Exclusion of *RPGRIP1* ins44 from Primary Causal Association with Early-Onset Cone–Rod Dystrophy in Dogs

Tatyana Kuznetsova,¹ Simone Iwabe,¹ Kathleen Boesze-Battaglia,² Sue Pearce-Kelling,³ Yim Chang-Min,² Kendra McDaid,¹ Keiko Miyadera,¹ Andras Komaromy,¹ and Gustavo D. Aguirre¹

PURPOSE. Canine cone–rod dystrophy 1 (*cord1*) has been previously mapped to CFA15, and a homozygous 44-bp insertion in exon 2 (Ins44) of canine *RPGRIP1* (*cRPGRIP1*^{Ins/Ins}) has been associated with the disease. However, from the recent identification of a significant discordance in genotype–phenotype association, we have reexamined the role of *cRPGRIP1* in *cord1*.

METHODS. Retinal structure and function was assessed by clinical retinal examination, noninvasive imaging, electroretinography, and histopathology/immunohistochemistry. *cRPGRIP1* splicing was analyzed by RT-PCR. Retinal gene expression was determined by quantitative RT-PCR (qRT-PCR). Five markers spanning the entire *cRPGRIP1* were identified and used for haplotyping.

RESULTS. Electroretinography demonstrated that cone responses were absent or present in *cRPGRIP1*^{Ins/Ins} individuals. Moreover, performance in vision testing and optical coherence tomography (OCT) were comparable in *cRPGRIP1*^{Ins/Ins} dogs, regardless of the cone ERG status. While histologic changes in retinal structure were minimal, immunohistochemistry demonstrated a lack of cone opsin labeling in *cRPGRIP1*^{Ins/Ins} dogs. cDNA analysis revealed that Ins44 disrupts a putative exonic splicing enhancer that allows for skipping of exon 2, while retaining the functional RPGR-interacting domain (RID) of the protein. New *cRPGRIP1* sequence changes were identified, including a 3-bp deletion affecting the 3' acceptor splice site of alternative exon 19c. The extended haplotype spanning *cRPGRIP1* was identical in *cRPGRIP1*^{Ins/Ins} dogs with and without retinal degeneration. Gene expression analysis showed that expression levels were not associated with Ins44 genotype.

CONCLUSIONS. The results indicated that *cRPGRIP1* Ins44 is an unlikely primary cause of *cord1*, and that the causal gene and mutation are likely located elsewhere in the critical disease interval. (*Invest Ophthalmol Vis Sci.* 2012;53:5486–5501) DOI: 10.1167/iovs.12-10178

Cone–rod dystrophies (CRDs) represent a group of progressive, inherited blinding diseases characterized by primary dysfunction and loss of cone photoreceptors preceding or accompanied by rod death. Humans with CRD exhibit reduced visual acuity followed by severe loss of central and color vision that often progresses to complete blindness. The onset of clinical disease ranges from early to late adulthood. Mutations in more than 20 genes have been associated with CRD in human patients (Retinal Network RetNet: <http://www.sph.uth.tmc.edu/Retnet/disease.htm> University of Texas Houston Health Science Center, Houston, TX).

Canine models have been identified for a number of inherited retinal degenerations.^{1,2} In terms of CRD, the standard wire-haired dachshund, miniature long-haired dachshund (MLHD), Glen of Imaal terrier, and pit bull terrier are the only dog breeds thus far affected, and the genes involved have been identified in all but pit bull terriers.^{3–6} Of these, a severe early-onset CRD (*cord1*) was described in MLHDs⁵ and the locus mapped to a region of canine chromosome 15 (CFA15) orthologous to human chromosome 14 (HSA14). This region includes *RPGRIP1*, which codes for the retinitis pigmentosa GTPase regulator (RPGR)–interacting protein, a ciliary protein important for maintenance and function of photoreceptor cilia.^{7,8} Mutations in *RPGRIP1* in humans are associated with Leber congenital amaurosis⁹ and recessive cone–rod dystrophy.¹⁰

Sequence analysis of *cRPGRIP1* in diseased dogs have identified a 44-nucleotide insertion in exon 2 (Ins44) that is proposed to generate a frameshift and premature stop codon in exon 3.⁵ The strong phenotype–genotype correlation between the mutation and the disease in the original study colony suggests that the aberrant gene product promotes *cord1* in MLHD. However, subsequent studies in a subset of the general MLHD pet population have shown that approximately 16% of apparently normal controls were, in fact, *RPGRIP1* Ins44 homozygous mutants (*RPGRIP1*^{Ins/Ins}).¹¹ Furthermore, ophthalmic and ERG testing of *RPGRIP1*^{Ins/Ins} MLHDs in the UK pet population have indicated that homozygosity is not invariably associated with early-onset *cord1*.¹² As well, the Ins44 insertion or its variant has also been found in dog breeds that are not predisposed to CRDs, for example, Beagle, English springer spaniel (ESS), Labrador retriever, and French bulldog.¹¹ Indeed, 42% of >1100 ESSs tested are *RPGRIP1*^{Ins/Ins} (provided in the public domain by the English Springer Spaniel Field Trial Association, http://www.essfta.org/Health_Research/prs_news.htm), yet less than 0.6% of

From the ¹Section of Ophthalmology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; the ²School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; and ³OptiGen LLC, Cornell Business and Technology Park, Ithaca, New York.

Supported by National Eye Institute, National Institutes of Health Grants EY-06855, -010420, -13132, -17549, -018705, P30 EY-001583, Foundation Fighting Blindness center grant and Career Development Award (KM), Van Sloun Fund for Canine Genetic Research, Hope for Vision, and The ONCE International Prize for R&D in Biomedicine and New Technologies for the Blind.

Submitted for publication May 10, 2012; revised July 10, 2012; accepted July 11, 2012.

Disclosure: T. Kuznetsova, None; S. Iwabe, None; K. Boesze-Battaglia, None; S. Pearce-Kelling, OptiGen LLC (E); Y. Chang-Min, None; K. McDaid, None; K. Miyadera, None; A. Komaromy, None; G.D. Aguirre, OptiGen LLC (C), P

Corresponding author: Gustavo D. Aguirre, School of Veterinary Medicine, University of Pennsylvania, 3900 Delancey Street, Philadelphia, PA 19104; gda@vet.upenn.edu.

FIGURE 1. Pedigree of research colony informative for *cRPGRIP1* Ins44. The dogs with clinical retinal degeneration are indicated by **. Dogs with absent 30-Hz ERG cone flicker responses are indicated by *oblique black arrows*; *oblique gray dashed arrow* indicates dog (R28) with markedly reduced but still present 30-Hz ERG cone flicker responses. All dogs with cone ERG abnormalities are *cRPGRIP1*^{Ins/Ins}, but two dogs with this genotype (R17 and R42) have cone responses.

18,129 dogs examined between 2000 and 2009 are affected with clinically evident inherited retinal degeneration.¹³

Together, these results suggest that there is not a simple cause and effect relationship between the *cRPGRIP1* Ins44 sequence change and *cord1*. While it remains possible that the Ins44 represents a predisposing factor for disease, it seems more likely that Ins44 is a benign polymorphism, and that a second mutation in *cRPGRIP1*, or a different gene within the mapped *cord1* critical region, is causal for disease. As such, it could interact with a recently identified second locus to determine the early-onset form of the disease.¹⁴

Previously we have characterized the structure, organization, and expression of the *cRPGRIP1* reference sequence along with five alternatively spliced variants and showed a complex 5'- and 3'-splicing pattern.¹⁵ With this information in hand, we set out to determine how *cRPGRIP1* mutations and alteration in expression of *cRPGRIP1* isoforms contribute to *cord1* pathogenesis in dogs.

MATERIALS AND METHODS

Study Dogs

Several different populations of dogs were used for the studies. In all cases, the research was conducted in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with Institutional Animal Care and Use Committee (IACUC) approval.

***cord1* Colony.** Experimental dogs were bred and maintained at the Retinal Disease Studies Facility (RDSF), Kennett Square, Pennsylvania. The colony was established by outcrossing two purebred MLHDs to unrelated mixed breed dogs (Fig. 1). Semen from one of the founders (C-UK1) was obtained from the Animal Health Trust (AHT), Lanwades Park, Newmarket, United Kingdom. C-UK1 is *RPGRIP1*^{Ins/Ins} and affected with an early-onset generalized retinal degeneration.⁵ The dog had 30-Hz cone flicker ERG responses that were very low in

amplitude, but not absent when first recorded, and decayed exponentially at later ages (C. Mellersh, personal communication, July 11, 2011; see Fig. 4 “Casper” in Turney et al.¹⁶ [2007]). C-UK1 was outcrossed to an unrelated normal, mixed breed female (N212), and intercross matings were subsequently performed.

The second colony founder (R10) was a female *RPGRIP1*^{Ins/Ins} MLHD affected with advanced end-stage retinal degeneration at 2.6 years of age (Figs. 1, 2A); the retinal vessels were attenuated or unapparent ophthalmoscopically, and the tapetal region of the fundus was hyperreflective. Clinically, R10 was severely impaired visually, with widely dilated pupils that were poorly responsive to light. Because of her advanced retinal degeneration and visual impairment, an ERG was not recorded. At the time of donation for research investigations, R10 had been bred to a male *RPGRIP1*^{+/+} (wild type) MLHD (R9) that had a severe visual impairment from a retinal disease clinically different from *cord1*, and considered to be acquired and nonhereditary (asymmetric fundus abnormalities: end-stage retinal degeneration in right eye and midstage disease in the left eye; the left eye had a flat retinal detachment with multiple retinal holes and multifocal retinal scars indicative of diffuse, healed chorioretinitis of inflammatory origin). Five progeny (R11–R15) resulted from this mating; all were *RPGRIP1*^{+/Ins}, clinically normal when first examined at 6 months of age, and remained normal subsequently (most recent examinations at 2.6–4.0 years of age). Subsequent matings among the progeny of these founders formed the basis of this study (Fig. 1).

Purebred and Other Dogs. In addition to the study colony, 20 MLHDs, 11 ESSs, and 1 English cocker spaniel (ECS) were included for genotype analysis of *cRPGRIP1*, and genotype-phenotype correlation (Table 1). The samples from the pet population were accompanied by documentation of ophthalmic examination and clinical diagnoses by board-certified veterinary ophthalmologists (diplomates, the American College of Veterinary Ophthalmologists [ACVO]) made through the Canine Eye Registration Foundation program. Thirty additional dogs, including 12 Siberian huskies, 8 Beagles, and 10 Papillons were also tested for Ins44. Of 10 Papillons analyzed, 5 were normal and 5 had retinal degeneration. The Siberian huskies and Beagles were from an X-linked retinal degeneration pedigree (XLPR1; 5-nt *RPGR*^{RF15} micro-

TABLE 1. Ins44/D3 Genotype and Clinical Retinal Phenotype for Dogs Examined

Dog	Ins44 Genotype*	D3 Genotype*	Clinical Retinal Phenotype-Inherited Retinal Degeneration	Clinical/ERG Examination Age (y)	Pathology-IHC, Age (y)	Breed†
Research colony						
R1	12	12	Normal fundus; no ERG performed	4.4		MLHDx
R2	12	12	Normal fundus; no ERG performed	4.4	4.4	MLHDx
R4	12	12	Normal fundus; rod/cone ERG responses	0.4		MLHDx
R5	22	22	Normal fundus; absent cone ERG	3.1/0.4		MLHDx
R6	12	12	No eye exam; rod/cone ERG responses	0.4		MLHDx
R7	12	12	No eye exam; rod/cone ERG responses	0.4		MLHDx
R8	12	12	Normal fundus; rod/cone ERG responses	2.9/0.4	2.9	MLHDx
R9	11	12	Acquired retinal disease—detachment, inflammation, degeneration/no ERG performed	2.8		MLHD
R10	22	22	Generalized retinal degeneration; no ERG performed	2.6		MLHD
R11	12	22	Normal fundus; no ERG performed	0.5		MLHD
R12	12	22	Normal fundus; rod/cone ERG responses	1.8		MLHD
R13	12	22	Normal fundus; rod/cone ERG responses	2.6		MLHD
R14	12	12	Normal fundus; no ERG performed	4.0		MLHD
R15	12	12	Normal fundus; no ERG performed	0.5		MLHD
R16	12	12	Normal fundus; rod/cone ERG responses	0.6/0.4		MLHDx
R17	22	22	Normal fundus; rod/cone ERG responses	1.9/1.8		MLHDx
R18	22	22	Normal fundus; absent cone ERG	0.6/0.4		MLHDx
R21	22	22	Normal fundus; absent cone ERG	0.6/0.4	0.7	MLHDx
R22	12	12	Normal fundus; rod/cone ERG responses	0.6/0.4	0.7	MLHDx
R25	11		Normal fundus; rod/cone ERG responses	0.4/0.5		MLHDx
R26	12	12	Normal fundus; rod/cone ERG responses	1.5/1		MLHDx
R27	12	12	Normal fundus (focal choroidal coloboma-OS); rod/cone ERG responses	1.4/1.4	1.6	MLHDx
R28	22	22	Normal fundus; cone ERG responses markedly reduced	1.5/1.5		MLHDx
R29	22	22	Normal fundus; absent cone ERG	1.5/1.3	1.5	MLHDx
R30	12		Normal fundus; rod/cone ERG responses	1.1/1		MLHDx
R35	12	12	Normal fundus; no ERG performed	1.4		MLHDx
R36	12	12	No eye exam; rod/cone ERG responses	1/0.7		MLHDx
R42	22	22	Normal fundus; rod/cone ERG responses	1/1		MLHDx
R46	22	22	Normal fundus; absent cone ERG	1/1		MLHDx
Pet population‡						
Caroline	22	22	Normal fundus	3.5		MLHD
Sandy	12	12	Normal fundus	4.4		MLHD
Clair	22	22	PRA suspicious	1.3		MLHD
Kaylee	12	12	Normal fundus	12		MLHD
Molson	22	22	PRA	4.4		MLHD
Corby	12	12	Normal fundus	8.8		MLHD
Josie	22	22	Normal fundus	1		MLHD
Maura	12	12	Normal fundus	6		MLHD
Miper	12	12	Normal fundus	6		MLHD
Bella	11	12	Normal fundus	1		MLHD
08-7988	11	12	Normal fundus	7.5		MLHD
08-7989	11	12	Normal fundus	2.1		MLHD
08-7991	11	12	Normal fundus	5.7		MLHD
Melody	11	11	Normal fundus	11.4		MLHD
Chloe	11	12	Normal fundus	10.9		MLHD
Kaylee	11	11	Normal fundus	1.2		MLHD
Tori	11	22	Normal fundus	1		MLHD
Spice	11	22	Normal fundus	1		MLHD
Brandy	12	12	Normal fundus	7.1		MLHD
Hamilton	11	22	Normal fundus	2		MLHD
George	22	22	PRA	13.8		ESS
Stoney	12	12	PRA	6.1		ESS
Minnie	22	22	PRA	9.9		ESS
Keah	22	22	PRA	15.5		ESS
Stella	22	22	PRA	6.2		ESS
Emma	22	22	PRA	2.8		ESS

TABLE 1. Continued

Dog	Ins44 Genotype*	D3 Genotype*	Clinical Retinal Phenotype-Inherited Retinal Degeneration	Clinical/ERG Examination Age (y)	Pathology-IHC, Age (y)	Breed†
Holly	22	22	PRA	13		ESS
Genesis	22	22	Normal fundus	7		ESS
Mackenzie	22	22	Normal fundus	11.4		ESS
Kayla	22	22	Normal fundus	11		ESS
Tucker	22	22	Normal fundus	8.3		ESS
Louie	22	22	Normal fundus	1.8		ECS

IHC, immunohistochemistry; PRA, progressive retinal atrophy (equivalent to generalized retinal degeneration).

* Mutant allele = 2; wild-type allele = 1.

† MLHD-purebred and MLHDx-crossbreed from study pedigree.

‡ Retinal status was assessed by indirect ophthalmoscopy after pharmacologic mydriasis; ERG was not performed.

deletion) derived from an affected male dog outcrossed to unrelated normal mix bred or purebred females as previously described.¹⁷

Clinical Assessment and In Vivo Retinal Imaging

Routine ophthalmic examinations, including biomicroscopy, indirect ophthalmoscopy, and direct/indirect fundus photography, were conducted on a regular basis throughout the study. In 3 dogs that were retained for breeding, in vivo imaging was done under general anesthesia by using a combined confocal scanning laser ophthalmoscope/spectral-domain optical coherence tomography (cSLO/sdOCT) instrument (Spectralis HRA/OCT, Heidelberg, Germany). En face imaging was done with near-infrared or shortwave autofluorescent modes with the 55° lens, and overlapping images were acquired. sdOCT scans were done with the 30° lens in groups of parallel linear (“raster”) scans. In each retinal quadrant centered on the optic disc, a 30 × 20-degree area was imaged with 49 sequential B-scans, each separated by 120 μm. As well, single, high-resolution averaged ($n = 100$) sequential B-scans were taken in a 30° area (line) on the four cardinal points and centered in the optic disc. For sdOCT volume scans, each scanned image was the average of nine.

Electroretinography

Standard Ganzfeld scotopic and photopic ERGs were recorded from anesthetized dogs as described, with a modified Ganzfeld dome fitted with the light-emitting diode (LED) light stimuli of a ColorDome stimulator (Diagnosys LLC, Lowell, MA).¹⁸ The ERG was recorded with custom-made Burian-Allen bipolar contact lens electrodes (Hansen Labs, Coralville, IA) in monopolar mode, and platinum subdermal needle electrodes (Grass Safelead needle electrodes; Grass Technologies, West Warwick, RI) by using the Espion E² computer-based system (Diagnosys Systems, Inc., Littleton, MA). The dogs were premedicated with subcutaneous acepromazine maleate (0.5 mg/kg) and atropine sulfate (0.02 mg/kg), and anesthetized with intravenous propofol followed by isoflurane inhalation anesthesia. Rod- and mixed rod-cone-mediated responses were recorded after 20 minutes of dark adaptation with scotopic single white flash stimuli of increasing intensities (from 0.000577 to 10.26 cd.s/m²). Following 5 minutes of light adaptation to a background illumination of 34.26 cd/m², 1-Hz single flash (from 0.00577 to 10.26 cd.s/m²) and 29.41-Hz flicker (from 0.00577 to 5.77 cd.s/m²) cone-mediated signals were recorded. Except for the brighter scotopic light stimuli (≥ 0.577 cd.s/m²), multiple responses were averaged.

Objective Vision Testing

We used an objective means for quantifiable assessment of vision in dogs. The obstacle-avoidance course is 3.6-m long and has six panels, which are varied in position in a random order for every test run. Details of the apparatus and method of testing have been

described; the testing modality permits quantitative detection of absent photopic vision in dogs with achromatopsia, and the recovery of cone function following gene therapy.^{18,19} The luminance used for testing has now been expanded to lower light levels, and vision is tested at 0.003, 0.2, 1, 65, and 646 lux.

Tissue Processing, Histology, and Immunohistochemistry

Eyes from selected dogs were collected immediately after enucleation and fixed in formaldehyde-containing Excalibur Alcoholic Z-Fix solution (Excalibur Pathology, Inc., Oklahoma City, OK) for 72 to 96 hours, and transferred to 70% ethanol. Tissue sections from the central (pupil-optic nerve) axis were cut in the dorsoventral plane and stained with hematoxylin-eosin (H&E). Unstained sections were deparaffinized by heat and xylene extraction before serial rehydration in decreasing ethanol concentrations. For immunohistochemistry, deparaffinized sections were incubated with primary antibodies in a solution 0.025% Triton X-100 and 1.5% normal goat serum in PBS overnight followed by incubation with appropriate fluorescent secondary antibodies (Alexa Fluor Dyes; Molecular Probes, Invitrogen, Carlsbad, CA). Primary polyclonal antibodies included antibodies against R/G cone opsin (anti-opsin red-green, 1:2000, catalog No. AB 5405; Chemicon [Millipore], Temecula, CA), human cone arrestin (1:10,000; LUMif; provided by Cheryl Craft, Doheny Eye Institute, University of Southern California, Los Angeles, CA), and rod opsin (1:1000, catalog No. MAB 5316; Chemicon [Millipore]); 4',6-diamidino-2-phenylindole (DAPI) stain was used to label cell nuclei. Slides were mounted with fluoromount G mounting media (catalog No. 01100-01; Southern Biotech, Birmingham, AL), cover-slipped, and examined by epifluorescence microscopy with a Zeiss Axioplan microscope (Carl Zeiss Mediatech, Oberkochen, Germany) by using transmitted light or epifluorescence. 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.

Sample Collection, DNA/RNA Extraction, cDNA Synthesis

Retinas were collected under sterile and RNase-free conditions within 1 minute after euthanasia and enucleation, frozen by immersion in liquid nitrogen, and stored at -70°C until used. Total RNA was isolated from canine tissues by using TRIzol (Invitrogen) and a single chloroform extraction. First strand cDNA was synthesized in 20-μL reactions by 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 by using the QIAamp DNA kit (Qiagen, Valencia, CA) following the manufacturer's directions.

FIGURE 2. Fundus photographs of selected research colony dogs taken at different ages. Only dog R10 (A) has generalized retinal degeneration with marked vascular attenuation and tapetal hyperreflectivity. The retinas in the other dogs are clinically normal: R10, R17, and R29 are *CRPGRIP1*^{Ins/Ins}; R13, R14, and R30 are *CRPGRIP1*^{+/Ins}. See Table 1 for details on clinical examination results and ages.

PCR Amplification and Sequencing

Primer sequences for all PCR experiments are represented in Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-DCSupplemental>). PCR reactions were performed on 50 ng genomic DNA or cDNA generated from 50 ng DNAase-treated retinal RNA, in a final volume of 20 μ L containing 1 $\times$ PCR buffer (Qiagen), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 μ M forward and reverse primers, and 1 unit Platinum *Taq* DNA polymerase (Invitrogen). Cycling conditions were 5 minutes initial denaturation at 95°C followed by 35 cycles of 95°C for 20 seconds (denaturation), 55°C for 30 seconds (annealing), 68°C for 1 min/1000 bp (elongation). When necessary, PCRs were optimized by adding 6% Dimethyl sulfoxide (DMSO). PCR products were analyzed on a 6% polyacrylamide gel followed by ethidium bromide staining. For sequencing purpose, PCR products were analyzed on a 1.5% agarose gel, extracted with NucleoTrap Gel Extraction Kit (Clontech, Mountain View, CA) and directly sequenced.

DNA Marker Selection

Sequence differences identified in *CRPGRIP1* in individual dogs of different breeds were evaluated to select critical informative markers for haplotype assignment.

3'RACE

The analysis was performed as previously described.¹⁵

Relative Quantification (ddCt) Assay

Real-time PCR was performed in a total volume of 25 μ L by using 96-well microwell plates on the Applied Biosystems 7500 Real-Time PCR System. Amplification data were analyzed with the 7500 Software version 2.0.1 (Applied Biosystems). All PCRs were performed in microwells by using cDNA generated from 50 ng DNAase-treated retinal RNA from 5-month-old (2 females) and 7-month-old (1 male) normal dogs, 7-month-old *RPGRIP1*^{Ins/Ins} (R18, male; R21, female) dogs, and 7-month-old *RPGRIP1*^{+/Ins} (R16, male; R22, female) dogs. The SYBR green platform was used for gene expression analysis of nine *RPGRIP1* splice variants by using a primer concentration of 0.15 μ M. Primer sequences for amplicons 1-10EX, 19-19aEX, 21-22EX, and 19c-2 are listed in Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-DCSupplemental>). Primer sequences for amplicons 2-5a, 2-5b, 2-5c and for the reference sequence used have been previously described.¹⁵ The TaqMan platform (Applied Biosystems) was used for the analysis of eight photoreceptor-expressed genes. *SAG* and *GFAP* primers and TaqMan Gene Expression Assay probes

FIGURE 3. ERG data for MLHD dogs with different *Ins44* genotypes. ERGs were recorded at 0.4 years of age (R4–R8; *top panel*), and at 0.4 years (R16–R22) or 0.3 years (R25; *lower panel*). All *cRPGRIP1*^{Ins/Ins} dogs are also homozygous for the D3 deletion. Among the *cRPGRIP1*^{Ins/Ins} dogs, R5, R18, and R21 lack recordable cone ERG responses, but these are present in R17.

(Cf02628845_m1 and Cf02655695_m1, respectively) were used in the analysis. Primer and probe sequences for *OPN1SW*, *OPN1LW*, *CNGB3*, *RHO*, *CNGA1*, and *CNGB1* have been previously validated and published (see Table S1 in Komaromy et al.¹⁸ [2010]). The *GAPDH* primers and TaqMan Gene Expression Assay probe (Hs02786624_g1) were used to normalize the cDNA templates.

Western Blot

Retinas (R18-*cRPGRIP1*^{Ins/Ins}, 0.6 years; R16-*cRPGRIP1*^{+/Ins}, 0.6 years; controls ($n = 2$), 7 and 16 weeks) were lysed in RIPA buffer (Abcam, Cambridge, MA) homogenized, and gently sonicated. Debris was pelleted at 13,500 rpm (15 minutes, 4°C) and the resulting cleared lysate was for Western blot analysis. Equal amounts of protein (35 µg per lane) were separated on a 4% to 12% Bis-Tris gel, transferred onto a nitrocellulose membrane, blocked with generic protein (milk), and probed with anti-*RPGRIP1* antibody Ab38 against the conserved *RPGR*-interacting domain (RID) of *RPGRIP1*^{7,20} at a 1:500 dilution. Secondary antibody used was horseradish peroxidase (HRP) anti-rabbit (31432; Thermo Scientific, Rockford, IL) at 1:1000 dilution. The membrane was imaged with a Kodak imaging station (Carestream Health, Inc., Rochester, NY).

RESULTS

Exclusion of *RPGRIP1* *Ins44* as the Primary Causative Mutation for *cord1*

To assess the role of *cRPGRIP1*^{Ins/Ins} in *cord1*, we used dogs from our research colony, formed from two unrelated MLHDs that were *cRPGRIP1*^{Ins/Ins} and had early-onset retinal degeneration (Fig. 1, C-UK1 and R10). The resulting pedigree of dogs studied is presented in Figure 1. The genotype of all dogs was confirmed by PCR.

Clinical Findings. Ophthalmic examinations were performed, and results of the most recent examination are presented in Table 1. All 16 *cRPGRIP1*^{+/Ins} dogs were clinically normal at all examination time points. Notably, eight *cRPGRIP1*^{+/Ins} dogs were examined at >1.5 years of age, and none showed any clinical evidence of retinal degenerative disease (Figs. 2B, 2C, 2F, and Table 1). Of the eight *cRPGRIP1*^{Ins/Ins} dogs produced for this study, four were descendants of founder male C-UK1 (R5, R17, R18, R21), and four were intercross progeny descending from both founders (R28, R29, R42, R46) (Fig. 1). In contrast to previous results in

FIGURE 4. ERG and objective vision testing in *CRPGRIP1^{Ins/Ins}* dogs. **(A)** Both dogs have rod-mediated responses that show minimal to no changes at the two time points examined. Cone 30-Hz flicker responses are absent in one of the dogs. **(B)** Objective vision testing evaluated with an obstacle avoidance course. The graph shows the transit time in seconds for dogs R42 (white bar) and R46 (black bar) as a function of ambient light intensity; results are shown for the 6-, 8-, and 10-month time periods. Both dogs show comparable and normal visual performance under scotopic and photopic illumination, and no change with time. Values in gray are the mean $\pm$ 1 SD for transit times in normal dogs.¹⁹

the original AHT colony,⁵ retinal degenerative changes were *not* observed in any of the dogs of any age, even those one year old or older (R42, R46, R28, R29, R17, and R5; Figs. 2D, 2E, and Table 1).

Electroretinography. ERGs were recorded from selected dogs to assess retinal functional abnormalities at different ages (Fig. 3, Table 1). Of the *CRPGRIP1^{Ins/Ins}* dogs, five (R5, R18, R21, R29, R46) had nonrecordable cone-mediated 30-Hz flicker responses and one had markedly reduced amplitude (R28). However, two dogs (R17, R42) had cone-mediated 30-Hz flicker responses that were normal in waveform, but with amplitudes that were slightly lower than some *CRPGRIP1^{+/Ins}* or wild-type controls (Fig. 3). In addition, rod-mediated, and mixed rod-cone responses were similar among *CRPGRIP1^{+/Ins}* and *CRPGRIP1^{Ins/Ins}* dogs, although the amplitude was slightly lower than what was observed in the limited number of wild-type control dogs. However, even within the *CRPGRIP1^{+/Ins}* group, there was variability of response amplitudes among dogs (e.g., compare the 30-Hz cone-mediated flicker responses of R8 and R16 [relatively higher amplitude] to those of dogs R6 and R22 [relatively lower amplitude] in Fig. 3).

Finally, to determine if there was any age-related decrement in ERG function, recordings were repeated in four *CRPGRIP1^{Ins/Ins}* dogs: R5, R17, R42, and R46. Such analysis revealed that responses remained virtually unchanged over time in dogs with demonstrable cone-mediated ERG responses (R17 between 0.7 and 1.8 years; R42 between 0.3 and 1 year). In dogs with nonrecordable cone-mediated ERG responses, rod-mediated responses remained consistent over time (R5 at 0.4 and 3.1 years; R46 at 0.3 and 1 year) (Figs. 3, 4, and Supplementary Fig. S1 [http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-/DCSupplemental]).

Objective Vision Testing. To objectively assess vision in *CRPGRIP1^{Ins/Ins}* dogs, dogs with recordable (R42) or nonrecordable (R46) 30-Hz cone-mediated responses were selected for training in an obstacle avoidance course. Testing was done every 4 to 8 weeks between 4 and 10 months of age, and ERGs were performed at 4-month and 1-year time points (Fig. 4). Both dogs performed equally well in the test, and there was no decrease in performance over time. Note that dogs with achromatopsia and total absence of cone function due to *CNGB3* mutations have significant increases in obstacle course

FIGURE 5. Retinal photomicrographs of 0.7-year-old *cRPGRIP1*^{+/Ins} (R22, **A1–A4**) or *cRPGRIP1*^{Ins/Ins} (R21, **B1–B4**) dogs. Images are taken from region near and inferior to the optic disc (*area 1*), and stained with H&E (**A1, B1**) or immunolabeled with anti-rod opsin (**A2, B2**), hCAR (**A3, B3**), or R/G opsin (**A4, B4**) antibodies. DAPI nuclear stain was used with the immunolabeling, but only shown in half of the images. In the central regions, retinal integrity is normal in the *cRPGRIP1*^{+/Ins} or *cRPGRIP1*^{Ins/Ins} dogs (**A1, A2**), and opsin immunolabeling is comparable although focal delocalization of opsin is present (**B2, arrow**). In the control, hCAR (**A3, oblique arrows**) and R/G opsin (**A4**) antibodies label the cones. In contrast, the *cRPGRIP1*^{Ins/Ins} dog shows that hCAR labels only the outer perinuclear region of cone somata (**B3, short arrows**) and synaptic terminals, but labeling of cone IS and OS is not present, and only one cone OS labels with R/G opsin antibody (**B4, arrow**). Calibration marker in **A1** = 20 μ m and applies to all figures except those highlighted by boxed region in **A3, A4, B3, B4**, which is shown to the *right* of the main panels (calibration marker = 5 μ m). GCL, ganglion cell layer; INL, inner nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium.

transit times at the 65 and 646 lux intensities, which are reversed to normal following corrective gene therapy.^{18,19}

Retinal Histopathology. To determine if *cRPGRIP1*^{Ins/Ins} was associated with retinal abnormalities, eyes from six study dogs, four *cRPGRIP1*^{+/Ins} and two *cRPGRIP1*^{Ins/Ins}, were examined; both *Ins44* homozygotes had absent cone ERG function. The four *cRPGRIP1*^{+/Ins} dogs (age range, 0.7–4.4 years) had normal retinal structure as illustrated for the 0.7-year-old dog (Figs. 5A, 6A). Retinas from the two *cRPGRIP1*^{Ins/Ins} dogs (aged 0.7 and 1.5 years) were similar, although specific regional abnormalities were observed (Figs. 5B, 6B). To further evaluate such abnormalities, morphologic assessment was made at six defined locations in the superior (S) and inferior (I) meridians: Superior area 1 (S1) and inferior area 1 (I1) were within 1000 μ m of the optic disc edge; superior area 2 (S2) and inferior area 2 (I2) were at the midpoint ($\pm$ 500 μ m) between the optic disc and ora serrata; and superior area 3 (S3) and inferior area 3 (I3) were within 1000 μ m of the ora serrata. Both *cRPGRIP1*^{Ins/Ins} dogs had normal to near-normal preservation of retinal structure near the optic disc (*area 1*; compare Figs. 5A1 to 5B1); however, the inner and outer segments were shortened and compressed, with occasional macrophages present in the subretinal space in areas 2 and 3 (Figs. 6B1, 6B2; 6C, respectively). These abnormalities were not progressive, as there was no further loss of photoreceptor cells during the 0.7- to 1.5-year time period (Fig. 7).

To evaluate the localization of photoreceptor proteins, single immunolabeling and DAPI nuclear staining were carried out following paraffin removal and dehydration of the sections. The fixative used permits excellent immunolabeling of rod opsin, while preserving labeling of cones with hCAR and R/G opsin antibodies.²¹ For other antibodies, it is marginally or not effective. The immunolabeling results of *cRPGRIP1*^{+/Ins} used as controls were comparable to those obtained previously in retinas of *cRPGRIP1*^{+/+} dogs (data not shown).

Retinal area 1 showed similar rod opsin immunolabeling in *cRPGRIP1*^{+/Ins} and *cRPGRIP1*^{Ins/Ins} dogs, with opsin labeling predominantly restricted to the outer segment layer (Figs. 5A2, 5B2). While an occasional rod cell showed opsin mislocalization in area 1 of *cRPGRIP1*^{Ins/Ins} dogs, areas 2 and 3 showed more extensive opsin mislocalization, with opsin also localized to the outer nuclear layer (ONL) and synaptic terminals (areas 2 and 3; Figs. 6B2, 6C). In the *cRPGRIP1*^{+/Ins} dog, antibodies directed against cones labeled the outer/inner segments, perinuclear region, and synaptic terminals (hCAR), or outer segments (R/G cone opsin) (Figs. 5A3, 5A4). However, in the *cRPGRIP1*^{Ins/Ins} dog, the cone outer and inner segments did not label with hCAR, and labeling was concentrated in the apical perinuclear region. In addition, R/G cone opsin-positive outer segments were rarely evident even in area 1, the region that appeared normal in H&E

FIGURE 6. Retinal photomicrographs of 0.7-year-old *cRPGRIP1*^{+/Ins} (R22, A1–A2) or *cRPGRIP1*^{Ins/Ins} (R21, B1–B2, C) dogs. Images are taken from *area 2*, midpoint between the optic disc and ora serrata (A1–A2, B1–B2), or from the periphery (*area 3*, C). Sections stained with H&E (A1, B1) or immunolabeled with anti-rod opsin (A2, B2, C) antibodies. DAPI nuclear stain was used with the immunolabeling, but only shown in half of the images. In the *cRPGRIP1*^{Ins/Ins} dog, there is progressive shortening and loss of rod outer segments, and opsin delocalization into the ONL (B2, C, oblique arrows and *, respectively). A subretinal macrophage is present in areas of rod outer segment shortening and loss (B1, arrowhead). Calibration marker in A1 = 20 μ m and applies to all figures. NFL, nerve fiber layer; PR, photoreceptor layer.

sections and had minimal rod opsin mislocalization (Figs. 5B3, 5B4).

In Vivo Retinal Imaging. Noninvasive in vivo retinal imaging using high-resolution sdOCT was undertaken to assess retinal integrity in dogs retained for breeding. For this we used one *cRPGRIP1*^{+/Ins} (R27) and two *cRPGRIP1*^{Ins/Ins} dogs, one with normal (R17) and one with absent (R5) cone ERG responses. Qualitatively, both the 30 $\times$ 20-degree area parallel linear scans of each quadrant centered on the optic disc (data not shown) and the single high-resolution averaged scans (Fig. 8) were normal and were similar to scans of the heterozygous control and other nonaffected dogs (data not shown). However, direct measurements of ONL thickness indicated that the heterozygous control had a thicker ONL in the S and I quadrants near the optic nerve head (S = 62 μ m per I = 53 μ m) than did the two *cRPGRIP1*^{Ins/Ins} dogs (R17: S = 50 μ m per I = 43 μ m; R5: S = 49 μ m per I = 41 μ m). These differences could be associated with the Ins44 genotype irrespective of the phenotype, or could result from aging, as the control dog was the youngest of the ones imaged. The histopathology and in vivo retinal

imaging studies in *cRPGRIP1*^{Ins/Ins} dogs demonstrated that photoreceptors are not progressively lost over time, at least within the time frame (between 1.5 and 3.5 years) of the current study.

Ins44 Mutation Produces Skipping of Exon 2, but Does Not Prevent Expression of RPGRIP1 RID Domain. The studies described above do not support a causative role for the Ins44 in *cord1*. However, owing to the vital role of RPGRIP1 in photoreceptor function, we decided to determine if Ins44 mutation is indeed associated with the generation of a stop codon, as originally predicted.⁵ Molecular analysis of *cRPGRIP1* retinal transcripts indicated that Ins44 was associated with exon 2 skipping, which eliminated the mutation from the transcript (Fig. 9A). Skipping of exon 2 in the 344-bp RT-PCR product was verified by direct sequencing of the product (Fig. 9B). In silico translation of the aberrant transcript, that is, the reference sequence without exon 2, predicts that it encodes a protein product nearly identical to the reference full-length protein, but with a truncated 5'-region and the putative start codon (first Met) now encoded by exon 5.

FIGURE 7. Outer nuclear layer counts from *crPGRIP1*^{Ins/Ins} (R21, R29) and *crPGRIP1*^{+/Ins} (R22, R8) dogs. Y-axis values represent the mean of three ONL nuclear layer thickness counts within a single microscopic field per location; for each dog, four defined retinal locations (two areas in the *superior* and two areas in the *inferior meridian*) are shown. There are no apparent differences between the two genotypes, or between dogs of different ages.

Because mutation-associated exon skipping is predominantly associated with disruption of an exonic splicing enhancer (ESE), we attempted to determine if Ins44 interrupts an ESE motif. To do so, we analyzed the sequence of exon 2 with the ESE prediction software RESCUE-ESE.²² Among the ESEs predicted in this region, one (hexamer GATGAG) is disrupted by the Ins44 (GATG-Ins44-AG), and therefore could provide a putative molecular mechanism leading to exon 2 skipping in *crPGRIP1*.

Western blot analysis was then undertaken on retinal tissue extracts from 7-month-old *crPGRIP1*^{+/Ins} and *crPGRIP1*^{Ins/Ins} dogs, and 7- and 16-week-old wild-type samples, using antibody Ab38 specific for the conserved RID domain of RPGRIP1 (Fig. 9C). Ab38 has been previously shown to identify approximately 33-, 45-, and 160-kDa protein isoforms in canine retina, but the 160-kDa isoform is clearly observed only upon longer exposure of the blot.²⁰ In our hands, two predominant RPGRIP1 immunoreactive isoforms with apparent masses of 37 kDa and 45 kDa were detected in retinal extracts of all samples tested, but the 160-kDa band was not visible. Thus, despite exon 2 skipping in *crPGRIP1*^{Ins/Ins} dogs, the resulting protein pattern detected by Ab38 is the same as in *crPGRIP1*^{+/+} and *crPGRIP1*^{+/Ins} retinal samples, and the RPGR-interacting domain, critical for RPGRIP1 function, is preserved.

Lack of Correlation between Ins44 and Retinal Gene Expression. Although our results indicate that production of the aberrant C-terminally truncated protein is highly unlikely, Ins44 could still affect the expression of *crPGRIP1* isoforms, and directly or indirectly contribute to the disease. To address this possibility, expression studies were carried out on total retinal RNA from *crPGRIP1*^{Ins/Ins} (R18, R21), *crPGRIP1*^{+/Ins} (R16, R22), and three nonaffected wild-type dogs to examine the relative expression level of nine *crPGRIP1* splice variants and other photoreceptor-specific genes. The samples analyzed came from littermate dogs (R16, R18, R21, R22) that had normal clinical retinal examination findings, but absent 30-Hz cone ERG signals (R18 and R21) (Figs. 1, 3). In addition, dogs R21 and R22 were used for the morphologic studies that showed cone and midperipheral/peripheral rod abnormalities in R22 (see Figs. 5, 6).

We first investigated the expression profile of nine *crPGRIP1* splice variants; owing to the substantial heterogeneity, gene expression data for each dog are presented

FIGURE 8. In vivo images of retinal structure of *crPGRIP1*^{+/Ins} (A) and *crPGRIP1*^{Ins/Ins} (B, C) dogs. Each row of images compares the near-infrared en face view (left panel) with a spectral domain OCT image from the same location. The vertical green arrow in each en face image indicates the specific location illustrated by sOCT and presented in the right-adjacent panel. Retinal layer organization is similar in all dogs INL, inner nuclear layer; ONL, outer nuclear layer.

individually (Fig. 10A). As expected, Ins44 homozygous dogs showed negligible 1/2-3EX transcript levels, while transcript levels in Ins44 heterozygotes were reduced by approximately 0.4- to 0.8-fold from control values. The lowest levels of 2-5a, 2-5b, 2-5c, and 21-22EX transcripts were observed in both *crPGRIP1*^{Ins/Ins} retinas and one *crPGRIP1*^{+/Ins} retina. Isoform 19c-2 was reduced in the two male dogs, irrespective of their Ins44 genotype. The other *crPGRIP1* isoforms showed expression levels comparable to normal control in female retinas and decreased expression level in male regardless of Ins44 genotype. The results suggest that Ins44 does not change the transcription of *crPGRIP1* but affects only splicing of exon 2, and excludes any evidence of nonsense-mediated decay pathway.²³⁻²⁵

FIGURE 9. Analysis of splicing pattern and protein expression of *cRPGRIP1* in *cRPGRIP1^{Ins/Ins}* dogs. (A) Analysis of splicing variants of *cRPGRIP1* by RT-PCR in *cRPGRIP1^{Ins/Ins}* and wild-type retinas. Primers 1EX, F and 5EX, R were used for the experiment (see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-/DCSupplemental>). In *cRPGRIP1^{Ins/Ins}*, exon 2 is “skipped” during splicing and is not included in the final processed mRNA. The truncated PCR product is indicated by *white arrow*. Heteroduplex formed during PCR from mixtures of DNA templates is indicated by *asterisk*. (B) Sequence of the truncated cDNA containing exons 1 and 3 spliced together. (C) Western blot analysis performed with Ab38 antibody against RPGRIP1 RID domain did not show a significant difference between *cRPGRIP1^{Ins/Ins}*, *cRPGRIP1^{+/Ins}*, and wild-type dogs. NTC, no template control.

FIGURE 10. Gene expression analysis in *Ins44*-homozygous and heterozygous dogs compared to normal wild-type control. (A) Relative quantification of nine *cRPGRIP1* splice variants expressed in the retina: 5- and 7-month-old controls ($n = 3$), 7-month-old *Ins44*-homozygous (R18, male; R21, female) and heterozygous (R16, male; R22, female) dogs. (B) Relative quantification of cone-expressed (*OPN1SW*, *OPN1LW*, *CNGB3*), rod-expressed (*RHO*, *CNGA1*, *CNGB1*), photoreceptor/pineal gland-specific (*SAG*), and astrocyte/Müller cell-specific (*GFAP*) gene markers to characterize photoreceptor gene expression in the corresponding dogs. Note: Because of heterogeneity in gene expression levels among the four siblings, the gene expression data are presented individually.

FIGURE 11. Analysis of *cRPGRIP1* splicing pattern in D3-homozygous dogs. (A) Localization of primers (F, Rev1-4) used in RT-PCR. (B) RT-PCR performed in wild-type dog produced multiple transcripts. Several new exons were identified (for exon boundaries see Supplementary Table S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-DCSupplemental>). In D3-homozygous retina, the number of transcripts detected is greatly reduced in comparison to normal control. PCR products detected are listed only by number because of space limitation. (C) Schematic representation of transcripts detected by RT-PCR in wild-type and D3-homozygous dogs. Asterisks represent position of a predicted stop codon. Note: Supplementary Table S2 indicates how transcript numbers in Figure 11C correspond to PCR product numbers in Figure 11B. (D) 3'RACE data show absence of the main transcript having alternative exon 19c. Asterisk shows PCR products heteroduplex.

We next examined the expression of three cone-specific (*OPN1SW*, *OPN1LW*, *CNGB3*), four rod-specific (*SAG*, *RHO*, *CNGA1*, *CNGB1*), and one inner retinal (*GFAP*) gene, the expression of which are known to be altered in a variety of inherited retinal degenerations. With the exception of *GFAP*, which showed increased expression in 3 of the 4 retinas examined (two homozygous [R18, R21] and one heterozygous [R22]), the expression patterns for these genes generally paralleled those of *cRPGRIP1* isoforms in that the two retinas with the lowest levels of *cRPGRIP1* expression (one homozygous and one heterozygous male) also had lower expression of rod- or cone-specific genes (compare Fig. 9A with Fig. 9B). Analysis of additional heterozygous dogs did not show sex-related differences (data not shown). The results showed that *Ins44* affects expression of only a restricted set of *cRPGRIP1* isoforms,

which normally have exon 2 in their structure. Moreover, the relative abundance of the remaining *RPGRIP1* transcripts, as well as transcripts from eight photoreceptor-specific genes analyzed, was not associated with *Ins44* genotype.

Exclusion of the *RPGRIP1* Gene from Direct Causal Association with *cord1*

Identification of a Novel Mutation Affecting Splicing of 3'-Terminal Alternative Exon 19C. We next assessed if alternative mutations in the *cRPGRIP1* gene could instead be responsible for the disease, either independently of or synergistically with *Ins44*. To this end, we identified a homozygous three-nucleotide deletion (D3) in *cRPGRIP1*^{*Ins/Ins*} dogs that eliminates

TABLE 2. Sequence Changes Identified in Canine *RPGRIP1* Gene

Location of Sequence Change	Nucleotide Change	Amino Acid Change	Breed
Promoter (P1)	–197C > T		Siberian Husky, Beagle
Intron 1	c.19+19T > A		MLHD/MLHDx, ESS, ECS
Exon 2	c.142_143ins44		MLHD/MLHDx, ESS, ECS
Intron 2	c.152+186_227del42		Siberian Husky
Intron2	c.152+227_228ins205		Siberian Husky
Exon 14	c.1740C > T	p.P580P	Siberian Husky
Exon 17	c.2548G > A	p.E850K	Siberian Husky, Beagle
Intron/exon 19c*	c.548-2_c.548del3		MLHD/MLHDx, ESS, ECS
Exon 24	c.3494T > C	p.V1165A	MLHD, Siberian Husky, Beagle
Exon 24	c.3531(GGGAGCCGA)2-7	p.1178(GAE)2-7	MLHD, Siberian Husky, Beagle, Papillon

MLHD-purebred and MLHDx-crossbred from study pedigree; Siberian Husky-derived outbred dogs are referred to as Siberian Husky in the table.

* Numeration is given according to the nucleotide sequence numbering (GenBank Accession No. HQ315863) <http://www.ncbi.nlm.nih.gov/>; provided in the public domain by the National Center for Biotechnology Information; for the rest, numeration is given according to the reference nucleotide sequence numbering (GenBank Accession No. HM021768).

the 3' acceptor splice site and the first nucleotide of alternative 3'-terminal exon 19c, which is common for the group of transcripts driven by an internal promoter and which potentially encodes proteins without RID.¹⁵ To address the possibility that the D3 sequence change reduces the efficiency of polyadenylation^{26,27} and *CRPGRIP1* splicing, we performed comparative RT-PCR analysis with cDNA generated from a polyT primer from wild-type and D3-homozygous dogs. From our previous 3'RACE data, the 3'UTR sequence following exon 19c was believed to be incomplete since a stretch of oligo-A (A₁₅) is present in the DNA sequence, suggesting possible mispriming of the polyT primer.¹⁵ Therefore, to estimate the putative size of the 3'UTR, we next used a forward primer designed to bind to exon 19 and four reverse primers (Rev1, Rev2, Rev3, Rev4) binding to different positions in the 3'UTR flanking exon 19c (Fig. 11A, Supplementary Table S1 [<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-DCSupplemental>]). Such primer design allows for detection of spliced RNA that has not yet been cleaved for polyadenylation. Each primer pair yielded a diverse population of products from wild-type dog cDNA (Fig. 11B). The F-Rev1 pair of

primers gave eight products (1s–8s), F-Rev2 produced six products (1s–6s), while F-Rev3 and F-Rev4 gave rise to only one product each (6s and 9s, respectively) (Figs. 11B, 11C; Supplementary Table S2 [<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10178/-DCSupplemental>]). These results indicated that different transcripts may derive from alternate use of polyadenylation signals in the 3'UTR flanking exon 19c. A 3'RACE experiment with a forward primer located in exon 19 showed four bands corresponding to the four *CRPGRIP1* transcripts with different alternative 3'-terminal exons (19a, 19c, 19d, or 24) as previously described¹⁵ (Fig. 11D).

The same RT-PCR analysis was carried out in a *CRPGRIP1* D3-homozygous dog. In addition to the correct splicing patterns, we observed several different splicing abnormalities, including exon 19c skipping and cryptic splicing that greatly reduced transcript diversity. A comparable 3'RACE analysis on 2 retinas of dogs homozygous for D3 and Ins44 showed no band corresponding to the main transcript-containing exon 19c (Fig. 11D), suggesting abnormal polyadenylation.

TABLE 3. *CRPGRIP1* Haplotype and Phenotype of *CRPGRIP1*^{Ins/Ins} Dogs

Sample ID	c.19+19T > A	Ins44	D3	p.V1165A	p.1178(GAE)2-7	Phenotype*	Age (y)	Breed
R5	22	22	22	11	33	NVL/absent cone ERG	1.8	MLHDx
R10	22	22	22	11	33	Retinal degeneration (late stage)	2.6	MLHD
R18	22	22	22	11	33	NVL, absent cone ERG	0.7	MLHDx
R21	22	22	22	11	33	NVL, absent cone ERG	0.7	MLHDx
Molson	22	22	22	11	33	PRA	4.4	MLHD
Josie	22	22	22	11	33	NVL	1	MLHD
Caroline	22	22	22	11	33	NVL	3.5	MLHD
Clair	22	22	22	11	33	"PRA suspicious"	1.3	MLHD
George	22	22	22	11	33	PRA	13.8	ESS
Minnie	22	22	22	11	33	PRA	9.9	ESS
Keah	22	22	22	11	33	PRA	15.5	ESS
Stella	22	22	22	11	33	PRA	6.2	ESS
Emma	22	22	22	11	33	PRA	2.8	ESS
Holly	22	22	22	11	33	PRA	13	ESS
Genesis	22	22	22	11	33	NVL	7	ESS
Mackenzie	22	22	22	11	33	NVL	11.4	ESS
Kayla	22	22	22	11	33	NVL	11	ESS
Tucker	22	22	22	11	33	NVL	8.3	ESS
Louie	22	22	22	11	33	NVL	1.8	ECS

Note: mutant allele = 2; wild-type allele = 1, exception is microsatellite polymorphism where number of repeats is shown. MLHD-purebred; MLHDx-crossbred from study pedigree. NVL, no visible lesions on ophthalmologic examination.

* Phenotypes range from normal, ERG abnormalities, to severe retinal degeneration.

We next investigated potential correlations between the *Ins44/D3* genotype and the disease phenotype. We used a set of 59 dogs, all of which had been previously genotyped for *Ins44*, and which included 47 MLHD or MLHD-crossbred dogs from the study pedigree and the pet population, 11 ESSs, and 1 ECS. All dogs were genotyped for the D3 variant (Table 1). All *cRPGRIP1^{Ins/Ins}* dogs studied also were homozygous for D3. Several, but not all, of these dogs had retinal cone functional abnormalities (study pedigree) or clinically evident retinal degeneration (colony founders and dogs from the same or other breeds in the pet population). Several *cRPGRIP1^{+/Ins}* or *cRPGRIP1^{+/+}* dogs were homozygous for D3; none of these had evidence of retinal dysfunction or disease. A further 30 dogs, comprising 12 Siberian Husky-derived crossbreds, 8 Beagles, and 10 Papillons were genotyped only for *Ins44*, and all were *cRPGRIP1^{+/+}*. As clinically normal dogs can be homozygous for both D3 and *Ins44*, we concluded that neither is sufficient to cause *cord1*.

Haplotype Analysis of *cRPGRIP1* for Disease Association. To further explore the association between *cRPGRIP1* and retinal disease, we extended the mutation analysis of *cRPGRIP1*, and the results provided data to develop a gene-specific haplotype for association studies. We searched for mutations in the coding region, promoter (P1, P2, 1000 bp each), and 3'UTR (600 bp) of this gene in two *cRPGRIP1^{Ins/Ins}* dogs (R5, R10) and several clinically normal wild-type dogs (MLHD, Siberian Husky, and Beagle breeds). In addition to *Ins44* and D3, eight other sequence changes were detected in the analysis. We also performed a genotyping pilot study of *cRPGRIP1* sequence changes in additional dogs of different breeds. Table 2 represents the summary of the results.

Five of these sequence changes were found in purebred or MLHD-derived dogs (MLHDx), and were chosen for haplotype analysis. The haplotype panel for analysis included: c.19+19T > A; c.142_143ins44 (*Ins44*); c.548-2_c.548del3 (D3); c.3494T > C, and c.3531(GGGAGCCGA)2-7 (Table 2). These markers covered a total of 63.7 kb of *cRPGRIP1* genomic region and were used for comparative haplotyping of 8 MLHD or MLHDx crossbreds (0.6–4.4 years), 10 ESSs (2.8–15.5 years), and 1 ECSs (1.8 years), all *cRPGRIP1^{Ins/Ins}*.

From previously published data of strong association of *Ins44* with *cord1*⁵, homozygosity for *Ins44* was an inclusion selection criterion in the group analyzed. The selected retinal phenotypes ranged from clinically normal, absent ERG 30-Hz cone function, or advanced retinal degeneration. Results of genotyping and phenotype characteristics are presented in Table 3. Without exception, all dogs had a single 5-locus homozygous haplotype (2-2-2-1-3; see Table 3). Results indicated that there is no association between extended haplotype of *cRPGRIP1* and retinal degeneration.

DISCUSSION

The original studies that established a causal association between *cRPGRIP1^{Ins/Ins}* and *cord1* have been carried out in a closed research colony in which affected dogs show ophthalmoscopic evidence of retinal disease at approximately 25 weeks of age, which progressed to advanced degeneration before 1 year of age.¹⁶ Thirty-Hz cone ERG responses are markedly reduced at 6 weeks of age, and by 30 weeks of age, both rod and cone ERG responses are barely detectable.¹⁶ Although there is considerable interanimal variation of cone responses, progression is the rule, not the exception.¹⁶ Histologic abnormalities have also been observed in the photoreceptors of affected dogs by 6 weeks of age, with shortening of the inner and outer segments, and opsin delocalization. These changes are progressive, resulting in an

approximately exponential decay in photoreceptor cell numbers in the outer nuclear layer with age. Similar abnormalities also have been reported in a colony of MLHDs in France that was derived from the AHT dogs.²⁸ All affected dogs have a homozygous 44-nucleotide insertion in the 3' end of exon 2 that is predicted to alter the reading frame, thereby introducing a premature stop codon that could presumably lead to a truncated or absent protein that lacks the RID domain and is causal to the disease.⁵ However, despite the highly consistent genotype-phenotype correlation in the original AHT research colony, significant discordance has been found in the general MLHD pet population, and the onset of disease is highly variable if at all present.^{11,12,14}

To more definitively address the role of *cRPGRIP1* in the disease, we established a research colony whose four founders included two affected dogs (*cRPGRIP1^{Ins/Ins}*) that had early-onset retinal degeneration, one of which came from the AHT research colony described above.^{5,16} Of the *Ins44* homozygous dogs produced, all six dogs examined after 1 year of age were ophthalmoscopically normal (age range: 1–3.5 years), a time at which advanced retinal degeneration would have been expected among the affected dogs in the original research colony.^{5,16} Cone-mediated 30-Hz flicker ERG responses were variable in *cRPGRIP1^{Ins/Ins}* dogs, being nonrecordable in five, normal in two, and markedly reduced in amplitude in one. Moreover, repeated ERGs in several of the dogs clearly indicated that these responses were stable and that rod-mediated responses were not lost over time. With the exception of the two colony founders that had retinal degeneration leading to early-onset blindness, the results indicated that the functional deficits are nonprogressive, at least in the time period of the study, and that ERG cone functional abnormalities, when present, do not predict photopic visual performance. The data were not consistent with the previously published phenotype of *cord1* dogs¹⁶ or *RPGRIP1* knockout (*RPGRIP1^{tm1Titi}* and *RPGRIP1^{nmf247}*) mice^{29,30} that have early and severe photoreceptor abnormalities. Together, these results indicated that *Ins44* in *RPGRIP1* is not the primary cause of the disease.

In the dog, the *Ins44* variant in exon 2 has been predicted to alter the reading frame of the transcript, thereby introducing a premature stop codon.⁵ We established that, instead of altering the reading frame, the *Ins44* mutation induces skipping of exon 2. Comparable expression of *RPGRIP1* in *cRPGRIP1^{Ins/Ins}*, *cRPGRIP1^{+/Ins}*, and *cRPGRIP1^{+/+}* retinas was found by Western blot analysis using an antibody against the RID domain, a finding that argues against introduction of a premature stop codon. Moreover, preservation of the RID domain, which is critical for *RPGRIP1*-*RPGR* interaction,⁷ means that the *Ins44* variant does not prevent expression and translation of transcripts bearing a normal 3' end that encodes RID, suggesting that the protein is likely functional. In our experiment, we only detected two highly abundant translated products of 37 kDa and 45 kDa. A higher molecular mass protein of approximately 160 kDa, previously observed only upon longer exposure of the blots,²⁰ was not found, probably because of proteolysis. Despite the fact that *cRPGRIP1* gives rise to multiple isoforms, there are no data so far to support their efficient translation, since some of them have been detected at very low expression levels.¹⁵ In addition, some *cRPGRIP* isoforms, such as 19c-1 and 19c-2, have no RID domain to be detected with Ab38, specific for the conserved RID.

Web-based analysis using RESCUE-ESE to identify putative ESEs suggests that inactivation of an ESE is the most likely mechanism underlying exon skipping in the *Ins44* variant in *cRPGRIP1* exon 2. Moreover, bioinformatics analysis of aberrant *cRPGRIP1* sequences also suggests that *Ins44* will

affect the N-terminus of some cRPGRIP1 isoforms.¹⁵ Previous studies have shown that certain RPGRIP1 isoforms undergo limited proteolysis, leading to the relocation and nuclear accumulation of a small N-terminal domain encoded by first three exons of *RPGRIP1*.³¹ In this scenario, alteration of the N-terminal sequence of cRPGRIP1 by Ins44 could affect nuclear import of the RPGRIP1 nuclear domain. Although the ultimate effect of such alterations in nuclear import is unclear, our data to date suggest that it does not cause disease or affect progression.

To further determine if changes in cRPGRIP1 isoforms and/or photoreceptor-specific gene expression could contribute to disease progression, we used quantitative RT-PCR (qRT-PCR) to examine retinal mRNA expression levels in wild-type control and dogs homozygous or heterozygous for Ins44. The homozygous dogs used showed absent cone ERG responses, and morphologic examination in one cone function-deficient dog identified abnormalities consistent with loss of cone outer segments, and regional rod opsin mislocalization. While the expression results for exon 2 were consistent in dogs with Ins44 (negligible in cRPGRIP1^{Ins/Ins}, and 0.4–0.8 reduction in cRPGRIP1^{+/Ins}), there was marked variability of expression for the other cRPGRIP1 isoforms, which was not dependent on the Ins44 genotype. Expression of other photoreceptor-specific genes was equally variable, and not associated with the Ins44 genotype. Initially, we suspected that the gene expression profiles could be sex dependent, but analysis of additional heterozygous dogs did not show any sex-related differences (data not shown).

As our results did not support the previously proposed mechanism that cRPGRIP1 Ins44 is largely truncated and thus dysfunctional, we next sought to identify additional or alternative mutations in *RPGRIP1* that could provide insight into its role in the clinical spectrum of retinal disease. To this end, we have reported nine previously uncharacterized sequence changes including three intronic mutations, a silent mutation in exon 14, missense mutations in exons 17 and 24, a microsatellite polymorphism in exon 24, and a deletion (D3) that removes the 3' splice site and first nucleotide of alternative exon 19c.¹⁵ Using RT-PCR and 3'RACE, we next demonstrated that the D3 mutation reduces the normal 3' splicing pattern, activates a cryptic splice site, and promotes generation of erroneous splicing isoforms containing "pseudoexon" 19c-4. As both Ins44 and D3 alter the splicing of cRPGRIP1 and defects in alternative splicing contribute to disease,^{32–34} we next examined whether the D3 deletion can modify the Ins44 disease phenotype by assessing their distribution among 59 dogs that were either clinically normal or had retinal degeneration. In most cases, cRPGRIP1^{Ins/Ins} dogs also are homozygous for D3, an indication that the two are in linkage disequilibrium. However, as clinically normal dogs can also be homozygous for both D3 and Ins44, we conclude that neither is sufficient to cause *cord1*; it is yet to be determined if the combined changes contribute to an increased risk for developing *cord1*.

We have also extended the cRPGRIP1 disease-association analysis by using five polymorphic markers spanning almost the entire gene sequence. Notably, all 19 Ins44 homozygous dogs (MLHDs, MLHDx, ESSs, and ECSSs) bore the same haplotype, regardless of their clinical phenotype. Moreover, the four ESSs without retinal degeneration ranged in age between 7 and 11.4 years, much older than the typical disease onset of either *cord1* or the ESS breed-specific retinal disease (Table 3). For cRPGRIP1^{Ins/Ins} MLHDs from the purebred pet population, one had overt retinal degeneration at 4.4 years, one was suspected to have degeneration (1.3 years), and two were normal (1 and 3.5 years). *cord1* was ruled out in the dogs clinically normal at or above 1 year of age, as typical *cord1*

cases in the original research colony have shown recognizable retinal degeneration by this age. Given that retinal degeneration in ESSs and MLHDs has each been recognized as autosomal recessive, and the same cRPGRIP1 extended haplotype occurs in clinically normal and retinal degenerate animals, it is unlikely that this gene is causally associated with the disease by itself.

Based on a previous study that has shown that not all cRPGRIP1^{Ins/Ins} dogs develop clinically recognizable retinal degeneration,^{11,12} a recent genome-wide association study in cRPGRIP1^{Ins/Ins} dogs stratified by phenotype has mapped an independent locus which, in the homozygous state, correlates with an early-onset phenotype.¹⁴ The newly identified locus is located approximately 35 Mb from *RPGRIP1* on CFA15, and the genes in its 1.49-Mb interval are being analyzed to determine a role as a *cord1* disease modifier (Keiko Miyadera et al., unpublished data). While the identification of the second locus does not necessarily lend further support to cRPGRIP1^{Ins/Ins} being causal to *cord1*, it is essential to unequivocally establish the disease gene at the first locus. Mapping of the first locus to a 14.15-Mb interval by Mellersh et al.⁵ benefited from the use of the research colony, which subsequently was shown to be fixed for the second locus.¹⁴ The involvement of the first locus is definitive, based on the perfect association with *cord1* in the AHT research colony. *RPGRIP1* is located within the mapped *cord1* region of 14.15 Mb on CFA15,⁵ as well as within the narrowed 1.74-Mb critical disease interval,¹¹ and has been the most likely disease candidate given the predicted severity of the insertion. However, despite its important functional role in photoreceptors, and its interactions with a number of ciliary proteins in visual cells,^{29,30,35} our study indicated that mutations in *RPGRIP1* are not the primary or sole cause of *cord1*, and the real causative mutation representing the locus initially mapped by Mellersh et al.⁵ is in physical proximity with *RPGRIP1* in the narrowed 1.74-Mb critical disease interval.¹¹ In such a case, cRPGRIP1 Ins44 could represent a benign polymorphism that has been fixed in some populations, for example, AHT research colony¹⁴ and many ESSs, or could be a *cord1* disease modifier. For that reason, identification of the direct causal gene/mutation will provide new insights into animal and human retinopathies.

Acknowledgments

The authors thank Cathryn Mellersh, Animal Health Trust, United Kingdom, for providing semen for one of the colony founders (C-UK1) with cRPGRIP1 Ins44 and affected with early-onset *cord1*; Paulo Ferreira for anti-RPGRIP Ab38; Sem Genini and Jessica Rowland for providing TaqMan probes for gene expression analysis; Gregory M. Acland, Jacob G. Appelbaum, and Leslie King for helpful discussions and comments; the RDSF staff for excellent animal care and technical support; Svetlana Savina for immunohistochemistry; and Mary Leonard of the Biomedical Art and Design facility at the University of Pennsylvania for illustrations.

References

1. Aguirre GD, Acland GM. Models, mutants and man: searching for unique phenotypes and genes in the dog model of inherited retinal degeneration. In: Ostrander EA, Giger U, Lindblad-Toh K, eds. *Dog and Its Genome*. Cold Spring Harbor, NY; 2006;291–325.
2. Miyadera K, Acland GM, Aguirre GD. Genetic and phenotypic variations of inherited retinal diseases in dogs: the power of within- and across-breed studies. *Mamm Genome*. 2012;23:40–61.
3. Goldstein O, Mezey JG, Boyko AR, et al. An ADAM9 mutation in canine cone-rod dystrophy 3 establishes homology with human cone-rod dystrophy 9. *Mol Vis*. 2010;16:1549–1569.

4. Kijas JW, Zangerl B, Miller B, et al. Cloning of the canine ABCA4 gene and evaluation in canine cone-rod dystrophies and progressive retinal atrophies. *Mol Vis*. 2004;10:223–232.
5. Mellersh CS, Boursnell ME, Pettitt L, et al. Canine RPGRIP1 mutation establishes cone-rod dystrophy in miniature long-haired dachshunds as a homologue of human Leber congenital amaurosis. *Genomics*. 2006;88:293–301.
6. Wiik AC, Wade C, Biagi T, et al. A deletion in nephronophthisis 4 (NPHP4) is associated with recessive cone-rod dystrophy in standard wire-haired dachshund. *Genome Res*. 2008;18:1415–1421.
7. Roepman R, Bernoud-Hubac N, Schick DE, et al. The retinitis pigmentosa GTPase regulator (RPGR) interacts with novel transport-like proteins in the outer segments of rod photoreceptors. *Hum Mol Genet*. 2000;9:2095–2105.
8. Kuznetsova T, Zangerl B, Aguirre GD. RPGRIP1 and cone-rod dystrophy in dogs. *Adv Exp Med Biol*. 2012;723:321–328.
9. Dryja TP, Adams SM, Grimsby JL, et al. Null RPGRIP1 alleles in patients with Leber congenital amaurosis. *Am J Hum Genet*. 2001;68:1295–1298.
10. Hameed A, Abid A, Aziz A, Ismail M, Mehdi SQ, Khaliq S. Evidence of RPGRIP1 gene mutations associated with recessive cone-rod dystrophy. *J Med Genet*. 2003;40:616–619.
11. Miyadera K, Kato K, Aguirre-Hernandez J, et al. Phenotypic variation and genotype-phenotype discordance in canine cone-rod dystrophy with an RPGRIP1 mutation. *Mol Vis*. 2009;15:2287–2305.
12. Busse C, Barnett KC, Mellersh CS, Adams VJ. Ophthalmic and cone derived electrodiagnostic findings in outbred miniature long-haired dachshunds homozygous for a RPGRIP1 mutation. *Vet Ophthalmol*. 2011;14:146–152.
13. Genetics Committee of the American College of Veterinary Ophthalmologists. *Ocular Disorders Presumed to Be Inherited in Purebred Dogs*. 5th ed. American College of Veterinary Ophthalmologists; 2010.
14. Miyadera K, Kato K, Boursnell M, Mellersh CS, Sargan DR. Genome-wide association study in RPGRIP1 (-/-) dogs identifies a modifier locus that determines the onset of retinal degeneration. *Mamm Genome*. 2012;23:212–223.
15. Kuznetsova T, Zangerl B, Goldstein O, Acland GM, Aguirre GD. Structural organization and expression pattern of the canine RPGRIP1 isoforms in retinal tissue. *Invest Ophthalmol Vis Sci*. 2011;52:2989–2998.
16. Turney C, Chong NH, Alexander RA, et al. Pathological and electrophysiological features of a canine cone-rod dystrophy in the miniature longhaired dachshund. *Invest Ophthalmol Vis Sci*. 2007;48:4240–4249.
17. Guyon R, Pearce-Kelling SE, Zeiss CJ, Acland GM, Aguirre GD. Analysis of six candidate genes as potential modifiers of disease expression in canine XLPRA1, a model for human X-linked retinitis pigmentosa 3. *Mol Vis*. 2007;13:1094–1105.
18. Komaromy AM, Alexander JJ, Rowlan JS, et al. Gene therapy rescues cone function in congenital achromatopsia. *Hum Mol Genet*. 2010;19:2581–2593.
19. Garcia MM, Ying GS, Cocores CA, Tanaka JC, Komaromy AM. Evaluation of a behavioral method for objective vision testing and identification of achromatopsia in dogs. *Am J Vet Res*. 2010;71:97–102.
20. Castagnet P, Mavlyutov T, Cai Y, Zhong F, Ferreira P. RPGRIP1s with distinct neuronal localization and biochemical properties associate selectively with RanBP2 in amacrine neurons. *Hum Mol Genet*. 2003;12:1847–1863.
21. Beltran WA, Hammond P, Acland GM, Aguirre GD. A frameshift mutation in RPGR exon ORF15 causes photoreceptor degeneration and inner retina remodeling in a model of X-linked retinitis pigmentosa. *Invest Ophthalmol Vis Sci*. 2006;47:1669–1681.
22. Fairbrother WG, Yeo GW, Yeh R, et al. RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Res*. 2004;32:W187–W190.
23. Baker KE, Parker R. Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Curr Opin Cell Biol*. 2004;16:293–299.
24. Maquat LE. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol*. 2004;5:89–99.
25. Neu-Yilik G, Gehring NH, Hentze MW, Kulozik AE. Nonsense-mediated mRNA decay: from vacuum cleaner to Swiss army knife. *Genome Biol*. 2004;5:218.
26. Cooke C, Alwine JC. Characterization of specific protein-RNA complexes associated with the coupling of polyadenylation and last-intron removal. *Mol Cell Biol*. 2002;22:4579–4586.
27. Niwa M, Rose SD, Berget SM. In vitro polyadenylation is stimulated by the presence of an upstream intron. *Genes Dev*. 1990;4:1552–1559.
28. Lheriteau E, Libeau L, Stieger K, et al. The RPGRIP1-deficient dog, a promising canine model for gene therapy. *Mol Vis*. 2009;15:349–361.
29. Won J, Gifford E, Smith RS, et al. RPGRIP1 is essential for normal rod photoreceptor outer segment elaboration and morphogenesis. *Hum Mol Genet*. 2009;18:4329–4339.
30. Zhao Y, Hong DH, Pawlyk B, et al. The retinitis pigmentosa GTPase regulator (RPGR)-interacting protein: subserving RPGR function and participating in disk morphogenesis. *Proc Natl Acad Sci U S A*. 2003;100:3965–3970.
31. Lu X, Guraju M, Oswald J, Ferreira PA. Limited proteolysis differentially modulates the stability and subcellular localization of domains of RPGRIP1 that are distinctly affected by mutations in Leber's congenital amaurosis. *Hum Mol Genet*. 2005;14:1327–1340.
32. Blencowe BJ. Alternative splicing: new insights from global analyses. *Cell*. 2006;126:37–47.
33. Matlin AJ, Clark F, Smith CW. Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol*. 2005;6:386–398.
34. Wang GS, Cooper TA. Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat Rev Genet*. 2007;8:749–761.
35. Roepman R, Wolfrum U. Protein networks and complexes in photoreceptor cilia. *Subcell Biochem*. 2007;43:209–235.