Cone versus Rod Disease in a Mutant Rpgr Mouse Caused by Different Genetic Backgrounds

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PURPOSE. To establish mouse models for RPGR-associated diseases by generating and characterizing an Rpgr mutation (in-frame deletion of exon 4) in two different genetic backgrounds (BL/6 and BALB/c).

METHODS. Gene targeting in embryonic stem (ES) cells was performed to introduce a in-frame deletion of exon 4 in the Rpgr gene (Rpgrex4Δ). Subsequently, the mutation was introduced in two different inbred mouse strains by successive breeding. Mutant and wild-type mice of both strains were characterized by electroretinography (ERG) and histology at five time points (1, 3, 6, 9, and 12 months). RPGR transcript amounts were assessed by quantitative RT-PCR. A variety of photoreceptor proteins, including RPGR-ORF15, RPGRIP, PDE6b/PrBP5, rhodopsin, and cone opsin, were localized on retinal sections by immunohistochemistry.

RESULTS. Mislocalization of rhodopsin and cone opsin was an early pathologic event in mutant mice of both lines. In contrast, RPGR-ORF15 as well as RPGRIP1 and PDE6b/PrBP5 showed similar localizations in mutant and wild-type animals. Functional and histologic studies revealed a mild rod-dominated phenotype in mutant male mice on the BL/6 background, whereas a cone-dominated phenotype was observed for the same mutation in the BALB/c background.

CONCLUSIONS. Both Rpgr mutant mouse lines developed retinal disease with a striking effect of the genetic background. Cone-specific modifiers might influence the retinal phenotype in the BALB/c strain. The two lines provide models to study RPGR function in cones and rods, respectively. (Invest Ophthalmol Vis Sci. 2010;51:1106–1115) DOI:10.1167/iovs.08-2742

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Degeneration of initially rod and later of cone photoreceptor cells is a common hallmark of the clinical and genetic heterogeneous disease retinitis pigmentosa (RP). The X-linked form of RP (XIRP) is the most severe form in terms of onset and progression; males are predominantly affected though occasionally carrier females may show variable expressivity of disease manifestations.1,2 Mutations in the retinitis pigmentosa GTPase regulator gene (RPGR) are the major cause and account for approximately 70% of all XIRP cases.3 The second major X-linked gene was also identified and was designated RP2.4 Expression of RPGR transcript isoforms were detected in many tissues, including retina, brain, lung, kidney, and testis, of several species.5 In humans as well as in mice, RPGR was described to consist of 19 constitutively expressed exons (RPGR1–19 variant).6,7 Exon 19 contains a conserved isoprenylation motif, indicating that this isoform might be posttranslationally modified.8 Several studies reported a high degree of alternative splicing, and many novel exons—some of them tissue specific—were discovered. The retina-abundant RPGR-ORF15 isoform has an alternative C termi

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influence of such modifying factors is limited. In addition, no animal model of retinal degeneration exists that displays a similar shift in the phenotype between rod and cone photoreceptors.

We generated a transgenic mouse model with an in-frame deletion of exon 4 of Rpgr. This alteration mimics mutations in patients with RP that lead to exon 4 skipping. In addition, we bred the mutation in two different mouse strains (BL/6 and BALB/c). This study describes the detailed characterization of the retinal phenotypes in the two genetic backgrounds. We found both mouse strains developing retinal disease but strikingly either rod or cone dominated. To the best of our knowledge, this is the first example of different photoreceptor-specific (i.e., rods vs. cones) phenotypes in two different mouse strains carrying the same Rpgr mutation.

**Materials and Methods**

**Animals**

All experiments were performed in compliance with the National Institutes of Health guidelines, as approved by the Swiss cantonal veterinary office, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were born and maintained in controlled ambient illumination on a 12-hour light/12-hour dark cycle with an illumination of 60 lux. For breeding, male wild-type BL/6 animals were purchased from the Jackson Laboratory (Bar Harbor, ME). Male wild-type BALB/c animals were obtained from the Bundesinstitut für Risikobewertung (Berlin, Germany).

**Generation of Rpgr<sup>Δex4</sup> Mice**

For construction of the Rpgr<sup>Δex4</sup> targeting vector, a genomic region of 0.8 kb (intron 2 F 5' GCTCTAGACAGGTGCTGTGTTTACGTTTGG 3' to intron 3 R 5' AGCCGTCGACTCACTCTTGTAGCATGCCTTGA 3') was genotyped by sequencing using flanking primers (Rpe65_F 5' AGCACGTACTCGTATTACCAT- 3' to intron 3 R 5' CCGCTCGACTATGTCTCACACTGTCGAG 3'). The murine Rpgr gene was amplified from the 129 mouse strain and cloned into the 5' multiple cloning site of the neomycin cassette of the pTG1-vector (Transgenics, Berlin, Germany). Subsequently, a second recombinant construct was generated using a 12-hour light/12-hour dark cycle with an illumination of 60 lux. For breeding, male wild-type BL/6 animals were purchased from the Jackson Laboratory (Bar Harbor, ME). Male wild-type BALB/c animals were obtained from the Bundesinstitut für Risikobewertung (Berlin, Germany).

**Genotyping of Rpgr and Rpe65**

Genotyping was performed by PCR on DNA isolated from tail biopsies. For Rpgr, two different forward primers were used to amplify a wild-type specific (exon 4, 5'TGAAACCTTGCTGGAGGA 3') and a mutant specific product (neomycin cassette, 5' AGCACGTACTCGTATTACCAT- 3') respectively. A third common reverse primer is located in intron 4 (5' agcacgtacctcgtttaa 3'). The polymorphism in Rpe65 was genotyped by sequencing using flanking primers (Rpe65_F 5'TGACAGGAATAAGAGAC 3' and Rpe65_R 5' ATTACCATCATCTTCTCCA 3').

**Morphometric Analysis on Plastic Sections**

Mice were anesthetized with CO<sub>2</sub> and subsequently killed by cervical dislocation. Eyes were marked at the superior pole, removed, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C overnight. Eyes were bisected on the optic nerve head into a superior and an inferior part, washed in cacodylate buffer, poststained in osmium tetroxide for 1 hour, dehydrated, and embedded in resin (Epon 812; Sigma-Aldrich, Buchs, Switzerland). Tissue sections of 0.5 μm were stained with toluidine blue and analyzed under a microscope (Carl Zeiss AG, Feldbach, Switzerland).

For morphometric analysis of wild-type and mutant mice of both strains, sections from the inferior central retina containing the optic nerve head and the ora serrata on both sides were chosen for retinal measurements. For each eye, three sections were examined. Four areas were chosen from each section (periphery temporal, center temporal, center nasal, and periphery nasal). The central areas were defined as located 15° temporally and nasally from the optic nerve head (0°), and the temporal region was chosen 30° from the ora serrata of each side. Measurements of the thickness of the outer nuclear layer (ONL) and the length of the rod inner segment (RIS) and rod outer segment (ROS) were performed, each in triplicate. Additionally, the number of nuclei in the ONL was counted in an area of 1000 μm<sup>2</sup> (20 × 50 μm). The mean value of ROS, RIS, and ONL thickness and mean number of nuclei in the ONL for each area was calculated (2 ± n = 5 per genotype). For quantification of cones, cone nuclei were counted in the whole area of one picture taken at 40× magnification. Statistical analysis was performed using the 95% confidence interval (CI) or Student’s t-test.

**Electroretinography**

ERG methodology has been described previously. Mice were dark adapted (>2 hours), pupils were dilated by tropicamide 0.5% and atropine 1%, and mice were anesthetized by injection of xylazine (15 mg/kg body weight) and ketamine (100 mg/kg). The recording electrode was a monopolar contact lens electrode, and a subcutaneous fixed silver needle was the reference and ground electrode. For recording, the mouse was placed into a commercially available Ganzfeld bowl (Toennies Multiliner Vision, Höchberg, Germany), with the examined eye facing the back of the globe. The signal was amplified by 10,000 with a bandpass filter from 1 to 300 Hz. Oscillatory potentials were obtained by bandpass filtering from 100 to 300 Hz.

In the dark-adapted state, a flash series consisting of 8 steps started at −4.0 log cd·s/m<sup>2</sup> and reached 0.48 log cd·s/m<sup>2</sup>. For a-wave recording, six additional flash energies were applied ranging from 1.0 to 2.5 log cd·s/m<sup>2</sup>. These high-intensity stimuli were provided by a photoflash mounted in the Ganzfeld bowl. After recording the a-wave, the background light (1.5 log cd·m<sup>−2</sup>) was turned on, and the photopic ERG was recorded (1.2, 1.4 log cd·s/m<sup>2</sup>; average of 10 recordings at 15 Hz). Subsequently, the animals were further light adapted for 10 minutes, and the photopic ERG was recorded again using a series of flash energies (−0.3, 0.7, 1.2, 1.4, 1.7, and 2.0 log cd·s/m<sup>2</sup>). The b-wave amplitude was determined from a-wave trough to b-wave peak, behind the last prominent oscillatory potential. For the determination of b-wave amplitudes and implicit times, the third oscillatory potential was chosen because of low variability. Statistical analysis was performed using ANOVA for repeated measurements, if applicable. Single recordings were tested by the two-tailed t-test. The critical P value was set to 0.05.

**Generation Isoform-Specific RPGR Antibody**

An epitope within the hRPGR-ORF15 protein (1<sup>110</sup>HTYQKKSVT-NTQQNGKKE<sup>117</sup>) (National Center for Biotechnology Information accession nos. NP_0003519 and NP_001050025) was chosen to generate the isoform-specific antibody AbC2.1 (Eurogentec, Seraing, Belgium). The antisera was affinity-purified against the cognate peptide accord-
Quantitative Real-Time PCR of Rpgr Transcript

To study the expression of Rpgr in wild-type and mutant animals of the Rpgr<sup>ATXS</sup> strains, a quantitative real-time PCR assay was performed. Retinal RNA was extracted from BL/6 (n = 5 for each wild-type and mutant) and BALB/c (n = 2 wild-type; n = 3 mutant) animals, and cDNA was generated and analyzed from each animal. A gene expression assay (TaqMan; ABI, Rotkreuz, Switzerland) was ordered in which mouse RPGR sequences specific for exons 8 and 9. Expression levels were normalized to 18S ribosomal RNA as endogenous control. Experiments were determined and pooled from five technical replicates, with amplification curves starting between 25 and 28 cycles (ABI HT 7900; TaqMan). Data were analyzed using the SDS 2.2 software (ABI). Expression levels were not significantly different between wild-type and mutant animals in both strains. Error bars represent 95% CI.

Immunohistochemistry

Animals were anesthetized by inhalation of CO<sub>2</sub> or by injection of ketamine-xylazine and subsequently were killed by cervical dislocation. Eyes were removed and either directly embedded in optimal cutting temperature compound (Tissue Tek; Digitaitec, Horgen, Switzerland) or fixed in 4% paraformaldehyde in PBS and subsequently cryoprotected by treatment with sucrose in ascending concentrations (10% wt/vol, 20% wt/vol, and 30% wt/vol in PBS) at 4°C. Sections of 8 µm were cut on a cryostat and air-dried (CM3050s; Leica Microsystems, Glattbrugg, Switzerland). After blocking with 10% normal goat serum (NGS) sections were incubated overnight with primary antibodies. Primary antibodies used were anti-PrBP (1:4000, MWL cone opsin, kindly provided by Christian Grimm, University Hospital Zurich, Switzerland) were applied to fixed eyes of wild-type and mutant animals of the Rpgr<sup>ATXS</sup> BL/6 (n = 4/genotype) and BALB/c (n = 2/genotype) strain. An anti-PrBP antibody (kindly provided by Rick Cote, University of New Hampshire, Durham, NH) was used on fixed sections (n = 2/genotype) in a 1:200 dilution. For detection of proteins in the connecting cilium, unfixed eyes were used as other conditions were found to quench the signal. RPGR AbC2 was used in a 1:25 and RPRGIP Ab58<sup>12</sup> (kindly provided by Ronald Roepman, Nijmegen, Netherlands) in a 1:200 dilution (n = 2/genotype). As markers for the connecting cilium and the centriole/basal body, acetylated α-tubulin (Sigma) and γ-tubulin (Abcam, Cambridge, UK) were used (n = 2/genotype), respectively. Slides were photographed with a Zeiss microscope equipped with ApoTome technology. For each antibody comparable acquisition times for wild-type and mutant retinas were used. Images were processed with graphics editing software (Photoshop; Adobe, Munich, Germany) by applying the same parameters for each one.

RESULTS

Generation of Rpgr Mutant Mice in Two Different Genetic Backgrounds

We used gene-targeting technology to generate a mouse line with a deletion mutation in the Rpgr gene. In the gene-targeting construct, exon 4 was replaced by a neomycin cassette, leading to an in-frame deletion of 63 bp of the Rpgr transcript and 21 amino acids of the RPGR protein (Fig. 1A). The mutant mouse line was obtained after injection of two independently targeted ES clones (C2 and F10). The C2 and F10 ES clones were selected after testing for correctly targeted Rpgr gene copies (Fig. 1B). From both ES cell lines we obtained chimeric animals. Germline transmission was confirmed for both and resulted in viable and fertile hemizygous male mice.

**Figure 1.** Generation of Rpgr mutant mouse lines. (A) Scheme of wild-type allele and targeting construct of the Rpgr gene and the four known interaction partners of the RCC1-like domain (RLD). Exon 4, which is located within the RLD, was replaced by a neomycin cassette (Neo) in the construct. This leads to a lack of exon 4 in the transcript of the targeted allele (Rpgr<sup>ATXS</sup>). Direction of transcription of neomycin is the same as for Rpgr. (B) Digestion with BamHI and subsequent Southern blot analysis with a probe depicted in (A) resulted in a fragment of 7.8 kb for wild-type (+) and a truncated fragment (6.5 kb) for mutant (−) Rpgr. This ES clone (C2) and a second one (F10) were chosen for further injection into blastocysts. (C) RT-PCR on mouse retinal cDNA using forward and reverse primers in exons 2 and 5 (as indicated in A). In the mutant animals only transcripts skipping exon 4, and thus showing 63-bp deletions, were found. In wild-type animals only correctly the spliced products was detected. (D) Quantitative RT-PCR of Rpgr<sup>ATXS</sup> BL/6 and BALB/c wild-type and mutant mouse retinal cDNA. Expression levels of Rpgr (indicated in relative units) were not significantly different between wild-type and mutant animals in both strains. Error bars represent 95% CI.
The mutation was first bred into the pigmented BL/6 strain (C2 and F10 line) for at least eight generations. Several studies reported on modifier genes influencing retinal degeneration and light damage susceptibility in BALB/c mice. Thus, we decided to breed the \textit{Rpgr}^{H9004\text{Ex4}} mutation into the albino BALB/c strain (C2 line) for another seven to eight generations.

Expression of Wild-type and Mutant \textit{Rpgr}

Deletion of exon 4 in the mutant mice was verified by conventional RT-PCR using forward and reverse primers in exons 2 and 5, respectively. In wild-type mice of both backgrounds, the expected band of 430 bp was found (Fig. 1C). Mutant animals specifically showed a lower band corresponding to the deletion of 63 bp of exon 4. The bands were verified by sequencing. The deletion of exon 4 does not alter the reading frame but would lead to a lack of 21 amino acids in the predicted RLD (Fig. 1A). Expression levels of wild-type and mutant \textit{Rpgr} transcripts were analyzed by quantitative RT-PCR on mouse retinal cDNA (Fig. 1D). The probe (TaqMan; ABI) detected the exon 8/9 boundary downstream of the genomic neomycin cassette insertion site. Comparable expression levels of wild-type and mutant transcripts were found in both strains, indicating that the deletion of exon 4 does not influence \textit{RPGR} transcript levels. These results suggest that \textit{Rpgr} is equally expressed in wild-type and mutant animals.

Assessment of Retinal Function by Electroretinography

For assessment of functional consequences of the \textit{Rpgr}^{H9004\text{Ex4}} mutation in the two mouse lines, mutant male mice and their littermates were subjected to ERG measurements. The \textit{Rpgr}^{H9004\text{Ex4}} BL/6 line displayed a slightly progressive loss of amplitude of the scotopic a-wave from the age of 3 months onward (Fig. 2). The scotopic b-wave remained unaffected at all ages examined, whereas the photopic b-wave was marginally reduced but only at 9 months of age. Surprisingly, functional measurements revealed a strikingly different result in the \textit{Rpgr}^{H9004\text{Ex4}} BALB/c background (Fig. 3). Here, the cone system was affected already at 1 month of age, as indicated by the reduction of the photopic b-wave in mutant animals (Fig. 3C). Simultaneously, the amplitudes of the scotopic a-wave were reduced. At 3 months, the \textit{Rpgr}^{H9004\text{Ex4}} BALB/c line showed a cone-rod phenotype compared with the isolated mild rod phenotype in \textit{Rpgr}^{H9004\text{Ex4}} BL/6 mice at a comparable age (Figs. 2B, 3B). Over time, the decrease in the rod photoreceptor response in \textit{Rpgr}^{H9004\text{Ex4}} BALB/c mice was progressive, whereas the scotopic b-wave was not affected at any time point examined. Given that the reduction of the a-wave was mild in both strains, this effect might not have been observed in the b-wave responses because this effect could have been masked by sum-
mation effects during transmission. It is important to emphasize the stable photopic b-wave in wild-type animals until 12 months of age (Fig. 3C) though the wild-type scotopic responses significantly declined during aging. This age-related retinal degeneration is well known to occur in BALB/c and, to a lesser extent, in BL/6 mice.34

Retinal Morphology
To determine morphologic changes accompanying the retinal dysfunction indicated by ERG examinations, the same five time points of ERG measurements were chosen for histologic analyses of the retina (1, 3, 6, 9, and 12 months). In both lines, retinal morphology was properly organized in mice at the age of 1 month, indicating normal retinal development (Figs. 4A, B). In both lines (Rpgr<sup>ΔEx4</sup> BL/6 and Rpgr<sup>ΔEx4</sup> BALB/c), no obvious differences in retinal architecture were identified between wild-type and mutant animals. The age-related retinal degeneration naturally occurring in BALB/c mice was again evident from 6 months onward.

For the detection of subtle changes within the retina that might reflect the reduction in the rod and cone responses in the ERG, a detailed morphometric analysis was performed. In Rpgr<sup>ΔEx4</sup> BL/6 animals, a tendency toward reduction of the thickness of the outer nuclear layer (ONL), outer segments (OS), and inner segments (IS) was found in mice aged 6 months or older (Supplementary Fig. S1; all Supplementary Figures are available at http://www.iovs.org/cgi/content/full/51/2/1106/DC1). Overall, these subtle changes in retinal morphology fit well with the observed mild decrease in the photoreceptor response on ERG. In the Rpgr<sup>ΔEx4</sup> BALB/c line, no differences between wild-type and mutant mice were apparent up to 3 months (Supplementary Fig. S2). We cannot exclude mutation-specific effects at later stages, which might be masked by the age-related retinal degeneration.

The defect in the cone system in Rpgr<sup>ΔEx4</sup> BALB/c identified by ERG prompted us to test whether this defect is caused by the loss of cone photoreceptor cells. Analysis of the histologic sections of the BALB/c strain (Fig. 4B) suggested that in older animals (9 and 12 months) the number of cones might be reduced (data not shown). Cone numbers of wild-type animals of 1 month and 12 months were similar, indicating that cones are not prone to age-related degeneration as are rods (Fig. 4B; Supplementary Fig. S2). These observations are consistent with the cone dysfunction identified in ERG measurements and might further emphasize the effect of the Rpgr<sup>ΔEx4</sup> mutation on cones in BALB/c mice.

Localization of RPGR-ORF15, Interacting Proteins, and Ciliary Markers
We next investigated the localization of wild-type and mutant protein in the retina. A set of antibodies against RPGR, its interaction partners, and ciliary and other photoreceptor-spe-
specific proteins was applied on retinal sections. The RPGR antibody was directed against the ORF15 isoform (AbC2.1) and produced strong and specific signals. Specificity was shown by blocking with the cognate peptide (Supplementary Fig. S3). An intense punctate staining pattern was observed in the area of the connecting cilium with AbC2.1 (Figs. 5A–D). This localization of RPGR-ORF15 has been described in mouse using a different set of antibodies.35 Comparison of the staining between wild-type and mutant revealed the same pattern. Thus, the mutation had no influence on localization of the protein. The staining pattern observed with this antibody closely resembled β-tubulin, a marker for the basal body, indicating that the ORF15 isoform specifically localizes there (Figs. 5I–J). Additionally, we investigated the localization of RPGRIP1. As expected, RPGRIP1 was found along the ciliary axoneme in wild-type and mutant retinas, similar to acetylated β-tubulin (Figs. 5E–H, M–P). An antibody against PDE6β/PrBP, which was the first known interaction partner of RPGR, stained the inner segments of rods and cones in both mouse lines, as described by others36 (Supplementary Fig. S4). The integrity of the ciliary structures and components was not disturbed in mutant photoreceptors, as suggested by staining with the ciliary markers γ-tubulin and acetylated α-tubulin. Thus, mislocalization of RPGR or gross disarrangement of connecting cilia is not the cause of retinal dysfunction and degeneration in these two mouse lines.

Mislocalization of Visual Pigments in Rpgr Mutant Mice

Several mouse models with defects in known or putative proteins of ciliary transport pathways show aberrant localization of components of the phototransduction cascade, such as rhodopsin, cone opsin, arrestin, and transducin.57–40 For RPGR, several lines of evidence exist that it also might be involved in ciliary transport. In addition, in a knockout mouse model for Rpgr and in a canine XIPRA2 model, alterations in the localization of visual pigments were described.31,42 We stained retinas with antibodies against rhodopsin and cone opsin and analyzed mice at 1 month of age to elucidate primary defects before or simultaneous with the occurrence of functional impairments. These experiments revealed mislocalization of cone opsin in both mutant mouse strains (Figs. 6B, D). In addition to the localization in cone outer segments (COS), it was found in the outer nuclear layer and the cone synapses (Figs. 6B, D, arrow). Similarly, rhodopsin was partially mislocalized in the perinuclear region of rod photoreceptor cells from mutant male mice of both lines (RpgrΔEx4 BL/6 and RpgrΔEx4 BALB/c; Figs. 6F, H). To test whether other proteins might be affected by a localization defect, we additionally stained dark-adapted retinas for arrestin and transducin. These proteins were shown to localize in the IS and OS in the dark, respectively.43 In contrast to cone opsin and rhodopsin, these proteins showed no aberrant localization in mutant mice (Supplementary Fig. S5).

Evaluation of Rpe65 as Modifier Gene

Based on the different phenotypes occurring in BL/6 and BALB/c mice, both carrying the same RpgrΔEx4 mutation, we assumed that a strain-specific modifier must exist. To test whether the RPE65 polymorphism at position 450 of the amino acid sequence has an effect on the phenotype in the BALB/c line, we designed a breeding scheme to obtain BALB/c mice carrying the BL/6 (methionine) variant at position 450. Wild-type and mutant male mice at the age of 5 to 6 weeks underwent phenotype analysis by ERG. We still observed a cone-rod phenotype in mutant animals carrying the BL/6 variant (methionine) of RPE65 in an otherwise highly homogenous BALB/c background (<0.5% BL/6 after 8 generations; data not shown). Therefore, RPE65 alone is not responsible for this modifying effect.
DISCUSSION

In this study we showed that two mouse strains carrying the same Rpgr\textsuperscript{A32x4} mutation display strain-independent but also strain-specific characteristics of disease manifestations. Our findings indicate an important role of the genetic background on the degeneration of rod and cone photoreceptor cells as well as RPGR function. Moreover, the BALB/c line provides the first model for cone-rod degeneration because of an Rpgr mutation in mice.

Influence of the Genetic Background on the Disease Phenotype

The most striking finding of our study was the manifestation of different phenotypes in the two mouse lines carrying the same...
Rpgr mutation. We observed that Rpgr<sup>Ex4</sup> BL/6 mice presented with a mild rod phenotype, whereas the Rpgr<sup>Ex4</sup> BALB/c strain developed an early-onset cone-rod phenotype. This result indicates that genetic modifiers act on the physiologic homeostasis of rod and especially cone photoreceptors in addition to the Rpgr<sup>Ex4</sup> mutation. Variability in clinical manifestations was found in carrier females with the same mutation in exon 8 and in dizygotic twins with an RPGR-ORF15 mutation.47,48 One sibling had RP, and the other had a cone-rod dystrophy. This situation is reminiscent of the phenotypic variability of our Rpgr<sup>Ex4</sup> mutant mice. To date, the effect of the genetic background has only been described to alter onset, severity, and progression of retinal degeneration.43 To the best of our knowledge, we describe for the first time an impact of the genetic background on the primarily affected photoreceptor cell type (i.e., rods vs. cones) because it was also observed in human patients. One previously identified modifier gene for retinal degeneration in mice is Rpe65. The variant with a leucine at position 450 of this protein renders BALB/c mice more susceptible to light-induced retinal degeneration by altering rhodopsin regeneration kinetics compared with mice carrying methionine at this position (e.g., BL/6).49 Introducing the methionine at position 450 into the BALB/c background allowed us to elucidate the influence of this polymorphism on the phenotype of Rpgr<sup>Ex4</sup> mice. Based on this experiment, we found that the BL/6 variant of Rpe65 alone has no significant effect on the cone phenotype of Rpgr mutant mice of the BALB/c line.

In humans, it is known that cone-specific RPGR isoforms exist.50 Similarly, strain-specific RPGR isoforms predominately expressed in cone photoreceptors might contribute to the different phenotypes. To date, no such strain- or cone-specific RPGR isoforms have been described in mice. Similarly, a strain-specific single nucleotide polymorphism or mutation in a cone-specific gene provides another explanation for the differences between BL/6 and BALB/c mice. Another potentially protective factor in BL/6 retinas might be the pigment. However, because we did not observe a difference in the two lines regarding the impairment of the rod system but only for cones, we consider this explanation rather unlikely. Additional genetic analyses, such as QTL mapping, are necessary to map and identify the respective modifiers. Identification of such genes might help to better understand the distinct molecular characteristics of rod and cone photoreceptors.

**Defect of Ciliary Transport but Not Ciliary Structure**

We showed that RPGR and RPGRIP1 are correctly localized in mutants and that the ciliary structure is not obviously compromised. Thus, retinal disease might rather be explained by an altered protein function caused by different disease-associated mutations. This is supported by the observed mislocalization of rhodopsin and cone opsin early in disease. Mislocalization preceded the decline in the scotopic a- and b-waves at approximately 1.5 months of age (P40). Cone responses also declined; however, this effect was not quantified. Compared with the canine and murine models, the Rpgr<sup>Ex4</sup> BALB/c line represents a model for cone-rod degeneration. Our mouse lines expressing a transcript that leads to a deletion of 21 amino acids in the predicted RLD can be regarded as a model for disease-associated human mutations (in-frame deletions and insertions as well as missense mutations), leading to altered RPGR proteins in patients, which account for approximately 48% of all mutations described (http://rpgr.hgu.mrc.ac.uk).

**Disease Course of Retinal Degeneration from RPGR Mutations**

In humans, mutations in RPGR lead to XLRP, a severe early-onset rod degeneration, in some cases followed by cone degeneration. To date, three different splice site mutations in intron 4 were reported in patients with XLRP.29-47,48 All them occurred in the splice donor site (positions +1 and +3) and are supposed to lead to omission of exon 4 in the respective transcripts. Indeed, exon skipping was demonstrated for the mutation at position +3.49 Exon 4 is part of the coding region of the RCC-1-like domain (RLD).5 The amino acid residues are 100% identical in human and mouse, and a significant portion of mutations are located in the RLD. In addition, it was shown to bind several interaction partners such as RPGRIP1,12 SMC1 and SMC3,55 and PDE6β.14 Of importance, it has been shown that missense mutations within the RLD abolished its binding to RPGRIP1 isoforms.12

Patients with cone-dominated phenotypes and RPGR mutations were also described, illustrating the clinical heterogeneity of RPGR-related retinopathies. Two naturally occurring canine models develop a mild late-onset or very severe early-onset disease, depending on the mutation.49 Nevertheless, in both models, rods were primarily affected with cones involved later. These models thus reflect the classical pathogenesis of RP because rod degeneration precedes cone cell death.

In addition, two Rpgr mouse lines were described. An Rpgr knock-out mouse line exhibited mild retinal degeneration involving both rods and cones. The ERG was taken at a single time point (6.5 months); thus, no discrimination of rod and cone photoreceptors can be made over time. Still, the cone phenotype was more pronounced. Of note, the mouse is on a mixed BL/6J1 background. Thus, either the lack of the RPGR default variant leads to affection of both rod and cone photoreceptors or the mixed genetic background has an impact on the additional involvement of cones that was not present in our Rpgr<sup>Ex4</sup> BL/6 mice. Transgenic mice with expression of a truncated RPGR-ORF15 protein revealed rapid photoreceptor degeneration, in contrast to the slow degeneration in the knockout line. ERG measurements displayed a decrease of the scotopic a- and b-waves at approximately 1.5 months of age (P40). Cone responses also declined; however, this effect was not quantified. Compared with the canine and murine models, the Rpgr<sup>Ex4</sup> BALB/c line represents a model for cone-rod degeneration. Our mouse lines expressing a transcript that leads to a deletion of 21 amino acids in the predicted RLD can be regarded as a model for disease-associated human mutations (in-frame deletions and insertions as well as missense mutations), leading to altered RPGR proteins in patients, which account for approximately 48% of all mutations described (http://rpgr.hgu.mrc.ac.uk).
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