**Jagged 1 Rescues the Duchenne Muscular Dystrophy Phenotype**

**Graphical Abstract**

**Highlights**
- Escaper GRMD dogs show that a normal lifespan is possible without muscle dystrophin
- *Jagged1*, a Notch ligand, is upregulated in mildly affected dystrophin deficient dogs
- *Jagged1* overexpression can rescue the phenotype of dystrophin deficient zebrafish

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**In Brief**
The study of two exceptional dogs that escaped from the severe phenotype associated with dystrophin deficiency unveils a genetic modifier that allows functional muscle and normal lifespan despite the complete absence of dystrophin.

**Accession Numbers**
GSE69040
Jagged 1 Rescues the Duchenne Muscular Dystrophy Phenotype

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SUMMARY

Duchenne muscular dystrophy (DMD), 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 reveals 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.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked disorder caused by mutations in dystrophin (Hoffman et al., 1987), which affects 1 in 3,500 to 5,000 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 the 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 CK levels, and lack of 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 do these escaper dogs have a fully functional muscle without dystrophin? Skeletal muscle of DMD patients undergoes 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 three 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 of 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—to 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 27 Mb 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, Figure S1). No other genomic regions showed any signs of linkage (Figure S2). Thus, convergent IBD, association, and linkage analyses all pointed to the same 27 Mb 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 ten samples (Figure 2A). Of these, 65 genes were also differentially expressed between escapers and wild-type dogs (Table S1), implicating them in a possible compensatory mechanism active in only the escaper dogs. Only one of these 65 genes, \textit{Jagged1}, is located under the association peak on chromosome 24. \textit{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 \textit{Jagged1} locus (including 3 KB 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 \( \sim \)1,300 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 \textit{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 \textit{Jagged1} (cfa24:11655709, Figure 3A). Sanger sequencing of the \textit{Jagged1} candidate escaper variant was performed in the escaper extended pedigree, including the first escaper (M1M4), his offspring, and a sibling’s offspring (M1M5) (Figure S3). 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 \textit{Jagged1} variant, while both escapers were heterozygous. Thus, the novel \textit{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 6 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 (Figures 3A and 3B). 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 (EMSAs) 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 WT 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 whether 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 WT sequence or the escaper variant fused to a luciferase reporter. Luciferase vectors containing either 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 3-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.
Figure 3. Variant Located in the Jagged1 Promoter of Escaper GRMD Dogs
(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 WT probe.
(F) Luciferase reporter assay showing activity of WT and E genotype vectors in both 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.

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, 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* = 4.4 × 10⁻⁶ for *jagged1a*, *p* = 4.4 × 10⁻⁶ for *jagged1b*, Figure 4A). Genotypic analysis revealed that about 75% of dystrophin-null fish injected with *jagged1a* and 60% of those injected with *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 WT 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 WT 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 4 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

![Figure 4. Functional Analysis of *jagged1* Expression](image_url)

(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* compared to non-injected *sapje* fish. In red are dystrophin-null fish with a WT phenotype, recovered by *jagged1* overexpression. (C) Immunofluorescence of *jagged1a* and *jagged1b* overexpression in the *sapje* fish. WT, 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 WT fish (*n* = 10) even without dystrophin. Photographs were taken at 20x magnification. (D) *Jagged1* protein levels in the muscle of cardiotoxin injured mice one, four, and seven 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 WT, two escaper, and two affected GRMD dogs. Error bars indicate SEM (*n* = 2, three replicates).
proliferation assay using myogenic cells from biopsies ofagematched 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 associated to the mild phenotype observed in a muscular dystrophy mouse model on the MRL (Murphy Roths Large) “superhealing” background. These mice show enhanced muscle regeneration and reduced dystrophic pathology. This healing phenotype was 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 4 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 Jagged1 overexpression resulted in endothelial branching of vasculature processes; while conversely, Jagged1 endothelial deletion blocked angiogenic growth in Jagged1 eKO mice (Pedrosa et al., 2015). Indeed, Jagged1 overexpression leads 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 Jagged1 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 Jagged1 ligand is not a viable therapeutic option, as external Jagged1 weakens Notch signaling even more than dystrophin-deficiency (Xiao et al., 2015). 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.

**EXPRESSIMENTAL PROCEDURES**

GRMD dogs were classified for this study in two groups based on full ambulatory capacity and survival age. The escapers group included the 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 9.5 years old and shows full ambulation. The affected group included the GRMD 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 were confirmed to carry the GRMD mutation. DNA from GRMD dogs with and without the affected phenotype was genotyped using the Illumina canine 170,000 SNP array and was 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 v4.1274) 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 WT, escaper, and affected dogs were assessed for phenotypic changes at 4 days post-fertilization (4dpf). Methods for cell growth assay and cardiotoxin injury are described in Supplemental Experimental Procedures. Supplemental Experimental Procedures are available as supplemental materials.

**ACCESSION NUMBERS**

The accession number for the gene expression data reported in this paper is GEO: GSE69049.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.10.049.

**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


Wellcome Trust Case Control, C.; Wellcome Trust Case Control Consortium (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447, 661–678.


Figure S1. Chromosome 24 Linkage Results, Related to Figure 1

(A) Linkage analysis of the escapers’ pedigree identified a 27 Mb linkage peak on chromosome 24 with a maximal parametric dominant LOD score of 3.31. A smoothed linkage curve (red) is shown on top of the single SNP LOD (gray). (B) Non-dog related Jagged1 homologs all reside under this linkage peak. Shown are alignments of the human, bovine, rat, mouse, and frog Jagged1 refseq sequences, as well as the two zebrafish homologs used in this study.
A single linkage peak was identified in the escapers’ pedigree, mapping to chromosome cfa24:3,073,196-30,066,497 (CanFam2).
Figure S3. GRMD Dogs’ Pedigree and Genotype, Related to Figure 3
All dogs were genotyped for the GRMD mutation and for the jagged1 variant (G>T at Chr24: 11,655,709). Escaper dogs are: M1M4 and H3M10.
Supplemental Information

Jagged 1 Rescues the Duchenne Muscular Dystrophy Phenotype

**Supplemental Methods**

**GRMD dogs**
All animals were housed and cared for in the University of São Paulo and genotyped at birth for the GRMD mutation as previously described (Honeyman et al., 1999). GRMD dogs were identified by microchip implants. Animal care and experiments were performed in accordance with animal research ethics committee of the Biosciences Institute, University of São Paulo (034/2005).

**GRMD dog phenotype**
Typically, 80% of GRMD dogs die before 18 months old and severely affected dogs do not survive past 5 years (Zucconi et al., 2009). At 4 months of age all severely affected GRMD dogs show clear clinical signs resulting from muscle degeneration with difficulty to ambulate. The two dogs here called escapers are clearly distinguishable from the other GRMD dogs: they survived and remained fully ambulatory beyond the known range of the GRMD phenotypic variability. Given this major phenotype difference, 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 1/2 years old and shows full ambulation (can walk and run). Affected group: dogs that died before 5 years old with difficulties in walking, deglutition, respiratory failure or cardiomyopathy as well as stillbirths and neonatal death (confirmed to carry the GRMD mutation).

One offspring that carried the candidate mutation (K2M11) and died at age 6 months from ingestion of a foreign object showed a similar phenotype as the two escaper dogs and was fully ambulatory. Hence, he is potentially an escaper, consistent with his genotype, but his early death makes it impossible to confirm this. He was classified as an affected dog for the genomewide association analysis, based on the phenotype criteria described above.

**Comparison of affected and escaper animals in the pedigree using the Illumina 170K canine array**
DNA from all GRMD dogs from the pedigree (Supplemental Figure 3) was isolated from blood and genotyped using the Illumina 170K canine HD array. Badly genotyped individuals and lowly genotyped SNPs were filtered out using Plink (PLINK http://pngu.mgh.harvard.edu/purcell/plink/), leaving 2 escapers, 31 affected (offspring of M1M4 and M1M5). EMMAX (Kang et al., 2010) was used to simultaneously correct for population structure and relatedness in the pedigree using a linear mixed model approach (Kang et al., 2010) applied to a binary phenotype (escaper vs. affected) as described in the EMMAX documentation. 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
Linkage analysis was performed on the same SNP dataset described above. First, we used PedCut (Liu et al., 2008) to identify a smaller, computationally feasible sub-pedigree, without loss of information, as the original pedigree size was computationally intractable for linkage analysis. The resulting sub-pedigree was composed of 15 dogs: two escapers and 13 affected dogs. MERLIN (Abecasis et al., 2002) 1.1.2 was used to first remove inconsistent genotypes and then calculate LOD scores (logarithm of the odds ratios) using a dominant parametric model with complete penetrance.

mRNA expression profiling
Total mRNA was extracted from two escaper, four affected GRMD dogs, and four wild-type dogs, all age-matched muscle biopsies. Sample labeling and array hybridization were performed according to the Two-Color Microarray-Based Gene Expression Analysis—Low Input Quick Amp Labeling—protocol (Agilent Technologies) using the SurePrint Canine 4x44K (Agilent Technologies) Microarray (GEO Platform GPL11351). Samples were labeled with Cy5 and a single RNA from a wild-type individual was labeled with Cy3 and used as a common reference on all arrays. A dye-swap technical replicates approach was also applied, where all samples were labeled with Cy3 and the reference RNA was labeled with Cy5. Labeled cRNA was hybridized using Gene Expression Hybridization Kit (Agilent). Slides were washed and processed according to the Agilent Two-Color Microarray-Based Gene Expression Analysis protocol (Version 5.5) and scanned on a GenePix 4000 B scanner (Molecular Devices, Sunnyvale, CA, USA). Fluorescence intensities were extracted using Feature Extraction (FE) software (version 9.0; Agilent). Averaged values of dye-swap technical replicates were used for further analysis. Genes differentially expressed between wild-type, escaper, and affected animals were identified with the Significance Analysis of Microarray (SAM) statistical approach (Tusher et al., 2001), using the following parameters: one-class unpaired responses, t-statistic, 100 permutations. False discovery rate (FDR) was 5%. Gene Expression data were deposited at GEO database under Accession Number GSE69040.

Whole genome sequencing and variant calling
Whole-genome sequencing was performed to 30x depth of three dogs (two escapers and one severely affected related dog). Samples were sequenced on an Illumina HiSeq 2000, sequencing reads were aligned to the CanFam 3.1 reference sequence using BWA (Li and Durbin, 2010). SNPs and indel variants were called following GATK (McKenna et al., 2010) Best Practices recommendations (DePristo et al., 2011); including base quality score recalibration, indel realignment, duplicate removal, HaplotypeCaller, variant quality score recalibration, and variant filtration using standard hard filtering parameters. Variants were called in the jagged1 gene including the 3KB regions upstream and downstream of the gene (chr24:11654000-11696000, canfam3).

Variants were then filtered aiming to find a new mutation present only in the escaper GRMD dogs and not in the affected siblings or previous canine SNP data sets (Axelsson et al., 2013) using a custom PERL script. Variants were lifted-over to human genome using UCSC Genome Browser (Kent et al., 2002). Variants present in muscle regulatory regions (ENCODE) (Rosenbloom et al., 2013) were considered of interest if they had not previously been reported in other unrelated dogs. To verify the segregation of the variant, genomic DNA from GRMD dogs related to the first escaper was amplified by PCR using the following primers: forward primer 5’-ACCCAACCTTTTCTGCAGCTC-3’ and reverse primer 5’-CATAGCCAAGGTCGAAGGAA-3’, with a 55 °C annealing
temperature and 35 cycles. PCR products were purified using ExoSap (Affymetrix) and sequenced at The Molecular Genetics Core Facility at Boston Children’s Hospital.

Electrophoretic mobility shift assay (EMSA)
The duplex DNAs obtained by annealing of complementary oligonucleotides were either biotin labeled or unlabeled competitors. Probes sequence were: WT: CTCCTTTTATTTCAGCGGAACCTAAAGAAGTCTC and for the variant CTCCTTTTATTTCAGCTGGAACCTAAAGAAGTCTC. Biotin-labeled probes (0.5pmol) were incubated with C2C12 nuclear extract (Active Motif) on ice for 20min in the reaction buffer (10mM Tris (pH 7.5), 50mM KCl, 1mM EDTA, 1mM DTT, 50% glycerol, 50ng/ul of poly (dI.dC) and 10ug/ul of bovine serum albumin). For competition experiments, unlabeled competitor DNAs in 100-fold molar excess over the labeled probe were included in the binding reactions. The supershift assay was performed with 5ug of anti-myogenin F5D (Santa Cruz Biotechnology) mouse monoclonal antibody incubated 30 min at room temperature. Anti-mouse IgG (Abcam) was used as control antibody. Samples were loaded onto 6% DNA Retardation Gels (Invitrogen) and separated at 10 V/cm in 0.5 TBE (45 mM Tris, 45 mM borate, 1 mM EDTA). Transfer to positive charged nylon membrane (GE – Hybond-N+) was performed in 0.5 TBE at 5°C for 1 hour at 380 mA. Membrane crosslink was performed at 120mJ/cm². Detection of the biotin-labeled DNA was carried out by chemiluminescence using LightShift Chemoluminescent EMSA kit (Thermo Scientific) following manufacturer’s instructions.

Luciferase reporter assays
The wild type and GRMD dog Jagged1 promoter region containing the G>T change at dog chr24:11644709 were amplified from affected and escaper dogs DNA. Amplicons were then cloned into the pGL4.10 vector (Promega). Human MYOD1 (NCBI Ref. NM_002478.4) and MYOG (NM_002479.5) EST open reading frame clones (Open Biosystems) were amplified and cloned into the pIRES-2a-hrGFP expression plasmid (Stratagene). The luciferase reporter assay was performed by first plating 10,000 HEK293T or C2C12 cells/well into 96-well plates. The following day the cells were transfected using Lipofectamine 3000 reagent with 90 ng of affected or escaper 3'UTR jagged1-luc reporter constructs and 100 ng of Myogenin or MyoD overexpression plasmid and 10ng of renilla as internal control. Forty-eight hours after transfection the cells were lysed in Reporter Lysis Buffer (Promega), and 20 µL of whole cell lysate were assayed with 25 µL of luciferase substrate using the Dual Reporter Assay (Promega). Luciferase levels were measured on a plate luminometer. Luciferase measurements were normalized to the renilla luciferase control on each well. Experimental samples were run and analyzed in triplicate.

Zebrafish lines
Zebrafish were housed in the Boston Children’s Hospital Aquatics facility (Director Christian Lawrence) under the animal protocol number: 09-10-1534R and maintained as breeding stocks as previously described (Lawrence and Mason, 2012).

Sapje Genotyping
Genomic DNA extracted from injected fish (Meeker et al., 2007) and controls was amplified with for sapje mutation: forward primer 5'-CTGGTTACATTCTGAGAGACTTTC-3' and reverse primer 5'-AGCCAGCTGAACCAATTAACCTCA-3', with a 52 °C annealing temperature and 35 cycles. PCR products were purified using ExoSap (Affymetrix) and sequenced at The Molecular Genetics Core Facility at Children’s Hospital Boston.
Zebrafish *jagged1* overexpression

Fertilized one-cell stage eggs from a *sapje* heterozygous fish mating were injected with 20 pg of mRNA of either one of the zebrafish *jagged1* gene copies: *jagged1a* or *jagged1b*. Plasmid constructs were kindly provided by Dr. Itoh from Nagoya University (Yamamoto et al., 2010) and linearized by NotI restriction digestion. mRNA was synthesized with SP6 message machine kit (AMBIION) and purified with mini Quick Spin Columns (Roche). Overexpression of zebrafish *jagged1* was confirmed by western blot for HA tag (data not shown). Zebrafish injected with either mRNA and non-injected controls and assessed for phenotypic changes at 4 days post fertilization (4dpf). Each injection was performed four times. Approximately 200 embryos were injected at each dosage in four separate experiments.

Zebrafish Birefringence assay

The typical *sapje* dystrophic muscle phenotype was detected by using a birefringence assay, a technique used to analyze muscle quality due to the unique ability of highly organized sarcomeres to rotate polarized light. The birefringence assay is performed by placing anesthetized embryos on a glass polarizing filter and covering them with a second polarizing filter. The filters are placed on a bottom-lit dissection scope and the top polarizing filter is twisted until the light refracting through the zebrafish's striated muscle is visible.

Zebrafish Immunostaining

Immunostaining was performed in 4dpf embryos. Embryos were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight and dehydrated in 100% methanol. After rehydration, 4 dpf embryos were incubated in 0.1% collagenase (Sigma) in PBS for 60 min. Blocking solution containing 0.2% saponin was used for 4 dpf embryos. Anti-slow muscle myosin heavy chain antibody (F59, Developmental Studies Hybridoma Bank; 1:50) was used. The embryos were placed in 3% methyl cellulose or mounted on a glass slide and observed with fluorescent microscopes (Nikon Eclipse E1000 and Zeiss Axioplan2).

Western Blot

Muscle sample proteins were extracted using RIPA buffer with proteinase inhibitor tablets (Roche). Samples were centrifuged at 13,000g for 10 minutes to remove insoluble debris. Soluble proteins were resolved using electrophoresis with Novex 4–20% Tris-Glycine gels (Life Technologies), and transferred to nitrocellulose membranes (Hybond; Amersham Biosciences). All membranes were stained with Ponceau (Sigma-Aldrich) to evaluate the amount of loaded proteins. Blots were blocked for 1 hour in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and reacted overnight with the following primary antibodies: anti- *jagged1* (C-20, Santa Cruz Biotechnology, 1:1000). Horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, 1:1000) was used to detect immunoreactive bands with Pierce ECL 2 (Thermo). Anti-beta actin antibody (HRP – ABCAM) was used as loading control.

Cell growth assay

GRMD and wild-type myoblasts were plated at 96-well plates in three different concentrations: 100, 1000 and 10000 cells/well. All samples were plated in triplicate. Cells were maintained in DMEM-HG (Dulbecco’s modified Eagle’s medium with high glucose; Gibco) supplemented with 20% (v/v) FBS (fetal bovine serum; Gibco) and anti-
anti (Gibco) at 37°C and 5% CO₂ for one week. Cell growth was measured using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT – Promega) following manufacture’s protocol. Absorbance was recorded using the Synergy2 plate reader (BioTek).

Cardiotoxin injury
All mouse procedures were approved by the Boston Children’s Hospital Animal Care and Use Committee (IACUC) under the animal protocol number 12-10-2287R. Wild type (C57B6/J) adult (2-4 month) male mice were house under pathogen free conditions. Mice were injured using an injection of 50 µl of 10 µM cardiotoxin isolated from Naja mossambica mossambica snake venom (Sigma-Aldrich, C-9759) into the right quadriceps muscle. The left, contralateral quadriceps muscle served as mock injected (PBS) control.

References
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