Study of Gene-Targeted Mouse Models of Splicing Factor Gene Prpf31 Implicated in Human Autosomal Dominant Retinitis Pigmentosa (RP)

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PURPOSE. Pre-mRNA processing factor 31 (PRPF31) is a ubiquitous protein needed for the assembly of the pre-mRNA splicing machinery. It has been shown that mutations in this gene cause autosomal dominant retinitis pigmentosa 11 (RP11), which is characterized by rod-cell degeneration. Interestingly, mutations in this ubiquitously expressed gene do not lead to phenotypes other than retinal malfunction. Furthermore, the dominant inheritance pattern has shown incomplete penetrance, which poses interesting questions about the disease mechanism of RP11.

METHODS. To characterize PRPF31 function in the rod cells, two animal models have been generated. One was a heterozygous knock-in mouse (Prpf31P216A/H11001/H11002) carrying a point mutation p.A216P, which has previously been identified in RP11 patients. The second was a heterozygous knockout mouse (Prpf31−/−). Retinal degeneration in RP11 mouse models was monitored by electroretinography and histology.

RESULTS. Generation of the mouse models is presented, as are results of ERGs and retinal morphology. No degenerative phenotype on fundus examination was found in Prpf31P216A/H11001 mice. Prpf31P216A/H11001 and Prpf31−/− genotypes were embryonic lethal.

CONCLUSIONS. The results imply that Prpf31 is necessary for survival, and there is no compensation mechanism in mouse for the lack of this splicing factor. The authors suggest that p.A216P mutation in Prpf31 does not exert a dominant negative effect and that one Prpf31 wild-type allele is sufficient for maintenance of the healthy retina in mice. (Invest Ophthalmol Vis Sci. 2009;50:5927–5933) DOI:10.1167/iovs.08-3275

Retinitis pigmentosa (RP) is a set of hereditary retinal disorders leading to progressive loss of vision with age. Characterized by degeneration of rod photoreceptors, the first symptoms are night blindness and loss of peripheral vision, followed by severe loss of sight in later stages of the disease.1 With a worldwide prevalence of approximately 1 in 4000,1,2 retinitis pigmentosa is one of the most frequent forms of inherited retinopathies. Thus far, more than 40 genes have been identified associated with this disease. Most of these genes are specifically expressed in rod photoreceptors and retinal pigment epithelium (RPE) cells. However, in some cases RP is caused by mutations in ubiquitously expressed genes, such as the pre-mRNA processing factor (PRPF) genes PRPF3, PRPF8, PRPF31, and PAP-1.3–6

The purpose of the work reported here was to gain understanding of the disease mechanism of retinitis pigmentosa 11 (RP11) through the study of gene-targeted animal models of Prpf31. The human gene comprises 14 exons and encodes a 61-kDa protein of 499 amino acids, which is highly conserved in eukaryotes.7 It is essential for splicing, taking part in the assembly of the pre-mRNA splicing machinery, and maintaining the stability of the U4/6. U5 tri-snRNP (small nuclear ribonucleoprotein) complex.7,8 Through binding to U4 snRNA, PRPF31 is thought to play a role in the spliceosome activation.9–11 Products of other splicing factor genes implicated in retinitis pigmentosa (PRPF3, PRPF8, PAP-1) also associate with the U4/6. U5 tri-snRNP. PRPF3, PRPF31, and PAP-1 are U4/U6 specific, and PRPF8 binds to the U5 tri-snRNP.9,10,12

Although PRPF31 is essential for splicing,7 mutations in this ubiquitously expressed gene do not lead to any other symptoms except for retinal degeneration in patients. To date, nearly 40 mutations have been reported in PRPF31 in RP11-linked families and sporadic cases3–13–17; two-thirds lead to frameshift mutations, and the remaining one-third are missense mutations. It has been demonstrated through a study of a number of frameshift mutations that mutant RNA is degraded by nonsense-mediated decay and does not contribute to aberrant protein production.13 One characteristic feature associated with mutations in PRPF31 is the nonpenetration of symptoms and retinal changes in some obligate carriers of the disease gene.3,18 It was noted that symptomatic members of a family inherited the same wild-type allele from the unaffected
parent, whereas asymptomatic siblings consistently inherited the other wild-type allele. Comparing mRNA levels of the wild-type PRPF31 between the symptomatic and asymptomatic family members revealed a higher level of PRPF31 expression in asymptomatic persons that modulated the clinical manifestation of the disease. Therefore, the clinical symptoms are a result of coinheritance of the mutation in the PRPF31 gene, along with a low-expressing wild-type allele.

In this study, we report the generation and characterization of two mouse models for RP11, Prpf31A216P/+ and Prpf31A216P/A216P. Neither of the models developed a perceptible RP phenotype. Therefore, we suggest that the p.A216P mutation in Prpf31 does not exert a dominant negative effect and that one Prpf31 wild-type allele is sufficient for the maintenance of healthy retina in mice. We demonstrate that homozygous Prpf31A216P/+ and Prpf31+/+ mice were embryonic lethal, implying a minimum level of this factor necessary for survival.

**Materials and Methods**

**Generation of the RP11 Mouse Model, Maintenance, and Breeding**

All mouse procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Prpf31A216P/+ knock-in mouse was generated in collaboration with GenOway (Lyon, France). The gene-targeting vector consisted of murine PrpF31 exons 6 to 12, where exon 7 carried an alteration of the 216th codon from alanine to proline (GCA to CCT). To enable the generation of a conditional knockout, exon 7 was flanked by LoxP sites, which are sensitive to Cre recombinase. This gene-targeting construct was introduced into mouse embryonic stem (ES) cells of the 129S2/Sv strain, where it integrated into the genome by homologous recombination. The modified ES cells were inserted into a blastocyst, which was then implanted into the uterus of a female mouse from the 129S2/Sv strain. The chimeric pups with modified germline were backcrossed with the C57BL/6j strain. The chimeric pups with modified germline were backcrossed with the C57BL/6j strain. The chimeric pups with modified germline were backcrossed with the C57BL/6j strain. The chimeric pups with modified germline were backcrossed with the C57BL/6j strain. The chimeric pups with modified germline were backcrossed with the C57BL/6j strain.

**Genotyping**

Prpf31A216P/+ and Prpf31A216P/− mice were identified by PCR on genomic DNA (Expand Long Template PCR System; Roche, Burgess Hill, UK) using primers in exons 4 and 8 (forward primer, 5′-CTCCTGAGTAG-GGAGGTCTTGC; reverse primer, 5′-GTAGAACAGAAAGCAGAGGTTCTG). The 3.6 kb amplicon from Prpf31A216P/− mice was subsequently digested with the EcoRV (Promega, Southampton, UK) restriction enzyme to distinguish between the Prpf31A216P/+ and wild-type (undigested) alleles. For the generation of a mouse model, we selected the p.A216P mutation because of the severe phenotype noted in a large RP11 family. Additionally, this mutation was chosen because of the high conservation of this residue among eukaryotes,3,7,22 resulting in a low-expressing wild-type allele.

**Electroretinography and Scanning Laser Ophthalmoscopy**

Electroretinography (ERG) and scanning laser ophthalmoscopy (SLO) were performed according to previously described procedures,20,21 Mice were dark-adapted overnight and anesthetized with ketamine (66.7 mg/kg body weight) and xylazine (11.7 mg/kg body weight) by subcutaneous injection. The pupils were dilated with tropicamide eye drops, and single-flash ERG responses were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Light adaptation was accomplished with a background illumination of 30 candela (cd) per square meter starting 10 minutes before photopic recording. Single white-flash stimulation ranged from 10 to 25 cd·s/m², divided into 10 steps of 0.5 and 1 log cd·s/m². Ten responses were averaged with an interstimulus interval (ISI) of either 5 seconds or 17 seconds (for 1, 3, 10, and 25 cd·s/m²). Band-pass filter cutoff frequencies were 0.3 and 300 Hz. Stainless steel needle electrodes were served reference (forehead) and ground (tail) electrodes, and ring electrodes made of gold wire (0.5 mm in diameter) served as active electrodes. ERG responses were recorded from both eyes simultaneously.

Subsequent to ERG recording, mice were examined with a confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph; Heidelberg Engineering, Heidelberg, Germany). Laser wavelengths used for images of the fundus were 830 nm (infrared channel), 514 nm (red-free channel), and 488 nm with a barrier filter at 500 nm (for autofluorescence imaging and fluorescein angiography). Fluorescein angiography was performed using a subcutaneous injection of 75 mg/kg body weight fluorescein-Na (University Pharmacy, University of Tübingen, Tübingen, Germany). During the procedure, a 78-D ophthalmoscopic lens (Volk Optical, Mentor, OH) was inserted into the optical pathway between the scanning laser ophthalmoscope and the eye.

**SDS-PAGE and Western Blot Analysis**

To detect the full-length and the potential truncated mutant Prpf31 protein in Prpf31A216P/A216P mouse retinas, a polyclonal antibody was raised in rabbit against the N-terminal peptide FIRDKYSKRFPELES (Eurogentec, Liege, Belgium). Total protein was extracted from mouse retinas by lysis in urea buffer (8 M urea, 50 mM dithiothreitol, and 50 mM Hepes). Aliquots containing 10 μg total protein were loaded onto a 12% SDS-PAGE gel. After they were transferred onto nitrocellulose membrane, the blots were blocked with 5% (wt/vol) powdered milk in PBS, Tween 0.1% (vol/vol) powdered milk in PBS (1× PBS, Tween 0.1%; Sigma, Poole, UK). Blots were then probed with the N-terminal Prpf31 and, after washing, with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). The signal was detected with enhanced chemiluminescence reagent (Amer sham Biosciences, Uckfield, UK) according to the manufacturer’s instructions.

**Results**

**Generation of Prpf31A216P/+ Mouse Model**

For the generation of a mouse model, we selected the p.A216P mutation because of the severe phenotype noted in a large RP11 family. Additionally, this mutation was chosen because of the high conservation of this residue among eukaryotes, in which human and mouse Prpf31 proteins are 99% identical. Heterozygous p.A216P mice (Prpf31A216P/−) were generated by creating a gene-targeting construct carrying the pathogenic...
change in exon 7 of Prpf31 (Fig. 1A). The mice were genotyped by amplification of genomic DNA and restriction enzyme digestion (Fig. 1B). Prpf31A216P/H11001 mouse line was generated on a mixed 129S2/Sv and C57BL/6J background (see Materials and Methods).

The knock-in heterozygote crosses with wild-type animals resulted in 137 pups, of which 65 were genotyped as Prpf31A216P/H11001 and 72 as wild type. Average litter size was six to seven pups. Prpf31A216P/H11001 mice were indistinguishable from their wild-type littermates growing and breeding normally. Wild-type and Prpf31A216P/H11001 littermates were examined at different times for retinal degeneration by studying retinal histology, electrophysiology, fundus imaging, and apoptosis staining.

Histologic and Functional Analysis of Prpf31A216P/+ Mice

Histologic examination of the retinas of wild-type and Prpf31A216P/+ mice was performed until the mice were 18 months of age. Comparison of wild-type and Prpf31A216P/+ mouse retinas did not reveal any gross changes throughout the neural retina. Specifically, no differences were noted in the length of the photoreceptor outer segments or in the outer nuclear layer thickness (Fig. 2A).

To obtain a detailed functional characterization of the Prpf31A216P/+ retinas, we performed electroretinography (ERG) and fundus examination with SLO. ERG was carried out in scotopic and photopic conditions. Eight mice were subjected to single-flash ERG at different time points up to 18 months of age. We did not observe any statistically significant differences in the ERG responses between the wild-type and the knock-in animals (Figs. 2B, C). Additionally, under fundus examination with SLO, no signs of retinal degeneration were seen in Prpf31A216P/+ retinas (Fig. 2D). Analysis of the retinal vasculature revealed no changes in the inner retina or choroid up to the age of 18 months. Both Prpf31A216P/+ and wild-type mouse retinas displayed the same autofluorescence patterns.

Apoptotic Assays of Prpf31A216P/+ Mice

Because the lifespan of mice may be too short to document the retinal degeneration resulting from Prpf31 mutations, we investigated cellular events that may precede observable tissue deterioration, including changes in biochemical pathways that may induce cell death at later stages. We thus measured the occurrence of apoptosis and oxidative stress in the retinas of Prpf31A216P/+ and wild-type mice. Up to 18 months, no significant differences were observed in the number of apoptotic nuclei in the outer nuclear layer between the retinas of Prpf31A216P/+ and wild-type mice (Fig. 2E). TUNEL-positive nuclei were quantified on the most central sections of the retina (containing the optic nerve), and at least 40 sections were analyzed under each experimental condition. At the age of 18 months, the difference in TUNEL staining between the wild-type and mutant mice was not statistically significant (2.3 ± 0.9 TUNEL-positive nuclei/section in mutant mice and 2.7 ± 0.8 in wild-type mice; two-tailed t-test; P = 0.432).

Oxidative stress in the retina was investigated with avidin staining, and it also showed no significant differences (data...
**FIGURE 2.** Phenotyping of Prpf31$^{A216P/+}$ mice. (A) Histologic sections of the retina from wild-type and Prpf31$^{A216P/+}$ mutant mice at 18 months. (B) Scotopic single-flash ERG measurements of the wild-type (black) and Prpf31$^{A216P/+}$ mutant mice (red) at 18 months. A set of recordings obtained with different stimulus light intensities that were stepwise increased from $10^{-4}$ cd/s/m$^2$ (dim light, top trace) to $25$ cd/s/m$^2$ (bright light, bottom trace). The overlay panel represents superposition of the responses from a representative wild-type and mutant mouse. (C) ERG b-wave amplitude data plotted against light intensity. The gray boxes indicate the 25% to 75% quantile range, the whiskers indicate the 5% and 95% quartiles, and the cross indicates the median of the data. (D) SLO images of the eye fundus of 18-month-old mice. AF, autofluorescence assessment (to examine photoreceptor damage); RF, red-free native image of the retina; FA, fluorescein angiography (to examine retinal blood vessels). (E) Quantification of the TUNEL-positive nuclei in wild-type and Prpf31$^{A216P/+}$ retinas of the 18-month-old mice. P values resulted from two-tailed t-tests between mutant and wild-type mouse samples.
not shown). Therefore, up to the age of 18 months, the p.A216P mutation in the Prpf31 gene in mice did not increase either programmed cell death or oxidative stress in the mouse retina.

**Homozygous Crosses of Prpf31A216P/+ Mice**

Given that no retinal phenotype was observed in Prpf31A216P/+ mice, we sought to analyze retinal degeneration in homozygous Prpf31A216P/A216P mice. Of the Prpf31A216P/+ cross-breeding, 42 mouse pups were born (9 wild-type, 33 heterozygous Prpf31A216P/+; none were homozygous for the p.A216P mutation). The breeding results were analyzed by χ² statistical tests, which indicated that the lack of homozygous Prpf31A216P/+ animals was not due to chance (χ² = 17.57; df = 2; two-tailed P = 0.0002). Subsequently, cross-breeding were established to analyze Prpf31A216P/A216P mice at embryonic day 10. Of 26 embryos, no homozygotes for the p.A216P mutation were found; 5 embryos were wild-type, and 23 were heterozygous, implying that the Prpf31A216P/A216P embryos die early during embryonic development (χ² = 16.08; df = 2; two-tailed P = 0.0003).

**Generation and Characterization of Homozygous Prpf31 Knockout Mouse**

The lack of retinal phenotype in the Prpf31A216P/+ knock-in mice may be attributed to partial activity of the mutant Prpf31 protein. It is likely that the p.A216P mutation reduces the activity of Prpf31 without abolishing it completely. To verify this possibility, a knockout mouse was generated by which heterozygous knockout mice should have shown a more severe phenotype than Prpf31A216P/+ knock-in mice.

To take advantage of the LoxP sites flanking exon 7 in Prpf31A216P/+ mice (Fig. 1A), a heterozygous knockout was created by Cre-mediated excision of exon 7. This heterozygous knockout mouse (Prpf31A216P) was generated by breeding with the Cre-expressing line BALB/c-Tg(CMV-Cre)1Cgn/J. Given that exon 7 contains 170 nucleotides, deleting it created a predicted frameshift leading to nine novel codons followed by a premature stop codon in exon 8 (Fig. 3A). Such a truncated protein would contain 185 amino acids and have a size of 26 kDa. Alternatively, the occurrence of the premature stop codon in exon 8 might have led to transcript elimination caused by nonsense-mediated decay.

Because of the Cre-mediated excision, the mutant allele was expected to be 897 bp shorter (Fig. 1A). The truncated allele was detected in Prpf31A216P mice by PCR amplification (Fig. 3B). The absence of exon 7 was additionally verified on retinal mRNA, which was PCR amplified, yielding two products from wild-type and mutant alleles. These amplicons were subsequently sequenced, demonstrating that exon 7 is absent in the mutant Prpf31 mRNA (Fig. 3C), which may lead to a 185-amino acid protein. Western blot analysis of retinal tissues from Prpf31A216P and control mice revealed the wild-type Prpf31 protein at the expected size of 61 kDa. However, the truncated mutant protein was absent in Prpf31A216P mouse retinas (Fig. 3D). This indicated that removal of exon 7 led indeed to nonsense-mediated mRNA decay, as assumed earlier.

Thus far, Prpf31A216P mice up to the age of 12 months have been analyzed using ERG and histology. No significant differences were noted in either of the two analyses between the wild-type and knockout mice (data not shown). As expected, Prpf31A216P crossing did not result in any homozygous knockout offspring, further confirming embryonic lethality in such mice.

**DISCUSSION**

Mutations in PRPF31 underlying an autosomal dominant form of retinitis pigmentosa (RP11) raise some interesting issues about the molecular nature of the disease. First, mutations in the ubiquitously expressed splicing factor gene cause a retinopathy with no other accompanying symptoms in patients with RP11. Second, research on this autosomal dominant form of RP has led to a scientific debate about the possible pathologic mechanism in this dystrophy. Do the mutations exert a dominant negative effect present only in the photoreceptor cells?26,27 or is the pathology related to haploinsufficiency?19,28 To investigate these questions in vivo,
two mouse models—Prpf31A216P/Prpf31A216P and Prpf31A216P/Prpf31A216P—were developed. No retinal abnormalities were observed in either of the models. Nevertheless, the p.A216P change was shown to have an effect on the functioning of the Prpf31 protein, because mice homozygous for this mutation were embryonic lethal. This result suggests that the p.A216P mutation in Prpf31 renders the protein dysfunctional or that it reduces its function to such a point that the Prpf31A216P/Prpf31A216P genotype is lethal. At the same time, this mutation does not affect the stability of the Prpf31 protein, as shown in previous studies by Derry et al.32 Lethality of Prpf31A216P/Prpf31A216P mice also implies that no other protein can fully compensate for the loss of Prpf31.

Other mouse models of human retinal degeneration that showed no retinal phenotype are ushers syndrome models for USH1C (harminin)30–32 USH1D (cadherin 23),30–32 USH1F (protocadherin 15),30,32 and USH1G (suns).30–35 As in the case of Prpf31, expression of these particular genes is not restricted to the retina, which may be important in the discrepancy of retinal phenotype between mouse models and human patients. Additionally, some mouse models for autosomal dominant retinitis pigmentosa demonstrate a very mild phenotype in a heterozygous state, in which only homozygous knockout mice develop a retinal degeneration representative of the disease severity in human patients. One such example is the mouse model for RP10, in which only Impdb1−/− null mice were reported to develop retinal degeneration.34 Equally, in an adRP1 mouse model, retinas of Rp1−/− null mice show clear degeneration, whereas retinas of Rp1+/+ mice demonstrate normal photoreceptor morphology with relatively small ERG changes up to the age of 16 months.35 In the case of RP11 mouse models reported here, homozygous animals are embryonic lethal; therefore, unfortunately, the study of retinal degeneration in such mice cannot be performed. These examples clearly demonstrate difficulties in modeling of human retinal diseases using mice. Recently, a new autosomal recessive RP gene, EYS56–59—which is disrupted in many species, including rodents—was discovered. EYS is essential for the proper functioning of the retina in human, yet it is not expressed in mice, indicating that the EYS protein is not required for normal functioning of the mouse retina.

In addition to PRPF31, three other splicing factor genes, PRPF3, PRPF8, and PAP-J, which are also part of the tri-snRNP, have been found to cause retinitis pigmentosa.1–6 No signs of retinal degeneration were observed in heterozygous Prpf3 knockout mice,57 unlike the RP11 mouse models described in this article. Additionally, in parallel with our findings, homozygous Prpf3 knockout mice were also embryonic lethal.37 It is interesting that the RP-related splicing factors are implicated in the same splicing step and, therefore, that the disease mechanism may be associated with inefficient spliceosome assembly. Nevertheless, one cannot rule out the possibility that these splicing factors have an additional, yet undiscovered, role in the retina. Some of the mutations might have a gain-of-function effect in this tissue that may not be seen in other organs. However, the fact that there are four splicing factors implicated in retinitis pigmentosa reduces the chances of each of them having a unique function in the retina that is implicated in the RP phenotype.

Recently, it has been demonstrated in yeast that environmental stress (e.g., ethanol, amino acid deprivation) induces unique spliceosomal responses.38 It has been proposed that the environmental factors may provoke posttranslational regulation of the splicing proteins, which alter splicing of specific transcripts. Such mechanisms may also be present in photoreceptor cells, which endure high levels of oxidative stress resulting from prolonged exposure to light.39 Additionally, it has been demonstrated that mutations in core spliceosomal components may elicit transcript-dependent splicing alterations in yeast.40 Similarly, PRPF3, PRPF8, PRPF31, and PAP-J mutations may affect a distinct group of retinal transcripts, which could explain the unique retinal phenotype.

Why mutations in the ubiquitously expressed splicing factor genes cause retinal phenotype in humans remains unanswered. One hypothesis is related to the endogenous high rod opsin turnover within a limited time interval,31 which creates a strong demand for a robust splicing machinery. It is, therefore, conceivable that any defect in splicing may lead to aberrant production of this protein, causing severe damage of rod cells. Finally, it is important to note that the retina is a nondividing tissue and that photoreceptors differentiate during the embryonic stage and persist throughout life. Therefore, long-term accumulation of biochemical defects increases photoreceptor fragility.

Although the anticipated retinal phenotype in the Prpf31A216P/+ mice was not observed, this study contributes to the understanding of the mechanism of RP11. The results show that the p.A216P mutation in Prpf31 does not have a dominant negative effect in mice, which was shown for the first time in vivo. Considering high conservation of the protein between human and mouse (99%), it is reasonable to extrapolate this finding in patients affected by the p.A216P mutation. Therefore, we would like to suggest that the disease manifestation in RP11 families is essentially attributed to haploinsufficiency rather than to the dominant negative effect of PRPF31 mutations. Additionally, the finding of partial penetrance in most RP11 families also favors haploinsufficiency as a basis for the disease because the dominant negative effect of a mutant protein is more likely to lead to a disease phenotype in all carriers of the disease allele.42–45

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References


