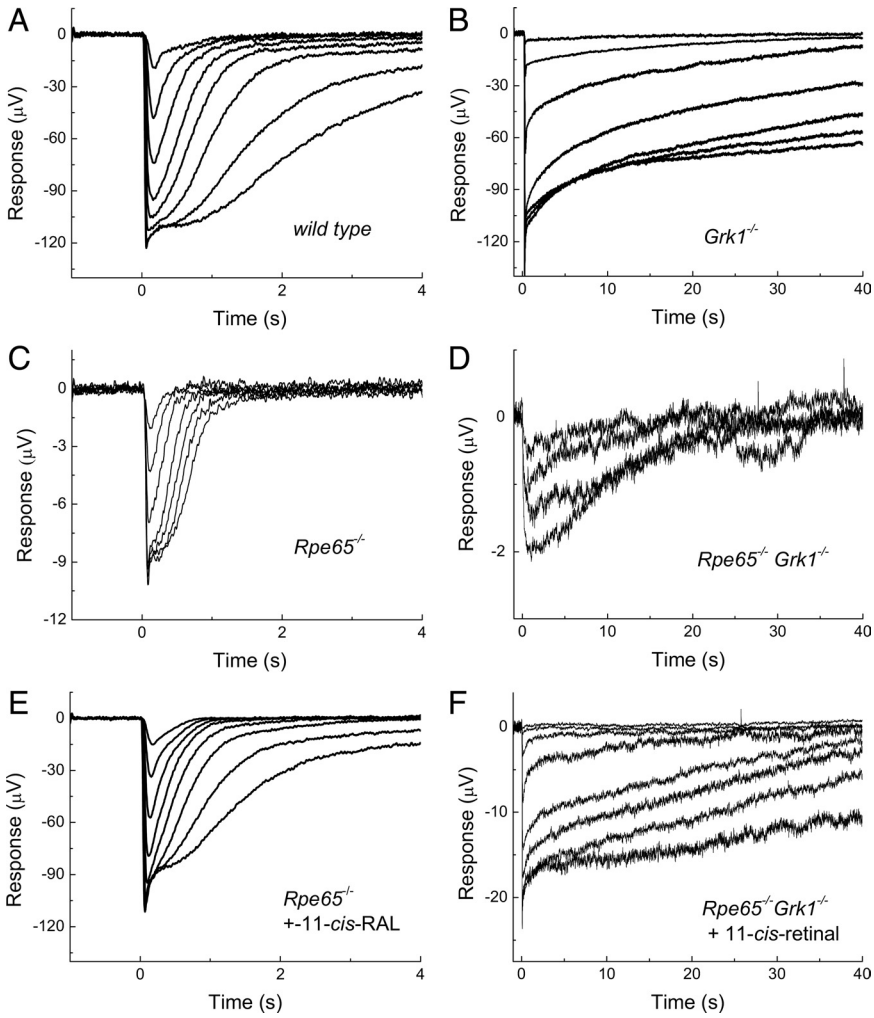

Figure 5. ***A–F***, Response families of transretinal ERG recordings from WT (***A***), *Grk1*$^{-/-}$ (***B***), *Rpe65*$^{-/-}$ (***C***, ***E***), and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ (***D***, ***F***) mice. Responses in ***E*** and ***F*** were recorded after application of 11-*cis*-retinal. Flashes of white light were delivered at time 0 in 0.5 log incremental steps except for the last three traces in ***C***, which were delivered in twofold increments by doubling the flash duration. The dimmest flashes were delivered at −1.5 log (***C***, ***D***), and −4.5 log (***A***, ***B***, ***E***, and ***F***) unit attenuation.

where R is the transient-peak amplitude of response, R_{max} is maximal response amplitude, I is flash intensity, and I_o is flash intensity estimated to produce half-maximal response. Comparison of sensitivity measured with white light and with calibrated 500 nm light in wild-type retina revealed that the intensity of unattenuated white light at 10-ms duration was equivalent to 2.8×10^5 photons μm^{-2} at 500 nm. To describe the kinetics of the dim-flash response, the time-to-peak (T_{peak}) was determined as the time from the mid-point of the test flash to the peak of the response, and the recovery time constant (τ_{rec}) was determined by fitting the final response decline with a single-exponential-decline function.

Histology. The eyes were enucleated and immersion-fixed in a solution of 60% methanol, 30% chloroform, and 10% acetic acid overnight at 4°C, and dehydrated over several hours before being embedded in paraffin in transverse orientation. The eyes were sectioned at 7 μm thickness and mounted on poly-L-lysine-coated slides. The sections were stained with 1% toluidine blue in deionized water, dehydrated, and coverslipped with mounting medium (Permount; Fisher Scientific). Central areas of the retina (within 100–300 μm of the optic nerve) were photographed for documentation. Images were acquired on a Zeiss microscope (Axioplan 2). Cell nuclei were counted by two individuals without knowledge of the identity of the tissues.

Phosphorylation measurements. Retinae (two per sample) were homogenized in 8 M urea and digested with Asp-N (25 ng; Sigma) in 100 μl of Tris buffer (10 mM, pH 7.6). Supernatants were collected by centrifugation (120,000 × *g*) and analyzed online with an LCQ mass spectrometer (Thermo-Finnigan Instrument Systems) (Hurley et al., 1998; Ablonczy et al., 2000). The data were acquired by repetitive scanning and corrected with a factor for the decreased detection efficiency of the C-terminal phosphopeptides, as measured from synthesized standards [the peak area of mono-phosphopeptides was multiplied by 1.35 (Hurley et al., 1998)].

Statistics. For all experiments, data were expressed as mean ± SEM. Data were analyzed using a two-tailed Student *t* test, accepting a significance level of $p < 0.05$.

Results

Opsin phosphorylation in *Rpe65*$^{-/-}$ mice depends on GRK1

Rod opsin in mouse models lacking 11-*cis*-retinal, such as the *Rpe65*$^{-/-}$ and *Lrat*$^{-/-}$ mice, is known to be mono-phosphorylated (Ablonczy et al., 2002; Fan et al., 2008). We measured the rod opsin phosphorylation levels in WT, *Rpe65*$^{-/-}$, *Grk1*$^{-/-}$ and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice to determine whether GRK1 is the kinase responsible for the phosphorylation of opsin in *Rpe65*$^{-/-}$ mice. Phosphorylation was quantified by cleavage of the C terminus of the opsin protein followed by mass-spectrometric analysis (Ablonczy et al., 2002) (Fig. 1). The light-adapted WT mice showed 65.5 ± 2.7% ($n = 3$) rod opsin phosphorylation, of which 28.6 ± 1.4% ($n = 3$) was mono-phosphorylation. There was essentially no phosphorylation in dark-adapted WT mice. The *Rpe65*$^{-/-}$ mice showed 15.6 ± 1.5% ($n = 3$) phosphorylated opsin, all mono-phosphorylated, independent of light exposure. This phosphorylation was not specific to a single site, but distributed over the major sites observed in light-activated mouse rhodopsin (serines 334, 338, and 343 with some evidence of threonine 342; data not shown). The *Grk1*$^{-/-}$ mice showed no significant phosphorylation regardless of light exposure, as reported previously (Chen et al., 1999). Most important, the deletion of GRK1 in RPE65-deficient mice (*Rpe65*$^{-/-}$ *Grk1*$^{-/-}$) also ablated opsin phosphorylation in darkness or light. Therefore, GRK1 underlies the phosphorylation of rod opsin in *Rpe65*$^{-/-}$mice.

Rpe65$^{-/-}$ mice show rapid retinal degeneration in absence of GRK1

To determine how the lack of opsin phosphorylation affects the survival of photoreceptors, we quantified opsin levels in 2-month-old, cyclic-light-reared mice by measuring the absorption spectra of regenerated pigments after incubation of retinal homogenates with 11-*cis*-retinal. All data were normalized to WT levels (Fig. 2*A*). The absence of GRK1 alone (*Grk1*$^{-/-}$ mice) was more deleterious to the retina than the absence of chromophore (*Rpe65*$^{-/-}$ mice), both of these degenerations having been described previously (Redmond et al., 1998; Chen et al., 1999). However, the absence of both GRK1 and RPE65 (*Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice) exacerbated degeneration, with the opsin levels reduced further compared with *Rpe65*$^{-/-}$ and *Grk1*$^{-/-}$ mice.

These data were confirmed by Western blot analysis (data not shown). Therefore, opsin phosphorylation, albeit at low levels of only 15%, does provide considerable protection against retinal degeneration.

We analyzed the retinal morphology in 2-month-old WT, *Rpe65*$^{-/-}$, *Grk1*$^{-/-}$ and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice raised in cyclic-light conditions (Fig. 2*B*). The outer segments of rod photoreceptors in WT mice were long and organized. The outer segments were of intermediate length in *Rpe65*$^{-/-}$ and *Grk1*$^{-/-}$ mice, and were short and the least organized in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice. The rows of photoreceptor nuclei in the outer nuclear layer (ONL) were also significantly reduced in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice compared with WT, *Rpe65*$^{-/-}$, and *Grk1*$^{-/-}$ mice ($p < 0.005$) (Fig. 2*C*). These data further confirm the severe degeneration of retinae lacking both GRK1 and RPE65.

To examine the age-dependency of the degeneration, we compared opsin levels in 1-, 2-, 4-, and 6-month-old mice (Fig. 2*D*). The *Rpe65*$^{-/-}$ mice retained a constant level of opsin over 6 months at ~50–60% of WT. The *Grk1*$^{-/-}$ retinae degenerated steadily over the 6 month period, with only 8.5 ± 1.5% ($n = 3$) opsin remaining at 6 months of age. In *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice, opsin was barely detectable at 4 months, indicating accelerated retinal degeneration. However, given that only 15% of opsin in the *Rpe65*$^{-/-}$ mouse is phosphorylated, the severe aggravation of degeneration in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice was surprising.

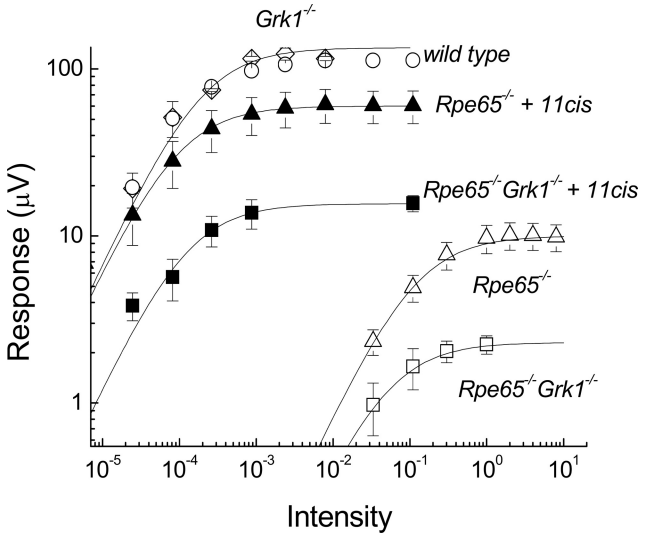

Figure 6. Amplitude of transretinal ERG a-wave as a function of flash intensity in WT (○; $n = 3$), *Grk1*$^{-/-}$ (□ $n = 3$), *Rpe65*$^{-/-}$ (Δ; $n = 7$), *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ (□; $n = 9$), *Rpe65*$^{-/-}$ with 11-*cis*-retinal (▲; $n = 9$), and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ with 11-*cis*-retinal (■; $n = 6$) retinae. The intensity-response data are fitted by Equation 1. Data from *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice are from animals raised in cyclic-light conditions. Data are presented as mean ± SEM ($n = 8$).

Lack of GRK1 causes retinal degeneration even in the absence of α-transducin

The slow retinal degeneration in RPE65-deficient mice (*Rpe65*$^{-/-}$) is caused by the constant activation of transducin by bare opsin, which contains 10–20% mono-phosphorylation, and degeneration can be suppressed by deleting the α-subunit of rod transducin (i.e., the *Rpe65*$^{-/-}$*Gnat1*$^{-/-}$ mouse) (Woodruff et al., 2003). To determine whether the rapid degeneration in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice is due to increased activation of the transducin pathway by fully unphosphorylated opsin, we generated and examined the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ *Gnat1*$^{-/-}$ mouse. The deletion of α-transducin alone had no adverse effects on retinal morphology and the opsin levels in 2-month-old *Gnat1*$^{-/-}$ mice were comparable to WT, as reported previously (Fig. 3*A*) (see also Calvert et al., 2000). Surprisingly, blocking the transducin pathway only partially rescued the degeneration in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice as the opsin level at 2 months of age improved only to 54.2 ± 4.9% ($n = 3$) of WT (vs ~10% in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$mice). Furthermore, at 4 and 6 months of age, this degeneration was accelerated (Fig. 3*B*). Examination of the ONL thickness and outer segment length in 6-month-old *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ retinae compared with age-matched *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ and WT retinae (Fig. 3*C*) confirmed the incomplete rescue of retinal degeneration by the deletion of α-transducin. This result is in contrast with the complete block of retinal degeneration by the deletion of α-transducin in RPE65-deficient mice (Woodruff et al., 2003) and indicates that in the absence of GRK1, degeneration occurs by a mechanism unrelated to transducin signaling. Indeed, a comparable, light-independent decrease in opsin levels in 2-month-old *Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice in cyclic light (75.7 ± 10.3%) and in constant darkness (71.6 ± 8.0%) was observed (Fig. 3*A*), supporting this notion.

ERG responses show severe loss of rod function in the absence of GRK1

To correlate the levels of the light-evoked rod response in these models, we recorded transcorneal scotopic ERGs from 2-month-old animals (Fig. 4). Previous studies from *Rpe65*$^{-/-}$ mice have shown that their cones have almost completely degenerated by that age (Rohrer et al., 2005; Znoiko et al., 2005) and their small ERG responses are triggered by rods (Seeliger et al., 2001). The *Rpe65*$^{-/-}$, *Grk1*$^{-/-}$*Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mice all had undetectable rod a-waves (Fig. 4) and reduced or undetectable b-waves compared with WT mice, in the order of progressive severity. This reduction in sensitivity is consistent with the large accumulation of opsin in the absence of RPE65, which should lower quantum catch and also produce constitutive activation of the transduction cascade by opsin. These results demonstrate that, consistent with our morphological studies above, rod function was more severely affected in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice compared with *Rpe65*$^{-/-}$. However, transcorneal ERG recordings did not allow us to distinguish between the effect of opsin-phosphorylation loss and that of retinal degeneration in reducing rod function.

We therefore initially attempted single-cell, suction-pipette recordings. Unfortunately, the combination of severe degeneration and slow response-termination in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ rods (Fig. 5, Table 1) made these recordings impossible. Instead, we used ERG recordings from isolated whole retina to assess rod function. We isolated the photoreceptor component (a-wave) of the ERG response by pharmacologically blocking synaptic transmission in the retina, thus eliminating all other components (see Materials and Methods for details). In principle, the a-wave response to a saturating flash contains a dominant rod- and a minute cone-component (Wang et al., 2009). However, the negligible cone component, together with the rapid cone degeneration in the absence of RPE65, allowed us to treat the a-wave as coming purely from rods.

We found no reduction in the saturated response amplitude and in the sensitivity (defined as the reciprocal of the half-saturating flash intensity) of 1-month-old, dark-reared *Grk1*$^{-/-}$ mouse rods compared with those from cyclic-light-reared WT mice (Fig. 5, compare *A*, *B*; see also Fig. 6, Table 1), suggesting limited degeneration at this age (Chen et al., 1999). The response–

Table 1. Parameters of transretinal ERG recordings

Genotype	R_{max} (μV)	Sensitivity (I_o)	T_{peak} (ms)	τ_{rec} (ms)
Wild type (DL, $n = 3$)	112 ± 2	(1.1 ± 0.1) × 10^{-4}	179 ± 2	414 ± 39
Grk1 $^{-/-}$ (DD, $n = 3$)	118 ± 9	(1.4 ± 0.4) × 10^{-4}	159 ± 11	17,871 ± 4910
Rpe65 $^{-/-}$ (DL, $n = 7$)	11 ± 2	(1.4 ± 0.3) × 10^{-1}	111 ± 5	111 ± 10
Rpe65 $^{-/-}$ *Grk1* $^{-/-}$ (DL, $n = 9$)	2.2 ± 0.2	(8.3 ± 2.5) × 10^{-2}	231 ± 41	17,610 ± 2216
Rpe65 $^{-/-}$ *Grk1* $^{-/-}$ (DD, $n = 8$)	2.7 ± 0.5	(1.8 ± 0.8) × 10^{-2}	241 ± 84	22,051 ± 8516
Rpe65 $^{-/-}$ (DL, 11 *cis*, $n = 9$)	61 ± 13	(1.6 ± 0.4) × 10^{-4}	190 ± 12	251 ± 17
Rpe65 $^{-/-}$ *Grk1* $^{-/-}$ (DL, 11 *cis*, $n = 6$)	15 ± 2	(3.9 ± 1.1) × 10^{-4}	192 ± 19	21,522 ± 5314
Rpe65 $^{-/-}$ *Grk1* $^{-/-}$ (DD, 11 *cis*, $n = 5$)	12 ± 1	(3.7 ± 0.6) × 10^{-4}	171 ± 7	27,941 ± 9207

Mice were reared in constant darkness (DD) or cyclic light (DL). "11*cis*" indicates application of 11-*cis* retinal before recordings. Sensitivity was determined as described in Materials and Methods. R_{max}, Maximal (saturating) response of a-wave; T_{peak}, time to peak; τ_{rec}, recovery time constant. Values are mean ± SEM.

recovery time constant in *Grk1* $^{-/-}$ mice increased 43-fold compared with WT mice (Table 1), as expected from the critical role of GRK1 in response termination (Chen et al., 1999).

The saturated response amplitude in *Rpe65* $^{-/-}$ mice showed a 10-fold decrease from WT (Fig. 5, compare *A*, *C*; see also Fig. 6, Table 1). In addition, their response kinetics were significantly accelerated (Table 1), and their sensitivity declined $>10^3$-fold (Fig. 6, Table 1). To determine the role of opsin in this decline of rod function, we incubated *Rpe65* $^{-/-}$ retina with exogenous 11-*cis*-retinal before recordings to convert free opsin into visual pigment. After this treatment, the *Rpe65* $^{-/-}$ a-wave increased by 5.5-fold in saturated amplitude and by 880-fold in sensitivity, and showed significant slowing of its kinetics (Fig. 5, compare *C*, *E*; see also Fig. 6, Table 1). These changes represent the effect of bare opsin on rod function in *Rpe65* $^{-/-}$ mice (Dizhoor et al., 2008). Notably, both saturated amplitude and sensitivity of the *Rpe65* $^{-/-}$ a-wave did not recover to WT levels after treatment with 11-*cis*-retinal (Fig. 6; Table 1), consistent with residual effects due to the shortening of rod outer segments (Fig. 2*C*) and the reduced opsin levels (Fig. 2*A*) in the *Rpe65* $^{-/-}$ retina.

Consistent with their more severe retinal degeneration (Fig. 2), the a-wave of the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice showed a further fivefold decrease in saturated amplitude from that of *Rpe65* $^{-/-}$ (Fig. 5, compare *C*, *D*; see also Fig. 6, Table 1). Surprisingly, *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ rods were 1.7-fold more sensitive than *Rpe65* $^{-/-}$ rods despite their more severe degeneration (Table 1) and had more than twofold lower opsin content (Fig. 2*A*). The response kinetics of *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ rods were comparable to those of *Grk1* $^{-/-}$ rods (Table 1), indicating that the lack of phosphorylation of the residual visual pigment still dominated response termination. To determine the role of opsin in the decline of *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ rod function, we again used 11-*cis*-retinal. Following regeneration of the rod visual pigment with 11-*cis*-retinal, the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ a-wave increased by 7-fold in saturated amplitude and by 220-fold in sensitivity (Table 1). This increase in sensitivity is still four times less than the 880-fold increase in sensitivity of *Rpe65* $^{-/-}$ rods. This result indicates that the reduction in total free opsin due to degeneration rather than the absence of opsin phosphorylation in *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice is the determining factor for rod sensitivity. Once the opsin activation was removed by 11-*cis*-retinal, the sensitivity of *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ rods was 2.4-fold lower than that of *Rpe65* $^{-/-}$ rods (Table 1), also consistent with the lower level of opsin in the former. Likewise, the saturating response amplitude of *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ rods was four times lower than that of *Rpe65* $^{-/-}$ rods and approximately eight times smaller than the saturating response in WT rods (Fig. 6; Table 1), consistent with the severe degeneration of the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ retina.

We conclude that, as a surprising consequence of degeneration, the reduced level of opsin (hence a reduced opsin activity that more than overcomes the consequence of the lack of opsin phosphorylation) in *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ retina compared with *Rpe65* $^{-/-}$ retina results in a slight increase in sensitivity. Finally, raising *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ animals in darkness also produced an approximately fivefold increase in sensitivity (Table 1), presumably by allowing 9-*cis*-retinal visual pigment to gradually accumulate in the outer segments (Fan et al., 2003). Following treatment with 11-*cis*-retinal, there was no significant difference in the function of rods from *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ animals raised in darkness or in cyclic light (Table 1), indicating that light exposure did not affect the extent of degeneration.

Discussion

The relatively slow rod photoreceptor degeneration in *Rpe65* $^{-/-}$ mice has been something of a puzzle. A similar degeneration is noted in the *Lrat* $^{-/-}$ mouse, also lacking 11-*cis*-retinal. As both models show a low but constant level of opsin phosphorylation, we designed these experiments to determine whether GRK1 is the kinase involved and whether its removal would accelerate the rod photoreceptor degeneration. Our data show that GRK1 is indeed the kinase underlying opsin phosphorylation in *Rpe65* $^{-/-}$ mice and this phosphorylation apparently provides some protection against degeneration. The surprising result is the large extent of degeneration in the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mouse, particularly with time.

It is well established that GRK1 phosphorylates serines and threonines in the C terminus of activated rhodopsin and this phosphorylation initiates the rhodopsin inactivation of the visual cascade (Maeda et al., 2003). Our current study shows, however, that GRK1 is capable of phosphorylating rod opsin in the *Rpe65* $^{-/-}$ mouse even in the absence of light. Presumably, the constitutively active opsin in the absence of chromophore (Fan et al., 2005) is in a form that is weakly recognized and acted upon by the kinase.

Photoreceptor degeneration is greatly accelerated in the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mouse when compared with the *Rpe65* $^{-/-}$ mouse, as measured by morphology, opsin levels, and photoreceptor function. Our original hypothesis was that the phosphorylation, even at only a 10–20% level, would provide some protection against degeneration in the *Rpe65* $^{-/-}$ mice, presumably by inactivating opsin and thus preventing the continuous activation of transducin. The lack of phosphorylation should increase the overall activity of opsin, but the high level of degeneration, particularly as the animal aged, was unexpected.

Several lines of evidence indicate that the more severe degeneration in the *Rpe65* $^{-/-}$*Grk1* $^{-/-}$ mice compared with *Rpe65* $^{-/-}$ mice cannot be explained solely by the increase in opsin activity in the absence of phosphorylation. As only ~15% of opsin is phosphorylated in the *Rpe65* $^{-/-}$ rods, deletion of GRK1 would result in a corresponding 15% increase of unphosphorylated op-

sin. Thus, even if phosphorylated opsin is completely inactive, the opsin activity would be expected to increase at most by a modest 15% upon deletion of GRK1. In addition, our biochemical studies demonstrated that the opsin content in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice is only one third that of *Rpe65*$^{-/-}$ mice. Thus, the opsin activity in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice would be expected to be lower based on their substantially reduced opsin content compared with *Rpe65*$^{-/-}$ mice. We conclude that constitutive opsin activity in the absence of chromophore is not the sole mechanism for photoreceptor degeneration in the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice. Instead, we hypothesized that the more severe degeneration in the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice, particularly in the older animals, is due to the lack of GRK1. Our results indicate that the consequences of this defect increase with the age of the animal, which may explain why *Grk1*$^{-/-}$-associated degeneration was not observed in a previous study of dark-reared 2-month-old *Grk1*$^{-/-}$ mice (Chen et al., 1999).

To test whether the photoreceptor degeneration in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice was due to activation of the visual transduction pathway using α-transducin, we investigated the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ mouse. Degeneration progressed in the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ retina over time even though opsin signaling through rod transducin was silenced. In contrast, the *Rpe65*$^{-/-}$ retina does not degenerate on a *Gnat1*$^{-/-}$ background (Woodruff et al., 2003). The block of signal transduction in the absence of α-transducin allowed us to rule out opsin producing activation of the transduction cascade as the source of degeneration in the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$*Gnat1*$^{-/-}$ retina. Therefore, we believe that the degeneration in the triple knockout is due to the deletion of GRK1. This result was confirmed by the light-independent degeneration in *Grk1*$^{-/-}$ retina when GNAT1 was deleted.

If phosphorylation, and therefore the binding of arrestin, is eliminated, the opsin could conceivably interact with other G-proteins, triggering other signaling processes that exacerbate retinal degeneration. Multiple interactions of a single G-protein-coupled receptor (GPCR) with a variety of G-proteins have been identified in other GPCR systems (Bouvier, 1990). The mechanism of GPCR activation of G-proteins is highly conserved. Indeed, certain chimeras of rhodopsin and the β_2-adrenergic receptor have been shown to activate G_s (Yamashita et al., 2001; Kim et al., 2005). A second possibility is that GRK1 has a role not yet discerned that could involve the phosphorylation of a protein other than opsin, thus affecting another signaling pathway. Indeed, Carman et al. (1999) have pointed out that GRK may well have other interactions in addition to phosphorylation in G-protein signaling cascades. Deletion of GRK1 may change the expression of other critical proteins. This area certainly merits further investigation. Finally, although Hao et al. (2002) have proposed an apoptotic pathway in light-induced retinal degeneration that does not involve transducin, the pathway observed in our studies is light independent.

Surprisingly, despite their more severe degeneration, *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ rods were more sensitive than *Rpe65*$^{-/-}$ rods. Our biochemical results demonstrated that the *Rpe65*$^{-/-}$ rods have threefold higher opsin content than *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ rods. This difference in opsin levels will affect sensitivity in two ways. On the one hand, their higher opsin level will result in higher quantum catch, increasing the sensitivity of *Rpe65*$^{-/-}$ rods compared with *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ rods. On the other hand, however, their higher opsin level will also result in higher opsin activity, decreasing the sensitivity of *Rpe65*$^{-/-}$ rods compared with *Rpe65*$^{-/-}$ *Grk1*$^{-/-}$ rods. The lower sensitivity of *Rpe65*$^{-/-}$ rods indicates that the desensitization due to opsin activity outweighs the increase in quantum catch. This result is consistent with the nonlinear relation between loss of quantum catch and reduction in sensitivity following a bleach (Jones et al., 1996; Luo and Yau, 2005). Thus, as a surprising consequence of the more severe degeneration in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice compared with *Rpe65*$^{-/-}$ mice, the total opsin activity was actually lower in the double knock-out. Treatment with exogenous chromophore will result in increased sensitivity first, by increasing quantum catch and second, by removing opsin activation. We found that following treatment with exogenous chromophore the sensitivity of the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ rods was 2.5-fold lower than that of the *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ rods, consistent with the relative levels of opsin in the two lines. The similarity between decline in opsin content and reduction in sensitivity is consistent with only a minor role for the activity of the additional 15% unphosphorylated opsin in the double knockout.

In summary, several conclusions can be drawn from this study. First, the phosphorylation of opsin in *Rpe65*$^{-/-}$ mice is due to the action of GRK1. Second, this phosphorylation is protective against the retinal degeneration caused by opsin activity. Third, deletion of GRK1 contributes to the increasing retinal degeneration with age in *Rpe65*$^{-/-}$*Grk1*$^{-/-}$ mice. Finally, the retinal degeneration driven by the lack of GRK1 is through a non-transducin-based signaling pathway.

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