An ADAM9 mutation in canine cone-rod dystrophy 3 establishes homology with human cone-rod dystrophy 9

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Purpose: To identify the causative mutation in a canine cone-rod dystrophy (crd3) that segregates as an adult onset disorder in the Glen of Imaal Terrier breed of dog.

Methods: Glen of Imaal Terriers were ascertained for crd3 phenotype by clinical ophthalmoscopic examination, and in selected cases by electroretinography. Blood samples from affected cases and non-affected controls were collected and used, after DNA extraction, to undertake a genome-wide association study using Affymetrix Version 2 Canine single nucleotide polymorphism chips and 250K Sty Assay protocol. Positional candidate gene analysis was undertaken for genes identified within the peak-association signal region. Retinal morphology of selected crd3-affected dogs was evaluated by light and electron microscopy.

Results: A peak association signal exceeding genome-wide significance was identified on canine chromosome 16. Evaluation of genes in this region suggested A Disintegrin And Metalloprotease domain, family member 9 (ADAM9), identified concurrently elsewhere as the cause of human cone-rod dystrophy 9 (CORD9), as a strong positional candidate for canine crd3. Sequence analysis identified a large genomic deletion (over 20 kb) that removed exons 15 and 16 from the ADAM9 transcript, introduced a premature stop, and would remove critical domains from the encoded protein. Light and electron microscopy established that, as in ADAM9 knockout mice, the primary lesion in crd3 appears to be a failure of the apical microvilli of the retinal pigment epithelium to appropriately invest photoreceptor outer segments. By electroretinography, retinal function appears normal in very young crd3-affected dogs, but by 15 months of age, cone dysfunction is present. Subsequently, both rod and cone function degenerate.

Conclusions: Identification of this ADAM9 deletion in crd3-affected dogs establishes this canine disease as orthologous to CORD9 in humans, and offers opportunities for further characterization of the disease process, and potential for genetic therapeutic intervention.

Cone-rod dystrophies are severe hereditary retinal diseases characterized by primary dysfunction and loss of cone photoreceptors accompanying or preceding that of rods. The typical age of clinical onset in affected humans ranges from early to late adulthood; autosomal dominant, recessive, and X-linked forms of the disease occur. Multiple mapped human loci are recognized, including over a dozen causative genes (RETNET). A clinically similar disorder, termed canine cone-rod dystrophy 3 (crd3), segregates in the Irish Glen of Imaal Terrier (GIT) breed of dog as an adult onset trait of previously undetermined mode of inheritance. This disease becomes evident ophthalmoscopically in affected dogs as young as 3 years of age, and progresses to end-stage retinal degeneration over several years. Concomitantly, the dogs develop visual problems; these usually manifest first as difficulties avoiding obstacles in dim light, and worsen over several years to apparent total blindness. Its mode of inheritance has been difficult to establish from natural populations, because of the multiple inbreeding loops in natural pedigrees. Previous candidate gene studies have excluded unc-119 homolog (C. elegans) (UNC119 [HOR4]), cone-rod homebox (CRX), peripherin 2, retinal degeneration, slow (PRPH2), interphotoreceptor matrix proteoglycan 1 (MPG-J), tissue inhibitor of metalloproteinase 3 (TIMP3), and retinitis pigmentosa GTPase regulator interacting protein (RPGRIPI) as the causative loci (unpublished data).

The GIT is a numerically small breed originating from the Wicklow Mountains region of Ireland. Although the breed has been known internationally since the 19th century, and was first registered in 1934, the present-day population derives from a very small number of founders from the 1970s. It is thus a genetic isolate, with a small gene pool, significant inbreeding, and pedigrees with multiply overlapping
generations. This population structure predicts extensive linkage disequilibrium flanking the mutation site, and creates an ideal opportunity to map the causative locus by a genome-wide association study (GWAS), as has been demonstrated and exploited for other canine traits [1–8].

The present study reports results of a GWAS that found significant association to crd3 on canine chromosome 16 (CFA16), and led to identification of a deletion mutation in the canine ADAM9 gene that cosegregates with the disease. The mutation removes approximately 23 kb of genomic sequence, including exons 15 and 16, and results in a premature stop codon in exon 17. The mutant protein translated from this transcript is predicted to be truncated, lacking the last 287 amino acids of the C-terminus, part of the cysteine-rich domain, the complete epidermal growth factor (EGF)-like domain, the transmembrane domain, and the cytoplasmic tail. The association of this deletion mutation in canine ADAM9 with crd3 establishes that this canine disease is orthologous to human CORD9 [9].

METHODS

Animal use: All procedures involving animal care were conducted in accordance with the guidelines of the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the US Public Health Service (Public Health Service Policy on Humane Care and Use of Laboratory Animals).

Sample collection: Blood was collected for DNA extraction from a) privately owned crd3-affected and non-affected purebred GIT dogs; b) mixed breed dogs derived from GIT founders and maintained as a study colony as part of an NIH-sponsored project (EY006855) at the Retinal Disease Studies Facility (RDSF) in Kennett Square, PA; and c) from 80 privately owned pedigreed dogs from breeds not known to segregate crd3 (Table 1).

| Breed number | Breed                      | Number tested |
|--------------|----------------------------|---------------|
| 1            | American Cocker Spaniel    | 5             |
| 2            | American Eskimo            | 6             |
| 3            | Australian Cattle Dog      | 5             |
| 4            | Border Collie              | 5             |
| 5            | Chesapeake Bay Retriever   | 5             |
| 6            | Chinese Crested            | 5             |
| 7            | Elkhound                   | 3             |
| 8            | English Springer Spaniel   | 5             |
| 9            | Entlebacher Mountain Dog   | 5             |
| 10           | Golden Retriever           | 5             |
| 11           | Italian Greyhound          | 5             |
| 12           | Labrador Retriever         | 5             |
| 13           | Nova Scotia Duck Tolling   | 5             |
| 14           | Siberian Husky             | 5             |
| 15           | Tibetan Terrier            | 5             |
| 16           | Portuguese Water Dog       | 6             |

Total 80

All dogs were homozygous normal.

| TABLE 1. BREEDS TESTED FOR THE PRESENCE OF ADAM9 CRD3-MUTATION. | Breed number | Breed                      | Number tested |
|---------------------------------------------------------------|--------------|----------------------------|---------------|
| 1 American Cocker Spaniel                                     | 5            |
| 2 American Eskimo                                             | 6            |
| 3 Australian Cattle Dog                                       | 5            |
| 4 Border Collie                                               | 5            |
| 5 Chesapeake Bay Retriever                                    | 5            |
| 6 Chinese Crested                                             | 5            |
| 7 Elkhound                                                    | 3            |
| 8 English Springer Spaniel                                    | 5            |
| 9 Entlebacher Mountain Dog                                    | 5            |
| 10 Golden Retriever                                           | 5            |
| 11 Italian Greyhound                                          | 5            |
| 12 Labrador Retriever                                         | 5            |
| 13 Nova Scotia Duck Tolling                                   | 5            |
| 14 Siberian Husky                                             | 5            |
| 15 Tibetan Terrier                                            | 5            |
| 16 Portuguese Water Dog                                       | 6            |
| **Total**                                                     | **80**       |

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extending from the optic disc to the ora serrata, and the trimmed quadrants were separately placed into the third fixative (2% osmium tetroxide in 0.1 M Na cacodylate buffer) for 1 h at 4 °C, or on ice. The quadrants were then dehydrated in increasing concentrations of ethanol and embedded in an epoxy resin (PolyBed 812; Polyscience, Warrington, PA), sectioned at 1 μm (Supercut 2065 micrometre; Leica, Deerfield, IL), stained with azure II (Sigma, Atlanta, GA) and methylene blue (Electron Microscopy Services, Hatfield, PA) and counterstained with paraphenylenediamine (Sigma). For each dog, 1 μm sections extending continuously from the optic disc to the ora serrata of superior, inferior, and temporal meridians were evaluated using a light microscope.

From selected epoxy embedded blocks, after an area of interest was first determined by light microscopy, a 1–1.5 mm square pyramid was trimmed; ultrathin sections (approximately 60 nm) were cut with a 3 mm diamond knife on a Reichert Ultracut 701701 ultra microtome (Reichert-LABTEC, München, Austria) and floated onto nickel thin bar grids. The grids were then stained for 20 min with aqueous 2% uranyl acetate, washed with degassed MQ water (Milli-Q-Synthesis A 10 System, Model=ZMQP6VFTI, Millipore Corporations, Burlington MA) and then stained with Sato modified lead stain for 7 min and washed as above. Grids were examined with an FEI Tecnai T20 BioTwin transmission electron microscope and images collected with a high-resolution thermally cooled Gatan Orius dual-scan CCD camera (Gatan, Inc.; Warrendale, PA).

Whole-genome association study:

Group design—Nineteen crd3-affected purebred GIT dogs were selected that were the least related to each other as possible, based on maximizing the number of grandparents contributing to the selected cases. Two GIT-derived, mixed-breed crd3-affected dogs were added to bring the total number of cases to 21. A total of 22 unaffected purebred GIT dogs were similarly selected as the least related to each other, but matched pairwise to the affected dogs (i.e., siblings or parents of affecteds).

Single nucleotide polymorphism genotyping—Samples were genotyped using Affymetrix Version 2 Canine single nucleotide polymorphism (SNP) chips and the standard Affymetrix GeneChip Mapping 250K Sty Assay protocol (Affymetrix Inc., Santa Clara, CA), using 250 ng genomic DNA. Genotypes were called using the MAGIC algorithm [13] on batched sets of the Affymetrix probe results files (CEL files).

Association analysis—Genotype calls were tested for association with disease phenotype using Fisher’s exact test to test for under/over-representation of the disease phenotype in one homozygote class versus the rest (i.e., versus a class pooling heterozygotes with homozygotes for the opposite allele). This test was done twice, pooling heterozygotes with either homozygote in turn. A Bonferroni correction for multiple tests was used to set the genome-wide significance threshold at \(-\log_{10}(p)\geq6.39 \{0.05/(60,245*2)\}\). Genotype calls for chromosome 16 were assembled into haplotypes to identify the minimal linkage disequilibrium (LD) interval.

Candidate genes evaluation: Candidate-gene screening was done by exon scanning (Table 2). ADAM9 evaluation was done by amplification of overlapping fragments covering the complete coding region of the gene (Table 3), from normal and affected retinal cDNA (12 weeks and 13.4 weeks old, respectively) prepared with Reverse Transcriptase enzyme (ThermoScript, Invitrogen, Carlsbad, CA) and 2 μg retinal RNA following the manufacturer’s protocol. Twenty ng of DNA or cDNA was denatured at 95 °C for 2 min, and 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min/1000 bp were performed in a thermal cycler (MJ Research, Watertown, MA). An additional final extension time of 5 min at 72 °C ensured full-length products. When necessary, PCRs were optimized by increasing the annealing temperature to up to 64 °C. The reactions were performed using GoTaq® Green Master Mix (Promega, Madison, WI) in a final volume of 25 μl. PCR products were run on 1.8% agarose and stained with ethidium bromide (2 μg/ml in a water bath). Single specific PCR products were extracted using the Qiagen PCR extraction kit (Qiagen) and eluted in 10 mM Tris–HCl (pH 7.5). Some products were extracted from the gel using a Qiagen gel extraction kit (Qiagen). A PCR product (200 ng/1000 bp) was mixed with 8 pmol of, either forward or reverse primer, and sequencing was performed using the Applied Biosystems Automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Each PCR product was sequenced with the forward and reverse primer. Sequences were then analyzed and compared using Sequencher 4.2.2 software (Gene Codes Corp., Ann Arbor, MI).

ADAM9 mutation identification: To further characterize canine ADAM9, 33 primer pairs were designed to amplify and sequence the genomic interval between exon 14 and exon 17 from one normal and two affected dogs (Table 4).

Mutation screening: To identify crd3-affected, -carrier, and -normal dogs, a multiplex PCR was designed to amplify the normal and mutated alleles in one PCR reaction. One primer pair, specific to the normal allele, was located within the deleted sequence, and one primer pair specific to the mutant allele flanked the deletion.

RNA expression: RNA was extracted from a 13.4-weeks-old crd3-affected retina and from 8.6-, 10.4-, and 12-weeks-old normal retinas, as well as 7.7- and 15.7-weeks-old normal brains and a 22.1-weeks-old normal spleen. For Northern analysis, 10 μg of total RNA was mixed with 10 μg/ml ethidium bromide and 3X gel loading buffer (Ambion, Austin, TX) in a final volume of 10 μl, heated at 65 °C for 10 min, chilled on ice for 2–3 min, and loaded on a 1% agarose-formaldehyde denaturing gel; 3 μg of 0.24–9.5 kb RNA ladder was used as a size marker (Invitrogen). The gel ran with continuously circulating 1X Mops running buffer (Ambion)
| Primer pair | Gene name | Forward primer name | Forward primer sequence | Reverse primer name | Reverse primer sequence |
|-------------|-----------|---------------------|-------------------------|---------------------|-------------------------|
| 1           | C8orf4    | C8orf4F1            | AATCAACCTGATAAGCCAGAAGA | C8orf4R1           | TAAATGGGCTCCTCCAAATATCAGA |
| 2           | C8orf4    | C8orf4F2            | CAATCCTACCGTAACAGGCAAGAG | C8orf4R2           | GAAAGAAGGGGAGAAGGACAGGC |
| 3           | C8orf4    | C8orf4F3            | GTGTTGCGTGTCAACCAAGTCTTC | C8orf4R3           | CATCTCCTGCTCCATCCCTGGA |
| 4           | IDO1      | IDO1F1              | AGTTCTCTTGGAGCAACTCCCAT | IDO1R1             | ACTCTGGTCTAATTTTGGCTAG |
| 5           | IDO1      | IDO1F2              | TGTGAAAGGAGACGGTAGGAGTG | IDO1R2             | TAAATGCAGGACGAGGATGGAAG |
| 6           | IDO1      | IDO1F3              | GGGTGTATGAGATACATGGTCTCT | IDO1R3             | AGGCTTGGTCTAGGTTGAAAG |
| 7           | IDO1      | IDO1F4              | GATGGACGCAACAATAAGTACTG | IDO1R4             | TACCCCTAAAGCAGGAAAGGAC |
| 8           | IDO1      | IDO1F5              | GCAGTAAACACCTGAAAACCTGAA | IDO1R5             | TGATGGTCTAATTTTGGCTAG |
| 9           | IDO1      | IDO1F6              | TCAAGCTTAAATGAGGTTGAAAT | IDO1R6             | CCAAGGACCCATCAGAAGAC |
| 10          | IDO1      | IDO1F7              | GGGAACCTTCTCTCACTCCCTCC | IDO1R7             | CTACCTCCTGCTCCCTACTCG |
| 11          | IDO1      | IDO1F8              | CGAAGAGAAACACGAGAAGGAGA | IDO1R8             | TACTATAGGAAGGAGCAGAT |
| 12          | IDO1      | IDO1F9              | TCCTTTTCTCTCCCATCTTCTT | IDO1R9             | TTGTCTACGACGAGGATGTTT |
| 13          | IDO1      | IDO1F10             | TGATTTCTTTTCTCTCACAAGT | IDO1R10            | CCAAGCCTTTTCTCTTACCTT |
| 14          | IDO1      | IDO1F11             | GGGAAGAGAAATACGAGCAAGA | IDO1R11            | CAGATGAAATGGTGAGAGGAT |
| 15          | TM2D2     | TM2D2F1             | GGAATCTGAGTATCGAAAGGAC | TM2D2R1            | ATTTGTTGAGAAGTAGCTAC |
| 16          | TM2D2     | TM2D2F2             | GACAGAAGACACTCCAAAGTAA | TM2D2R2            | GCTCAGAAACAAAGACAGGTT |
| 17          | TM2D2     | TM2D2F3             | TCCACATGCTGACCATCCTAA | TM2D2R3            | AAGCTATTTTCTCCAGAGTTATC |
| 18          | TM2D2     | TM2D2F4             | CACAGAGGACACTCAGATCAT | TM2D2R4            | CCAAGAAAGCAGAACGACAAA |
| 19          | HTRA4     | HTRA4F1             | AGAAAGGCTTCTGAGGGAGGAT | HTRA4R1            | GAGGAGTGAAGGAGGCTAG |
| 20          | HTRA4     | HTRA4F2             | CTCCTCCTCTCCCATCCCTCCT | HTRA4R2            | GCAGTAGAAACACGAGAGCA |
| 21          | HTRA4     | HTRA4F3             | CGTGTCGTTGTGAGGTTCTTGA | HTRA4R3            | CGTAGGAAGGGAAGAGCAGTTT |
| 22          | HTRA4     | HTRA4F4             | TGGCTATAAATACCTACAGGAGT | HTRA4R4            | GCAGACTTTAAGGACAGCAAG |
| 23          | HTRA4     | HTRA4F5             | TGTTGTAGGCTCTGAGAATAATCTGA | HTRA4R5         | ACCTCTACCTGCTTCTCTCTT |
| 24          | HTRA4     | HTRA4F6             | CACTGAGGACTGAGTGGGAGA | HTRA4R6            | CAGACTAGGCTCTGAGGAAAG |
| 25          | HTRA4     | HTRA4F7             | CGTTGCTACGGGACTGAGGAA | HTRA4R7            | AGGTCAGGGGTTGAGAAAACAC |
| 26          | HTRA4     | HTRA4F8             | GTGTTGGTTGTTGATGATGAGT | HTRA4R8            | GTCACTCGGCCCTTCTCTT |
| 27          | HTRA4     | HTRA4F9             | GTTGGTGTGGTGTTGATGAGT | HTRA4R9            | AAGAAGGAGAAGAACTGGGAAC |
| 28          | HTRA4     | HTRA4F10            | GAAGAGGTGAGGAGAGGAGGAC | HTRA4R10           | GACCTCAGTGGTATTTGGGAAG |
| 29          | HTRA4     | HTRA4F11            | GTGTCCTGACCTCCCTCCTA | HTRA4R11           | TCCACATGCTTATTCAGTCTT |
| 30          | HTRA4     | HTRA4F12            | AATCTGACACTTGAGGGAG | HTRA4R12           | GCACATCCTCAACACATATTACA |
| 31          | HTRA4     | HTRA4F13            | ATGGCTTTTCTCTCCCTCTCT | HTRA4R13           | CATTTTGCCACATCTCAAACAA |
| 32          | LETM2     | LETM2F1             | TCTTCTTTATCCCACTATTTG | LET1R              | CCTTCTTCAATCCGCCAATAGT |
| 33          | LETM2     | LETM2F2             | CAAAGTGGACAGTTCCTTCTCTTA | LET2R           | TGCTCAAGTCGAGCCAAGCTC |
| 34          | LETM2     | LETM2F3             | CCAAGTGACCACTCCGAAATAA | LET3R              | TCCCTCTTGGTTGCTCATACTGA |
| 35          | LETM2     | LETM2F4             | ACTGGGAGACACACAAACAAACGTA | LET4R           | GCAGTTAAGAATCACAACAA |
| 36          | LETM2     | LETM2F5             | TCTTCTCTCTCTTCTCTCTCT | LET5R              | ATCTGGGAGACACCCGTTAT |
| 37          | LETM2     | LETM2F6             | AGGAGTTATGGTAGGAAAGCAA | LET6R              | TTGTTGATGAGAAAGGAGTGT |
| 38          | LETM2     | LETM2F7             | CCAAGACAGAAGAGAGTAGCAGA | LET7R              | TTTTACAGTGGTGTGGAGTAC |
| 39          | PPAPDC1B  | PPAPDC1BF1          | GCCTTCCCTCCGCGATTTCCC | PP1R               | GTCTCAGGGCAAATTTTGAGCTC |
| 40          | PPAPDC1B  | PPAPDC1BF2          | CTTATTGGGGGGGGTTGTTTGGT | PP2R               | CAGGGGAGGGGAGGGAGGAG |
| 41          | PPAPDC1B  | PPAPDC1BF3          | GTCTACCTTGCTTCTTTCTG | PP3R               | CAACCTCTTCTCTTCTCTGAA |
| 42          | PPAPDC1B  | PPAPDC1BF4          | CGGAATGATGAGGAGGCTTCT | PP4R               | TCAAGGAATGAAAATTTGAGGCA |
| 43          | PPAPDC1B  | PPAPDC1BF5          | TTCTCACCAGTACGAGTCTGCCAGT | PP5R           | CACAGGAGAAGTACAAAGAGCC |
| 44          | DDHD2     | DDHD2F1             | GGGTGGAGGAGGGAAAGAAAGATA | DDR1              | TCAAGAGAAGAGACCTTGGTACAA |
| 45          | DDHD2     | DDHD2F2             | GGGAGAGCAGCGGAGAGTTTACATT | DDR2              | CGGAGCGGGATGAGGAAACAGTAA |
| 46          | DDHD2     | DDHD2F3             | GAGGCGAGAGCGGAGGTAGGAAAG | DDR3              | ACAAGCAGAGACATTCAAGAAGA |
| 47          | DDHD2     | DDHD2F4             | GTGTTGCTCTCTCCCTCTCT | DDR4              | AGGCTTGGTCTAATTTTGGCTAG |
| 48          | DDHD2     | DDHD2F5             | TCCTCAAGGACAGTCAGAAGGA | DDR5              | TGAAATATACGAAAAAGGAGCAG |
| 49          | DDHD2     | DDHD2F6             | AGCTTTTTTATTTTTCTGCTCGA | DDR6              | AAATTGTTGGCGATCTGAAAC |
| 50          | DDHD2     | DDHD2F7             | CAAGCTTGACTTGTATGCTGGGGAAT | DDR7              | AATCAAGATCATGAAAGAGGCTA |

**Note:** The table provides primer pairs and sequences used to screen candidate genes within the minimal LD interval. Each row represents a specific primer pair targeting a different gene, with the forward and reverse primers listed for each. The sequences are given in nucleotide form.
| Primer pair | Gene name | Forward primer | Reverse primer |
|-------------|-----------|----------------|----------------|
| 51          | DDHD2     | DDG8           | DDR8           |
| 52          | DDHD2     | DDR9           | DDR10          |
| 53          | DDHD2     | DDR11          | DDR12          |
| 54          | DDHD2     | DDR13          | DDR14          |
| 55          | DDHD2     | DDR14          | DDR15          |
| 56          | DDHD2     | DDR16          | DDR17          |
| 57          | DDHD2     | DDR18          | DDR19          |
| 58          | LSM1      | LSM1_F1        | LSM1_R1        |
| 59          | LSM1      | LSM1_F2        | LSM1_R2        |
| 60          | LSM1      | LSM1_F3        | LSM1_R3        |
| 61          | LSM1      | LSM1_F4        | LSM1_R4        |
| 62          | LSM1      | LSM1_F5        | LSM1_R5        |
| 63          | STAR      | STAR1F         | STAR1G         |
| 64          | STAR      | STARF2         | STARR3         |
| 65          | STAR      | STARF3         | STARR4         |
| 66          | STAR      | STARF4         | STARR5         |
| 67          | STAR      | STARF5         | STARR6         |
| 68          | STAR      | STARF6         | STARR7         |
| 69          | EIE4BP1   | EIE4F1         | EIE4F2         |
| 70          | EIE4BP1   | EIE4F2         | EIE4F3         |
| 71          | EIE4BP1   | EIE4F4         | EIE4F5         |
| 72          | EIE4BP1   | EIE4F5         | EIE4F6         |
| 73          | EIE4BP1   | EIE4F6         | EIE4F7         |
| 74          | EIE4BP1   | EIE4F7         | EIE4F8         |
| 75          | BRF2      | BRF1           | BRF1R1         |
| 76          | BRF2      | BRF2R2         | BRF2R3         |
| 77          | BRF2      | BRF3           | BRF4           |
| 78          | BRF2      | BRF5           | BRF6           |
| 79          | GPR124    | GPR124F1       | GPR124R1       |
| 80          | GPR124    | GPR124F2       | GPR124R2       |
| 81          | GPR124    | GPR124F3       | GPR124R3       |
| 82          | GPR124    | GPR124F4       | GPR124R4       |
| 83          | GPR124    | GPR124F5       | GPR124R5       |
| 84          | GPR124    | GPR124F6       | GPR124R6       |
| 85          | GPR124    | GPR124F7       | GPR124R7       |
| 86          | GPR124    | GPR124F8       | GPR124R8       |
| 87          | GPR124    | GPR124F9       | GPR124R9       |
| 88          | GPR124    | GPR124F10      | GPR124R10      |
| 89          | GPR124    | GPR124F11      | GPR124R11      |
| 90          | GPR124    | GPR124F12      | GPR124R12      |
| 91          | GPR124    | GPR124F13      | GPR124R13      |

Table 2: Continued.
| Primer pair | Gene name | Forward primer name | Forward primer sequence | Reverse primer name | Reverse primer sequence |
|-------------|-----------|---------------------|-------------------------|---------------------|-------------------------|
| 101         | GPR124    | GPR124F14           | TAAATGGGAAAGGATGGGACAG  | GPR124R14           | AGTAGCCAGAAGGGAACCTGAGTC|
| 102         | GPR124    | GPR124F15           | CACCCTTCATCCACTGCTTGT  | GPR124R15           | CCCCTGCACACTGACTCTTG   |
| 103         | GPR124    | GPR124F16           | CCCCTGACGCTACCAACCTG   | GPR124R16           | GTCGTGACTTTGCGCCCTT    |
| 104         | GPR124    | GPR124F17           | CTCACACGACGAGTCTGAC    | GPR124R17           | GAGAGGGTGGAAGGATACCTAT |
| 105         | ERLIN2    | ERLF1               | GCATAAGGGCAAATCCAAATAG | ERLR1               | GATGGCTTTCTTGGTTCGTAG |
| 106         | ERLIN2    | ERLF2               | GTTGTGCGCTAGCCTTGTGTT  | ERLR2               | GTTGGTGACACTAGGGGAGCAG |
| 107         | ERLIN2    | ERLF3               | GAACACCTCTACGTTAGGATTGC| ERLR3               | TGTTGGTAAACACCCAAACC  |
| 108         | ERLIN2    | ERLF4               | CTGTCGTTGATGTTTCCAGAGTG| ERLR4               | CAGAAGGCAAGGAACTTGTTG |
| 109         | ERLIN2    | ERLF5               | ATGAACTCTGCTGTCTTTTGCT| ERLR5               | CTGTCCTCCAGTCTCTGT    |
| 110         | ERLIN2    | ERLF6               | CTAGAATGGGACAGGAGCATAA | ERLR6               | AAAAGTGATTTCTGAGATTTTC |
| 111         | ERLIN2    | ERLF7               | GTGTCCTGCTCTTCTTACG    | ERLR7               | CAACACACTGCTCCTAGC    |
| 112         | ERLIN2    | ERLF8               | TTATATGCGCTAGGCAAAGAAGAT| ERLR8               | AATGGGAGACTCAAAACTCACT|
| 113         | ERLIN2    | ERLF9               | TGTGACTTAGGAGGAGGTTAGGG| ERLR9               | ATCTGCGGCGGAGTGAATG   |
| 114         | ERLIN2    | ERLF10              | TACTCAGCTCCAGGACTCTCAAG| ERLR10              | CTACACCTGAATGACCAACAA |
| 115         | ERLIN2    | ERLF11              | GGAGGGGAAAGGAGGATTAGCAT| ERLR11              | CTAAAGGGAGGGGCAATAACTCT|
| 116         | ERLIN2    | ERLF12              | GCACACCCTATTAGTACCTCCGTCAC| ERLR12              | TGCTTACCTGATAAGACCCCAAA|
| 117         | ERLIN2    | ERLF13              | CTGTTGGCTCTCTGTTCAATA  | ERLR13              | GGAACCTGCTGACTCCCATGAT |
| Primer pair | Forward primer name | Forward primer sequence | Reverse primer name | Reverse primer sequence | Observed size in normal (bp) | Observed size in affected (bp) |
|-------------|---------------------|-------------------------|--------------------|-------------------------|----------------------------|-------------------------------|
| 1           | ADAM9_cDNA_F3       | GTTGAGTGGAAACCTGCG      | ADAM9_cDNA_R3      | GGCTCTCTCTCTCATAGTT     | Exon 6                     | 610                           |
| 2           | ADAM9_cDNA_F2       | ATTCATCCATGCTCTCA       | ADAM9_cDNA_R2      | TACAAAGTTCCCCAACACA     | Exon 9                     | 482                           |
| 3           | ADAM9_cDNA_F4       | TGTCTGGCACAGACCCG       | ADAM9_cDNA_R4      | TGGAAACATCACATCGTTG     | Exon 14                    | 832                           |
| 4           | ADAM9_cDNA_F1       | GAGGGATGAGGACACC        | ADAM9_cDNA_R1      | CATTGGTGGCTTTTACGG      | Exon 16                    | NO                            |
| 5           | ADAM9_cDNA_F6       | TGGTATTGGGAACTGGGC      | ADAM9_cDNA_R6      | TACTTTCGTTGGTGGGGA      | Exon 22                    | NO                            |
| 6           | ADAM9_cDNA_F5       | ATGCATGGCAAAAAATCA      | ADAM9_cDNA_R5      | ATCCAGTGGCGAGTCAATTA    | Exon 20                    | 541                           |
| 7           | ADAM9_cDNA_F7       | TGTCACCCCCAGTGTTTTT     | ADAM9_cDNA_R7      | GCTCCACCTCTCCGTAT       | Exon 18                    | 283                           |
| 8           | ADAM9_cDNA_F8       | TGTGCAGTGTCTGCAAG       | ADAM9_cDNA_R8      | CACTTCGCTGATCCCTTTA     | Exon 18                    | NO                            |
| 9           | ADAM9_cDNA_exon1R    | GATATAAGACACCTGTTCT     |                     |                         | Exon 1                     | 389                           |

NO=not observed.
| Primer pair | Forward primer name | Forward primer sequence | Forward primer location | Reverse Primer name | Reverse primer sequence | Reverse primer location | Observed size in normal (bp) | Observed size in affected (bp) |
|-------------|---------------------|-------------------------|-------------------------|---------------------|-------------------------|-------------------------|-----------------------------|-----------------------------|
| A. Initial sequencing of the region |
| 1 | deletion_F1 | CGAGGAAAAACCAAGC AATGTGAT | Exon 14 | deletion_R1 | gagaagcaaatgaaataa gaga | Intron 14 | 521 | 521 |
| 2 | deletion_F2 | ctctctgagtaggagagaggt | Intron 14 | deletion_R2 | tcaagaacactctcttttgc | Intron 14 | 627 | 627 |
| 3 | deletion_F3 | ctcggagagagaggt | Intron 14 | deletion_R3 | gccaaggaatgaaaaacaa | Intron 14 | 691 | NO |
| 4 | deletion_F4 | cggcagctgagagagacc | Intron 14 | deletion_R4 | aagcagagagagagacc | Intron 14 | 691 | NO |
| 5 | deletion_F5 | ccagataggtctgtctgtcag | Intron 14 | deletion_R5 | atactctgactactgactc | Intron 14 | 612 | NO |
| 6 | deletion_F6 | tgtggtgttagtctctgtcctga | Intron 15 | deletion_R6 | gagaaggtgagagagact | Intron 15 | 610 | NO |
| 7 | deletion_F7 | tcttagcagacgacagactcct | Intron 15 | deletion_R7 | cagcagctgacactacatc | Intron 15 | 622 | NO |
| 8 | deletion_F8 | atctgtgctacactcctctgtgct | Intron 15 | deletion_R8 | cattcagagactctgactc | Intron 15 | 611 | NO |
| 9 | deletion_F9 | aatgagaaggctagtctgt | Intron 15 | deletion_R9 | aatcagctgactactgactc | Intron 15 | 999 | NO |
| 10 | deletion_F10 | tctaaatgagagagagagctc | Intron 15 | deletion_R10 | tctctctctctctctctc | Intron 15 | 602 | NO |
| 11 | deletion_F11 | tgctgtcgactactcctctctc | Intron 16 | deletion_R11 | tctctctctctctctctc | Intron 16 | 688 | NO |
| 12 | deletion_F12 | tgtgtctgctgctgctgctgct | Intron 16 | deletion_R12 | cactactactctctctctc | Intron 16 | 759 | NO |
| 13 | deletion_F13 | cactactactctctctctc | Intron 16 | deletion_R13 | cactactactctctctctc | Intron 16 | 795 | NO |
| 14 | deletion_F14 | aatgagaaggctagtctgt | Intron 15 | deletion_R14 | aatgagaaggctagtctgt | Intron 15 | 774 | NO |
| 15 | deletion_F15 | tctctctctctctctctc | Intron 16 | deletion_R15 | tctctctctctctctctc | Intron 16 | 805 | NO |
| 16 | deletion_F16 | tctctctctctctctctc | Intron 16 | deletion_R16 | tctctctctctctctctc | Intron 16 | 835 | NO |
| 17 | deletion_F17 | aatgagaaggctagtctgt | Intron 16 | deletion_R17 | aatgagaaggctagtctgt | Intron 16 | 796 | 796 |
| 18 | deletion_F18 | cactactactctctctctc | Intron 16 | deletion_R18 | cactactactctctctctc | Intron 16 | 798 | 798 |
| 19 | deletion_F19 | cactactactctctctctc | Intron 16 | deletion_R19 | cactactactctctctctc | Intron 16 | 729 | 729 |
| 20 | deletion_F20 | aatgagaaggctagtctgt | Intron 16 | deletion_R20 | aatgagaaggctagtctgt | Intron 16 | 718 | 718 |
| 21 | deletion_F21 | aatgagaaggctagtctgt | Intron 16 | deletion_R21 | aatgagaaggctagtctgt | Intron 16 | 787 | 787 |
| 22 | deletion_F22 | aatgagaaggctagtctgt | Intron 16 | deletion_R22 | aatgagaaggctagtctgt | Intron 16 | 822 | 822 |
| 23 | deletion_F23 | cactactactctctctctc | Intron 16 | deletion_R23 | cactactactctctctctc | Intron 16 | 769 | 769 |
| 24 | deletion_F24 | cactactactctctctctc | Intron 16 | deletion_R24 | cactactactctctctctc | Intron 16 | 739 | 739 |
| 25 | deletion_F25 | cactactactctctctctc | Intron 16 | deletion_R25 | cactactactctctctctc | Intron 17 | 752 | 752 |
| Primer pair | Forward primer name | Forward primer sequence | Forward primer location | Reverse Primer name | Reverse primer sequence | Reverse primer location | Observed size in normal (bp) | Observed size in affected (bp) |
|-------------|---------------------|------------------------|------------------------|--------------------|------------------------|------------------------|-----------------------------|-------------------------------|
| 26          | deletion_F26        | caagccaaaggtaggtctga   | Intron 14              | deletion_R26       | tcactccacagttgaaaac   | Intron 14              | 459                         | 459                           |
| 27          | deletion_F27        | tgactgaaccaggaaggttg   | Intron 14              | deletion_R27       | tgaagcagggagaaagg       | Intron 14              | 462                         | 462                           |
| 28          | deletion_F28        | acetggtgctgcttttagg    | Intron 14              | deletion_R28       | gecctgctggtgacattaa    | Intron 14 and Intron 16 | 487                         | 487                           |
| 29          | deletion_F29        | ctggagcaataggtgctgata  | Intron 14              | deletion_R29       | aaccccaaaacaaaaacaaaaa | Intron 14 and Intron 16 | 649                         | 649                           |
| 30          | deletion_F30        | atcagctgtgaggtgctgata  | Intron 14 and Intron 16| deletion_R30       | cggtaaaagaaaaacaggct    | Intron 14 and Intron 16 | 421                         | 421                           |
| 31          | deletion_F31        | ggaaggttgaggaaggtgaa   | Intron 14 and Intron 16| deletion_R31       | tggagataaaggaagaaaaaggtc | Intron 16              | 476                         | 476                           |
| 32          | deletion_F32        | aagaggaagggagagagacgt  | Intron 16              | deletion_R32       | tgagagagagagagagagagag  | Intron 16              | 658                         | 658                           |
| 33          | deletion_F33        | cacaagctcaacttctctgt    | Intron 16              | deletion_R33       | tggctctctctctctctctctct | Intron 16              | 479                         | 479                           |
| 34          | deletion_F29        | ctggagcaataggtgctgata  | Intron 14              | deletion_R31       | tggagcaataggtgctgata   | Intron 16              | NO                          | 1,515                         |

A. Initial analysis of the region. B. Refined analysis of the region. NO=not observed: PCR failed to amplify a fragment.
for 16 h at 21 V. After three 5-min washes in DEPC-treated water, 20 min in 0.05 N NaOH, and a 15-min soak in 10X SSC, transfer to a nylon-based membrane (GeneScreen Plus; NEN Life Science, Boston, MA) was done with 10X SSC buffer using a standard protocol. Full transfer was confirmed by exposing the gel to UV light. The membrane was washed in 2X SSC for 2 min, and RNA was cross-linked to the membrane (exposure was 0.12 J/cm2; Stratalinker UV Crosslinker; Stratagene, La Jolla, CA). An ADAM9 probe was produced by amplification of normal retinal cDNA using primers located on the 5′ UTR and exon 6, to produce a probe 610 bp long. The product was then cloned (TOPO TA cloning kit, Invitrogen), and the probe was labeled with [α-32P]dCTP using a RadPrime DNA Labeling System (Invitrogen) according to the manufacturer’s protocol, then used for blot hybridization. Hybridization was performed using Ultrasound solution (Ambion, Austin, TX) following the manufacturer’s protocol, and the blot was exposed to X-ray film at −70 °C for 4 day with two intensifying screens. Loading control was achieved by hybridizing a canine-specific beta-actin probe to the membranes under the same conditions and exposing it to X-ray film for 2 h.

RESULTS

Phenotypic evaluation: Clinically, GIT dogs affected by crd3 were diagnosable by ophthalmoscopic examination as young as 3 years of age, although some affected dogs did not show lesions until much older. With one exception, none of the dogs diagnosed as clinically non-affected when examined at ages over 8 years have since proven to be homozygous for the ADAM9 mutation. The exceptional case was a 10 years-old GIT that presented with an unusual geographic pattern of pigmented disturbance in the fundus of both eyes, but with no overt evidence of the retinal thinning or vascular attenuation expected in a crd3-affected dog of this age. Other than this dog, disease was recognized ophthalmoscopically in all crd3-affected dogs by 5 years of age. Two distinctly different clinical phenotypes, however, were observed. In the majority of cases, disease first became apparent as a subtle but generalized alteration in the fundus, as is typically seen in many other forms of late-onset hereditary retinal degeneration in dogs. In these cases, subtle but generalized hyperreflectivity of the tapetal fundus, and retinal vascular attenuation, were the predominant signs of early disease, and these changes worsened appreciably within 12–24 months of initial observation. In a minority of cases, the initial ophthalmoscopic change detected was confined to the area centralis (mid-temporal tapetal fundus), as a discrete, distinctly hyperreflective lesion, with no accompanying ophthalmoscopic evidence of generalized retinal disease. In these dogs, the central lesion remained unchanged for at least 12 months after initial detection, but over a longer period (2–4 years), it did eventually progress to generalized retinal involvement.

The electroretinogram (ERG) of a 12-weeks-old, crd3-affected dog (Figure 1B) was indistinguishable from normal (Figure 1A), showing normal rod- and cone-mediated responses to light stimuli. At about 15 months of age, ERG dysfunction was detected as reduced 30 Hz cone flicker responses (Figure 1C), and at later ages by continued deterioration of both cone and rod responses (Figure 1D,E). At all ages, the loss of cone function was more prominent than that of rods. Vertical calibration bar=100 µV; horizontal=200 ms for rod flicker; and other responses are 100 ms.

Figure 1. Electroretinograms of normal and affected dogs. Electroretinograms (ERGs) from a 7 weeks old normal dog (A), a 12 weeks old dog affected with canine cone-rod dystrophy 3 (crd3; B), a crd3-affected dog aged 1.2 years (C), a 2 years old crd3-affected dog (D), and a 4.9 years old crd3-affected dog (E). Each vertical panel presents electroretinogram (ERG) responses to a red flash, a blue flash, a white flash, 5 Hz low-intensity white flashes (Rod), and 30 Hz high-intensity white light flicker (Cone). Short vertical arrows under the Rod and Cone flicker responses indicate the onset of the flickering light stimuli. Red and White traces represent mixed rod-cone responses, Blue and Rod traces are rod-specific, and Cone traces are cone-specific. Responses of the 12-weeks-old crd3-affected dog appear normal (B), but by 15 months of age, cone dysfunction is detected as reduced 30 Hz flicker responses (C), and is followed at later ages by continued deterioration of both cone and rod responses (D, E). At all ages, the loss of cone function is more prominent than that of rods. Vertical calibration bar=100 µV; horizontal=200 ms for rod flicker; and other responses are 100 ms.
photoreceptor OS and the retinal pigment epithelium (RPE; Figure 2D, arrows); IS and OS appeared disarrayed and disorganized; and structural abnormalities of cone IS and OS were at least as or more severe than those of rods. At later ages, distinct thinning and reduction of cell numbers in the ONL was observed (Figure 2 E,F). At 5 years of age (Figure 2F), cones and rods were severely reduced in number, with only a few nuclei remaining in the ONL, most of which appeared rod-like.

By electron microscopy, in the crd3-affected retina, the apical microvilli of the RPE showed very little of the normal investiture of rod and cone outer segments (Figure 2 H,I). Mostly these microvilli formed a flattened, entangled mat between the RPE cell bodies and the distal OS tips of the
photoreceptors. This mat appeared to correspond to the gap seen between the RPE and the OSL by light microscopy.

Whole-genome association analysis: Of the approximately 127,000 loci represented on the Affymetrix vs2 SNP chip, 60,245 passed the quality control incorporated into the MAGIC algorithm. One affected sample was contaminated and was excluded from the analysis. Comparison of two duplicated samples, to assess the consistency of the MAGIC program, found 98.4% of the calls to be identical in the first duplicate, and 93.3% in the second duplicate, for an average of 95.8%, and most mismatched calls were between an unclustered call and a defined call.

Fisher Exact analysis, comparing genotypes of cases to controls, identified an association signal on CFA16 (Figure 3) extending over 6 Mb and including 28 SNPs that exceeded the Bonferroni corrected significance threshold (-Log10(p) range=6.39–10.09, Table 5). The peak p-value (-Log10(p)=10.09) was shared by six SNPs comprising an interval of approximately 4.4 Mb (CFA16: 22,690,750–27,122,415). All affected dogs were homozygous at these SNP loci, suggesting a recessive mode of inheritance. With that in mind, all genotype calls on CFA16 were aligned to identify a homozygosity block, that is, where all genotype calls for all affected dogs were homozygous, between

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Figure 3. Results of genome-wide association study in canine cone-rod dystrophy 3. The statistical signal (y-axis, negative Log10 [Fisher exact test 2-tailed probability]) for association between canine single-nucleotide polymorphism (SNP) genotype and canine cone-rod dystrophy 3 (crd3) phenotype, plotted against SNP chromosomal location (A), demonstrates a distinct peak on canine chromosome 16 (CFA16). Green dots are SNPs for which the association signal exceeded the Bonferroni threshold for genome-wide significance. Chromosome X is represented by the numbers 39 and 40. Homozygosity analysis of SNP genotypes (B), in the region of CFA16 yielding the peak association signal, reveals heterozygosity throughout the interval in 21 nonaffected control dogs, and demonstrates a 2.74 Mb homozygosity block in 20 crd3-affected dogs. Genotypes are color coded as follows: pink and green represent the major and minor genotypes observed in affecteds, respectively; yellow is heterozygous; and white is missing data. Black lines border the 2.74 Mb homozygosity block. Refseq genes screened as potential positional candidates for crd3 in the present study (arrowheads), and ADAM family genes identified within the crd3 minimal linkage disequilibrium interval (arrows) are indicated with annotation and order consistent with the CanFam2 canine genome assembly (C, not drawn to scale).
positions 27,854,074 and 30,597,700—a 2.74 Mb interval (Figure 3; Appendix 1). The block of homozygosity was not observed in controls except for one dog (dog number 17 in Appendix 1). This dog, when first examined at 10 years of age, had retinal lesions that were regarded as incompatible with a diagnosis of crd3, but in retrospect were likely to represent an unusually delayed and mild form of crd3 disease. Thus, this homozygosity block represents the region of absolute linkage disequilibrium (LD) of crd3 in the GIT population studied.

Homozygosity analysis: To evaluate the power of association mapping by homozygosity, we analyzed genotype calls from just the 20 affected dogs (59,408 SNP calls from autosomal chromosomes) to identify homozygosity blocks greater than 1.0 Mb. Five such blocks were identified where all 20 affected dogs were homozygous for the same allele, and 10 blocks where 19 of the 20 were homozygous for the same allele (Table 6).

Candidate gene screening: The crd3 minimal LD interval (CFA16: 27,854,074 - 30,597,700) was identified as homologous to part of human chromosome 8p11, although the gene order is rearranged between human and dog (data not shown). Although CORD9, a human autosomal recessive cone-rod dystrophy, had previously been mapped to this human interval [14], the CORD9 gene had not yet been identified at the time of the current study. Comparison of genes common to the CORD9 mapped interval and the crd3 LD interval identified 31 potential candidate genes. Ten of these were completely screened (chromosome 8 open reading frame 4 [C8orf4], indoleamine 2,3-dioxygenase 1 [IDO1], TM2 domain containing 2 [TM2D2], leucine zipper-EF-hand containing transmembrane protein 2 [LETM2], phosphatidic acid phosphatase type 2 domain containing 1B

| Number | Location       | -Log10(p) | Location       | -Log10(p) |
|--------|----------------|-----------|----------------|-----------|
| 1      | 28600467       | 10.09     | 25601422       | 6.39      |
| 2      | 28926066       | 10.09     | 27844483       | 7.76      |
| 3      | 29299541       | 10.09     | 27845657       | 8.88      |
| 4      | 30342464       | 10.09     | 27854074       | 7.76      |
| 5      | 30480010       | 10.09     | 28286469       | 9.34      |
| 6      | 29748980       | 10.09     | 28301327       | 9.03      |
| 7      | 28286469       | 9.34      | 28592790       | 9.03      |
| 8      | 30509498       | 9.34      | 28600467       | 10.09     |
| 9      | 28301327       | 9.03      | 28926066       | 10.09     |
| 10     | 28592790       | 9.03      | 29543182       | 8.16      |
| 11     | 27845657       | 8.88      | 30022978       | 8.46      |
| 12     | 29949196       | 8.46      | 30031243       | 8.46      |
| 13     | 29967119       | 8.46      | 30348569       | 8.19      |
| 14     | 30022978       | 8.46      | 29495970       | 8.16      |
| 15     | 30031243       | 8.46      | 29543182       | 8.16      |
| 16     | 30348569       | 8.19      | 29949196       | 8.46      |
| 17     | 29495970       | 8.16      | 29967119       | 8.46      |
| 18     | 29543182       | 8.16      | 30022978       | 8.46      |
| 19     | 31638702       | 8.12      | 30031243       | 8.46      |
| 20     | 30473690       | 7.93      | 30348569       | 8.19      |
| 21     | 27844483       | 7.76      | 30473690       | 7.93      |
| 22     | 27854074       | 7.76      | 30480010       | 10.09     |
| 23     | 30039251       | 7.67      | 30509498       | 9.34      |
| 24     | 31810810       | 7.28      | 31638702       | 8.12      |
| 25     | 31664333       | 6.97      | 31664333       | 6.97      |
| 26     | 31793958       | 6.97      | 31793958       | 6.97      |
| 27     | 31823035       | 6.61      | 31810810       | 7.28      |
| 28     | 25601422       | 6.39      | 31823035       | 6.61      |

Single Nucleotide Polymorphisms that passed Bonferroni correction, location on chromosome 16, and -Log10(p) values sorted by -Log10(p) values (A) and by location (B). Bolded are the single nucleotide polymorphisms (SNPs) with the highest -Log10(p) values.
PPAPDC1B, DDHD domain containing 2 (DDHD2), LSM1 homolog, U6 small nuclear RNA associated (S. cerevisiae) (LSM1), steroidogenic acute regulatory protein (STAR), eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), and ER lipid raft associated 2 (ERLIN2) and three (HtrA serine peptidase 4 (HTRA4), BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like (BRF2), and G protein-coupled receptor 124 (GPR124)) were partially screened, by exon scanning (Figure 2C; Table 2). All 10 were excluded from association with crd3.

**ADAM9 evaluation:** When a mutation in ADAM9 was established as associated with CORD9 [9], we evaluated its role in crd3-affected dogs. The human ADAM9 gene (NM_003816.2) has 22 exons and encodes an 819-amino acid protein. Blasting this sequence against CanFam2 identified 21 of the predicted canine exons but failed to identify the complete sequence of exon 4. This sequence was identified completely in the CanFam1 assembly. Six primer pairs were designed from CanFam2 to amplify overlapping retinal cDNA fragments of the complete canine ADAM9 coding sequence (Table 3, primer pairs 1 to 6). From normal retinas, all six primer-pairs each amplified a single product and alignment of the overlapping sequences established a 2,781 bp canine ADAM9 cDNA sequence (accession number HM590630) comprising 29 bp of 5′ UTR, 2,460 bp of coding region, and 292 bp of 3′ UTR.

From affected retinas, primer pairs 4 (exon 13 to 16) and 5 (exon 16 to 22) both failed to amplify a product. Subsequently, amplification with primer pair 7 (exon 14 to 18) gave 573 bp and 283 bp product from normal and affected dogs, respectively. Primer pair 8 (exon 15 to 18) failed to amplify cDNA from the affected dog, but yielded a 389 bp product from normal dogs. Sequence analysis of the 283 bp crd3-affected amplicon from primer pair 7 showed that exons 15 and 16 are absent from affected cDNA.

The genomic interval between exon 14 and exon 16 was partially amplified and sequenced from one normal and two affected dogs (Table 4). This interval spans over 40 Kb in CanFam2 (CFA16: 29,384,764–29,425,539). Initial screening used 25 primer pairs, evenly distributed (Table 4). Primer pairs 3 to 16 failed to amplify DNA in the two affected dogs. This suggested a deletion of at least 21 Kb, between intron 14 and intron 16. To refine the deletion points, PCR was done using an additional eight primer pairs, amplifying overlapping fragments (Table 4, F26/R26–F33/R33). Surprisingly, all eight primer pairs amplified fragments from the affected dog that were identical in size to those from the normal dog. Critical examination of the CanFam2 sequence revealed an LTR sequence in intron 14 (29,422,135–29,423,412) and another in intron 16 (29,398,914–29,400,191), with 99.5% identity (6 SNPs in 1,278 bp). That suggested that unbalanced recombination between the two LTRs might have led to a deletion of more than 23 Kb, resulting in a single fused copy of the LTR and deletion of exon 15 and 16 from the genome (Figure 4A). To test this hypothesis, PCR was undertaken with primers F29 (on intron 14, upstream from the first LTR) and R31 (on intron 16, downstream from the second LTR). This distance is >24 Kb in the normal genomic DNA, and in the proposed scenario should be only 1,515 bp in the affected dogs. The PCR failed to amplify DNA in the normal dog, and amplified the predicted size product from affected ones. The sequence of this product revealed a deletion of 23,221 bp between the LTR in intron 14 and the LTR in intron 16. This deletion included part of intron 14, all of exon 15, intron 15 and exon 16, and part of intron 16. The SNPs in the LTRs suggest that the unbalanced recombination took place between the first and

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**Table 6. Homozygosity evaluation.**

| Chromosome | Size of block >1.0 Mb |
|------------|-----------------------|
|            | 19/20 dogs homozygous to same allele | 20/20 dogs homozygous to same allele |
| 1          | 1.02                  | -                      |
| 12         | 1.25                  | -                      |
| 12         | 3.57                  | -                      |
| 12         | 1.19                  | -                      |
| 14         | 1.4                   | 1.4                    |
| 16         | 3.14                  | 2.74                   |
| 20         | 1.26                  | 1.26                   |
| 21         | 1.94                  | 1.94                   |
| 26         | 1.33                  | 1.33                   |
| 38         | 1.2                   | -                      |
| Total      | 10                    | 5                      |

Homozygosity analysis was done on genotype calls for crd3-affected dogs (n=20). Blocks of homozygosity for the same allele were measured, and those greater than 1.0 Mb were recorded. In bold is the homozygosity block that bears the mutation for crd3.
the second of these SNPs, and resulted in a chimeric, single LTR (Figure 4A).

**Population mutation screening:** Multiplex PCR was designed to amplify the normal and mutated alleles of canine ADAM9.

Figure 4. ADAM9 mutation in canine cone-rod dystrophy 3 (crd3) affected dogs. A schematic drawing, of part of the canine ADAM9 genomic sequence, aligning the normal and crd3 mutant alleles suggests a possible mutation mechanism. Bordered square boxes represent exons 14–17, lines between are introns; unbordered rectangular boxes represent long-terminal repeat sequences (LTR). Within the LTR are the nucleotides identified as single nucleotide polymorphisms (SNPs). A suggested mechanism of unbalanced recombination is illustrated, resulting in the deletion of part of intron 14, all of exon 15, intron 15, exon 16, and part of intron 16, as well as the formation of a single chimeric LTR. Arrows represent the location of primers used to identify the mutation. Primer pair F29/R31 amplifies the mutant allele and results in a 1,515 bp PCR product from genomic DNA. Primer pair F10/R10 amplifies the normal allele and results in a 602 bp PCR product from genomic DNA.

B: Gel electrophoresis of multiplex PCR reaction identifies ADAM9 alleles from normal (N), crd3-carrier (C), and crd3-affected (A) dogs. The lower, 602 bp band is the normal allele, amplified by primer pair F10/R10 located within intron 15, which is deleted in the affected allele. The upper 1,515 bp band is the mutant allele, amplified by primer pair F29/R31, which flanks the >23 kb sequence deleted in the affecteds. Both bands are present in the heterozygous carrier dog. The normal and crd3 mutant canine ADAM9 transcripts, and their corresponding predicted translation products, are aligned (C) to illustrate their differences schematically. The protein domains represented are those predicted by Swiss-Prot for the human ADAM9 protein. Exons 15 and 16 are missing from the mutant transcript, and a premature stop codon is introduced (arrow). The mutant protein translated from this transcript is predicted to be truncated, lacking the last 287 amino acids of the C-terminus, part of the cysteine-rich domain, the complete epidermal growth factor (EGF)-like domain, the transmembrane domain, and the cytoplasmic tail.
Table 7. Genomic PCR primers. Primer pairs, sequences, location and observed product sizes in normal, crd3-carrier, and crd3-affected DNA, used in multiplex analysis. NO=not observed.

| Primer pair | Forward primer name | Forward primer sequence | Forward primer location | Reverse primer name | Reverse primer sequence | Reverse primer location | Observed size from normal dog (bp) | Observed size from crd3-carrier dog (bp) | Observed size from crd3-affected dog (bp) |
|-------------|---------------------|-------------------------|-------------------------|---------------------|-------------------------|-------------------------|-----------------------------------|------------------------------------------|--------------------------------------------|
| 1           | F10                 | tctaaatggaggaagttgaa    | Intron 15               | R10                 | ttcttggctttgga         | Intron 15               | 602                               | 602                                      | NO                                         |
| 2           | F29                 | ctggagaatgggtgga         | Intron 14               | R31                 | tctctgtgggctgaa        | Intron 16               | NO (>24 Kb)                       | 1515                                     | 1515                                      |

(Table 7, Figure 4A,B). Primer pair 10F/10R, located within intron 15, will only amplify a 602 bp-long fragment from a normal chromosome. The affected allele is detected using primers F29/R31, which flank the deletion. All dogs included in the GWAS were genotyped. All 20 affected dogs were homozygous for the mutation; five control dogs were carriers (dogs 7, 9, 10, 12, and 13 in Appendix 1, of which 10 and 13 were obligate heterozygotes because they produced affected dogs); and 16 controls were homozygous normal. Dog number 17 in the control group, whose genotype in the minimal LD region was identical to that of affected dogs, was homozygous for the mutation, confirming this dog’s affected status.

Thirty-six further purebred GIT dogs, not part of the GWAS, were screened for the mutation. Ten of these that had been diagnosed as affected were homozygous for the mutation; 24 non-affected dogs were either homozygous normal, or heterozygous; and two dogs that were clinically non-affected when examined at young ages (1.2 and 2 years old) were homozygous for the mutation, and presumably will become affected at a later age. A subset of crd3-colony dogs was genotyped as well, and showed segregation of the ADAM9 mutation with the disease (data not shown). The ADAM9 mutant allele was not found in 80 dogs from 16 other different breeds (Table 1).

The association between the six most significant SNPs identified from the GWAS was further analyzed by comparing the genotype for each SNP with that at the ADAM9 mutant locus, and calculating the correlation coefficients. Two SNPs (rs22468640 and rs22463503, located 798 Kb and 472 Kb upstream from the mutation, respectively) were in complete linkage disequilibrium with the mutation, with \( r^2 = 1 \), while the other four had correlation coefficients between 0.929 and 0.976 (data not shown).

**ADAM9 mutation: in silico protein analysis:** The 2,781 bp canine ADAM9 mRNA ( accession number HM590630, Appendix 2) is predicted to encode an 819-amino acid protein, and comparison to the human ADAM9 protein database (Swiss-Prot) suggests that this protein includes a signal peptide (amino acids 1–28, translated from exon 1); a pro-domain (amino acids 29–211, exon 2–exon 7); a metalloprotease domain (amino acids 212–404, exon 7-exon 12); a disintegrin domain (amino acids 414–501, exon 12–exon 14); a cysteine-rich domain (amino acids 505–634, exon 14–exon 17); an EGF-like domain (amino acids 644–698, exon 17–exon 19); and a transmembrane domain (amino acids 698–718, exon 19). Amino acids 29–697 are extracellular, 698–718 are transmembrane, and 719–819 represent the cytoplasmic tail (Figure 4C; Appendix 2).

Amplification of the ADAM9 coding region from crd3-affected retina identified a 2,491 bp-long, transcript that included all exons except 15 and 16, which are deleted from genomic DNA (Appendix 2). The mutation causes a frame shift, and results in a premature stop codon at base number 6 of exon 17. The mutant protein translated from this transcript is predicted to be truncated and to lack the last 287 amino acids of the C-terminus. That would remove part of the cysteine-rich domain, the complete EGF-like domain, the transmembrane domain, and the cytoplasmic tail (Figure 4C; Appendix 2).

**RNA expression profile:** Full-length RNA expression was evaluated by northern blot assay to determine the ADAM9 mRNA transcript size and absolute level of expression in crd3-affected retinas, compared to normal ones. Northern blot analysis showed one transcript of about 4.0 Kb, highly expressed in non-affected retinas, and in lower levels in the brain and spleen (Figure 5). In the affected retinas, a smaller-size band was observed, which corresponded to the mutated allele, and the level of expression seemed slightly reduced, compared to normal.

**DISCUSSION**

Recent advances in canine genomics have increasingly highlighted and exploited the wealth of hereditary traits that the domesticated dog provides. Such traits range from morphology and behavior, through a panoply of genetic diseases and disease susceptibilities, and extend to broader aspects of population and genomic evolution [15–19]. One specific and highly productive focus within this broader field has been into the numerous hereditary retinal diseases that segregate in specific canine populations. Numerous genes causally associated with these retinal diseases have been identified [20,21] providing novel insights into the structural and functional mechanisms involved in normal and diseased retinas [22], and a suite of canine models for preclinical
evaluation of potential therapies for comparable human diseases [23–26].

Canine population structure comprises multiple discrete, essentially closed populations; the specific breeds of dog lend themselves to genetic studies in much the same way as do the isolated human populations that geneticists have appreciated for many years [27–30]. Genome-wide association studies (GWAS) of Mendelian and complex traits in isolated human populations typically use platforms yielding genotypes for more than 500,000 informative SNPs [31–33]. GWAS efficiency is facilitated by the large LD blocks in these human isolate populations. In particular, young isolates with relatively few founders show particularly extensive LD with few gaps [31].

The division of the canine population structure into multiply isolated subsets, each of fairly recent origin and characterized by significant inbreeding and a restricted number of founders, permits a GWAS to be undertaken with a relatively lower number of cases and controls, and a much less dense set of SNP loci [6,7,32–35] than has typically been needed in human populations. In particular, young isolates with relatively few founders show particularly extensive LD with few gaps [31].

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Positional candidate gene analysis identified a 23,221 bp deletion within the canine ADAM9 gene, leading to a loss of exons 15 and 16, a premature stop codon, and a predicted protein truncated at its c-terminus. This establishes crd3 as a true ortholog of human CORD9, in which four distinct ADAM9 mutations have been found [9]. The latter mutations are either nonsense mutations or frame-shift mutations leading to nonsense change, all within the pro-domain or the metalloprotease domain. The canine mutation resembles the human in that it causes a frame-shift and a nonsense change, which would lead to a truncated protein.

Initially, the Adam9−/− mouse was reported as having no evident major abnormalities during development or adult life [36], but subsequent reevaluation identified electroretinographic abnormalities, suggesting a progressive degeneration affecting both rods and cones [9]. Histologically, the retinas of these mice showed an abnormal gap between the photoreceptor outer segment distal tips and the RPE apical membrane, which electron microscopy revealed to be a failure of the RPE apical processes to invest the OS; instead, they formed a flattened distorted mat between the OS and the RPE [9].

The light microscopic and ultrastructural morphological abnormalities of crd3-affected canine retinas reported herein are clearly consistent with those observed by Parry et al. in the Adam9−/− mouse [9]. As in the mouse, the canine ADAM9 mutant retina exhibits disarray, disorganization, and progressive degeneration of the photoreceptors. Unique to these two orthologous diseases, and presumptively therefore involved in human CORD9, is the failure of RPE apical microvilli to invest the photoreceptors. This strongly suggests that this structural abnormality reflects the absence of a critical function served by ADAM9.
ADAM proteases form a still-growing family of transmembrane proteins. The human genome contains 25 ADAM genes, of which four appear to be pseudo-genes (ADAM1, ADAM3, ADAM5, and ADAM6) and nine are expressed in the retina (ADAM9, ADAM10, ADAM11, ADAM12, ADAM15, ADAM17, ADAM19, ADAM23, and ADAM33; UniGene). In contrast, the mouse and rat have 37 and 34 ADAM genes, respectively, many of which are specifically expressed in testis. In the canine genome, 30 ADAM genes are predicted (Genome), with the five additional genes, compared to human, apparently corresponding to mouse genes specifically expressed in testis. Most of the genes in this family lack a functional protease domain, and appear to code primarily for adhesion proteins. ADAM9 is one of a few that have both adhesion and proteolysis functions [37–39]. Most ADAMs feature a significant overlap of substrate specificities, which may explain why inactivation of individual ADAMs only rarely causes major phenotypes. With that said, careful evaluation is needed, as the phenotype alteration is sometimes overlooked [36].

Comparison of the predicted canine, human, and mouse ADAM9 proteins shows that the human and canine sequences are more similar to each other than are human and mouse sequences (92.6% and 78.4%, respectively). The differences reside predominantly in the cytoplasmic tail, where only 14.7% of the mouse amino acids are identical to the human, compared to a 93.1% identity for dog amino acids. Furthermore, the mouse cytoplasmic tail has 26 additional residues that are absent from both the human and canine proteins.

Identification of this ADAM9 mutation in crd3 provides a potentially important canine model for CORD9. The opportunity to undertake synergistic studies among orthologous murine and canine models and human patients has been very useful both for advancing knowledge of the disease mechanism in numerous disorders and for developing potential therapies. CORD9-affected human patients suffer childhood-onset visual acuity impairment, progressing over decades to major loss of central and then peripheral visual function [14]. Thus, disease severity clearly establishes a basis for considering genetic therapy. In the canine model, the retina is not fully developed until 8 weeks of age, the cone and rods are largely intact as late as 12 weeks postnatally, and the ERG shows normal responses up to one year of age. Thus, there is not only a prima facie case for therapeutic intervention based on disease severity, but also a significantly long-term window of opportunity.

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Appendix 1. Genotype calls from 20 crd3-affected cases and 22 control dogs, in a 6.1 Mb interval on canine chromosome 16.

The interval includes all SNPs yielding an association signal exceeding the Bonferroni corrected threshold for genome wide significance. Columns=dogs, rows=SNP loci. For each locus, homozygosity for the most frequent allele observed in cases is color-coded in pink for all dogs (i.e., cases and controls); green indicates homozygosity for the minor allele observed in cases; yellow indicates heterozygosity; and white (–)=no call. The crd3-absolute LD interval is boxed. Control dog 17 proved to be affected with an atypical form of crd3 (see text). To access the data, click or select the words “Appendix 1.” This will initiate the download of a compressed (pdf) archive that contains the file.

Appendix 2. Canine ADAM9 mRNA and predicted protein.

A. Canine ADAM9, normal allele. A1. ADAM9 mRNA sequence from normal dogs. Upper case indicates coding sequence, boxed are the first methionine and the stop codon. In bold are exons 15 and 16 that are missing in the affected dog. A2. Canine ADAM9, predicted protein from a normal dog. The protein is 819 amino- acids long. Features in the protein are as follow: Red sequence: signal peptide- 1–28 aa (translated from exon 1); Grey sequence: 29–211 aa (translated from exon 2- exon 7); Orange sequence: Metalloprotease domain: 212–404 aa (translated from exon 7-exon 12); Blue sequence: Disintegrin domain: 414–501 aa (translated from exon 12-exon 14); Pink sequence: Cystein rich domain: 505–634 aa (translated from exon 14-exon 17); Brown sequence: EGF-like domain: 644–698 aa (translated from exon 17-exon 19); Green sequence: Transmembrane domain: 698–718 aa (translated from exon 19). Amino acids 29–697: extracellular, amino acids 698–718: transmembrane; amino acids 719–819: cytoplasmic. B. Canine ADAM9, crd3 mutant allele. B1. Sequence of crd3 mutant canine ADAM9 mRNA. Boxed are the first methionine and the premature stop codon. ◊ indicates the location of exons 15 and 16 missing in the affecteds. Underlined is the stop codon in a normal dog. B2. Canine ADAM9 predicted mutant protein product. Colored are the domains. The last two amino acids are not present in the normal protein. The predicted protein is 532 amino- acid long. To access the data, click or select the words “Appendix 2.” This will initiate the download of a compressed (pdf) archive that contains the file.