A Large Animal Model for CNGB1 Autosomal Recessive Retinitis Pigmentosa

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Abstract

Retinal dystrophies in dogs are invaluable models of human disease. Progressive retinal atrophy (PRA) is the canine equivalent of retinitis pigmentosa (RP). Similar to RP, PRA is a genetically heterogenous condition. We investigated PRA in the Papillon breed of dog using homozygosity mapping and haplotype construction of single nucleotide polymorphisms within a small family group to identify potential positional candidate genes. Based on the phenotypic similarities between the PRA-affected Papillons, mouse models and human patients, CNGB1 was selected as the most promising positional candidate gene. CNGB1 was sequenced and a complex mutation consisting of the combination of a one basepair deletion and a six basepair insertion was identified in exon 26 (c.2387delA,2389_2390insAGCTAC) leading to a frameshift and premature stop codon. Immunohistochemistry (IHC) of pre-degenerate retinal sections from a young affected dog showed absence of labeling using a C-terminal CNGB1 antibody. Whereas an antibody directed against the N-terminus of the protein, which also recognizes the glutamic acid rich proteins arising from alternative splicing of the CNGB1 transcript (upstream of the premature stop codon), labeled rod outer segments. CNGB1 combines with CNGA1 to form the rod cyclic nucleotide gated channel and previous studies have shown the requirement of CNGB1 for normal targeting of CNGA1 to the rod outer segment. In keeping with these previous observations, IHC showed a lack of detectable CNGA1 protein in the rod outer segments of the affected dog. A population study did not identify the CNGB1 mutation in PRA-affected dogs in other breeds and documented that the CNGB1 mutation accounts for ~70% of cases of Papillon PRA in our PRA-affected canine DNA bank. CNGB1 mutations are one cause of autosomal recessive RP making the CNGB1 mutant dog a valuable large animal model of the condition.

Introduction

Retinitis pigmentosa (RP) is the leading cause of inherited blindness in humans affecting about 1 in 4,000 people [1]. It can be inherited in a dominant, recessive or X-linked fashion and shows considerable locus heterogeneity, with mutations in over 40 genes identified as causing non-syndromic RP (RetNet: https://sph.uth.edu/retnet/sum-dis.htm). Proteins encoded by these genes are necessary for a variety of functions within photoreceptors and their supporting cells. The age at onset and rate of progression of RP vary such that some patients have a history of night blindness from childhood while others may not notice symptoms until they are adults. The variability depends on the gene involved and the effect of the mutation on gene function, but there is also variability between patients with the same mutation [2,3]. Rod photoreceptors are affected initially, resulting in loss of night (rod-mediated) vision and constriction of the visual fields. Loss of cone-mediated (daytime and color) vision may occur secondarily to rod-loss, even when RP is caused by a mutation of a gene exclusively expressed in rods, and can lead to complete blindness.

Retinal dystrophies analogous to RP occur in dogs, with reports of such conditions in over 100 different breeds [4]. The canine RP equivalent is known as progressive retinal atrophy (PRA) [5,6]. The gene mutations underlying several forms of PRA have been identified and many have proven to be in genes analogous to those known to cause RP [7,8,9,10,11,12,13] or in some instances have suggested new candidate genes for investigation in RP patients [14,15,16].

Spontaneously occurring retinal dystrophies in canine models are of particular interest because the canine eye is similar in size to the human eye. This morphological similarity allows for identical surgical approaches for intravitreal and subretinal injection of therapeutic agents and testing for approaches such as implantation of intravitreal sustained-release devices. An additional advantage of canine models over rodent models is that the canine eye has regions of higher photoreceptor density (of both rods and cones), namely the area centralis and the visual streak that are somewhat analogous to the human macula [17]. In contrast, the retina of laboratory rodents lacks an equivalent region having an even...
density of photoreceptors across the retina [18]. Dogs with spontaneous mutations resulting in retinal dystrophies have proven to be important in preclinical assessment of therapies destined for use in human patients. For example, dogs with a mutation in RPE65 as a model for Leber congenital amaurosis type II were crucial for preclinical proof-of-concept gene therapy trials [19] which led to phase 1/2 human clinical trials [20,21,22]. The RPE65 mutant dog and other dog retinal dystrophy models have subsequently been used in several other preclinical trials for retinal gene and drug therapy [19,23,24,25,26,27,28,29,30,31]. Identification of the gene mutations underlying other forms of canine PRA may provide additional spontaneous canine models to allow study of disease mechanisms and proof-of-concept therapy trials.

The Papillon breed of dog was initially reported to have PRA in 1995 [32]. Studies of the phenotype of affected dogs suggested loss of rod electrophysiological responses but maintenance of cone-driven responses at least until late in the disease process [33,34]. Our unpublished studies of PRA in Papillons show a wide range in age of onset. This phenotypic variability could either suggest within-breed locus heterogeneity or could merely be the result of background genetic or environmental influences.

In this study we report a frameshift mutation in CNGB1 that is the cause of one form of PRA in Papillon dogs providing a large-animal model of autosomal recessive RP (RP45) due to CNGB1 mutations.

Results

Phenotypic Description

DNA samples were collected from 23 PRA-affected Papillons and 119 unaffected Papillons. The dogs had all been examined by a veterinary ophthalmologist. For example, dogs with a mutation in RPE65 as a model for Leber congenital amaurosis type II were crucial for preclinical proof-of-concept gene therapy trials [19] which led to phase 1/2 human clinical trials [20,21,22]. The RPE65 mutant dog and other dog retinal dystrophy models have subsequently been used in several other preclinical trials for retinal gene and drug therapy [19,23,24,25,26,27,28,29,30,31]. Identification of the gene mutations underlying other forms of canine PRA may provide additional spontaneous canine models to allow study of disease mechanisms and proof-of-concept therapy trials.

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Mapping of the Papillon PRA Locus

An initial genome-wide association analysis performed using PLINK software [36] and including the genotypes from 23 Papillons (9 cases, 4 obligate carriers and 10 controls) yielded no significant associations (data not shown). Because within breed locus heterogeneity for PRA in dogs is a common occurrence, and because we and others had noted a wide range of age of onset [32] between the affected dogs, we suspected that more than one form of PRA may be segregating in the Papillon breed. Therefore, we analyzed the genotyping data from a small family group of 3 affected dogs within our breeding colony that we felt were very likely to share the same gene mutation (pedigree in Figure 3). We compared the genotyping results of the 3 affected dogs with that of 2 obligate carriers from the family and 11 control dogs (an additional control was included in this analysis). Our pedigree analysis supported an autosomal recessive mode of inheritance (data not shown) so we performed homozygosity mapping using a custom written computer program. The program was set to identify regions of homozygosity containing runs of at least six SNPs in the cases and for which the control animals did not share homozygosity (see Materials and Methods). This revealed 13 such regions of homozygosity greater than 1.5 Mb but only 4 of these
regions contained obvious positional autosomal recessive RP candidate genes; CNGB1 (CFA2), RBP3 and RGR (CFA4), RD3 and CRB1 (CFA7) and TULP1 (CFA12) (Table 1). Figure 3 shows a section of the run of homozygosity surrounding CNGB1 and the p-values for each marker, resulting from a chi-square association test for multiple testing (the full region of homozygosity is shown in Table S4). These four regions were then subjected to haplotype construction, and haplotypes were examined within the small family group (data not shown). Only in the CFA2 region did the affected dogs from this family have a unique extended haplotype which was not present in the homozygous state in control (non-obligate carrier) dogs. Obligate carrier dogs each possessed one copy of this haplotype. Furthermore, after comparing the phenotype of the PRA-affected dogs in our colony with that reported for human families and mouse models with CNGB1 mutations, CNGB1 was considered the strongest candidate. Based on the haplotype analysis and phenotypic information, CNGB1 was selected to screen first for mutations.

Genomic Structure of Canine CNGB1

To establish the genomic structure of canine CNGB1 we sequenced cDNA from a control canine retinal library and genomic DNA from a control Papillon and compared these to the published canine genomic sequence for this region (CanFam2.0). From these comparisons we deduced the intron/exon boundaries for CNGB1 (Table 2) which differed from the predicted structure (Table 1). The assay was used to genotype 20 Papillons that had been diagnosed by a veterinary ophthalmologist to have PRA (not including purpose-bred colony dogs) and 119 Papillons whose owners reported no abnormal vision (Table 4). The mutation was identified in the homozygous state in 13 of 20 Papillons that had been diagnosed with PRA. Of the phenotypically normal Papillons, none were homozygous for the mutation and 20 were heterozygous for it. This indicates a 16.3% carrier rate, and a mutated allele frequency of ~31% in the Papillon breed, however, ascertainment bias almost certainly falsely inflates these values. In addition, we genotyped 33 dogs from 8 different breeds that had been diagnosed with PRA and 66 dogs from 9 different breeds that

The affected Papillon had a frameshift mutation in exon 26. This consisted of a 1 bp deletion (chr2: 61,502,597; c.2387delA) and a 6 bp insertion (between chr2: 61,302,599–61,502,600; c.2389_2390insAGCTAC). This mutation (c.2307delA;2389_2390insAGCTAC, which for simplicity we will refer to as CNGB1-fs26) is predicted to result in a premature stop codon, 17 bp downstream and is present in affected Papillons but not in unaffected Papillons or in the canine reference genome (UCSC Genome Browser CanFam2.0) (Figure 4).

Table 3 shows the SNVs and SNPs detected and Figure S1 shows the predicted canine protein amino acid sequence and alignment with other species. Primers for sequencing canine gDNA and cDNA are supplied in Table S1 and Table S2, respectively.

A genotyping assay for the mutation was developed (methods S1). The assay was used to genotype 20 Papillons that had been diagnosed by a veterinary ophthalmologist to have PRA (not including purpose-bred colony dogs) and 119 Papillons whose owners reported no abnormal vision (Table 4). The mutation was identified in the homozygous state in 13 of 20 Papillons that had been diagnosed with PRA. Of the phenotypically normal Papillons, none were homozygous for the mutation and 20 were heterozygous for it. This indicates a 16.3% carrier rate, and a mutated allele frequency of ~31% in the Papillon breed, however, ascertainment bias almost certainly falsely inflates these values. In addition, we genotyped 33 dogs from 8 different breeds that had been diagnosed with PRA and 66 dogs from 9 different breeds that

| Chr | Start Position1 | End Position1 | Size of Region (bp) | arRP Candidate Genes2 | Location of Candidate Genes1 |
|-----|----------------|--------------|---------------------|------------------------|-----------------------------|
| 6   | 3,330,209      | 12,765,968   | 9,435,759           | –                      | –                           |
| 28  | 10,867,526     | 19,744,585   | 8,877,059           | –                      | –                           |
| 4   | 34,171,819     | 42,900,776   | 8,728,957           | RGR                    | chr4:37,501,028–37,504,555 |
| 4   | 34,171,819     | 42,900,776   | 8,728,957           | RBP3                   | chr4:38,165,164–38,175,385 |
| 21  | 19,922,213     | 28,084,112   | 8,161,899           | –                      | –                           |
| 7   | 7,812,042      | 14,771,908   | 6,959,866           | CRB1                   | chr7:8,233,979–8,375,413   |
| 7   | 7,812,042      | 14,771,908   | 6,959,866           | RD3                    | chr7:12,835,467–12,835,739 |
| 2   | 37,571,313     | 43,948,453   | 6,377,140           | –                      | –                           |
| 12  | 3,051,458      | 8,379,562    | 5,528,104           | TULP1                  | chr12:7,639,870–7,647,308  |
| 7   | 3,000,316      | 7,077,039    | 4,076,723           | –                      | –                           |
| 37  | 23,673,475     | 26,349,135   | 2,675,660           | –                      | –                           |
| 14  | 8,954,008      | 11,562,422   | 2,608,414           | –                      | –                           |
| 32  | 11,212,687     | 13,539,152   | 2,326,465           | –                      | –                           |
| 2   | 60,980,617     | 62,836,928   | 1,846,311           | CNGB1                  | chr2:61,454,476–61,520,336 |
| 14  | 37,878,684     | 39,484,021   | 1,605,337           | –                      | –                           |

1. Locations are all in respect to UCSC Genome Browser CanFam2.0 (http://genome.ucsc.edu).
2. arRP - autosomal recessive retinitis pigmentosa. Gene abbreviations: RBP3 - retinol binding protein 3, RGR - retinal G protein coupled receptor, RD3 - retinal degeneration protein 3, CRB1 - crumbs homolog 1, TULP1 - tubby like protein 1, CNGB1 - cyclic nucleotide gated channel beta 1.

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were clinically normal, none of the dogs of non-Papillon breeds had the mutation (Table S3).

Immunohistochemistry Shows Lack of Detectable Full-length CNGB1 Protein in Affected Retina

To confirm that the CNGB1-fs26 mutation does disrupt CNGB1 expression in the homozygous animal, we performed immunohistochemistry (IHC) on retinal sections from an 8 week-old PRA-affected Papillon from the breeding colony that was homozygous for the CNGB1-fs26 mutation and compared it to retinal sections from an 8 week-old normal dog that was confirmed not to have the CNGB1-fs26 mutation. CNGB1 in other species codes for multiple transcripts via alternative splicing [39,40]. The CNGB1 locus has been described to code for four sensory transcripts; three retinal transcripts and one olfactory sensory transcript, as well as other splice variants expressed in kidney, brain, testes and spermatozoa [41,42,43,44]. The 5’ portion of the gene encodes two glutamic acid rich proteins (GARPs) while the full-length transcript encodes the CNGB1 protein. The position of the CNGB1-fs26 mutation is predicted to allow normal expression of the two GARPs but to disrupt production of the full-length CNGB1 protein. To test this prediction we used two CNGB1 antibodies: one that targets the amino terminal (GARP region) of CNGB1 and the GARPs and a second antibody that targets the carboxyl end of CNGB1 downstream of both the GARP region and the predicted premature stop codon in the mutant canine CNGB1 gene. The results from this study showed that while the rod outer segments of the wild-type retina were labeled by both antibodies (Figure 5 A, C), the rod outer segments of the PRA-affected Papillon were labeled with the amino terminal antibody but not the carboxyl terminal antibody (Figure 5 B, D). This provides strong evidence

Figure 3. SNP analysis in the CNGB1 region using a small family group of affected and unaffected Papillons. The SNPs are located in a 1.84 Mb region of homozygosity among the affected dogs (Only SNPs near the center of the region are shown). CNGB1 is located at chr2:61,454,476–61,520,336 in the reference genome (canFam2.0). SNP genotypes are given in columns for each dog, with the family group data displayed to the left of the black bar and all others to the right of the black bar. All affected dogs (1–3) share the same haplotype, obligate carriers (C1–2) each have one copy of the haplotype seen in the affected dogs and none of the unaffected dogs (U1–11) possess the haplotype seen in the affected dogs. A-Major allele, B-minor allele, as designated by Illumina (Illumin Inc, San Diego, CA).

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that the mutation disrupts production of full-length CNGB1 protein while still allowing expression of GARPs as predicted from the CNGB1-fs26 mutation.

We also performed IHC with a CNGA1 antibody. The rod cGMP-gated channel consists of both CNGA1 and CNGB1 subunits. Studies in mouse models have shown that lack of CNGB1 also disrupts trafficking of CNGA1 to the outer segments resulting in very reduced or absent CNGA1 protein [45,46]. As predicted by the mouse model, the retinal sections for the affected Papillon showed lack of detectable CNGA1 protein while in the retinal sections from the normal dog CNGA1 was appropriately expressed and correctly targeted to the rod outer segments (Figure 5 E, F).

Table 2. Intron and exon boundaries for canine CNGB1 gene.

| Exon | Location on Chr2 | Donor | Intron (size) | Acceptor |
|------|-----------------|-------|--------------|----------|
| Exon 1 | 61,454,476–61,454,528 | ATCTGAGCAAgtaagtcagg | 2353 bp | tgcctcaagGTCTGGGAT |
| Exon 2 | 61,457,062–61,457,243 | GAGCTCCTGAtgaagcaca | 2069 bp | acactccagCCACCTGAC |
| Exon 3 | 61,459,313–61,459,364 | GGCCCTAGGgtgagtgccg | 288 bp | tcttttcagAAATCCAGGA |
| Exon 4 | 61,460,708–61,460,750 | GGAGCTAAGGgtgagggcag | 184 bp | ttcttctcagATGGACTTGG |
| Exon 5 | 61,460,935–61,460,965 | GGAGACCCGGgtgagtcct | 1238 bp | ccctgtcagGTCTGGGCCC |
| Exon 6 | 61,462,249–61,462,324 | AACTCTCAAAGtgtaagctaaa | 352 bp | tcactctcagGACCAGAGAG |
| Exon 7 | 61,467,677–61,467,716 | TTGGACACAGGtacaggggag | 685 bp | ccacttgcagGAGCCTGACT |
| Exon 8 | 61,470,096–61,470,125 | CAGGGAGCAGgtctgttctg | 1686 bp | tcccatgcagGAGCCTGACT |
| Exon 9 | 61,473,781–61,473,861 | CCGCTGACCAgtgagtcctg | 3776 bp | tcccctgcagTCTGCATCCT |
| Exon 10 | 61,480,379–61,480,415 | AGGAGGCAAAgtgagtgcct | 5744 bp | ggggtcacagTGTCCTGCTG |
| Exon 11 | 61,485,513–61,485,620 | TGGAAACACAGgtgagggcag | 2472 bp | ggcatttcagATGGACATGC |
| Exon 12 | 61,491,093–61,491,250 | CCAAAGCCCTgtgagtccag | 1047 bp | catcccacagGAAGCTGCCC |
| Exon 13 | 61,492,998–61,492,456 | CCGTGACCAAGtgagttgc | 288 bp | tgcctcaagACCTGTGAG |
| Exon 14 | 61,493,460–61,493,548 | AGACATCATTTgtgagttgct | 2723 bp | ttccttgtagACACAGAAA |
| Exon 15 | 61,494,272–61,494,322 | TCTGTTCAAAGgtgagttgc | 2723 bp | gggatttcagATGGACATGC |
| Exon 16 | 61,494,596–61,494,682 | CTGTTTGAGGgtgagttgct | 1872 bp | cccctcgagGAGCCTGACT |
| Exon 17 | 61,497,435–61,497,557 | TGGAAACACAGgtgagggcag | 2472 bp | ggcatttcagATGGACATGC |
| Exon 18 | 61,502,583–61,502,724 | GATCGAAGCAGgtgagttgc | 2723 bp | ttccttgtagACACAGAAA |
| Exon 19 | 61,503,429–61,503,588 | GGCATGCTGGgtagctggg | 2723 bp | ttccttgtagACACAGAAA |
| Exon 20 | 61,505,461–61,505,558 | TCTGTTCAAAGgtgagttgc | 2723 bp | gggatttcagATGGACATGC |
| Exon 21 | 61,509,659–61,509,742 | GTGAAAGGAggtagggcag | 29303 bp | ttccttgtagACACAGAAA |
| Exon 22 | 61,508,746–61,508,864 | GAGAAATAGgtgagggcag | 2508 bp | ttccttgtagACACAGAAA |
| Exon 23 | 61,509,123–61,509,269 | AGAAGGCCAGgtctgttctt | 1660 bp | ttccttgtagACACAGAAA |
| Exon 24 | 61,515,890–61,516,109 | GCTGTTTGAGGgtgagggcag | 2472 bp | ggcatttcagATGGACATGC |
| Exon 25 | 61,518,719–61,519,006 | GGGCCGAG - 3'UTR | 100 bp | tggatttttagGCCAAGAGCT |

1. Locations are all in respect to UCSC Genome Browser CanFam2.0 (http://genome.ucsc.edu).
2. Capital letters are exonic DNA sequences and lower case bases are intronic regions.
End of coding region marked in Exon 33 row by underlined TGA.
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Discussion

We used homozygosity mapping of SNP microarray genotyping data from PRA-affected Papillons to identify regions where the affected dogs had runs of homozygosity spanning greater than 1.5 Mb and for which control dogs showed allelic variability. Positional candidate genes were identified mapping to those regions and haplotype analysis revealed only one region (on CFA2) in which obligate-carrier dogs had one copy of the haplotype observed in affected dogs, and none of the unrelated control dogs were homozygous for the haplotype observed in affected dogs. CNGB1 was selected as the most promising positional candidate gene because of its location within the CFA2 region and of the similarity of the phenotype of CNGB1 retinal dystrophy in mice and humans to that of the PRA-affected Papillons in our breeding.
colony (early lack of rod function and yet a slow retinal degeneration). The canine genome assembly (CanFam2.0) on the UCSC Genome Browser had incorrect exon predictions for CNGB1. We established the normal gene structure by sequencing cDNA from a retinal library developed from a control dog (GenBank KC527595). Sequencing of the confirmed exons and nearby flanking intronic regions of CNGB1, in affected and phenotypically normal Papillons, revealed a frameshift mutation (CNGB1-fs26) that segregated with disease status in our breeding colony and was not present in the homozygous state in any unaffected Papillons. A missense variation was also detected in the affected dogs (p.P72L). This converts a proline to leucine but at a residue that in some species is a leucine and in others a proline, making it unlikely that this would have a major effect on the function of CNGB1 in the dog [47]. Furthermore, this amino acid change is likely to be due to a polymorphism rather than a causative mutation.

Figure 4. Papillon mutation in CNGB1 exon 26. Sanger dideoxy-sequencing traces for part of CNGB1 exon 26 are shown for an unaffected (A) and an affected (B) Papillon. Panel C shows the codon and amino acid alignment inferred from the traces in panels A and B, for the unaffected sequence (top) and affected Papillon mutation sequence (bottom). The complex Papillon mutation includes a 6 bp AGCTAC insertion between reference bases chr2:61,502,599–61,502,600 (yellow highlight in panel C and within yellow box in panel B) and an adenine deletion at chr2: 61,502,597 (red highlight in panel C and red triangle in panel B). The deletion causes a frameshift and premature stop codon within seven residues, including the two new, inserted codons.

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| Location | Exon Position on Chr2 | cDNA change | Reference allele | Variant allele | cDNA bp | Protein change | SNP number |
|----------|------------------------|-------------|-----------------|---------------|---------|---------------|-----------|
| Exon 2   | 61,457,096             | c.27G>A     | G               | A             | G       | –             | rs22870567 |
| Exon 2   | 61,457,220             | c.151G>A    | G               | A             | A       | p.E69K        | rs22870569 |
| Exon 3   | 61,459,353             | c.215C>T    | C               | T             | C       | p.P72L        | –         |
| Exon 33  | 61,518,754             | c.3378C>A   | C               | A             | A       | –             | –         |
| Exon 33  | 61,518,816             | c.3440T>C   | T               | C             | C       | p.L1165P      | –         |
| Exon 33  | 61,518,910             | c.3534G>A   | G               | A             | A       | –             | –         |

1. Locations are all in respect to UCSC Genome Browser CanFam2.0 (http://genome.ucsc.edu).
2. Reference allele from UCSC Genome Browser CanFam2.0.
3. Variant allele from sequenced gDNA of Papillons.
4. Protein change due to the SNV found in either Papillons and/or cDNA. Change is in respect to CanFam2.0.
5. SNP number from Broad Institute SNP collection (http://www.broadinstitute.org/mammals/dog/snp2).

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Interestingly, the CNGB1X-1 mouse knockout model, which lacks all retinal CNGB1 products, has shown that GARP proteins are necessary in outer segment disk development and the structural integrity of the rod outer segments [46]. Interestingly, the CNGB1X-1 mice have a weak rod response that is detectable on single cell recording. This activity originates from a low level of homomeric CNGA1 channel formation in the rod outer segments. Despite the presence of the channels and the resulting weak light-induced rod response, these mice have severe rod photoreceptor degeneration. It was suggested that degeneration may be due to rod structural, rather than functional, failure [46]. Another mouse model, CNGB1X-26, has an engineered mutation that leads to a deletion of exon 26 resulting in a frameshift that introduces a premature stop codon in the first triplet in exon 27 of CNGB1. The CNGB1X-26 mouse has a complete absence of mRNA for full-length CNGB1 and thus a complete absence of CNGB1 protein in the retina consists of an N-terminal “GARP region” and a C-terminal “channel domain”. GARP1 is of low abundance whereas GARP2 is more highly expressed [46]. The functions of the GARP subunits are still being explored. GARP2 does bind to PDE6 and may act to reduce dark level noise [54]. It is also postulated to have a structural role in the rod outer segment [46] where it interacts with peripherin-2 at the rod outer segment disk rim [55]. As discussed further below GARP2 may also play a role in control of the opening of the CNG channel [56]. Other than these three splice variants expressed in the retina, CNGB1 also has splice variants expressed in the olfactory epithelium (CNGB1b – which encodes a shorter protein than expressed in the retina lacking the GARP region), kidney, brain and testes [41,42,43,44].

Table 4. PRA type 1 genotypes and clinical status for 139 Papillons.

| Genotype | Clinical Status |
|----------|----------------|
|          | PRA affected | Unaffected | Total |
| CNGB1 M/M | 13          | 0          | 13    |
| CNGB1 M/+  | 3           | 20         | 23    |
| CNGB1+/-   | 4           | 99         | 103   |
| Total      | 20          | 119        | 139   |

1. Genotyping results: (+/-) means wild-type CNGB1 sequence. M = mutant (c.2387delA;2389_2390insAGCTAC) genotype.
2. Not including colony dogs to avoid inflation of mutation presence in the general population of Papillon dogs.

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Figure 5. Immunohistochemistry on frozen retinal sections from age (8 wk) and sex matched (female) control and affected dogs. Images on the left are from the control dog and images on the right are from the affected dog. Panels A and B are stained with GARPs and CNGB1. C and D are stained with CNGB1 C-terminal antibody (green) and DAPI. GARP proteins and CNGB1 are present in both the control and affected samples. Panels E and F are stained with CNGB1 antibody (green) and DAPI. CNGB1 is not detected in the affected sample, presumably due to necessity for CNGB1 to form functional channels and normal trafficking. Size bar: 20 μm. OS- photoreceptor outer segment, IS – photoreceptor inner segment, OPL – outer plexiform layer, INL – inner nuclear layer, IPL – inner plexiform layer, INL – inner nuclear layer.

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GARP2 has on channel opening, such that if cGMP cannot bind to CNGB1 the CNG channel is silent [56]. More recently resequencing of candidate RP genes led to the identification of a simplex RP patient homozygous for a missense substitution in CNGB1 (c.2957A>T; p.N986I) resulting in substitution of a conserved amino acid 7 codons upstream of the mutation identified by Bariel et al. and also in the CNBD [61]. Kondo et al. used homozygosity mapping to screen known arRP genes in Japanese RP patients and in one patient identified a mutation at the donor site of exon 32 (c.344+1G>A) of CNGB1 [62]. Subsequently, Becirovic et al. performed exom trapping experiments to investigate the effect of the mutation. Their studies suggest that the mutation leads to skipping of exon 32 and replacement of the last 170 amino acids by 68 unrelated amino acids [63]. The probands in the Bariel et al. and Kondo et al. studies had night blindness from a young age and were diagnosed with RP in their 30’s [60,62].

The CNGB1-fs26 mutation identified in Papillon dogs was present in 13 of the 20 PRA-affected Papillons tested. This suggests that there is at least one additional PRA locus segregating within the breed. Within-dog breeds genetic heterogeneity for PRA is becoming more evident [13,16]. Additional studies will be required to find the gene mutation(s) responsible for the other form(s) of PRA segregating in Papillons.

The early onset of loss of rod function in the CNGB1-fs26 mutant dog, coupled with a slow retinal degeneration that we have observed in our colony dogs, seems to accurately parallel the described disease course in human patients as well as the comparable mouse model (CNGB1X-26). Recently recombinant adeno-associated viral vector-mediated gene therapy to deliver a normal copy of CNGB1 to CNGB1−/− mice was made available for CNGB1 to CNGB1−/− mice with RP in their 20s [60,62].

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Spectral Domain-Optical Coherence Tomography

Assessment of retinal morphology was performed by Spectral Domain-Optical Coherence Tomography (SD-OCT; Spectralis OCT+HRA Heidelberg Engineering Inc., Heidelberg, Germany). Dogs were anesthetized as described for ERG, the pupil dilated with 1% topical tropicamide (Mydriacyl, Alcon Laboratories, Honolulu, HI, USA), a lid speculum fitted and the eye positioned in primary gaze using a stay suture in the inferior perilimbal conjunctiva. High-resolution cross-section images obtained by line and volume scanning and images from the same region of the central retina of affected and control (wild-type) Papillons were assessed.

Animal Use and Sample Collection

A pregnant female Papillon dog that had been diagnosed with PRA and had been mated with a PRA-affected Papillon stud dog was donated to the Michigan State University Comparative Ophthalmology laboratory with the consent of the owner to allow the study of the phenotype of PRA in the breed. This female and her offspring were used to establish a small breeding colony of dogs. The colony was kept under standard laboratory housing with 12:12 hr light:dark cycles.

Blood samples from client-owned Papillon dogs were donated with owner consent. DNA was extracted from blood samples using a commercial DNA extraction kit with a modified protocol (Qiagen Sciences, Germantown, MD). Briefly, a red blood cell lysis buffer (0.32 M sucrose, 10 mM Tris, 5 mM MgCl₂) was added in a 2 step fashion to whole blood (3X volume and then 2X volume, respectively). Cell lysis solution (Qiagen Sciences, Germantown, MD) was added to lyse the white blood cells followed by addition of protein precipitation solution (Qiagen Sciences, Germantown, MD), isopropanol DNA precipitation and a 70% ethanol wash step.

The retina from a mixed breed dog was dissected from an enucleated eye and placed in an RNA stabilization buffer (Invitrogen, Carlsbad, CA). A PRA-affected female Papillon from the research colony and 9 affected and 15 unaffected dogs from 9 different breeds. Twenty-four Papillons (9 cases, 15 controls) were genotyped for the mutation in CNGB1. For each individual SNP, if the affected dogs shared the same homozygous genotype, then the unaffected dogs were compared and rows identified where 95% of the unaffected dogs did not share the same genotype as the affected dogs. Upon those criteria being met, the affected and unaffected groups were considered ‘different’ by the algorithm. Identified SNPs were then sorted into groups formed by having a level of adjacency of at most four SNPs apart. Only sections with 6 or more SNPs meeting the above criteria were marked for further analysis. These regions were then sorted by size of the region and all regions 1.5 MB or larger were inspected for arRP candidate genes using the University of California, Santa Cruz (UCSC) Genome Browser [37] (http://genome.ucsc.edu/). Four regions of particular interest (on CFA2, CFA4, CFA7, and CFA12) were then subjected to haplotype construction, using fastPHASE [65]. Haplotypes were manually examined for shared regions in related affected family members.

DNA Sequencing

The UCSC Genome Browser (http://genome.ucsc.edu/) CanFam2.0 was used in conjunction with the cDNA sequences to identify the exons for the CNGB1 gene. Primers (Table S1) were designed flanking the entire exon and the splice sites using Primer3 (http://frodo.wi.mit.edu/). Sanger dideoxy-sequencing was done by an ABI 3730 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) at Michigan State University’s Research Technology Support Facility.

CNGB1 Genotyping Assay

A restriction enzyme digest was designed to quickly screen dogs for the mutation in CNGB1 (see methods S1). This assay was used to test for the presence of the mutation in 139 Papillon dogs, 33 PRA affected dogs from 8 different breeds and 66 unaffected dogs from 9 different breeds.

Genome-Wide Association Mapping

Two-hundred sixty-two single nucleotide polymorphisms (SNPs) were genotyped on Illumina Canine HD BeadChips. Initial genome-wide association analysis was conducted using the genome analysis toolset PLINK [36]. Initially, all SNPs with a minor allele frequency (MAF) of <5% and with missing genotype calls of >10% were removed from the analysis. The final data set consisted of 116,235 markers. All 24 individuals genotyped successfully for over 90% of the SNPs and were retained in the analysis. One of the control dogs was removed from the PLINK analysis but was used in the run of homozygosity analysis. The final genotyping rate was >99.8%. Chi-square association mapping was conducted in PLINK, and correction for multiple testing was achieved using the Max(T) permutation procedure (10,000 permutations) in PLINK.

Custom Sorting Program and Haplotype Construction

Homozygosity mapping was performed using a custom sorting program. An algorithm was written to search for blocks of SNPs where there was a difference in the calls between the affected and unaffected dogs. The high quality SNP data, generated from PLINK, was imported to a Microsoft SQL Server 2008R2 database (Microsoft Corporation, Redmond, WA) to take advantage of its efficient set theory based querying mechanism. The received data was formatted with dog identifiers as columns and SNPs as rows. Indexing the data provided a method to measure continuity. The query used scalar-valued functions to assess the criteria described below and attached a flag to each row identified. For each individual SNP, if the affected dogs shared the same homozygous genotype, then the unaffected dogs were compared and rows identified where 95% of the unaffected dogs did not share the same genotype as the affected dogs. Upon those criteria being met, the affected and unaffected groups were considered ‘different’ by the algorithm. Identified SNPs were then sorted into groups formed by having a level of adjacency of at most four SNPs apart. Only sections with 6 or more SNPs meeting the above criteria were marked for further analysis. These regions were then sorted by size of the region and all regions 1.5 MB or larger were inspected for arRP candidate genes using the University of California, Santa Cruz (UCSC) Genome Browser [37] (http://genome.ucsc.edu/). Four regions of particular interest (on CFA2, CFA4, CFA7, and CFA12) were then subjected to haplotype construction, using fastPHASE [65]. Haplotypes were manually examined for shared regions in related affected family members.

Immunohistochemistry

A PRA-affected female Papillon from the research colony and an unaffected mixed breed female control dog were humanely euthanized at 8 weeks of age. The eyes were enucleated and the right eye was fixed in paraformaldehyde following a previously describe protocol [66].

Frozen sections were immunolabeled with either a rabbit anti-mouse CNGB1 N-terminal (kindly provided by Dr. Stephen Pittler), rabbit anti-human CNGB1 C-terminal (Sigma-Aldrich, St Louis, MO) or mouse monoclonal CNGA1 ([67] kindly provided by Dr. Bob Molday) antibody. Sections were blocked with 10% horse serum (Sigma-Aldrich, St Louis, MO) for 2 hours at room temperature and labeled with primary antibodies (dilutions of: N-terminal CNGB1 1:100, C-terminal CNGB1 1:300 and CNGA1 1:10) overnight at 4°C. Secondary antibodies (anti-rabbit or anti-mouse Alexa Fluor 488, 1:500) (Invitrogen Molecular Probes, Carlsbad, CA) were placed on the sections for 2 hours at room temperature. All sections were counterstained with the nuclear stain DAPI (Invitrogen Molecular Probes, Carlsbad, CA).

Sections were imaged using a fluorescent microscope (Nikon Eclipse 80i, Nikon Instruments Inc., Melville NY) using commercial image capture software (MetaVue, Molecular Devices, Sunnyvale CA).
Supporting Information

Figure S1 CNGB1 amino acid alignments. Sequence alignments performed using muscle alignment in SeaView software ([68]). Single nucleotide polymorphism and variants found in Papillon gDNA sequencing are numbered (1: p.E69K, 2: p.P72L, 3: p.L1165P) and the changed amino acid is underlined. 2: p.P72L is the SNV that has only been seen in Papillons with the mutation and is marked with red text. The CNGB1X-26 mouse stop codon is highlighted in red. The Papillon mutation is highlighted in green. The human mutations are marked with red text. The arrow which represents a splice mutation ([62]). Epitopes for N-terminal antibody (mouse, highlighted in purple) and C-terminal antibody (human, highlighted in teal).

Table S1 Primers for genomic DNA sequencing.

Table S2 Primers for cDNA sequencing.

Table S3 Non-Papillon breeds tested for CNGB1 mutation.

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Table S4 The entire region of homozygosity surrounding the site of the CNGB1 mutation. (XLS)

Methods S1. (DOCX)

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Author Contributions

Conceived and designed the experiments: PAW LPS JF JTB KGE. Performed the experiments: PAW LPS JF JT. Analyzed the data: KJE AVF PAW JPS JF. Contributed reagents/materials/analysis tools: KJE AVF. Wrote the paper: PAW JPS JF.

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