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Jagged 1 rescues the Duchenne muscular dystrophy phenotype

Natassia M. Vieira[1,2,3], **Ingegerd Elvers**[4,5], **Matthew S. Alexander**[1,2,6], **Yuri B. Moreira**[7], **Alal Eran**[2], **Juliana P. Gomes**[3], **Jamie L. Marshall**[1,2], **Elinor K. Karlsson**[4], **Sergio Verjovski-Almeida**[7,8], **Kerstin Lindblad-Toh**[4,5,*], **Louis M. Kunkel**[1,2,9,†,*], and **Mayana Zatz**[3,†,*]

[1]The Division of Genetics and Genomics, Boston Children's Hospital, Boston, MA, USA 02115

[2]Department of Pediatrics and Genetics, Harvard Medical School, Boston, MA, USA 02115

[3]Human Genome and Stem Cell Center, Biosciences Institute, University of São Paulo, São Paulo, Brazil 05508-090

[4]Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA 02142

[5]Science for Life Laboratory, Department of Medical Biochemistry and Microbiology, Uppsala University, Box 597, SE-751 24, Uppsala, Sweden

[6]The Stem Cell Program at Boston Children's Hospital, Boston, MA 02115

[7]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil, 05508-000

[8]Instituto Butantan, São Paulo, Brazil, 05508-050

[9]The Manton Center for Orphan Disease Research at Boston Children's Hospital, Boston, MA, USA 02115

Summary

Duchenne muscular dystrophy, caused by mutations at the dystrophin gene, is the most common form of Muscular Dystrophy. There is no cure for DMD and current therapeutic approaches to restore dystrophin expression are only partially effective. The absence of dystrophin in muscle results in dysregulation of signaling pathways which could be targets for disease therapy and drug discovery. Previously we identified two exceptional Golden Retriever Muscular Dystrophy (GRMD) dogs that are mildly affected, have functional muscle and normal lifespan despite the complete absence of dystrophin. Now, our data on linkage, whole genome sequencing and transcriptome analyses of these dogs compared to severely affected GRMD and control animals

[†]Corresponding authors: LMK- kunkel@enders.tch.harvard.edu, MZ- mayazatz@usp.br.
[*]Co-senior authors

Author Contributions

NMV, IE, MSA, YBM, SVA, LMK, KLT and MZ designed the study. NMV, YBM, MSA and JLM performed experiments. NMV, IE, YBM, EKK, AE, and KLT performed data analysis and interpretation. NMV, IE, KLT and MZ wrote the paper with input from the other authors.

reveal that increased expression of Jagged1 gene, a known regulator of the Notch signaling pathway, is a hallmark of the mild phenotype. Functional analyses demonstrate that Jagged1 overexpression ameliorates the dystrophic phenotype, suggesting that Jagged1 may represent a target for DMD therapy in a dystrophin-independent manner.

Keywords

DMD; *Jagged1*; muscle; dystrophin; genetic modifier

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked disorder caused by mutations in *dystrophin* (Hoffman et al., 1987), which affects 1 in 3500 to 5000 boys (Axelsson et al., 2013; Mendell et al., 2012). Deficiency of muscle dystrophin causes progressive myofiber degeneration and muscle wasting (Hoffman et al., 1987). The first symptoms are usually evident at 3–5 years of age with loss of ambulation between 9 and 12 years. Death occurs in the second or third decade due to respiratory or cardiac failure. While there are several treatments under development or currently in use, particularly corticotherapy, which aims to ameliorate symptoms and slow down the disease progression, there is still no cure for DMD (Bushby et al., 2010; Guiraud et al., 2015). Allelic to DMD, Becker muscular dystrophy (BMD) is caused by mutations that do not affect the reading frame of the dystrophin transcript; the result is a semi-functional, truncated dystrophin protein (Koenig et al., 1989). DMD muscle shows a complete absence of dystrophin, whereas in the BMD muscle there is a variable amount of partially functional dystrophin (Monaco et al., 1988). Differently from DMD, where most boys carrying null mutations show a severe phenotype, BMD patients show a variable clinical course. Genotype/phenotype correlation studies suggest that the severity of the phenotype is dependent on the amount of muscle dystrophin or site of the mutation/deletion in the dystrophin gene (Koenig et al., 1989; Passos-Bueno et al., 1994; Vainzof et al., 1990)

DMD therapeutic approaches currently under development aim to rescue *dystrophin* expression in the muscle (Fairclough et al., 2013). Pre-clinical and clinical studies include exon-skipping (Goemans et al., 2011; Mendell et al., 2013; van Deutekom et al., 2007), AAV-delivery of μ-dystrophin (Mendell et al., 2010), and nonsense suppression to induce “readthrough” of nonsense mutations (Kayali et al., 2012). While AAV-delivery led to μ-dystrophin expression in skeletal muscle, T-cell immunity against dystrophin epitopes was reported (Mendell et al., 2010). Also the success of the dystrophin-based therapies relies on the quality of the recipient muscle. This requires the development of dystrophin-independent therapies to improve the muscle condition targeting the altered signaling pathways.

To explore the efficiency of the different therapeutic approaches for DMD, there is a need for animal models that mimic the human condition. However, animal models of dystrophin-deficiency show differences in skeletal muscle pathology in response to dystrophin-deficiency (Bassett and Currie, 2004; Chapman et al., 1989; Im et al., 1996; Kornegay et al., 1988; Zucconi et al., 2010). The dystrophin-deficient fish model (*sapje*) shows some

phenotypic variability, but nearly all fish die during the first weeks of life and all show abnormal muscle structure as measured by birefringence under polarized light (Bassett and Currie, 2004). The *mdx* mouse is the most widely used animal model for DMD, even though its mild phenotype does not mimic severe human DMD symptoms (Bulfield et al., 1984). The most similar to the human condition is the golden retriever muscular dystrophy (GRMD) dog (Bassett et al., 2003; Cooper et al., 1988; Kornegay et al., 1988; Sicinski et al., 1989). These animals carry a point mutation on a splicing site that causes the skipping of exon 7 and a premature stop codon, resulting in the absence of dystrophin. GRMD dogs and DMD patients share many similarities in disease pathogenesis, including early progressive muscle degeneration and atrophy, fibrosis, contractures, and grossly elevated serum creatine kinase (CK) levels (Kornegay et al., 1988; Sharp et al., 1992). Early death may occur within the first weeks of life but usually occurs around 1–2 years of age as a result of respiratory failure or cardiomyopathy. The great majority of GRMD dogs do not survive beyond age two. In the Brazilian GRMD colony at Biosciences Institute at the University of São Paulo, we have described two exceptional dogs presenting a very mild phenotype clearly distinguishable from other affected dogs despite the absence of muscle dystrophin. Histopathological and immunohistochemistry analysis of their muscle showed typical features of a dystrophic process with variability in fiber size, splitting, degeneration, and infiltrating connective tissue (Zucconi et al., 2010).

These two exceptional, related GRMD dogs (here called ‘escapers’) remained fully ambulatory with normal lifespans, a phenotype never reported before for GRMD. They fall outside the known GRMD phenotypic range of variability, differing significantly from typically affected dogs, despite their dystrophic muscle, absence of muscle dystrophin, elevated serum creatine kinase (CK) levels and no evidence of utrophin upregulation (Zatz et al., 2015; Zucconi et al., 2010). Most importantly, these GRMD dogs show that it is possible to have a functional muscle, in a mid-size dystrophin-deficient animal.

In this study, we set out to answer the following question: how these escaper dogs have a fully functional muscle without dystrophin? Skeletal muscle of Duchenne muscular dystrophy (DMD) patients undergo waves or cycles of degeneration followed by regeneration. Muscle repair is a regulated process that comprises different cell types and signaling molecules; but additional factors and genetic modifiers involved in DMD pathogenesis remain poorly understood, representing new potential therapeutic targets. Genetic modifiers have been reported in DMD patients with a slower progression, but none were associated with a nearly normal phenotype (Flanigan et al., 2013). Here, through 3 independent approaches, we identified a modifier gene, *Jagged1*, which can modulate the GRMD phenotype. Using a mixed model association and linkage analysis, we identified a chromosomal region associated with the escaper phenotype. One gene within this region showed altered expression when comparing muscle tissue from escaper and affected dogs. By whole genome sequencing we found a variant present only in escaper GRMD dogs that creates a novel *myogenin* binding site in the *Jagged1* promoter. Overexpression of *jagged1* in dystrophin deficient zebrafish rescues the dystrophic phenotype in this zebrafish model. This suggests that *Jagged1*, when increased in expression in muscle, can rescue dystrophin-

deficient phenotypes in two different animal models, pointing to a new potential therapeutic target.

Results

Escaper GRMD dogs share a common haplotype different from affected

To understand the genetic basis behind the escaper phenotype in GRMD dogs, we performed a genome wide mapping analysis comparing two related escaper GRMD dogs – the only two GRMD escapers reported to date - among 31 severely affected GRMD dogs from the same breeding population. All GRMD dogs were confirmed to carry the originally described point mutation (a change from adenine to guanine transition) in the intron 6 of the *dystrophin* gene. This mutation ablates a splicing site and exon 7 is skipped from the mature mRNA. The absence of exon 7 causes a premature stop codon at exon 8 (Cooper et al., 1988; Sharp et al., 1992). Based on survival age and functional capacity they were classified as escaper or affected (binary). All the dogs showing the standard range of phenotypic variability seen in GRMD dogs were classified as affected in this study. Our aim was to identify a single gene responsible for the milder phenotype seen in the two escaper dogs. We performed a two-step mapping analysis. First, we carried out an association study, utilizing the power of the many severely affected dogs expected to lack the modifier locus. This was followed by segregation analysis, taking advantage of the fact that the two escapers came from a well-defined pedigree, in which a transmission-based test could be used. All dogs were genotyped using the Illumina CanineHD 170K SNP array. We tested for association genome-wide using the mixed model approach implemented in EMMAX (Kang et al., 2010) to correct for population structure (Figure 1A) and identified strongly associated SNPs ($p<1x10^{-5}$) on chromosomes 24, 33 and 37 (Figure 1B). We then measured identity by descent (IBD) across the genome between the two escapers using Beagle (Browning and Browning, 2007). Only the associated SNPs on chromosome 24 also overlapped a segment of IBD in the two escapers, consistent with a single origin of the causative mutation (Figure 1B). The 27Mb segment showing both IBD and association with the escaper phenotype (CanFam2, cfa24:3,073,196–30,066,497) contains approximately 350 protein-coding genes. Linkage analysis using Merlin (Abecasis et al., 2002) strongly confirmed this region, with a maximal parametric LOD score of 3.31 (dominant inheritance model with complete penetrance, Supplemental Figure 1). No other genomic regions showed any signs of linkage (Supplemental Figure 2). Thus, convergent IBD, association, and linkage analyses all pointed to the same 27Mb region on chromosome 24 (Figure 1C).

Muscle gene expression profile of escaper and affected GRMD dogs

We then performed a genome-wide analysis for genes differentially expressed in muscle between the escapers and affected dogs. Using Agilent mRNA SurePrint Canine arrays, we compared muscle gene expression of the two escapers, four affected and four wild-type dogs at two years of age. We found very similar muscle gene expression patterns in the two escaper GRMD dogs, which were more similar to muscle from wild-type dogs than from the affected dogs. In total, 114 genes were found to be differentially expressed between escapers and affected GRMD dogs, as shown by unsupervised hierarchical clustering of all 10 samples (Figure 2A). Of these, 65 genes were also differentially expressed between escapers

and wild-type dogs (Supplemental Table 1), implicating them in a possible compensatory mechanism active in only the escaper dogs. Only one of these 65 genes, *Jagged1*, is located under the association peak on chromosome 24. *Jagged1* mRNA levels were two times higher in the escapers when compared to both wild-type and severely affected dogs (Figure 2B). Further protein level analysis confirmed the mRNA findings (Figure 2C).

Whole genome sequence of escaper dogs

To identify potentially causative variants behind the differential gene expression pattern observed in the escaper dogs, we performed whole-genome sequencing on three dogs (the two escapers and one severely affected related dog). We hypothesized that the compensatory variation would be novel, as the escaper phenotype had not previously been seen in GRMD dogs worldwide. We looked for variants located under the association peak on chromosome 24 and focused on the *Jagged1* locus (including 3KB upstream and downstream of the gene) in search for a variant present only in the escapers and not in the affected GRMD dogs. A total of ~1300 variants were detected within the escaper-associated region on chromosome 24. All variants were lifted over to the human genome and those present in muscle enhancer regions near the promoters of the two isoforms of *Jagged1* expressed in skeletal muscle (Figure 3A) (Hoeppner et al., 2014), were further analyzed. Since the escaper variant was hypothesized to be novel, all variants detected in previous extensive canine sequencing efforts (Axelsson et al., 2013) were excluded. After this filtering, only a single point variant was found to follow the escaper haplotype; a heterozygote G>T change in the promoter region of *Jagged1* (cfa24:11655709, Figure 3A). Sanger sequencing of the *Jagged1* candidate escaper variant was performed in the escaper extended pedigree, including the first escaper (M1M4), his offspring and a sibling's offspring (M1M5) (Supplemental Figure 3). We also sequenced key breeders of the kennel and found that the variant is specific to the escapers' pedigree and was introduced in a single outcross (B1F3 mate). All affected dogs lacked the *Jagged1* variant, while both escapers were heterozygous. Thus, the novel *Jagged1* mutation segregates with the escaper phenotype in this family. Four additional individuals carried the candidate variant: three were stillborn puppies and the fourth was a GRMD puppy that died at six months of age from an accidental ingestion of a foreign object. This puppy (K2M11) was fully ambulatory with a similar phenotype to the two escaper dogs, but he was classified as affected in the mapping analysis since we cannot predict his adult phenotype with confidence.

Functional analysis of Jagged1 variant

To understand the effects of the escaper variant, we performed different functional analyses. This candidate variant was found to be conserved across 29 eutherian mammals, suggesting a regulatory potential for this region (Figure 3A–B). Transcription factor binding site analysis, using TRAP (Manke et al., 2010) and TRANSFAC (Matys et al., 2006) revealed that this G>T change creates a novel myogenin binding site (Figure 3C), with a high information content for the mutant allele (T) in the myogenin consensus binding motif (Figure 3D). Myogenin is a muscle-specific transcription factor involved in muscle differentiation and repair (Wright et al., 1989). To determine whether the variant affects DNA binding by myogenin, we carried out electrophoretic mobility shift assays (EMSA) using muscle cell nuclear extracts and biotin-labeled oligonucleotide probes, containing

either the wild-type (Wt) or escaper (E) genotype. The oligonucleotide probe containing the escaper T allele robustly bound the myogenin protein, whereas an oligonucleotide probe containing the wild-type G allele did not bind at all (Figure 3E). A competition assay showed that an unlabeled escaper probe efficiently competed with the binding of the labeled escaper probe. In contrast, the unlabeled Wt probe had no effect on the binding activity of the labeled escaper probe, indicating a specific interaction between the escaper allele and myogenin (Figure 3E). To evaluate if the novel myogenin binding site found in the escaper dogs was driving the increased expression of *Jagged1*, we performed a luciferase reporter assay using *Jagged1* upstream promoter sequences containing either the wild-type sequence or the escaper variant fused to a luciferase reporter. Luciferase vectors containing either wild-type (Wt) or escaper sequence were transfected into muscle cells (myoblasts) and human embryonic kidney cells (HEK293T) along with constructs that overexpress either myogenin or another E-box myogenic factor (MyoD) as control. On HEK293K cells, overexpression of myogenin was able to activate the expression of the escaper *Jagged1* reporter three-fold, but showed no activation of the Wt reporter (Figure 3F). As predicted, the overexpression of MyoD did not activate either the Wt or escaper *Jagged1* luciferase reporter (Figure 3F). Similarly, myoblasts (that endogenously express myogenin) transfected with the escaper vector showed a similar luciferase activation that was three times higher than the Wt vector, notwithstanding the presence of overexpression vectors (Figure 3F). These results demonstrate that the creation of the novel myogenin binding site in the escaper *Jagged1* promoter is essential for driving the increase of *Jagged1* expression in the escaper dog skeletal muscles.

In vivo overexpression of Jagged1 rescues sapje muscle phenotype

To evaluate if the overexpression of *Jagged1* can ameliorate the dystrophic muscle phenotype in other species, we used the severely affected dystrophic *sapje* zebrafish DMD model. Muscle phenotype was assayed using birefringence, where fish are placed under a polarized light and dystrophin negative fish show a decrease in the amount of light as indicative of muscle tearing or muscle fiber disorganization. In four separate experiments we injected approximately 200 fertilized one-cell stage eggs from *sapje* heterozygous fish matings with mRNA of either one of the zebrafish *jagged1* genetic copies of the mammalian *Jagged1* gene: *jagged1a* or *jagged1b*. In all experiments, an average of 24% of the non-injected *sapje* fish exhibited a typical affected dystrophic, patchy birefringence phenotype. This proportion is within the 21–27% expected range of affected fish of a heterozygous *sapje* mating. In contrast, fish injected with either *jagged1a* or *jagged1b* showed a significantly lower percentage of fish with poor birefringence ($p=1.31\times10^{-6}$ for *jagged1a*, $p=4.4\times10^{-5}$ for *jagged1b*, Figure 4A). Genotypic analysis revealed that about 75% of dystrophin null fish injected with *jagged1a* and 60% of *jagged1b* had normal birefringence, which demonstrated a common rescue from the muscle lethality phenotype (Figure 4B). These results indicate that increasing *jagged1* expression rescues most dystrophin-null fish from developing the abnormalities typically seen in dystrophin-null muscle. To further evaluate the *jagged1a* and *jagged1b* overexpression *sapje* fish we performed immunostaining on individual fish bodies, using a myosin heavy chain (MHC) antibody to evaluate muscle structure. In wild-type fish, MHC was clearly expressed and showed that muscle fibers were normal. Interestingly, MHC staining of *jagged1* mRNA-injected

dystrophin-null rescued fish showed normal myofiber structure; similar to that of wild-type fish, whereas affected, non-injected dystrophin-null fish demonstrated clear muscle abnormalities (Figure 4C).

Jagged1 expression during muscle regeneration and cell proliferation in mice and dogs

When examining the effect of *Jagged1* on muscle regeneration in normal mice, we found that *Jagged1* expression is upregulated at day four after cardiotoxin-induced injury in mouse tibialis anterior muscle (Figure 4D). We also determined that *Jagged1* is elevated during myoblast muscle differentiation *in vitro (*Figure 4E). To examine whether muscle cells from escaper dogs proliferate faster than cells from severely affected dogs, we performed a proliferation assay using myogenic cells from biopsies of age-matched dogs. Escaper dogs' muscle showed typical dystrophic features (Zucconi et al., 2010) as evidenced by cycles of degeneration and regeneration, which is not seen in normal muscle. Because of these cycles and consistent activation, myogenic cells from affected GRMD dogs are expected to divide less frequently. We show that muscle cells from escaper dogs divide significantly faster than those from affected dogs (Figure 4F). These results are consistent with previous findings that show that overexpression of the Notch intracellular domain (NICD) expands the proliferative capacity of activated muscle satellite cells *in vitro* and *in vivo* (Wen et al., 2012).

Discussion

Animal models for DMD are important tools for developing new therapeutic approaches. Among the different animal models for muscular dystrophy, the GRMD dog is the closest to the human condition. Both GRMD dogs and DMD patients have a severe phenotype as well as many phenotypic and biochemical similarities, including early progressive muscle degeneration and atrophy, fibrosis, contractures, and elevated serum creatine kinase levels. We identified two dogs that escaped from the typical severe phenotype associated with dystrophin deficiency. Using a combined approach of mapping and identity by descent we identified a candidate region of association with the escaper phenotype. Only one gene within this region showed altered expression in escaper and affected dogs: *Jagged1*. We found a candidate variant at an upstream, conserved position creating a new muscle-specific transcription factor binding site that drives *Jagged1* overexpression. *Jagged1* is also in the region mapped to the MRL phenotype, the muscular dystrophy mouse model on the "superhealing" MRL strain background. The causative genetic modifiers in the MRL strain, which has enhanced muscle regeneration and reduced dystrophic pathology, have been mapped to a region containing 49 genes that includes the *Jagged1* locus (Heydemann et al., 2012).

The role of *Jagged1* in skeletal muscle development and disease has yet to be fully elucidated. Jagged1 is a Notch ligand (Lindsell et al., 1995). The Notch signaling pathway represents a central regulator of gene expression and is critical for cellular proliferation, differentiation and apoptotic signaling during all stages of embryonic muscle development. The Notch pathway also plays an important role in muscle regeneration (Conboy and Rando, 2002; Wen et al., 2012) and overexpression of *Notch* has been shown to improve muscle

regeneration in aged mice (Conboy et al., 2003). Moreover, Notch signaling has been shown to be dysregulated in muscle satellite cells and dystrophin-deficient muscles from *mdx* mice (Jiang et al., 2014). Additionally, there is an even more pronounced dysregulation of Notch signaling in the muscle satellite cell in the severe *mdx/utrn* double knockout mice (dKO), that have early lethality at two to four months due to a breakdown of the diaphragm muscles (Church et al., 2014; Mu et al., 2015). Here we observed greater proliferative capacity of the escaper dogs' myoblasts suggesting that *Jagged1* overexpression might be involved in muscle cell proliferation and repair. These results are consistent with previous findings, which demonstrate that *Jagged1* overexpression stimulates cell proliferation, suggesting that *Jagged1*-based therapy might be able to induce regeneration in a tissue-specific manner (Collesi et al., 2008). Our data show that *Jagged1* expression is upregulated at day four after cardiotoxin-induced injury in mouse, a time point when myoblasts proliferate and fuse to promote muscle regeneration (Couteaux et al., 1988). Furthermore, Jagged1/Notch signaling has been shown to promote the expansion and differentiation capacity of bone marrow-derived stromal/stem cells (BMSCs) to promote skeletal regeneration (Dong et al., 2014). In endothelial cells, genetic *Jagged-1* overexpression resulted in endothelial branching of vasculature processes; while conversely, *Jagged-1* endothelial deletion blocked angiogenic growth in *Jagged-1* eKO mice (Pedrosa et al., 2015). Indeed, *Jagged-1* overexpression lead to the activation of vasculature progenitor cells from quiescence, in a manner similar to that of muscle satellite cell activation (Ottone et al., 2014). Thus, it is likely that the endogenous overexpression of *Jagged-1* that occurs in the muscles of the escaper dogs is driving myogenic cell proliferation and potential muscle growth that occurs in mesodermal lineages. A proof-of-principle experiment in which the Notch downstream transcription factor Rbp-jk was deleted in muscle satellite cells demonstrated that inhibition of Notch activation was detrimental to both muscle growth and muscle satellite cell expansion (Bjornson et al., 2012). All these findings suggest that *Jagged1* is likely to be a mediator of the regenerative process that is disrupted in dystrophin-deficient muscles, and has potential as a novel therapy target to mitigate DMD pathological progression.

Although the great majority of DMD patients show a severe course, exceptional cases of dystrophin-deficient patients with a milder phenotype have been identified. We have previously reported two patients carrying null mutations, with no skeletal muscle dystrophin present via immunofluorescent staining or western blot analysis and a milder course including the maintenance of ambulation well into their second decade of life (Zatz et al., 2014). More recently, a dystrophin-negative patient who remained ambulant until age 30 was also reported (Castro-Gago, 2015). Several other genetic modifiers are known to affect the severity of the clinical symptoms of Duchenne muscular dystrophy (*LTBP4, SPP1, TGFBR2*). However, none of these genetic variants have been shown to fully restore or delay substantially the symptoms of dystrophin-deficiency in DMD boys (Bello et al., 2012; Flanigan et al., 2013; Pegoraro et al., 2011; Piva et al., 2012). Furthermore, it would be of great interest to examine the genomes of DMD boys with varying clinical symptoms and determine if variants in *Jagged1* or other Notch signaling factors exist and are causative for any variation of the dystrophic disease progression. The Notch signaling pathway, specifically *Jagged1* overexpression, represents a novel therapeutic entry point for the treatment of DMD. Full restoration of Notch signaling must be achieved in the muscle

satellite cell if one expects to correct the dysregulated Notch-dependent signaling that is affected in dystrophin-deficiency (Church et al., 2014). Direct injection of exogenous, soluble Jagged-1 ligand is not a viable therapeutic option, as external Jagged1 weakens Notch signaling even more than dystrophin-deficiency (Xiao et al., 2013). Thus, one might envision finding a small molecule or transcription factor that could increase expression of *Jagged1* in all of the skeletal muscles of DMD patient.

There is currently no cure for DMD, and existing therapies aiming to rescue dystrophin expression are only partially effective. Here we show that the overexpression of *Jagged1* is likely to modulate the dystrophic phenotype in dystrophin-deficient GRMD dogs. We also show that overexpression of *jagged1* rescues the dystrophic phenotype in a severe DMD model, the *sapje* zebrafish. Our study highlights the possibilities of across-species analysis to identify and validate disease-modifying genes and associated pathways. These results suggest that *Jagged1* may be a new target for DMD therapeutic efforts in a dystrophin-independent manner, which will complement existing approaches. In addition, further investigation on the gene target, *Jagged1* will contribute to a better understanding of the disease pathogenesis and molecular physiology.

Methods Summary

GRMD dogs were classified for this study in two groups based on full ambulatory capacity and survival age. Escapers group: GRMD dogs that were fully ambulatory (can walk and run) at 9 years old. One escaper dog (M1M4) died at 11 years old from a cardiac arrest (Zatz et al., 2015) and the second one (H3M10) is now 9 ½ years old and shows full ambulation. Affected group: dogs that died before 5 years old with ambulatory difficulties, respiratory failure and cardiopathy; this group includes stillbirths, neonatal death and one dog that was full ambulatory when he died by ingesting a foreign object at 6 months-old (K2M11), all confirmed to carry the GRMD mutation. DNA from GRMD dogs with and without the escaper phenotype was genotyped using the Illumina canine 170,000 SNP array, and compared using association, linkage, and IBD mapping. The threshold for genome-wide significance for each association analysis was defined based on the 95% confidence intervals (CIs) calculated from the beta distribution of observed p values, as previously described (Wellcome Trust Case Control, 2007). The likelihood of the two escapers being Identity By Descent (IBD) at each SNP was estimated, based on haplotype frequencies in the full pedigree, using Beagle 4 (release v4.r1274) with default parameter settings (Browning and Browning, 2007). Linkage analysis was performed using MERLIN (Abecasis et al., 2002) 1.1.2 to first remove inconsistent genotypes and then calculate LOD scores (logarithm of the odds ratios) using a dominant parametric model with complete penetrance. Expression analysis from the same dogs was performed using Two-Color Microarray-Based Gene Expression Analysis. Genes differentially expressed between wild-type, escaper and affected animals were identified with the Significance Analysis of Microarray (SAM) statistical approach. False discovery rate (FDR) was 5%. Whole-genome sequencing was performed to 30× depth of three dogs (two escapers and one affected dog). Samples were sequenced on an Illumina HiSeq 2000, sequencing reads were aligned to the CanFam 3.1 reference sequence using BWA. Following GATK base quality score recalibration, indel realignment, duplicate removal, SNP and INDEL discovery was

performed. To assess myogenin binding to candidate mutation, EMSA was performed using biotin labeled or unlabeled competitors probes and the LightShift Chemoluminescent EMSA kit (Thermo Scientific) following manufacturer's instructions. Luciferase reporter assay was performed cloning wild type and GRMD dog Jagged1 promoter region containing the G>T change into the pIRES-2a-hrGFP expression plasmid (Stratagene). HEK293T or C2C12 cells were transfected with affected or escaper 3'UTR jagged1-luc reporter constructs and Myogenin or MyoD overexpression plasmid and renilla as internal control. Cells were lysed and assayed with luciferase substrate using the Dual Reporter Assay (Promega). Luciferase measurements were normalized to the renilla luciferase control on each well. Zebrafish were used for *jagged1* overexpression assay, where fertilized one-cell stage eggs from a *sapje* heterozygous fish mating were injected with mRNA of either one of the zebrafish *jagged1* gene copies: *jagged1a* or *jagged1b*. Zebrafish injected with either mRNA and non-injected controls were assessed for phenotypic changes at 4 days post fertilization (4dpf). Methods for cell growth assay and cardiotoxin injury are described in Methods (Supplemental).

Extended Methods are available as supplemental materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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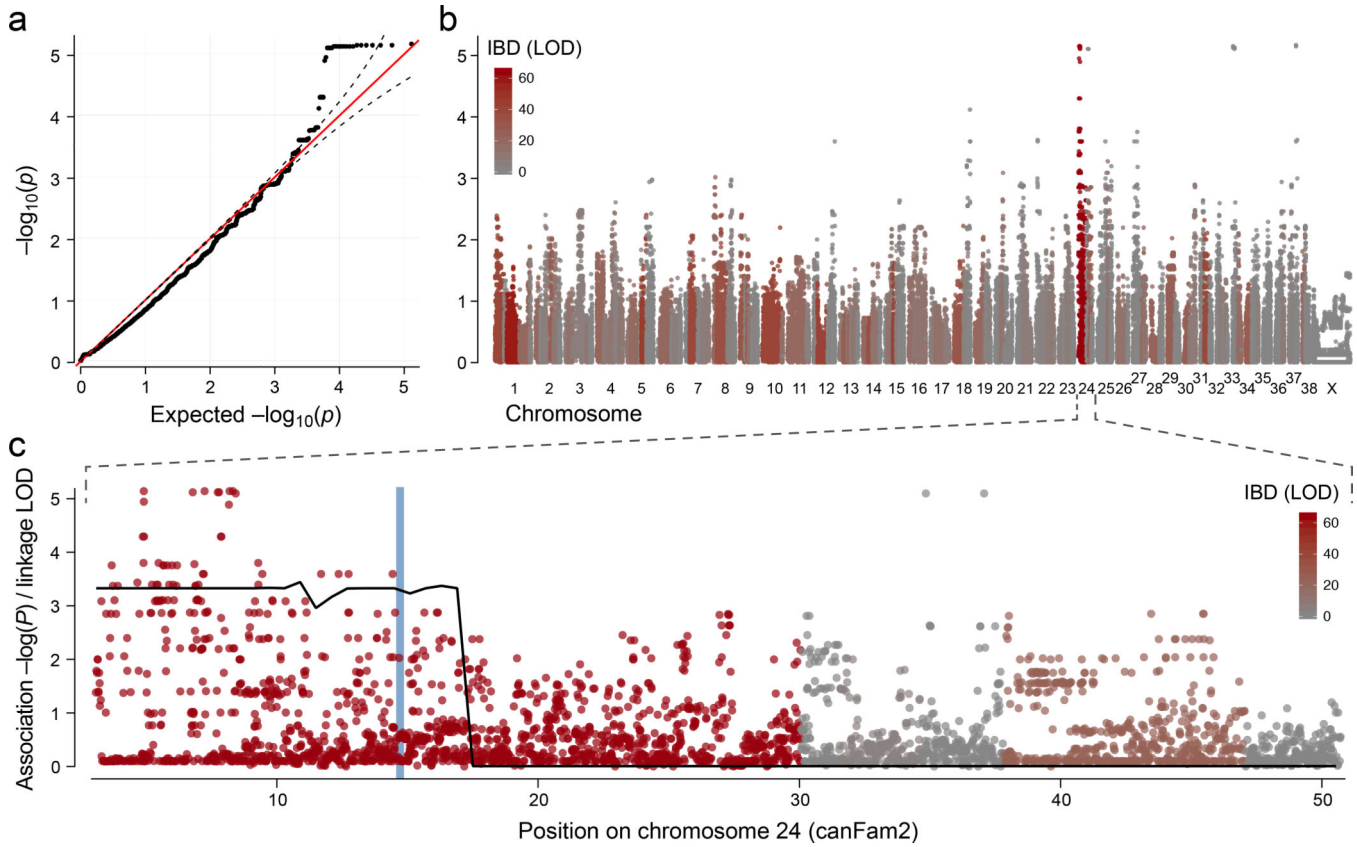

Figure 1. Combining association, linkage and identity-by-descent analysis identifies a 30Mb candidate region on chromosome 24

(a) A QQ plot of 129,908 SNPs tested for association identified 27 SNPs outside the 95% confidence intervals (dashed lines) and minimal stratification relative to the expected distribution (red line), suggesting the mixed model approach corrected for close relatedness among the two escapers and 31 severely affected GRMD dogs. (**b**) Only the association on chromosome 24 also falls in a region where the two escapers (sire and offspring) share a long haplotype likely to be identical-by-descent (IBD, red). Other peaks on chromosomes 24, 33 and 37 show no evidence of IBD (grey) and are most likely false positives due to the small sample size. (**c**) The mapped region extends 27Mb from the start of chromosome 24. Linkage analysis with Merlin (solid black line) detected a significant linkage peak (dominant parametric LOD > 3) overlapping the IBD/association peak which includes the putative driver gene *jagged1* (blue line) identified through gene expression profiling. See also Figures S1 and S2.

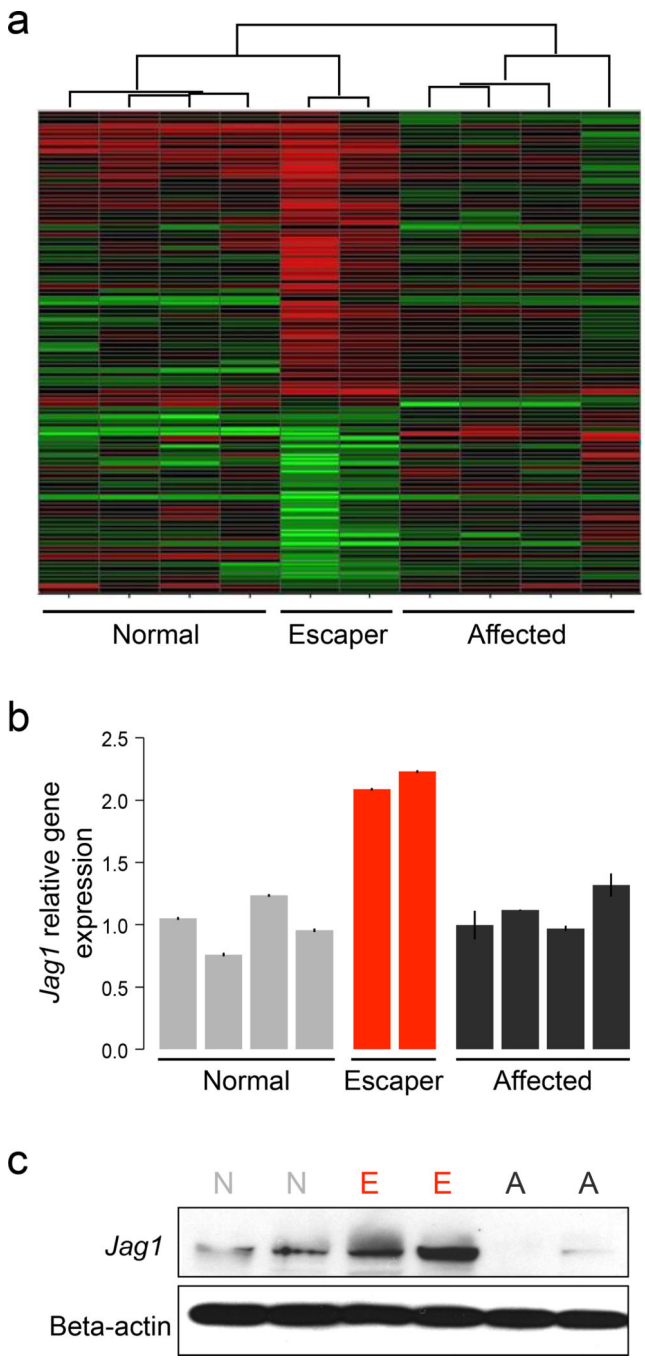

Figure 2. Altered *Jagged1* expression in escaper GRMD dogs

(a) mRNA microarray comparing muscle gene expression of escaper GRMD dogs with related severely affected and wild-type littermates. (**b**) mRNA expression of escaper dogs confirming the expression array findings. Relative *Jagged1* gene expression in muscle samples of escaper GRMD dogs as compared to related severely affected and wild-type dogs; bars indicate standard deviation from the mean. (**c**) *Jagged1* protein levels in the muscle of escaper GRMD dogs (E) as compared to severely affected (A) and wild-type dog muscle (N); Beta-actin is the loading control. See also Table S1.

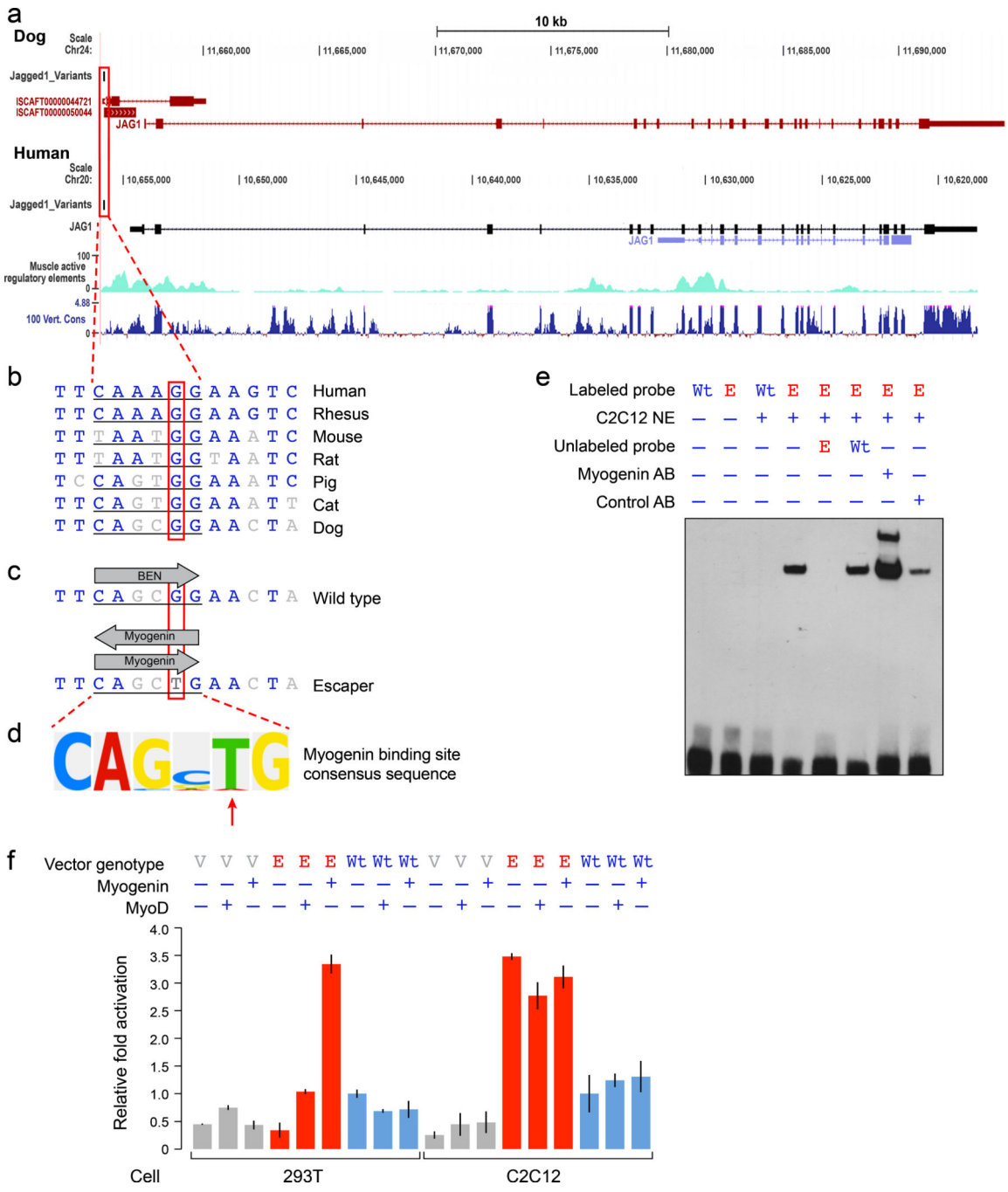

Figure 3. Variant located in the *Jagged1* promoter of escaper GRMD dogs

(a) Dog and Human *Jagged1* locus. Box: variant at dog chr24:11,644,709. (**b**) Conservation of the variant position. (**c**) Predicted transcription factor binding site at the region with the base pair change. (**d**) Consensus sequence of myogenin binding site, demonstrating the high information content of the T allele. (**e**) Electromobility shift assay (EMSA) showing myogenin binding to mutated probe (E) and not to the wild type probe (Wt). (**f**) Luciferase reporter assay showing activity of wild-type (Wt) and escaper (E) genotype vectors in muscle cells (C2C12) and embryonic kidney cells (293T) with *Myogenin* or *MyoD*

overexpression, as compared to empty vectors controls (V). Error bars indicate SEM (n = 3 replicates). See also Figure S3.

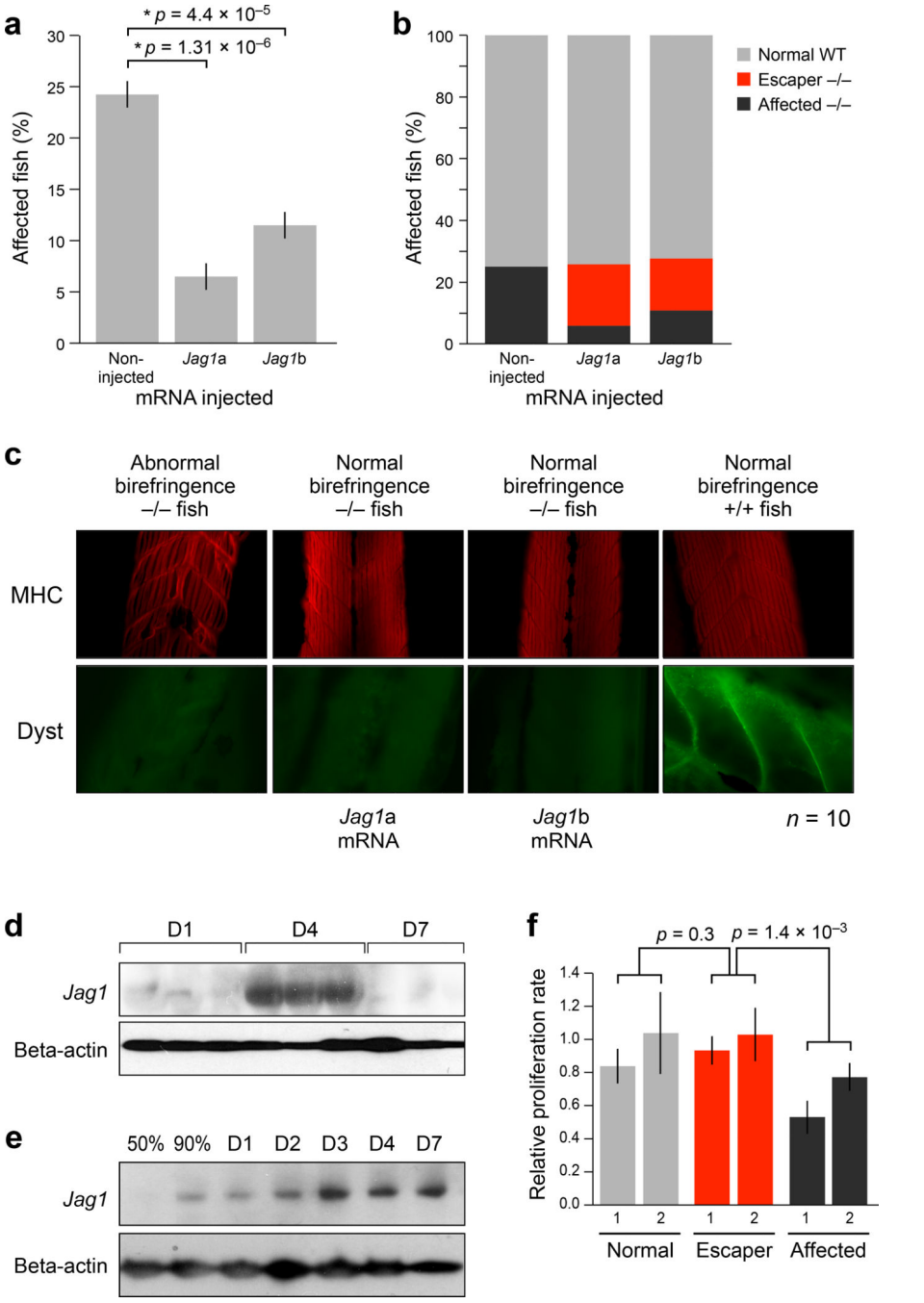

Figure 4. Functional analysis of *jagged1 expression*

(a) Percent affected *sapje* fish as determined by birefringence assay at 4 dpf. Note fewer affected fish in the *jagged1* injected *sapje* cohort. Four separate injection experiments were performed. (**b**) Genotype of *sapje* injected fish with *jagged1a* and *jagged1b* as compared to non-injected *sapje* fish. In red are dystrophin null fish with a wild-type phenotype, recovered by *jagged1* overexpression. (**c**) Immunofluorescence of *jagged1a* and *jagged1b* overexpression in the *sapje* fish. Wild-type, phenotypically affected homozygous fish for the dystrophin mutation and *jagged1a* and *jagged1b* injected with normal birefringence

(recovered) were stained for myosin heavy chain (MCH) and dystrophin antibodies. Note the organization of the muscle fibers in the recovered fish muscle comparable to the wild-type fish *(n=10)* even without dystrophin. Photographs were taken at 20× magnification. (**d**) *Jagged1* protein levels in the muscle of cardiotoxin injured mice 1, 4 and 7 days after injury. (**e**) *Jagged1* protein levels in muscle cells during *in vitro* muscle differentiation. (**f**) Muscle cell proliferation rate, as measured by MTT, of two wild-type, two escaper and two affected GRMD dogs. Error bars indicate SEM (n=2, three replicates).