**FGF4** retrogene on CFA12 is responsible for chondrodystrophy and intervertebral disc disease in dogs

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Chondrodystrophy in dogs is defined by dysplastic, shortened long bones and premature degeneration and calcification of intervertebral discs. Independent genome-wide association analyses for skeletal dysplasia (short limbs) within a single breed ($P_{\text{Bonferroni}} = 0.01$) and intervertebral disc disease (IVDD) across breeds ($P_{\text{Bonferroni}} = 4.0 \times 10^{-19}$) both identified a significant association to the same region on CFA12. Whole genome sequencing identified a highly expressed **FGF4** retrogene within this shared region. The **FGF4** retrogene segregated with limb length and had an odds ratio of 51.23 (95% CI = 46.69, 56.20) for IVDD. Long bone length in dogs is a unique example of multiple disease-causing retrocopies of the same parental gene in a mammalian species. FGF signaling abnormalities have been associated with skeletal dysplasia in humans, and our findings present opportunities for both selective elimination of a medically and financially devastating disease in dogs and further understanding of the ever-growing complexity of retrogene biology.

GWAS | inherited | genetic | dysplasia | chondrodysplasia

**Significance**

Chondrodystrophy, characterized by short limbs and intervertebral disc disease (IVDD), is a common phenotype in many of the most popular dog breeds, including the dachshund, beagle, and French bulldog. Here, we report the identification of a **FGF4** retrogene insertion on chromosome 12, the second **FGF4** retrogene reported in the dog, as responsible for chondrodystrophy and IVDD. Identification of the causative mutation for IVDD will impact an incredibly large proportion of the dog population and provides a model for IVDD in humans, as FGF-associated mutations are responsible for IVDD and short stature in human achondroplasia. This is a report of a second retrogene copy of the same parental gene, each causing complementary disease phenotypes in a mammalian species.


Conflict of interest statement: The University of California, California, Davis, has filed a provisional patent entitled: “Methods of Diagnosing Intervertebral Disc Disease and Chondrodystrophy in Canines,” on May 30, 2017.

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Data deposition: The sequence data reported in this paper has been deposited in the Sequence Read Archive (SRa Bioproject no. PRJA377155) and in the GenBank database (accession nos. MF040221 and MF040222).

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Skeletal dysplasia (SD), a general term to classify abnormalities of growth and development of cartilage and/or bone resulting in various forms of short stature, occurs in humans and dogs in many forms (19). With advances in molecular genetics, many of the diseases in humans are being reclassified based on the specific underlying causative mutations (20). To a lesser degree, progress has also been made in understanding the molecular nature of SD and the extreme interbreed limb-length variation observed in dogs (21–24). While the mutations causing some subtypes of SD in dogs have been determined, there are still many unexplained types of SD observed within and across dog breeds.

In 2009, the genetic basis for extreme differences in limb length in dogs was investigated by Parker et al. (25) using an across-breed genome-wide association approach. They determined that a FGF4 retrogene insertion on CFA18 (~25 Mb from the parental copy of the FGF4 locus was responsible for the “chondrodystasia” phenotype in a number of breeds, such as the bassett hound, Pemberle Welsh corgi, and dachshund. However, the FGF4 retrogene insertion on CFA18 failed to explain breeds such as the American cocker spaniel, beagle, and French bulldog, that in addition to dachshunds, were the breeds originally classified as chondrodystrophic based on histopathological and morphological analysis by Hansen (1) and Braund (3). The FGF gene family has similarly been implicated in SD in humans, with mutations in FGF3 found to be responsible for achondrodysplasia, the most common form of dwarfism, characterized by shortened limbs and abnormal vertebrae and IVDs (20, 26–30). FGF genes are involved in a number of embryological development processes, and specific levels of ligand and receptor are key for appropriate growth and development (31–33).

In this study, genome-wide association analysis in a cohort of Nova Scotia duck tolling retrievers (NSDTRs) with and without severe SD identified a significant association on CFA12 due to a 12-Mb associated haplotype, of which 1.9 Mb was found to be shared in chondrodystrophic breeds. Subsequent genome-wide association analysis of Hansen’s type 1 IVDD across breeds localized the same 1.9-Mb region on CFA12, suggesting that the locus responsible for SD in the NSDTR is also responsible for type 1 IVDD and the chondrodystrophic phenotype across dog breeds. A previous genetic investigation of IVDD in dachshunds and limb-length morphology in Portuguese water dogs both identified the same CFA12 locus; however, neither study reported a causative mutation (34, 35). Here, using Illumina paired-end genomic sequencing, we uncover a second FGF4 retrogene insertion (chr12: 33.7 Mb [Canis familiaris {canFam} 3]) in the canine genome and show that it is not only responsible for SD in the NSDTR, but also chondrodystrophy, including the predisposition to Hansen’s type 1 IVDD, across all dog breeds.

**Results**

**Genome-Wide Association Studies for SD and IVDD.** A form of SD is common in the NSDTR and is characterized by variable decrease in limb length and associated abnormalities, including long-bone bowing, physeal widening, and joint incongruity (Fig. 1 A and B). On physical examination, in addition to shorter limbs, SD dogs may also have valgus limb deformities and larger ears (pinnae). While SD is a common phenotype in the breed, the degree of severity is highly variable.

To determine a region of the genome associated with SD in the NSDTR, genome-wide association analysis was performed using 13 NSDTRs with severe SD and 15 NSDTR controls without severe SD. There were 41 single-nucleotide polymorphisms (SNPs) that were genome-wide significant with a $P_{\text{Bonferroni}}$ of <0.05, all present between chr12: 35,413,695 and 46,117,273 (top SNP chr12: 36,790,324 $P_{\text{Bonferroni}} = 0.01$) (canFam2) (Fig. 1C and SI Appendix, Fig. S1).

Underlying this strong association for SD in NSDTRs was an 12-Mb critical interval from chr12: 36–48 Mb (canFam2). Since the NSDTR SD phenotype is not uncommon in different dog breeds, we investigated haplotype sharing across breeds and observed that a portion of this associated haplotype was shared with two breeds of dog considered classically chondrodystrophic: the American cocker spaniel and beagle (1, 3). By plotting the minor allele frequency (MAF) across this interval for 7 American cocker spaniels, 14 beagles, and 13 SD-affected NSDTRs, the critical interval identified via GWAS for SD was shortened to a shared haplotype from chr12: 36.4–38.3 Mb (canFam2) (Fig. 2A).

To test the hypothesis that the same locus was responsible for SD and chondrodystrophy, a second genome-wide association study (GWAS) was performed using IVDD-affected cases ($n = 36$) and unaffected controls ($n = 31$) across 26 dog breeds (listed in SI Appendix, Table S6). The most highly associated SNP was located on CFA12 [chr12: 36,909,311 (canFam2)] with a $P_{\text{raw}} = 3.2 \times 10^{-15}$, $P_{\text{Bonferroni}} = 4.0 \times 10^{-10}$, and odds ratio of 32.67 (Fig. 2B). Observing linkage disequilibrium with the highest associated SNP using $r^2$ values >0.06, the critical interval identified via GWAS for IVDD overlaps with that seen when mapping MAF across breeds and SD in the NSDTR (Fig. 2C).

**Identification of FGF4 Insertion on CFA12.** To identify a causative variant for SD and IVDD, paired-end whole-genome sequences of two cases, one SD-affected NSDTR and one IVDD-affected dachshund, and 83 unaffected controls were investigated in the associated interval. The average coverage for these samples was 8.71x. There were 9,156 SNP variants and 7,877 insertion/deletion (indel) variants identified from chr12: 33.1–35.5 Mb (canFam3) [chr12: 36.1–38.5 Mb (canFam2)]; however, none segregated with the IVDD phenotype. The same interval was also investigated by visual inspection of BAM files to flag mate
retrogene insertion at FGF4 as that seen in the CFA12 genotypes were consistent with IVDD, 33 additional cases were genotyped for FGF4 (axis) for each genotype (i.e., FGF4 insertion (axis) and FGF4 insertion (axis) plotted by breed. SD-affected NSDTR (0.2) of the raw values (axis) and 0 (0.6) are known in the dog; however, they were associated (10); Table S1). Four large indels did not segregate when investigated in additional control genomes, while the remaining four were eliminated after PCR showed lack of segregation between cases and controls.

Visual inspection of the BAM files for read pairs mapping to a different chromosome location identified a region, located at approximately chr12: 33,710,200 (canFam3) that segregated with the two cases and two controls (Fig. S2). At this location, read mates mapped to chr18: 48.4 Mb (canFam3) and chr7: 68.3 Mb (canFam3) in the NSDTR and dachshund cases, but none of the controls. The reads that mapped to CFA18 aligned to parental FGF4, which was highly suggestive of a FGF4 retrogene insertion at this location. The reads that mapped to CFA7 were investigated by PCR and appear to mark a genome assembly error or a mutation within the dog used for the genome assembly (canFam3).

To investigate the potential FGF4 insert on CFA12, the region was PCR amplified using primers flanking the insertion site from genomic DNA of an IVDD-affected beagle. Wild-type dogs without the insert had a single 615-bp band, while dogs homozygous for the CFA12 FGF4 insertion had an ~4-kb product. Sanger sequencing showed the insertion on CFA12 is 3,209 bp long (GenBank accession no. MF040221) and includes parental FGF4 cDNA (i.e., FGF4 exons spliced without introns), as shown in the insert schematic comparing parental FGF4 to the CFA12 insert (Fig. 3). The insert also contains a majority of the predicted 5′-untranslated region (UTR), which includes the transcription start site (TSS) as the only PCR primers FGF4_TSS_F1 and FGF4_R1 yielded a product in RT-PCR using cDNA from neonatal beagle IVD (canFam3). The insertion location is intergenic between the 3′-UTR of OGFRL1 (~9.5 kb on the proximal side and ~350 kb to the RIMS1 gene on the distal side. It is also intronic to an unvalidated antisense transcript (NASEQ_A_0027291).

To compare the CFA12 FGF4 retrogene to the previously identified CFA18 FGF4 retrogene, it was necessary to obtain the full-length sequence of the CFA18 insertion (25). The cloned product was sequenced using the flanking and common internal primers (SI Appendix, Table S8), yielding a 2,665 bp insert (GenBank accession no. MF040222). While it contained the same length 5′-UTR and FGF4 cDNA as that seen in the CFA12 FGF4 insert, the 3′-UTR was shortened in comparison. The 3′-UTR of the CFA18 FGF4 insert was followed by a sequence containing 30 adenine and one guanine residues and a different target site duplication (TSD) sequence (AAC TCA GAC AGA G). The 5′-UTR shared between the two canine FGF4 retrogenes was compared with the human FGF4 gene and was 295 bp long and 91% identical at the nucleotide level. Within the highly conserved 5′-UTR that was transposed, there are 79 conserved transcription factor binding sites between dog and human (https://ecr.browser. dcode.org). There are also multiple DNaSe I hypersensitivity sites as well as H3K4me3 and H3K9ac marks (www.genome.ucsc.edu) within the human sequence.

Association of FGF4 Retrogene with SD, Height, and IVDD. To assay the insertions in additional dogs, insertion- and allele-specific PCR-based genotyping assays were developed for both the CFA12 FGF4 insertion and the previously identified CFA18 FGF4 insertion (Fig. 4). Twelve SD NSDTR cases from the GWAS were genotyped and were homozygous for the CFA12 FGF4 insertion, while all controls were heterozygous or wild type. Additionally, IVDD cases (n = 7) from the NSDTR breed were collected and were either homozygous mutant or heterozygous for the CFA12 FGF4 insertion (SI Appendix, Table S3). All NSDTRs tested for the CFA18 FGF4 insertion (n = 31) were wild type, including SD and IVDD cases. NSDTRs with known height (n = 20 males) at the withers were genotyped for the CFA12 FGF4 insertion to investigate the association of height with genotype status. Height and genotype were significantly associated in a dose-dependent manner (all P < 0.04) when comparing wild-type, heterozygous, and homozygous dogs (Fig. 4B).

To assess the significance of association of the CFA12 FGF4 insertion with IVDD across breeds, dogs used in the IVDD GWAS were genotyped for both insertions. All dogs’ genotypes were concordant with phenotype except for one case, a rottweiler (SI Appendix, Table S2). When associated with IVDD, the CFA12 FGF4 insertion was more highly associated than both the most highly associated SNPs from the GWAS, as well as the CFA18 FGF4 insertion (Fig. 4C). To further investigate the association of the CFA12 FGF4 insertion with IVDD, 33 additional cases were genotyped for
of 10 were heterozygous and 23 were homozygous for the CFA12
insertion on CFA12, semi-quantitative RT-PCR (semi-qPCR) was performed for genes across the IVDD-associated interval. Using cDNA derived from neonatal vertebral body (VB) and IVD, skeletal muscle, and testis, expression levels of genes across the CFA12-associated interval were assayed in a beagle case and cane corso or Labrador retriever control, including COL9A1, SMAP1, B3GAT2, OGFRL1, LINC00472, RIMS1, KCNQ5, and COL12A1. Expression differences between case and control were not apparent in these genes; however, we confirmed that all except RIMS1 are expressed in both neonatal VB and IVD, supporting that FGF4 inserted itself in a gene milieu conducive to expression in IVD. Semi-qPCR for total FGF4 (parental and retrogene products) in the same tissues showed increased expression across all tested tissue types in the case versus the control (SI Appendix, Fig. S3).

To evaluate the effect of the CFA12 FGF4 retrogene insertion on overall FGF4 transcript levels, quantitative RT-PCR was performed. A comparison between samples homozygous for the CFA12 FGF4 insertion and samples with only the parental copy of FGF4 (i.e., wild type for both the CFA12 and CFA18 FGF4 insertions) showed a 19.47x higher (P = 0.03) and 2.16x higher (P = 0.03) expression of FGF4 in neonatal IVD and VB, respectively (SI Appendix, Fig. S4).

**Discussion**

In this study, we report the identification of a FGF4 retrogene insertion in the dog genome responsible for chondrodystrophy across dog breeds, characterized by both short limbs and susceptibility to Hansen’s type I intervertebral disc disease. A region was identified on CFA12 due to association with a segregating form of skeletal dysplasia observed in the NSDTR. While NSDTRs can be variably affected, the use of severely affected dogs enabled identification of the locus through GWAS. Haplotype sharing with chondrodystrophic breeds and genome-wide association analysis for type I IVDD identified the same region on CFA12. Evaluation of mismapped mate pairs allowed the identification of an FGF4 retrogene, which leads to a ~20-fold increase in expression of FGF4 in neonatal intervertebral disc. Due to the embryonic expression pattern of FGF4, it is probable that these expression changes are also impacting endochondral ossification. This is the second FGF4 retrogene identified in dogs that affects limb length. While the FGF4 retrogene on CFA18 impacts limb length, the FGF4 retrogene on CFA12 explains the chondrodystrophic phenotype, which includes limb length and IVDD (significant odds ratio > 30).

**Fibroblast growth factor (FGF4)** is a growth factor gene expressed in specific tissues and at specific times throughout embryonic development in the mouse (36). FGF4 is highly expressed in the apical ectodermal ridge of the developing limb bud, as well as somites and the notochord that will form the vertebral column and IVDs (36–38). FGF signaling is required for chondrodystrophy, and the FGF4 retrogene is a potential factor contributing to the phenotype. The CFA12 FGF4 retrogene insertion is associated with a reduction in expression of FGF4 in neonatal IVD and VB, supporting the hypothesis that the FGF4 retrogene is a major contributor to chondrodystrophy in dogs.

### Table S4

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<th>p-value</th>
<th>Odds Ratio</th>
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<tr>
<td>CFA 12 FGF4 Insertion</td>
<td>67.32</td>
<td>2.3 x 10^-6</td>
<td>51.23 (46.69, 55.20)</td>
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<tr>
<td>CFA 18 FGF4 Insertion</td>
<td>23.51</td>
<td>1.2 x 10^-3</td>
<td>6.38 (5.99, 6.79)</td>
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**Fig. 4.** Association of FGF4 insertion genotypes with height and IVD. (A) Genotyping results for CFA18 and CFA12 FGF4 insertions. Arrows indicate wild-type (WT) band. Numbered lanes: ladder and 1–3, NSDTR; 4, beagle; 5, American cocker spaniel; 6, dachshund; 7, baset hound; 8, Pembroke Welsh corgi; 9, coton de Tulear; 10, cairn terrier; and 11, West Highland white terrier. (B) Height at the withers in inches (in) (y axis) by genotype (x axis) for 20 NSDTRs. Seven SD NSDTR cases were homozygous mutant for the CFA12 FGF4 insertion and their mean height was 18.22 in. Thirteen NSDTRs were unaffected with SD: 5 were wild type and had a mean height of 20.2 in; 8 were heterozygous for the CFA12 FGF4 insertion and had a mean height of 18.94 in. (C) Association of loci with IVDD.

**CFA12 FGF4 Retrogene Expression.** To investigate the gene expression environment in which FGF4 inserted on CFA12, semi-quantitative RT-PCR (semi-qPCR) was performed for genes across the IVDD-associated interval. Using cDNA derived from neonatal vertebral body (VB) and IVD, skeletal muscle, and testis, expression levels of genes across the CFA12-associated interval were assayed in a beagle case and cane corso or Labrador retriever control, including COL9A1, SMAP1, B3GAT2, OGFRL1, LINC00472, RIMS1, KCNQ5, and COL12A1. Expression differences between case and control were not apparent in these genes; however, we confirmed that all except RIMS1 are expressed in both neonatal VB and IVD, supporting that FGF4 inserted itself in a gene milieu conducive to expression in IVD. Semi-qPCR for total FGF4 (parental and retrogene products) in the same tissues showed increased expression across all tested tissue types in the case versus the control (SI Appendix, Fig. S3).

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for appropriate embryonic axial growth and segmentation, and FGF4/FGF8 murine hypomorphs are characterized by altered vertebral morphology and smaller limb buds (39, 40). Additionally, FGF8 hypomorphs are observed to have either hypoplastic or nonexistent external ear structures (41). In mice, creation of a gain-of-function FGF4 copy to replace an inactive FGF8 gene was able to rescue limb development; however, it also caused abnormal tissue deposition and postnatal polydactyly, highlighting that levels of FGF throughout embryonic development must be properly controlled for normal limb formation (31).

While the specific embryonic expression pattern of FGF4 in dogs with four to six copies of the gene is unknown, we hypothesize that the insertion site milieu on CFA12 versus CFA18 is contributing to differences in expression between the retrogenes, leading to the differences in phenotype.

A survey of retrogenes in the canine reference genome reported ~70 functional retrogenes in the dog; however, only the previous CFA18 FGF4 retrogene insertion has been reported to be associated with a disease-causing phenotype (25, 42). Similarly in humans, the formation of processed pseudogenes in general, as well as those that retain their intended function and cause disease, is rare (43–46).

Both copies of the canine FGF4 retrogenes have signatures of having arisen from RNA retrotransposed by LINE-1 integrase and reverse transcriptase including flanking TSDs and polyA tracts (class 1 templated sequence insertion polymorphism) (47). The insertion sites of the two FGF4 retrogenes are very different. The CFA18 site is within a LINE element and the CFA12 insertion site is intergenic between OGFRL1 and RIMS. The CFA18 FGF4 retrogene insertion was predicted to be expressed due to insertion near sequences with promoter properties (25). While the CFA12 FGF4 insertion is placed near a potential TATA box and RNA Pol II promoter, it is more likely that the CpG island included in the retrogene is driving expression (48–50). This hypothesis is supported by the finding that a majority of retrogene expression is actually due to genomic context and contribution of CpG islands, not through the use of nearby promoters (51). Human FGF4 shares the large CpG island observed in dogs and other species. Within the highly conserved 5′-UTR that was transposed, there are many conserved transcription factor binding sites between dog and human as well as multiple methylation marks further supporting that both CFA12 FGF4 and CFA18 FGF4 retrogenes contain the necessary components for transcription. To our knowledge, this is a unique documentation of a second retrogene insertion of the same parental gene resulting in a disease phenotype in a mammalian species. Due to the lack of resources available to identify these types of mutations, it is likely that there are other phenotype-inducing retrocopies present in the canine genome that have yet to be discovered.

Chondrodystrophy-associated mutation events occurred a very long time ago, as there are descriptions of short-legged dogs dating back over 4,000 y (52). In addition, both mutations occur concurrently in very unrelated dog breeds from diverse breed groupings and geographical locations. The fact that FGF4 has been retrotransposed twice in dogs in the last 3–4,000 y makes it likely that this has happened at other times. The large CpG island in the 5′-end of the parental FGF4 gene may enable phenotypic consequences more readily than for other retrogenes. Once the FGF4 retrogene appeared and produced an obvious phenotype, strong selection was likely applied to retain it, aided by the semidominant nature of the mutation.

The NSDTR is the smallest of the retriever dog breeds, and based on the association of the CFA12 FGF4 insertion with height, we hypothesize that the heterozygous phenotype is aesthetically desirable and that selection is maintaining the insertion at a relatively high allele frequency. Investigation of the CFA12 FGF4 insertion in additional breeds also showed high allele frequency in multiple small- and medium-sized dog breeds. In breeds also containing the CFA18 FGF4 insertion, there is an even more dramatic decrease in height (e.g., basset hound, Cardigan Welsh corgi, dachshund, etc.), supporting that both FGF4 retrogenes affect long-bone length.

In addition to segregating with height, the CFA12 FGF4 insertion also segregates with Hansen’s type I IVDD susceptibility. Of the IVDD cases genotyped for the CFA12 FGF4 insertion, all were homozygous mutant or heterozygous, except for one, suggesting that one additional copy of FGF4 on CFA12 is sufficient to cause type I IVDD. The single discordant case was a rottweiler, a breed that does not fit the chondrodystrophic phenotype. It is possible that there is another cause of IVDD in non,chondrodystrophic dog breeds occurring without endochondral ossification defects (10). IVDD-affected NSDTRs were also all either homozygous or heterozygous for the CFA12 FGF4 insertion. This supports the idea that while the CFA12 FGF4 insertion is semidominant with respect to height, it is dominant for altered IVDs. Given that the CFA18 FGF4 insertion is not found in the NSDTR and was inconsistently present in the IVDD cases tested, this further supports the idea that the identified insertion on CFA12 is causing both short limbs and Hansen’s type I IVDD in both the NSDTR and across dog breeds.

The breeds with a higher frequency of the CFA12 FGF4 insertion are the same breeds identified in the last 50 y as being predisposed to IVDD. Presence of the CFA18 FGF4 insertion is common in many breeds with IVDD, and it is possible that it may contribute to the disease; however, previous mapping within dachshunds, which are reported “fixed” for the CFA18 FGF4 insertion, show segregation of the associated haplotype on chromosome 12 with IVDD, supporting the idea that the CFA12 FGF4 insertion is the critical factor determining disease status (25, 34). Of particular interest is the lack of reports of IVDD cases in breeds such as the cairn terrier and West Highland white terrier, both of which have the CFA18 FGF4 insertion, but not the CFA12 FGF4 insertion. Similarly, the high incidence of IVDD in breeds such as the American cocker spaniel, beagle, and French bulldog that do not have the CFA18 FGF4 insertion but a high frequency of the CFA12 FGF4 insertion supports the idea that FGF4 specifically from CFA12 is contributing to the IVDD phenotype.

The segregation of the CFA12 FGF4 insertion within dog breeds presents an opportunity for improvement of animal health, as it is possible that the CFA12 FGF4 retrogene insertion is the critical factor determining disease status (25, 34). Some 12 with IVDD, supporting the idea that the CFA12 FGF4 insertion is the critical factor determining disease status (25, 34). Of particular interest is the lack of reports of IVDD cases in breeds such as the cairn terrier and West Highland white terrier, both of which have the CFA18 FGF4 insertion, but not the CFA12 FGF4 insertion. Similarly, the high incidence of IVDD in breeds such as the American cocker spaniel, beagle, and French bulldog that do not have the CFA18 FGF4 insertion but a high frequency of the CFA12 FGF4 insertion supports the idea that FGF4 specifically from CFA12 is contributing to the IVDD phenotype.

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The high mortality rate of IVDD and the high cost of surgery, identification of this susceptibility locus could provide a valuable tool for owners, breeders, and veterinarians for mitigating risk of intervertebral disc herniation and resulting myelopathy (9). This could be especially useful in breeds that have both the CFA12 and CFA18 FGF4 retrogene, as they could breed away from the CFA12 FGF4 retrogene, while still maintaining the aesthetically desirable shortness in stature contributed
by the CFA18 FGF4 retrogene. In breeds with only the CFA12 FGF4 retrogene, breeders will ultimately decide if prevention of
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133:33
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| 47:431
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