Molecular studies of phenotype variation in canine *RPGR*-XLPRA1

Tatyana Appelbaum, Doreen Becker, Evelyn Santana, Gustavo D. Aguirre

Section of Ophthalmology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA

Purpose: Canine X-linked progressive retinal atrophy 1 (XLPRA1) caused by a mutation in retinitis pigmentosa (RP) GTPase regulator (*RPGR*) exon ORF15 showed significant variability in disease onset in a colony of dogs that all inherited the same mutant X chromosome. Defective protein trafficking has been detected in XLPRA1 before any discernible degeneration of the photoreceptors. We hypothesized that the severity of the photoreceptor degeneration in affected dogs may be associated with defects in genes involved in ciliary trafficking. To this end, we examined six genes as potential disease modifiers. We also examined the expression levels of 24 genes involved in ciliary trafficking (seven), visual pathway (five), neuronal maintenance genes (six), and cellular stress response (six) to evaluate their possible involvement in early stages of the disease.
Methods: Samples from a pedigree derived from a single XLPRA1-affected male dog outcrossed to unrelated healthy mix-bred or purebred females were used for immunohistochemistry (IHC), western blot, mutational and haplotype analysis, and gene expression (GE). Cell-specific markers were used to examine retinal remodeling in the disease. Single nucleotide polymorphisms (SNPs) spanning the entire RPGR interacting and protein trafficking genes (*RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B*) were genotyped in the pedigree. Quantitative real-time PCR (qRT-PCR) was used to examine the expression of a total of 24 genes, including the six genes listed.
Results: Examination of cryosections from XLPRA1-affected animals of similar age (3–4 years) with different disease severity phenotype revealed mislocalization of opsins and upregulation of the Müller cell gliosis marker GFAP. Four to ten haplotypes per gene were identified in *RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B* for further assessment as potential genetic modifiers of XLPRA1. No correlation was found between the haplotypes and disease severity. During mutational analysis, several new variants, including a single intronic mutation in *RAB8A* and three mutations in exon 3 of *DFNB31* were described (c.970G>A (V324I), c.978T>C (G326=), and c.985G>A (A329T)). Expression analysis of stress response genes in 16-week-old predisease XLPRA1 retinas revealed upregulation of *GFAP* but not *HSPA5, DDIT3, HSPA4, HSP90B1,* or *HIF1A*. Western blot analysis confirmed GFAP upregulation. In the same predisease group, no significant differences were found in the expression of 18 selected genes (*RHO, OPN1LW, OPN1MW, RLBP1, RPGRORF15, RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31, RAB11B, CRX, RCVRN, PVALB, CALB1, FGFR1, NTRK2,* and *NTRK3*) involved in neuronal function.
Conclusions: Lack of association between haplotypes of *RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B* and the disease phenotype suggests that these genes are not genetic modifiers of XLPRA1. Upregulation of GFAP, an established indicator of the Müller cell gliosis, manifests as an important early feature of the disease.

Retinitis pigmentosa (RP) is the clinical rubric for a large, heterogeneous group of inherited retinal disorders characterized by progressive photoreceptor degenerative disease leading to vision loss [1]. Of these disorders, X-linked RP (XLRP) comprises some of the most severe forms of RP and accounts for approximately 10% to 20% of all RP cases [2,3]. To date, six disease loci (RP2, RP3, RP6, RP23, RP24, and RP34) on the X chromosome have been mapped (RetNet), and RP3 accounts for approximately 75% of XLRP cases [4,5]. Retinitis pigmentosa GTPase regulator (*RPGR*) is the disease gene of the RP3 locus (*RPGR*-XLRP) [6,7].

The *RPGR* gene produces multiple alternatively spliced transcripts, all of which encode an N-terminal RCC1-like domain that is structurally similar to the RCC1 protein [6], a guanine nucleotide exchange factor for the Ran GTPase. One major constitutive isoform spans exons 1 through 19 and carries a C-terminal isoprenylation site. The other major variant contains exons 1 to 14 and terminates with a large, alternative ORF15 exon (RPGR^orf15) [6-9]. The RPGR^orf15 isoform is expressed predominantly in photoreceptor connecting cilia and basal bodies [10,11] and appears to be play a critical role in retinal function as multiple disease-causing mutations have been identified in humans, dogs, and mice [6,12,13]. Furthermore, gene augmentation therapy with RPGR^orf15 preserves function and prevents degeneration in these diseases [14,15].

Correspondence to: Gustavo D. Aguirre, School of Veterinary Medicine, University of Pennsylvania, 3900 Delancey Street, Philadelphia, PA, 19104; Phone: (215) 898–9426; FAX: (215) 573–6050; email: gda@vet.upenn.edu

To date, the X-linked progressive retinal atrophy (XLPRA) dog is the only known naturally occurring large animal model of *RPGR* mutations [12]. The canine XLPRA phenotype has been linked to *RPGR*, and homology of canine XLPRA and human RP3 has been established [16,17]. Two disease-causing microdeletion mutations in exon ORF15 have been identified in canine *RPGR* [12]. One produces a premature stop (del1028–1032) resulting in a C-terminal truncation of 230 residues in XLPRA1. The second is a 2 nt deletion (del1084–1085) that causes a frameshift with the inclusion of 34 basic amino acids and truncation of the terminal 161 residues and is causal for XLPRA2. The phenotype associated with the frameshift mutation in XLPRA2 is severe and consistent, and manifests during retinal development; the phenotype resulting from XLPRA1 is expressed only after normal photoreceptor morphogenesis has been completed and has more gradual progression [12].

Extensive phenotypic diversity is observed in patients with *RPGR* mutations, between patients with different mutations, and between patients within families who have the same mutation [18-20]. Dogs with XLPRA1 also show remarkable phenotypic variability in clinical cases and, more surprisingly, within a closed research colony where all dogs are maintained in a constant environment, exposed to the same light intensities and cycles, and fed a uniform diet [21,22]. Such phenotypic variability cannot be simply explained by heterogeneity at the primary locus since all affected dogs inherited the same single mutant X chromosome, and the mutation is stably present through multiple generations [12,21]. Since genetic modifiers can alter the course of diseases, including *RPGR*-XLRP [18,23-25], we hypothesized that a modifier gene or genes contribute to the phenotypic variability observed in XLPRA1. Previously, we analyzed six genes (*RPGRIP1*, *RANBP2*, *NPM1*, *PDE6D*, *NPHP5*, and *ABCA4*) as genetic modifiers of XLPRA1 but excluded all [22]. As studies on RPGR-interacting proteins and those in the *RPGR*KO model suggest that RPGR functions in ciliary trafficking [9,26], here we focused on modifier genes that might also affect ciliary transport, thus potentiating the opsin mislocalization that contributes to XLPRA1 [27]. Candidate gene modifiers of XLPRA1 were chosen based on their contribution to ciliary formation, vesicular transport, and rod outer segment disc formation [28].

In the present study, we characterized retinas from XLPRA1 dogs with different degrees of disease for the expression of opsins and the Müller cell marker glial fibrillary acidic protein (GFAP), which responds to outer retinal stress and is one of the earliest genes upregulated in several non-allelic forms of inherited retinal degeneration [29,30].

We expanded the analysis of genetic modifiers of XLPRA1 to include genes that encode RPGR-interacting proteins (RPGRIP1L [25,31], DFNB31 [32], and RAB8A [33]) and proteins essential for cilia formation, ciliary trafficking, and cargo delivery (CEP290 [9], RAB11B [34], and CC2D2A [35]). We also examined the expression of 24 genes involved in ciliary trafficking, visual pathway, neuronal maintenance, and cellular stress response to evaluate their possible involvement in early stages of XPLRA1 disease.

METHODS

Ethics statement: The research was conducted in full compliance and strict accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

Pedigree resources and determination of the phenotypic status: Details of the origin and composition of the colony have been previously published [21,22,36]. Briefly, the colony was established by outcrossing a single XLPRA1-affected male Siberian husky to unrelated healthy female beagles shown to be free from inherited retinal degeneration based on test breeding to known homozygous affected dogs with other autosomal recessive diseases [37]. The carrier progeny were subsequently mated with mixed breed or purebred dogs of varied genetic background to produce informative hemizygous or homozygous affected males or females, respectively, and heterozygous females. All dogs were maintained under specific and standard conditions where all animals have the same exposure to cyclic light (12 h:12 h light-dark cycle), receive the same diet, and have the same medical procedures and vaccinations. A subset of the colony, consisting of 43 dogs (affected with the *RPGR* mutation (n = 24), carriers (n = 7), and healthy (n = 12)), was selected for the studies (Appendix 1). The dogs in the study were the same used in a prior analysis of potential candidate gene disease modifiers [22]. Dogs were included based on the results of serial clinical assessment of retinal disease status using indirect ophthalmoscopy and electroretinography (ERG). Morphologic criteria were used to establish grades of disease severity taking into account the animal's age and the degree and extent of disease [21]. Three grades were defined: Mild degeneration present only in periphery after 1.5 years of age or later, Moderate degeneration present only degeneration develops between 11 and 15 months of age, and Severe (photoreceptor degeneration stage 2 or more advanced, present centrally and peripherally) [21]. Of the 24 affected dogs (22 hemizygous males and two homozygous females), 14 were classified as

Severe, nine as Moderate, and one as Mild. The skewed phenotype distribution results from selective breeding to produce affected dogs that could be diagnosed at an early age to be ascertained for the linkage mapping and gene and mutation identification studies [12,17]. As the group of dogs with Mild disease contained only one dog, in the disease-association studies only those with Moderate and Severe disease were used for analysis.

Sample collection, DNA/RNA extraction, and cDNA synthesis: Retinas were collected from enucleated eyes under sterile and RNase-free conditions, frozen by immersion in liquid nitrogen, and stored at -70 °C until used. For terminal procedures, the dogs were anesthetized by intravenous injection of pentobarbital sodium (65 mg/ml solution was prepared under sterile conditions by the Penn Medicine Investigational Drug Service), the eyes enucleated, and the dogs immediately euthanatized with intravenous Euthasol (Virbac, Ft. Worth, TX). Total RNA was isolated from canine tissues using a modified TRIzol and single chloroform extraction protocol. Briefly, 1 ml of TRIzol (Invitrogen, Carlsbad, CA) was added to the retina, and 0.2 ml of chloroform (Sigma-Aldrich, Allentown, PA) was added after tissue homogenization; the tissue-Trizol-chloroform mix then was centrifuged at 1,484 ×g at 4 °C for 4 min. The clear aqueous upper phase was transferred to a fresh microfuge tube and mixed to 70% ethanol (1:1, V/V). Next, the sample was loaded on an RNeasy Mini spin column, and the RNA was further purified using an RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's directions. First-strand cDNA was synthesized in 20 µl reactions using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommendations. Genomic DNA was isolated from blood samples using the QIAamp DNA kit (Qiagen) following the manufacturer's directions.

Genotype and haplotype analysis within candidate genes: Six genes were chosen for analysis (*RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B*). Polymorphic sites consisting of SNPs were identified in selected genes based on the Broad Institute SNP database, the Dog Genome SNP Database, and SNP database or based on our own gene sequencing analyses. Genotyping was performed using direct sequencing of PCR products. PHASE software (v. 2.1.1) was used for haplotype reconstruction. Fisher's exact test (two-sided) was used for statistical analysis (p≤0.05). For this analysis, we posited that a candidate gene modifier would have a homozygous haplotype in dogs with Severe phenotype and heterozygous in those with a Moderate phenotype.

PCR amplification and sequencing: Primer sequences for the PCR experiments are shown in Appendix 2. PCR reactions were performed on 50 ng genomic DNA in a final volume of 25 µl containing 2x PCR master Mix (New England Biolabs, Ipswich, MA), 0.7 µM forward and reverse primers, and PCR-grade water. Cycling conditions were 2 min initial denaturation at 95 °C followed by 35 cycles of 95 °C for 20 s (denaturation), 52 °C for 30 s (annealing), and 68 °C for 30 s (elongation). For sequencing purposes, the PCR products were analyzed on a 2% agarose gel, extracted with the NucleoTrap Gel Extraction Kit (Clontech, Mountain View, CA), and directly sequenced.

Relative quantification (ddCt) assay: Real-time PCR was performed in a total volume of 20 µl in 96-well microwell plates on the Applied Biosystems 7500 Real-Time PCR System. All PCRs were performed using cDNA generated from 15 ng DNase-treated RNA from 16-week-old healthy retinas (n = 3) and XLPRA1-affected dogs (n = 6). The SYBR Green platform was used for gene expression analysis of 24 genes using a primer concentration of 0.2 µM. The primer sequences for real-time PCR are listed in Appendix 3. The *TBP* gene expression level was used to normalize the cDNA templates. Amplification data were analyzed with the 7500 Software version 2.0.1 (Applied Biosystems). Genes included for this analysis coded for proteins essential for cilia formation, ciliary trafficking, and cargo delivery, and genes involved in ciliary formation, visual pathway, neuronal maintenance, and cellular stress response; the genes are listed in Table 1.

Fluorescent immunohistochemistry: Retina from three XLPRA1-affected dogs and a healthy dog was used for immunohistochemistry (IHC). These dogs are not part of the genetic modifier pedigree. The procedures used for tissue collection, preparation, and sectioning have been previously described [38]. Cryosections were washed and treated with the primary antibodies in PBS solution (1X; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), 3% normal horse serum (NHS, Vector Laboratories, Burlingame, CA), 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.3% Triton X-100 (Sigma-Aldrich) overnight followed by incubation with appropriate fluorescent secondary antibodies (Alexa Fluor Dyes, 1:300; Molecular Probes, Eugene, OR). The following primary antibodies were used in the study: mouse anti-rhodopsin at 1:1,000 (MAB5316, Chemicon, Temecula, CA), rabbit anti-L/M opsin at 1:1,000 (AB5405, Millipore, Billerica, MA), goat anti-hCAR (human cone arrestin) at 1:1,000 (a gift from Dr. W. Beltran, University of Pennsylvania), and rabbit anti-GFAP at 1:1,000 (Z0334, Dako, Carpinteria, CA). 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) stain was used to label the cell nuclei. Slides were mounted with fluoromount G mounting media

(catalog No. 01100-01; Southern Biotech, Birmingham, AL), coverslipped, and examined with epifluorescence microscopy with a Zeiss Axioplan microscope (Carl Zeiss Meditech, Oberkochen, Germany). Images were digitally captured (Spot 4.0 camera; Diagnostic Instruments, Inc., Sterling Heights, MI) and imported into a graphics program (Photoshop; Adobe, Mountain View, CA) for display.

Western blot analysis: Western blotting was performed with multiplex infrared detection using secondary antibodies conjugated to IRDye® fluorescent dyes. Briefly, total protein extracts in equal amounts (50 µg) as determined with the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) were resolved using 4–15% acrylamide gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE gel; Bio-Rad Laboratories, Hercules, CA) in Tris/glycine/SDS buffer (BioRad). Resolved proteins were immunoblotted into nitrocellulose membranes (LI-COR Biosciences, Lincoln, NE) using the Wet/Tank Blotting

System (Bio-Rad) and blocked for 1 h at room temperature or overnight at 4 °C with 1× Odyssey Blocking Buffer (LI-COR). Immunoblots were probed overnight at 4 °C with unconjugated primary antibodies diluted in 1× Blocking Buffer. Membranes were then washed 3X with PBST (PBS containing 0.1% Tween 20 (Sigma-Aldrich) and incubated for 1 h at room temperature in goat anti-rabbit IRDye680RD- and goat anti-mouse IRDye800CW-conjugated immunoglobulin G (IgG) secondary antibodies (LI-COR) diluted 1/10,000 each in 1× Blocking Buffer containing 0.1% Tween-20. Immunoblots were washed three times in PBST, once in PBS, and scanned on the Li-COR Odyssey Fc Dual-Mode Imaging System with 700- and 800-nm channels. Normalization to ACTB and analyses were done using Image Studio Software provided by LI-COR. The primary antibodies used were rabbit anti-GFAP at 1:5,000 (Z0334, Dako) and anti-mouse ACTB at 1:10,000 (MAB1501, Millipore). Quantification of the proteins on western blot was performed with Li-COR Odyssey software.

Gene	Gene name	FC* XLPRA1 versus normal retina
RHO	rhodopsin	n.s.**
OPN1LW	opsin 1 (cone pigments), long-wave-sensitive	n.s.
OPN1MW	opsin 1 (cone pigments), medium-wave-sensitive	n.s.
RLBP1	retinaldehyde binding protein 1	n.s.
RPGRORF15	retinitis pigmentosa GTPase regulator	n.s.
RAB8A	RAB8A, member RAS oncogene family	n.s.
RPGRIP1L	RPGRIP1-like	n.s.
CEP290	centrosomal protein 290 kDa	n.s.
CC2D2A	coiled-coil and C2 domain containing 2A	n.s.
DFNB31	deafness, autosomal recessive 31	n.s.
RAB11B	RAB11B, member RAS oncogene family	n.s.
CRX	cone-rod homeobox	n.s.
RCVRN	recoverin	n.s.
PVALB	parvalbumin	n.s.
CALB1	calbindin 1	n.s.
FGFR1	fibroblast growth factor receptor 1	n.s.
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	n.s.
NTRK3	neurotrophic tyrosine kinase, receptor, type 3	n.s.
GFAP	glial fibrillary acidic protein	6.7
HSPA5	heat shock 70 kDa protein 5	n.s.
DDIT3	DNA-damage-inducible transcript 3	n.s.
HSPA4	heat shock 70 kDa protein 4	n.s.
HSP90B1	heat shock protein 90 kDa beta	n.s.
HIF1A	hypoxia inducible factor 1, alpha subunit	n.s.

TABLE 1. RNA EXPRESSION CHANGES OF RETINAL GENES IN 16 WEEK OLD XLPRA1 RETINAS.

*FC=fold change differences ** n.s.=non statistically significant differences
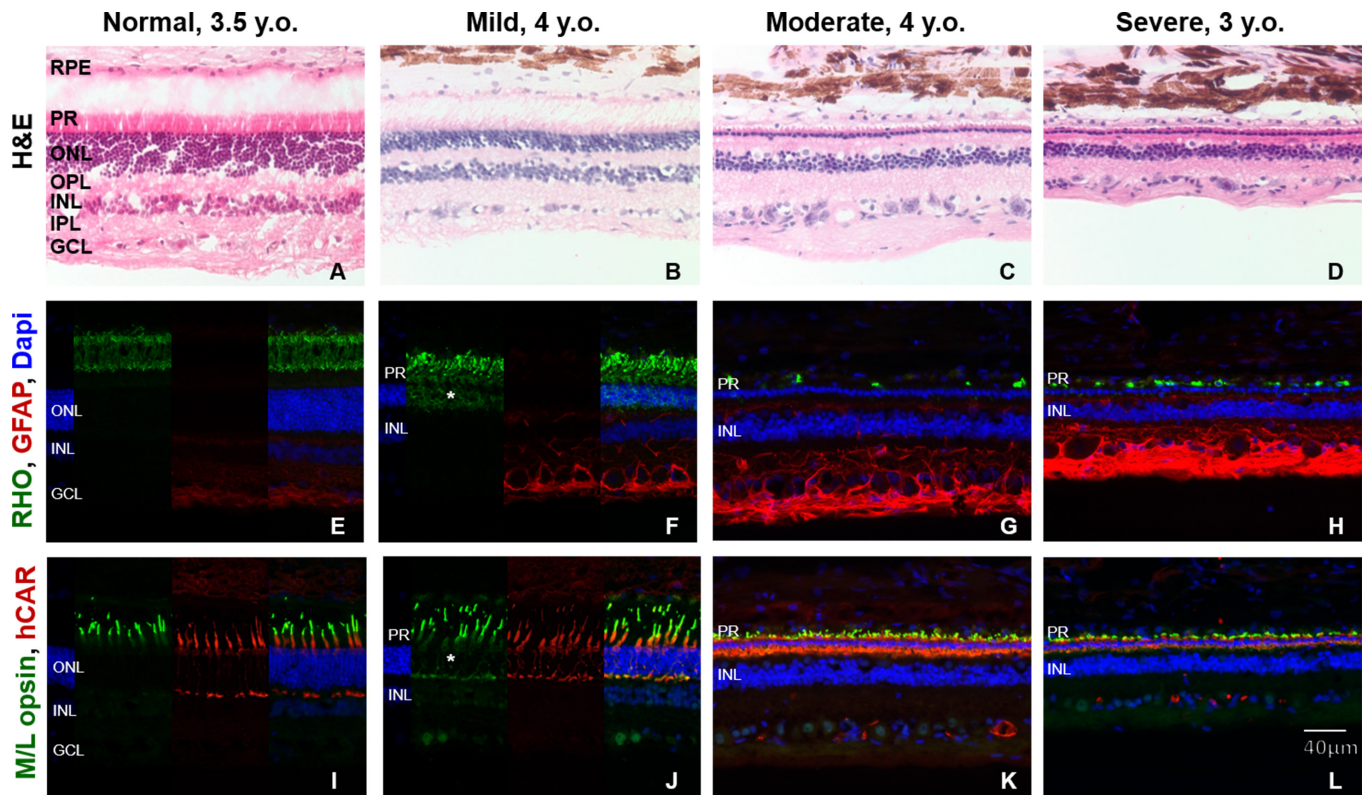
Figure 1. XLPRA1-affected retina: immunohistochemical characterization of different severity phenotypes. **A–D**: Hematoxylin and eosin (H&E)–stained retinal cryosections from the superior quadrant (6,000 μm from the ora serrata; area is approximately the midpoint between the disc and the ora serrata) in dogs of comparable ages that have different phenotype severity. **E–H**: Double immunolabeling with RHO and GFAP antibodies. **I–L**: Double immunolabeling with hCAR and M/L opsin antibodies. Where appropriate (**E**, **F**, **I**, **J**), the immunolabeling images show the individual blue, green, and red channels with the merged image on the right of each set to show the mislocalization of opsin (*). RPE= retinal pigment epithelium; PR = photoreceptors; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL= ganglion cells layer.

RESULTS

Characterization of XLPRA1-affected dogs with different severity phenotype: Examination of hematoxylin and eosin (H&E)-stained cryosections from XLPRA1-affected dogs of comparable ages (3–4 years) showed morphological alterations compatible with an ongoing disease process (Figure 1A–D). Disease was accompanied by decreased rod density and outer nuclear layer (ONL) thickness. Significant photoreceptor loss (ONL = 1 row) was observed in the youngest animal with Severe phenotype. To evaluate the localization of photoreceptor-specific proteins, double immunolabeling and DAPI nuclear staining were performed in the affected dogs. Immunolabeling with rod opsin showed mislocalization in Mild disease and loss of rods in Moderate and Severe disease. GFAP was upregulated in all disease phenotypes and was mostly present in the inner plexiform layer (IPL) and to a lesser extent in the outer plexiform layer (OPL; Figure 1E–H); increased GFAP expression was more prominent in Moderate

and Severe disease. Double immunolabeling with hCAR and M/L opsin antibodies showed cone outer segment disorganization and mislocalization of M/L cone opsin (Figure 1I–L), and disorganization and loss of the outer segments (OS) were observed in the remaining rods and cones.

Genotype-phenotype analysis in XLPRA1 pedigree: As demonstrated previously [27] and in the present study (Figure 1), XLPRA1 is characterized by defective protein trafficking that leads to mislocalization of opsins to the inner segment, nuclear, and synaptic layers. Thus, the presence of mutations that perturb protein trafficking could have severe functional consequences that could result in photoreceptor cell death. To this end, six genes (*RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B*) were investigated as candidate disease modifiers. The proteins encoded by these genes are involved in common and distinct pathways required for effective protein trafficking in cilia and cargo delivery, and some

(RPGRIP1L, DFNB31, and RAB8A) directly interact with RPGR.

Profiling sequence changes in RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31, and RAB11B genes: Polymorphic markers were identified by sequence analysis of randomly selected regions within the genes of interest in the XLPRA1-affected study dogs. In addition, details of SNPs from several public SNP databases used for genotype and haplotype analysis are presented in Appendix 2. As two mutations in *RPGRIP1L* (A229T and R744Q) have been previously shown to contribute to phenotypic variability in retinal degeneration [18,25], we first analyzed the affected RPGR mutant dogs for the presence of A229T and R744Q. Human and canine *RPGRIP1L* is predicted to have 90.0% amino acid sequencing homology (compare NM_015272 and XP_013964947); therefore, A229 and R744 are in the same position in the human and canine *RPGRIP1L* sequence. None of the dogs analyzed harbored either mutation.

Sequence analysis of other genes identified four new variants: a single intronic change in *RAB8A* and three in exon 3 of *DFNB31* in affected dogs. Sequence changes in *DFNB31* exon 3 include two missense [(c.970G>A (p.V324I) and (c.985G>A (p.A329T))] and one synonymous (c.978T>C (p.G326=)) change (Figure 2). We performed alignment of the DFNB31 orthologous sequences to see whether the missense mutations affected conservative amino acid residues (Figure 3). Analysis of the corresponding protein region in multiple species has identified valine and isoleucine in position 324 of DFNB31; thus, it is unlikely that the V324I missense change observed in canine DFNB31 is consequential. However, the canine sequence has alanine at position 329 of DFNB31 while all other analyzed orthologous sequences contain proline. Although alanine and proline are hydrophobic neutral amino acids, the A329T change found in DFNB31 replaces alanine with threonine, a polar and hydrophilic residue that could potentially affect the structure and function of the protein. However, A329T was not included in the genotype and haplotype analysis as A329T was present only in the founder (H2) that was a carrier for the mutation.

The DFNB31 variants (V324I and G326=) were frequently present in the XLPRA1-affected dogs of the pedigree, but neither was associated with disease phenotype (Appendix 4). Despite a high prevalence of V324I (69.6% of XLPRA1-affected dogs), this variant appeared to have been artificially accumulated in the pedigree as the founder (H2), and most females used for breeding harbored V324I and passed it on to their offspring. Therefore, we concluded that the V324I sequence change is unlikely to have any functional consequences.

Genotype distribution: Genotype frequencies for the SNPs abundant in the XLPRA1-affected dogs are shown in Appendix 4 and were compared for the Moderate and Severe phenotypes. The genotype distributions of all analyzed SNPs were not statistically different between these two groups.

Haplotype distribution: The polymorphic SNPs in Appendix 2 provide the basis for gene-specific haplotype association studies. These results are presented in Table 2 and Table 3. The number of haplotypes per gene varied from four (*CEP290*) to ten (*DFNB31*; Table 2). Next, we compared the frequencies of haplotypes in the Moderate and Severe phenotypes of XLPRA1-affected dogs (Table 3). The haplotype frequencies for the genes *RAB8A*, *RPGRIP1L*, *CEP290*, *CC2D2A*, *DFNB31*, and *RAB11B* were not statistically different between the two groups analyzed in this study, thus excluding all six genes as candidate modifiers of the disease phenotype observed in the XLPRA1 pedigree.

Gene expression of selected genes in the study model: Variations in gene expression can also be associated with disease [39,40], and the lack of gene expression data for components of the *RPGR* molecular network hinders our understanding of its role in phenotypic expression of XLPRA1. In this study, we examined the expression of *RPGRORF15*, *RAB8A*, *RPGRIP1L*, *CEP290*, *CC2D2A*, *DFNB31*, and *RAB11B* in six XLPRA1-affected predisease (16 week old) retinas to determine whether expression changes in gene expression might contribute to disease progression. To gain more in-depth understanding of the disease-induced changes in gene expression, we also examined the expression of selected genes involved in the visual pathway (*RHO*, *OPN1LW*, *OPN1MW*, and *RLBP1*), neuronal cells (*CRX*, *RCVRN*, *PVALB*, *CALB1*, *FGFR1*, *NTRK2,* and *NTRK3*), and cellular stress response (*GFAP*, *HSPA5*, *DDIT3*, *HSPA4*, *HSP90B1,* and *HIF1*).

Out of the total 24 genes examined, only *GFAP* gene expression was upregulated (6.7-fold increase) in all XLPRA1 animals compared to the control group (p<0.01, Table 1). The *GFAP* level in XLPRA1 in the predisease samples was highly variable and increased in expression early in other retinal degeneration disorders in dogs [29,30]. The estimated variance in the XLPRA1 group was 9.96 compared to variance of only 0.07 in the control group. Upregulation of the GFAP protein in the 16-week-old XLPRA1 retinas was confirmed with western blot analysis (Figure 4).

DISCUSSION

RPGR is an important component of the ciliary protein network, but details of the molecular function of RPGR in photoreceptors cilia are still incomplete. The protein complexes in which RPGR participates in the ciliary

compartment play key roles in the function and maintenance of photoreceptor cells. For example, RPGR is associated through NPHP1, NPHP5, RPGRIP1, RPGRIP1L, RAB8A, CEP290, and SMC1/3 with the nephrocystin protein network and ciliary transport [9,41-44], through DFNB31 to the Usher protein network [32], and through NPM1 to the centrosomal protein network [45]. Defects of proteins in these complexes lead to photoreceptor dysfunction, cell death, and retinal degeneration.

The mislocalization of opsin is thought to contribute to the pathophysiology of photoreceptor degeneration. Changes in opsin localization have been reported for many diseases and injuries where photoreceptor cell death occurs, including human and animal models of retinal degeneration [13,27,46,47]. Opsin mislocalization is a feature of XLPRA1

and XLPRA2 and other canine retinal degeneration disorders (Figure 1 and [27,30,48]). Similar to several other RPGR disease models [13,49], opsin mislocalization was detected in XLPRA1 before any discernible photoreceptor degeneration. Since mutations in ciliary trafficking genes can contribute to ciliary transport defects, six genes (*RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B*) were selected based on their interaction with RPGR or involvement in ciliary transport and investigated as candidate genetic modifiers of XLPRA1.

The XLPRA1 pedigree is small, and its inbred affected population is not ideal for linkage disequilibrium (LD) analysis. Because of these limitations, we compared gene-specific genotypes and haplotypes frequencies in Severe versus Moderate affected dogs to determine whether there
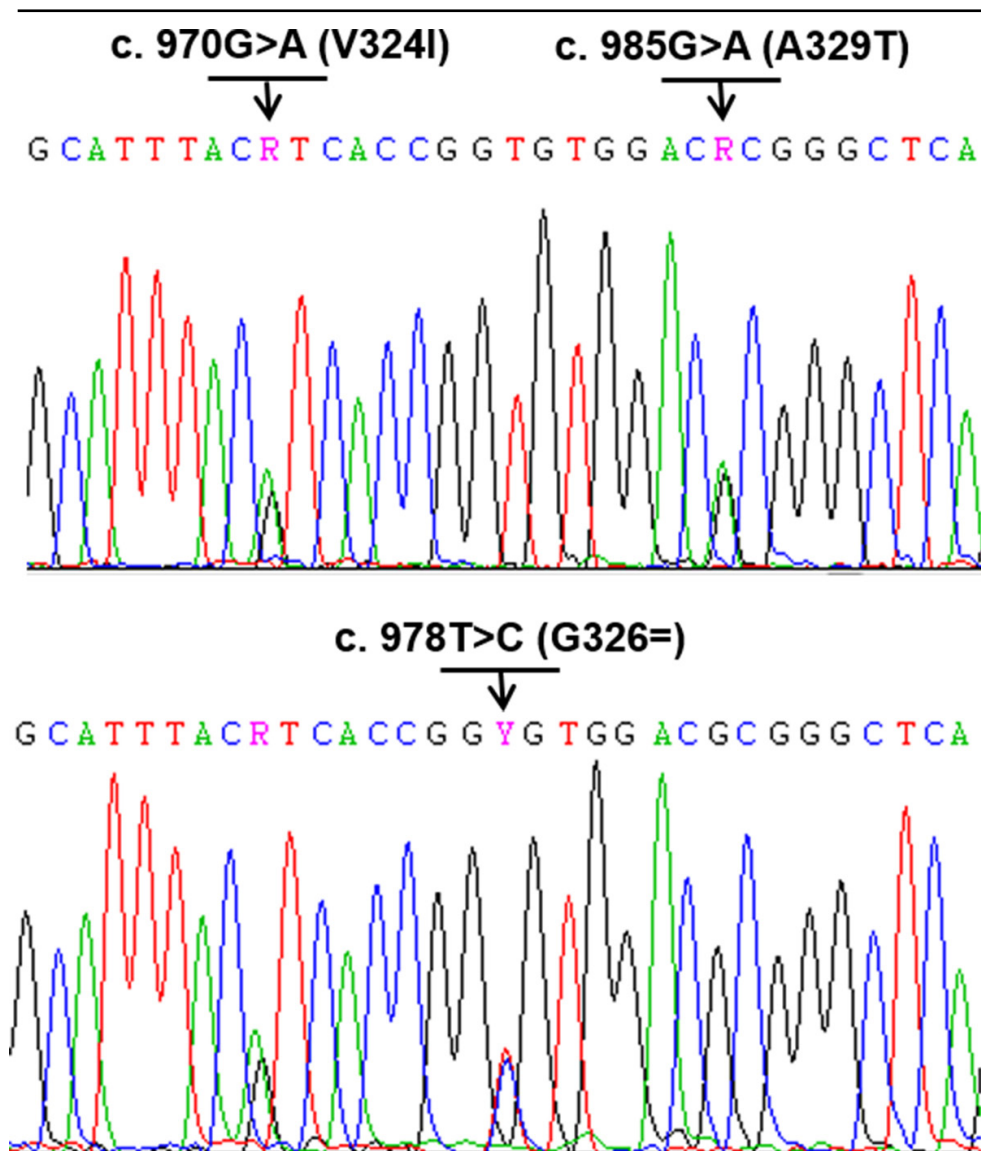

Figure 2. Chromatograms showing the variants detected in the canine *DFNB31* gene (exon 3). c.970G>A and c.985G>A result in a substitution of valine for isoleucine at position 324 and alanine for threonine at position 329 of *DFNB3*1, respectively (top). c.978T>C does not result in a change in the amino acid sequence (bottom). In both chromatograms, the relevant area is marked with an arrow.
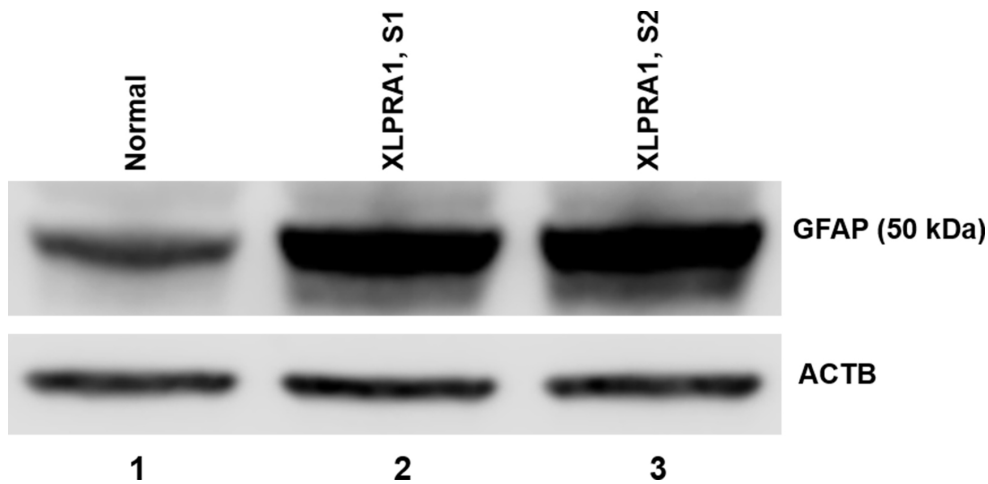
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                                            *
                               *         *
H. sapiens        TIRGGAEYGLGIYITGVDPGSEAE    316
P. troglodytes    TIRGGAEYGLGIYITGVDPGSEAE    393
M. mulatta        TIRGGAEYGLGIYITGVDPGSEAE    316
C. lupus          TIRGGAEYGLGIYVTGVDAGSEAE    334
B. Taurus         TIRGGAEYGLGIYVTGVDPGSEAE    316
M. musculus       TIRGGAEYGLGIYITGVDPGSEAE    317
R. norvegicus     TIRGGAEYGLGIYITGVDPGSEAE    315
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Figure 3. Multiple alignment of the *DFNB31* orthologous sequences. The alignment shows that vertebrate *DFNB31* orthologs have either valine or isoleucine (marked by a single asterisk) in the protein region corresponding to position 324 of the canine *DFNB31* and proline or alanine (marked by double asterisks) at position 329.

was a putative correlation between genotype or haplotype and disease phenotype. By carrying out this strategy, we found that moderately and severely affected dogs had no differences in genotypes and haplotype frequencies for *RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B.* Furthermore, these six genes were not variably expressed in

predisease XLPRA1 retinas suggesting that the gene products do not serve as modifiers of disease phenotype.

During mutational analysis, three novel sequence variants were discovered in *DFNB31* (V324I, G326, and A329T). All three were located in exon 3, which encodes part of the PDZ2 domain responsible for binding to the C-terminal of RPGR[orf15] [32]. V324I was present in 69.6% of

TABLE 2. HAPLOTYPES RECONSTRUCTED IN THE XLPRA1 PEDIGREE.

Gene	Haplotype							Gene	Haplotype						
RAB8A	1	2	3	4	5	6	7	*CC2D2A*	1	2	3	4	5	6	7
chr20:46336246	C	C	C	C	C	C	A	chr3:64841219	A	A	A	A	T	T	T
46344912	C	C	C	C	G	G	G	64854752	C	G	T	T	C	C	C
46349143	T	T	C	C	T	C	T	64859223	G	T	C	T	G	T	T
46351756	G	A	G	A	G	G	G	64861317	A	A	A	A	G	A	G

Gene	Haplotype										Gene	Haplotype									
RPGRIP1L	1	2	3	4	5	6					*DFNB31*	1	2	3	4	5	6	7	8	9	10
chr2:62092489	C	C	C	G	G	G					chr11:68620384	A	A	A	A	A	G	G	G	G	G
62122046	A	A	A	T	T	T					68642086	T	T	T	G	G	T	T	T	T	T
62138129	A	A	C	A	A	A					68658508	A	A	A	A	A	G	G	G	G	G
62152007	G	G	G	G	A	A					68671284	T	T	C	T	T	T	T	C	C	C
62163783	C	T	T	T	C	T					68671292	G	A	G	A	A	G	G	G	G	G
62166939	C	C	C	T	T	T					68680635	A	G	A	G	G	G	A	G	G	A
											68698265	C	C	C	C	G	G	C	C	G	C

Gene	Haplotype				Gene	Haplotype						
CEP290	1	2	3	4	*RAB11B*	1	2	3	4	5	6	7
chr15:29199693	G	G	G	C	chr20:52957965	C	C	C	C	G	C	A
29220545	G	G	A	A	52960719	C	C	C	C	C	G	G
29228039	T	T	C	C	52963299	T	T	C	C	T	C	T
29247843	A	G	A	A	52965822	G	A	G	A	G	G	G
29265700	T	T	C	C								
29279083	G	G	A	A								

Figure 4. Representative western blot analysis for GFAP on two XLPRA1-affected retinas (S1 and S2, 16 weeks) versus the age-matched healthy retina (16 weeks). Quantification of proteins on western blot performed with Li-COR Odyssey software showed upregulation of GFAP in S1 and S2 retinas of 2.3 and 2.5 times, respectively.

XLPRA1-affected dogs, suggesting at first that it could be associated with disease. However, on closer examination we found that this mutation artificially accumulated in the

	RAB8A	RPGRIP1L	CEP290	CC2D2A	DFNB31	RAB11B
Sample ID						
			Severe			
H2	1,2	1,2	1,1	1,2	1,4	1,2
H64	3,4	2,2	2,4	2,3	4,10	3,4
H104	3,5	3,6	2,2	2,3	6,6	3,5
H105	5,5	2,2	2,2	3,7	2,6	5,5
H78	5,5	2,5	2,3	7,7	4,10	5,5
H79	1,5	2,5	2,3	7,7	2,9	1,5
H82	1,5	2,5	1,2	2,7	2,9	1,5
H143	5,7	4,5	1,2	2,6	6,10	5,7
H35	3,5	2,5	3,4	2,2	3,3	3,5
H38	3,7	2,5	1,4	2,2	6,7	1,6
H71	2,3	3,5	1,4	1,1	4,10	2,3
H72	6,7	2,5	1,2	2,7	4,10	6,7
H73	2,5	3,5	1,2	1,1	5,10	2,5
H118	3,7	5,5	2,4	2,2	6,10	3,7
			Moderate			
H29	5,5	3,6	2.3	3,7	4,9	5,5
H31	5,5	3,5	1,3	1,5	4,10	5,5
H130	3,3	2,5	1,2	2,2	2,6	3,3
H131	3,3	2,5	2,4	4,4	4,6	3,3
H81	5,5	3,3	1,3	5,5	4,4	5,5
H208	2,5	2,4	2,2	2,7	2,6	2,5
H59	4,7	2,5	2,3	2,3	9,10	4,7
H201	2,5	5,5	1,1	2,3	8,10	2,5
H202	3,7	5,5	1,2	3,3	2,9	3,7

TABLE 3. HAPLOTYPES DISTRIBUTION IN **XLPRA1** AFFECTED DOGS WITH DIFFERENT SEVERITY PHENOTYPE.

pedigree. Additional analysis of DFNB31 orthologous sequences excluded a putative harmful effect of V324I.

In addition to the seven genes involved in protein trafficking (*RPGRORF15, RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B*), we analyzed the expression of a subset of genes necessary for the visual pathway (*RHO, OPN1LW, OPN1MW,* and *RLBP1*), neuronal cell maintenance (*CRX, RCVRN, PVALB, CALB1, FGFR1, NTRK2,* and *NTRK3*), molecular chaperons (*HSPA4, HSP90B1*), unfolded protein response (*HSPA5, DDIT3*), cellular response to hypoxia (*HIF1*), and Müller cell gliosis (*GFAP*) in early XLPRA1. Although opsins are mislocalized in early XLPRA1 [27], we found that their gene expression was not impaired in 16-week-old predegenerate retinas. Aside from *GFAP*, other genes were not differentially expressed. Notably, GFAP expression was already upregulated at 16 weeks of age, when the XLPRA1 retinas are morphologically normal [21]. GFAP expression in Müller cells in the mammalian retina is normally low but is upregulated in a variety of degenerative conditions, including retinal trauma, diabetic retinopathy [50], choroidal neovascularization [51], retinal detachment [52], glaucoma [53], and age-related macular degeneration [54].

The results obtained in this study clearly demonstrate that even when mutated retinas appear morphologically normal, the Müller cells respond with a dramatic increase in GFAP expression in the early phase of XLPRA1. We also identified *GFAP* as highly variable in expression across predisease XLPRA1 retinas. Could *GFAP* gene expression variability lead to or be a marker of phenotypic diversity of XLPRA1? It is possible. Recent publications describe a complex interplay between Müller cells and retinal microglia in pathological conditions [55,56] that mediate adaptive responses within the retina and may be relevant to amplifying and coordinating an inflammatory response. The severity of the photoreceptor degeneration in XLPRA1-affected dogs may be associated with increased retinal inflammation. Therefore, an inflammatory response in the XLPRA1 retina should be further investigated.

GFAP typically increases with disease progression but decreases in some models in advanced stages of degeneration [27,57]. When assessing retinal remodeling events in XLPRA1-affected dogs with different severity phenotype, we observed markedly higher levels of GFAP in all disease stages in comparison to the healthy retina (Figure 1E–H). In addition, GFAP immunolabeling showed Müller glia reactivity increased with severity of the disease. This increase could be a sign of a sustained disturbance in retinal homeostasis in the disease.

In conclusion, this study reports a lack of association between the *RAB8A, RPGRIP1L, CEP290, CC2D2A, DFNB31,* and *RAB11B* genes and disease severity phenotype in canine XLPRA1. Selected genes responsible for neuronal cell maintenance and cellular stress response were not differentially expressed in predisease retinas except *GFAP*, which appears to be as an important early feature of the disease.

Current perspectives on the genetic analysis of canine XLPRA1: The search for potential genetic modifier(s) of the XLPRA1 phenotype will continue. As the next step, we will conduct a genome-wide scan in the XLPRA1-affected pedigree to search for loci associated with the disease phenotype.

APPENDIX 1. XLPRA1 PEDIGREE.

To access the data, click or select the words "Appendix 1." H2 is a Siberian husky founder that was outcrossed to dogs from various breeds. The following abbreviations were used: (B) beagle, (N) mixed breed control, and (E) Norwegian elkhound-derived outcross. Severely affected dogs are in black, moderately affected dogs in dark gray, and mildly affected dogs in crosshatch pattern. Squares represent male and circles represent female. Dotted circles are carrier females that were not included in the phenotype analysis. (Figure was modified from our previously published article [22]).

APPENDIX 2. CHARACTERISTICS OF THE SNP MARKER PANEL AND ASSOCIATED PCR PRIMERS SEQUENCES

To access the data, click or select the words "Appendix 2." Note: *SNP positions are given according to UCSC Genome Browser on Dog May 2011 (Broad/canFam3) assembly; ** Mutation position in the protein

APPENDIX 3. REAL-TIME PCR PRIMERS USED IN THE STUDY.

To access the data, click or select the words "Appendix 3."

APPENDIX 4. GENOTYPE FREQUENCIES IN XLPRA1 AFFECTED DOGS WITH DIFFERENT SEVERITY PHENOTYPES.

To access the data, click or select the words "Appendix 4."

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