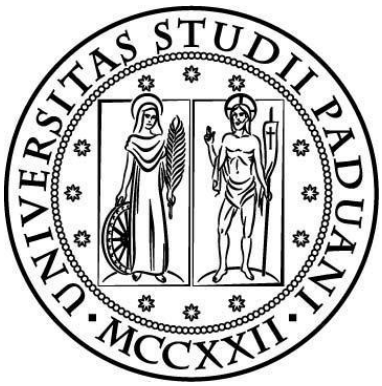
Università degli Studi di Padova

Dipartimento di Biologia

Corso di Laurea in Biotecnologie



Elaborato di Laurea

Jagged1 Rescues Duchenne Muscular Dystrophy Phenotype

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SOMMARIO

ABSTRACT

La distrofia muscolare di Duchenne, provocata da mutazioni a livello del gene distrofina, è la forma più comune di malattia genetica muscolare. Ad oggi non è disponibile alcuna cura definitiva e l'unica terapia in uso è quella sintomatica. L'assenza di distrofina nel muscolo altera alcune vie di segnalazione cellulare, che potrebbero diventare targets terapeutici per nuovi farmaci. Due cani GRMD mostravano un fenotipo attenuato, muscolatura funzionale e normale durata della vita pur avendo completa assenza di distrofina. Una regione cromosomica associata con il fenotipo "escaper", identificata usando un modello misto di analisi di linkage e di associazione, conteneva il gene *Jagged1* con espressione alterata tra gli escapers e i cani affetti. Il sequenziamento dell'intero genoma ha dimostrato che la variante genetica presente negli escapers crea un sito di legame per il fattore trascrizionale miogenina nel promotore di *Jagged1*. Questo gene se sovraespresso a livello del muscolo induce il recupero funzionale non solo nei cani GRMD ma anche in zebrafish *sapje*, un altro modello di distrofia muscolare. È noto che *Jagged1* regola la via di segnalazione cellulare Notch; come modificatore genetico potrebbe diventare un nuovo target terapeutico per la cura delle distrofie muscolari.

1 STATO DELL'ARTE

1.1 La distrofia muscolare di Duchenne

La forma di distrofia muscolare più frequente e severa è la distrofia muscolare di Duchenne (DMD), una malattia recessiva legata al cromosoma X. La patologia colpisce 1 su 4000-6000 individui di sesso maschile. Il gene per la distrofina, lungo 2.5 Mb e localizzato nel braccio corto del cromosoma X, codifica una proteina di 425 kDa con 79 esoni, molto lunga se paragonata ad altri prodotti genici. Ciò chiaramente aumenta la probabilità che avvengano mutazioni spontanee a livello di tale gene. Delezioni e duplicazioni sono responsabili del 65% dei casi di DMD e si concentrano in due hotspots mutazionali all'estremità N-terminale e nel dominio centrale a bastoncello. Le mutazioni alterano il registro di lettura del gene, portando a completa assenza di proteina distrofina. Nei soggetti femminili può esserci un meccanismo di compensazione, dato che è presente l'allele funzionale sull'altro cromosoma X. La distrofina connette citoscheletro e membrana plasmatica; è espressa prevalentemente a livello del muscolo scheletrico, cardiaco e respiratorio. Essa fa parte del complesso DAPC (Dystrophin-Associated Protein Complex), essenziale per ammortizzare le contrazioni muscolari, perciò la sua assenza diminuisce la resistenza meccanica del sarcolemma. Le miofibre degenerano progressivamente e solo inizialmente la loro perdita è compensata da parziale rigenerazione ad opera delle cellule satelliti del muscolo. Con il progredire della malattia prevale la degenerazione e le miofibre vengono rimpiazzate da tessuto connettivo ed adiposo. I primi sintomi sono di solito evidenti già da tre a cinque anni di età, con un decorso clinico severo e progressivo che porta a perdita di capacità ambulatoria tra nove e dodici anni. La morte avviene per arresto respiratorio o cardiaco e sopraggiunge solitamente nella seconda o terza decade di vita.

1.2 Meccanismi molecolari alla base della miogenesi

Il muscolo scheletrico mostra una straordinaria capacità di adattamento ai cambiamenti, siano essi fisiologici come la crescita o patologici come il danno tissutale. I processi che rendono possibili le modifiche del muscolo adulto sono attribuiti alle cellule satelliti, una popolazione cellulare localizzata tra la lamina basale e la membrana plasmatica della miofibra. Se il muscolo è in salute, le cellule satelliti si trovano in uno stato quiescente non proliferativo. In risposta a differenti stimoli esse si attivano, cominciano a proliferare ed esprimono specifici marcatori miogenici (MRFs, Myogenic Regulatory Factors). MyoD è un fattore trascrizionale critico per la determinazione delle cellule satelliti in mioblasti e per la loro successiva uscita dal ciclo cellulare e trasformazione in miociti mononucleati. La miogenina, altro fattore di trascrizione, promuove la

conversione in miotubi, mediata da adesione dei miociti tra di loro o a fibre preformate, allineamento delle loro membrane e fusione. La divisione delle cellule satelliti è asimmetrica, per assicurare da una parte la rigenerazione muscolare e dall'altra il mantenimento di un pool di cellule a carattere staminale. MyoD e miogenina sono fattori trascrizionali costituiti da un dominio di legame al DNA con struttura elica-giro-elica e un dominio attivatore (AD) in grado di interagire con altre proteine e guidare la loro attività. La sequenza consenso che media l'interazione di questi fattori con il DNA è 5'-CANNTG-3', definita E-box, presente nel promotore di molti geni specifici del muscolo. L'azione di questi fattori segue un preciso ordine temporale schematizzabile. Inizialmente MyoD regola il processo di miogenesi comprendente la formazione sia delle miofibre sia delle cellule satelliti attorno ad esse. Dopo che i miociti sono usciti dal ciclo cellulare, l'attivazione di miogenina non incontra ostacoli e permette l'espressione massiccia delle proteine contrattili.

1.3 Terapia e ricerca

La terapia standard per la DMD rimane basata su glucocorticoidi, in particolare prednisone, non privo di effetti collaterali. Di recente è stata approvata come farmaco una piccola molecola, l'ataluren, dotata di bassa tossicità. Essa rende possibile il *readthrough* dei ribosomi, i quali proseguono nella traduzione aggirando il codone di stop prematuro creato da una mutazione presente nel 10% dei pazienti distrofici. Il farmaco è disponibile per pazienti ancora ambulanti di età superiore a 5 anni e che portino la suddetta mutazione nonsenso. Sono in corso di sviluppo vari approcci di terapia genica: l'inserzione mediata da vettori virali adeno-associati di una forma di distrofina ridotta ma funzionale scatena purtroppo una risposta immunitaria cellulare e anticorpale sia contro l'antigene del capside virale sia contro la proteina esogena. Gli stessi vettori virali possono veicolare anche l'utrofina, una proteina funzionalmente e strutturalmente simile alla distrofina prodotta durante le fasi precoci dello sviluppo muscolare fetale e della rigenerazione muscolare. Mantenendo alta la sua espressione nell'adulto si potrebbe compensare l'assenza di distrofina e proteggere la funzionalità del muscolo. Una tecnologia emergente è il gene editing basato sulla tecnica CRISPR/Cas9, che accoppia l'endonucleasi Cas9 con corti RNA guida (sgRNA) per correggere piccole mutazioni del gene distrofina portando alla produzione di proteina quasi di lunghezza intera. Se le mutazioni sono di maggiore portata il sistema CRISPR/Cas9 permette lo *skipping* dell'esone mutato durante lo *splicing* e la produzione di distrofina di dimensioni inferiori, con la possibilità di inserire la porzione mancante sotto forma di cDNA. Questa tecnica innovativa rende dunque pensabile la correzione del difetto a livello genetico.

1.4 Animali modello

È chiaramente necessario disporre di animali modello che mimino le condizioni umane della patologia. I topi *mdx* condividono con i pazienti umani affetti da DMD l'assenza di distrofina, ma il loro fenotipo attenuato non riproduce i sintomi severi della malattia nell'uomo. Il modello di zebrafish *sapje* mostra variabilità fenotipica, ma tutti gli esemplari presentano struttura del muscolo anomala se saggiata mediante birifrangenza e la maggiorparte di essi muore nel corso delle prime settimane di vita. Se lo scopo di una ricerca è la terapia genica per l'uomo, questi due animali modello hanno dimensioni troppo ridotte rispetto al modello umano, limitando la valutazione di variabili quali motilità cellulare e diffusione di farmaci. Un eccellente omologo genetico per la DMD umana è il cane GRMD (Golden Retriever Muscular Dystrophy), un animale di taglia media. Gli animali affetti portano una mutazione puntiforme a livello del sito accettore di splicing nell'introne 6 del gene distrofina, la quale causa excisione dell'esone 7 e un codone di stop prematuro, risultando in assenza di distrofina muscolare. Come i pazienti DMD, anche i cani GRMD mostrano degenerazione precoce e progressiva del muscolo, atrofia muscolare, fibrosi, contratture. Le somiglianze biochimiche comprendono alti livelli nel siero di creatin chinasi muscolare, indicativa di danno alla membrana cellulare. I cani però possono avere difficoltà nella deglutizione, a differenza degli umani, e la perdita di capacità ambulatoria non è sempre presente. I cani GRMD sono lievemente affetti fino a tre mesi di età, i sintomi si aggravano progressivamente fino ai sei mesi per poi stabilizzarsi. La morte sopraggiunge solitamente a uno o due anni di età conseguentemente ad arresto respiratorio o cardiaco, ma può avvenire anche nelle prime settimane di vita. La maggioranza dei cani GRMD non supera i due anni di età. La variabilità clinica tra cani GRMD di diverse colonie è elevata e dovuta a modificatori genetici concentratisi durante gli incroci. Similmente anche il fenotipo della DMD nei pazienti umani è influenzato da polimorfismi genetici.

1.5 Introduzione all'approccio sperimentale

Tra i cani GRMD appartenenti alla colonia brasiliana fondata nell'anno 2000 all'Istituto di Bioscienze dell'Università di Saõ Paolo sono stati identificati due esemplari recanti un fenotipo chiaramente distinguibile da quello degli altri affetti. Ringo, nato nel 2003, è in grado di correre, saltare, e aprire una porta. Sulflair, suo discendente nato nel 2006, mostra anch'esso un fenotipo attenuato. Analisi istopatologiche di biopsie muscolari dei due cani mostrano le tipiche caratteristiche di un processo distrofico in corso, quali dimensione variabile delle miofibre, fenditure, necrosi, nuclei centrali, fibre rotondeggianti, degenerazione e infiltrazioni di tessuto connettivo. La distrofina muscolare è assente. I livelli nel siero di creatin chinasi muscolare sono elevati.

Da qui in poi ci si riferisce a questi due cani GRMD con il termine "escapers", a indicare l'eccezionalità del loro fenotipo. Comprendere quali siano i meccanismi in grado di proteggere questi cani dagli effetti deleteri della mutazione a livello del gene distrofina è di enorme interesse per lo sviluppo di nuove terapie.

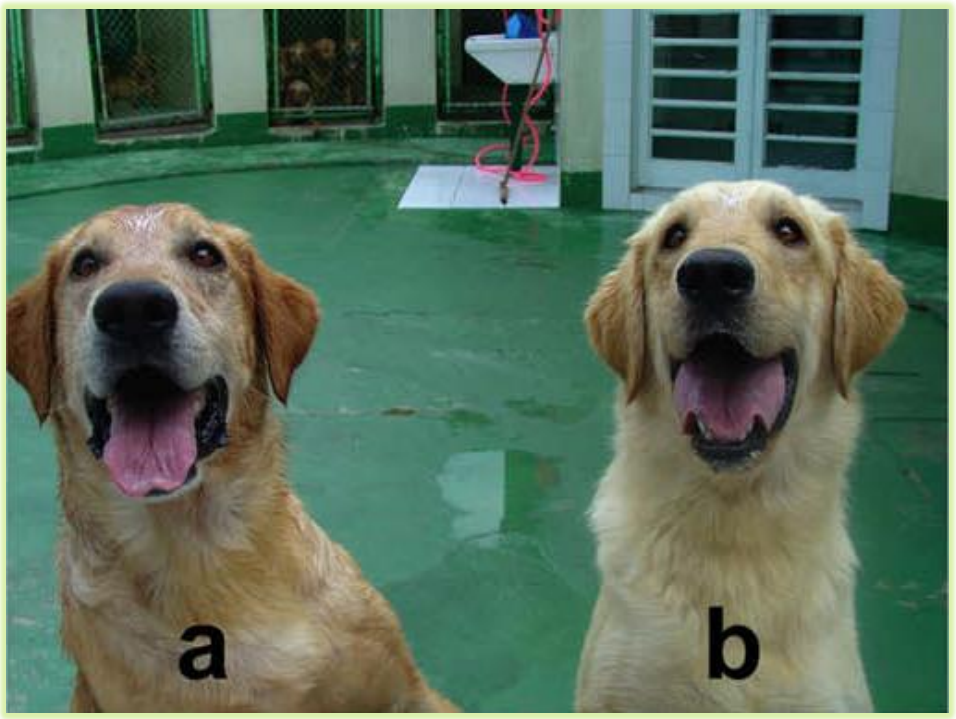

Foto dei due cani GRMD escapers analizzati in questo studio: a) Ringo, b) Sulflair.

2 APPROCCIO SPERIMENTALE

2.1 Genotipo di cani escapers e affetti

2.1.1 Studio di associazione genome-wide

Il DNA estratto dal sangue di tutti i cani GRMD appartenenti al pedigree di Ringo e Sulflair viene genotipizzato utilizzando l'array ad alta definizione Illumina 170K canine, il quale sfrutta l'appaiamento di frammenti del genoma a sonde rappresentanti più di 170mila SNPs della sequenza genomica di riferimento CanFam2.0. Le sonde sono lunghe 50 nucleotidi e sono fissate a delle biglie, in maniera da condurre l'ibridazione in soluzione. L'ultimo nucleotide incorporato è legato ad un fluoroforo, per visualizzare l'avvenuta ibridazione tra i frammenti di DNA genomico e le sonde. Dopo aver scartato i segnali d'ibridazione di bassa qualità rimangono i dati dei due cani escapers e di trentuno cani affetti. L'approccio EMMAX, correggendo la struttura della popolazione costituita da un numero limitato di esemplari strettamente imparentati, permette di ottenere dall'array di SNPs un modello di distribuzione del fenotipo nella popolazione basato su un fenotipo binario: escaper vs. affetto. L'intervallo di confidenza applicato è pari al 95%. Utilizzando il software Beagle 4 si stima la possibilità dei due escapers di essere identici per discendenza (IBD) ad ogni SNP, vale a dire di aver ereditato il tratto genomico corrispondente.

2.1.2 Analisi di linkage

Il pedigree iniziale viene ridotto con l'utilizzo del software PedCut a due esemplari escapers e tredici affetti, ottenendo senza perdita di informazioni un sub-pedigree più adatto all'analisi di linkage. La statistica più efficiente per rilevare la concatenazione è rappresentata dal LOD score. I valori sono calcolati servendosi del programma MERLIN, adatto per gestire un alto numero di loci in pedigree di modeste dimensioni, utilizzando un modello parametrico dominante con penetranza completa.

2.2 Profilo di espressione genica muscolare di cani escapers e affetti

L'mRNA totale viene estratto dalle biopsie muscolari dei due cani escapers, di quattro individui affetti e quattro wild-type. L'analisi di espressione per i geni differenzialmente espressi è condotta usando il microarray Agilent SurePrint Canine 4x44K. Su questa piattaforma le sonde oligonucleotidiche sono depositate base per base con metodo a stampante utilizzando la tecnologia SurePrint. Esse sono lunghe una sessantina di nucleotidi, in maniera da garantire un'elevata sensibilità, e la loro sequenza è disegnata al fine di essere specifica per ogni trascritto di cane noto. La tecnologia Two-Color prevede di creare un

cRNA marcato; per i campioni è usato il colorante Cy5 che emette luce rossa, mentre per il controllo costituito da mRNA di cane wild-type è usato il colorante Cy3 che emette luce verde. Inoltre si fa in parallelo un esperimento di dye-swap invertendo i colori, in modo da avere una sicurezza maggiore. L'ibridazione, attraverso il kit Gene Expression Hybridization di Agilent, è condotta overnight in una camera apposita in condizioni controllate. La temperatura è mantenuta costante, come anche la concentrazione salina. Si effettuano dei lavaggi diminuendo la concentrazione di sali per eliminare i targets legati in maniera non specifica. Lo scanner GenePix 4000B ad altissima risoluzione fa una scansione con luce monocromatica ad alta energia, ottimizzata per ottenere il massimo assorbimento. Un fotomoltiplicatore con software Feature Extraction raccoglie la luce emessa e la converte in un numero. Si fa una media tra i valori ottenuti nei due esperimenti di dye-swap. Il programma informatico SAM (Significance Analysis of Microarray) permette di individuare i geni la cui espressione è differenziale nei diversi campioni in maniera statisticamente significativa, con errore (false discovery rate) tollerato pari al 5%. I geni che rimangono sono in numero notevolmente ridotto rispetto al numero di partenza. È costruita una Heat Map che visualizza i geni differenzialmente espressi in cani escapers, wild-type e affetti, dove i risultati in numero sono convertiti in colori e i geni con profili simili sono raggruppati gerarchicamente.

2.3 Sequenziamento whole-genome dei cani escapers

Il genoma intero dei due cani escapers e di un cane affetto è sequenziato con lo strumento Illumina HiSeq 2000 con copertura 30X, cercando varianti presenti solo negli escapers localizzate sotto il picco di associazione nel cromosoma 24 e focalizzandosi nel locus *Jagged1*, incluse 3000 basi a monte e a valle del gene. La tecnologia Illumina sfrutta una cella di flusso con depositati cluster di ampliconi corrispondenti alla libreria genomica di interesse e l'aggiunta di dNTPs terminatori fluorescenti bloccati in maniera reversibile al 3'. Ogni ciclo prevede l'incorporazione del dNTP corretto, l'acquisizione della fluorescenza e lo sblocco del dNTP. L'allineamento delle sequenze è fatto sul genoma riferimento di cane CanFam3.1. Le varianti identificate nella regione in cui ci si è focalizzati sono pari a circa 1300, sotto forma di mutazioni SNPs e indels. Vengono filtrate per scartare quelle precedentemente riportate in altri studi e sovrapposte al genoma umano servendosi dell'UCSC Genome Browser. Le varianti presenti nelle regioni regolatorie del muscolo sono considerate di interesse se non ancora riportate. La regione candidata dei cani appartenenti all'intero pedigree è risequenziata mediante la tecnica Sanger dopo amplificazione con PCR.

2.4 Analisi funzionale della variante di *Jagged1*

2.4.1 Analisi dei siti di legame per fattori di trascrizione

Il metodo TRAP è applicato per analizzare in silico la variazione nucleotidica e predire le modifiche in senso quantitativo sulla forza di legame al DNA di fattori di trascrizione. Il database TRANSFAC comprende le sequenze consenso a livello di DNA specifiche per i fattori trascrizionali.

2.4.2 Saggio EMSA

Oligonucleotidi di DNA marcato con biotina e contenenti la sequenza dei cani wild-type e degli escapers sono incubati in ghiaccio per 20 minuti con estratti nucleari della linea cellulare muscolare murina C2C12. Per i due esperimenti di competizione si utilizzano sonde oligonucleotidiche con la sequenza degli escapers e dei wild-type non marcate e presenti in eccesso molare di 100 volte rispetto alle sonde marcate. Il saggio di supershift è condotto incubando per 30 minuti a temperatura ambiente con anticorpo monoclonale di topo anti-miogenina. L'anticorpo di controllo è immunoglobulina Ig anti-topo. I campioni sono separati su gel di poliacrilammide al 6% e trasferiti su membrana di nylon. La membrana è incubata con streptavidina coniugata con perossidasi di rafano (HRP). Il substrato della perossidasi permette di rilevare la chemioluminescenza.

2.4.3 Saggio della luciferasi

La regione promotore contenente la sequenza wild-type o escaper è amplificata e clonata in vettori pGL4.10, ottenendo la fusione del gene reporter a valle di essa. MyoD e MyoG umani sono clonati in vettori di espressione pIRES-2a-hrGFP. Cellule appartenenti alle linee muscolare murina C2C12 e renale umana HEK293T sono depositate in piastre da 96 pozzetti alla concentrazione di 10'000 cellule per pozzetto. La trasfezione agevolata da lipofectamine è effettuata con i vettori soli o insieme ai costrutti sovraesprimenti miogenina MyoG. Il fattore MyoD serve come controllo. Quarant'otto ore dopo le cellule sono raccolte, lisate e depositate sulla piastra per luminometro. Viene fornito il substrato per la luciferasi e acquisita la bioluminescenza. Immediatamente dopo è fornito il substrato della renilla e acquisita una seconda bioluminescenza, la quale permette di normalizzare i valori.

2.5 La sovraespressione in vivo di *Jagged1* recupera il fenotipo muscolare di *sapje*

2.5.1 Analisi del fenotipo mediante saggio di birifrangenza

Il saggio di birifrangenza si basa sull'abilità unica della struttura organizzata del sarcomero di ruotare il piano della luce polarizzata. L'esperimento è condotto servendosi di un microscopio, ponendo embrioni anestetizzati sopra un filtro di vetro polarizzatore e coprendoli con un secondo filtro. Viene applicata una luce polarizzata ed è fatto ruotare il vetrino superiore fino a che la luce non diventa visibile.

In quattro esperimenti separati sono iniettati in 200 embrioni *sapje* allo stadio di una cellula derivati da incroci tra *sapje* eterozigoti costrutti plasmidici esprimenti le isoforme di *Jagged1* presenti in zebrafish, *jagged1a* e *jagged1b*. La sovraespressione è confermata tramite western blot e il saggio di birifrangenza è condotto quattro giorni dopo la fecondazione.

2.5.2 Analisi del genotipo

Il DNA genomico estratto dagli esemplari zebrafish viene amplificato tramite PCR con primer a cavallo della regione con la mutazione *sapje*. I prodotti di PCR sono sequenziati all'interno di una struttura del Children's Hospital di Boston.

2.5.3 Immunofluorescenza

Embrioni di zebrafish wild-type, omozigoti per la mutazione in distrofina fenotipicamente affetti, e iniettati con *jagged1a* e *jagged1b* che mostrano recupero del fenotipo sono sottoposti ad immunofluorescenza quattro giorni dopo la fecondazione. Vengono fissati in paraformaldeide, deidratati in metanolo, reidratati e incubati con collagenasi. Sono utilizzati anticorpi contro la distrofina e contro la catena pesante della miosina. L'osservazione è condotta con microscopio a fluorescenza.

2.6 Espressione di *Jagged1* durante rigenerazione muscolare e proliferazione cellulare in topi e cani

2.6.1 Danno da cardiotossina

In topi wild-type maschi adulti appartenenti al ceppo C57B6/J vengono inoculati in vivo nel muscolo quadricipite destro 50µl di cardiotossina 10µM, isolata dal veleno del cobra *Naja mossambica mossambica*. Come controllo è iniettato tampone salino fosfato (PBS) a livello del muscolo quadricipite sinistro.

2.6.2 Western blot

Si analizzano i livelli di proteina *Jagged1* in biopsie muscolari dei due cani escapers, confrontandoli con i livelli presenti nel muscolo di due esemplari wild-type e due affetti. La beta-actina è utilizzata come controllo.

Le proteine sono estratte dal tessuto muscolare servendosi del tampone RIPA, che porta a lisi cellulare e solubilizzazione delle proteine. I campioni sono centrifugati, risolti in elettroforesi su gel di poliacrilammide al 20% e trasferiti su membrana. La colorazione con Ponceau serve a valutare l'efficienza del trasferimento. La membrana è incubata con anticorpo primario anti-*jagged1* e secondario coniugato a perossidasi di rafano. L'anticorpo anti-beta-actina è utilizzato come controllo di caricamento, dato che questa proteina è espressa in maniera costitutiva in tipi cellulari differenti.

2.6.3 Saggio di differenziamento muscolare

Su terreno proliferativo sono piastrate cellule della linea muscolare murina C2C12. Raggiunta un'alta percentuale di confluenza tra cellule il terreno viene cambiato con uno contenente meno nutrienti nel siero. Le proteine sono estratte dalle cellule, separate su gel di poliacrilammide e i loro livelli vengono visualizzati mediante western blot.

2.6.4 Saggio di proliferazione cellulare

Mioblasti prelevati da biopsie muscolari di cani wild-type, escapers e affetti sono depositati in piastre da 96 pozzetti alle concentrazioni di 100, 1'000 e 10'000 cellule/pozzetto. Il terreno impiegato è il proliferativo DMEM-HG (Dulbecco's Modified Eagle's Medium with High Glucose) supplementato con 20% di siero fetale bovino (FBS). La proliferazione cellulare è misurata attraverso il kit MTT Cell Proliferation Assay: alle cellule viene fornito sale di tetrazolio e solo quelle vitali metabolicamente attive sono in grado di ridurlo, facendo comparire il colore violetto, rilevato misurando l'assorbanza.

3 RISULTATI

3.1 Genotipo di cani escapers e affetti

3.1.1 Studio di associazione genome-wide

Sui 129'908 SNPs testati per verificare la loro co-segregazione con il fenotipo di interesse solo 27 cadono al di fuori dell'intervallo di confidenza del 95%, dimostrando che l'approccio EMMAX è stato in grado di correggere la struttura della popolazione comprendente esemplari strettamente imparentati.

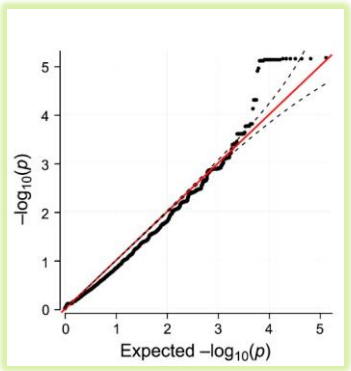

Figura 1a. QQ plot di 129'908 SNPs testati nello studio di associazione.

SNPs fortemente associati sono identificati a livello dei cromosomi 24, 33 e 37, ma solamente quelli situati sul cromosoma 24 mostrano evidenza di IBD, sovrapponendosi ad un lungo segmento IBD condiviso dai due cani escapers.

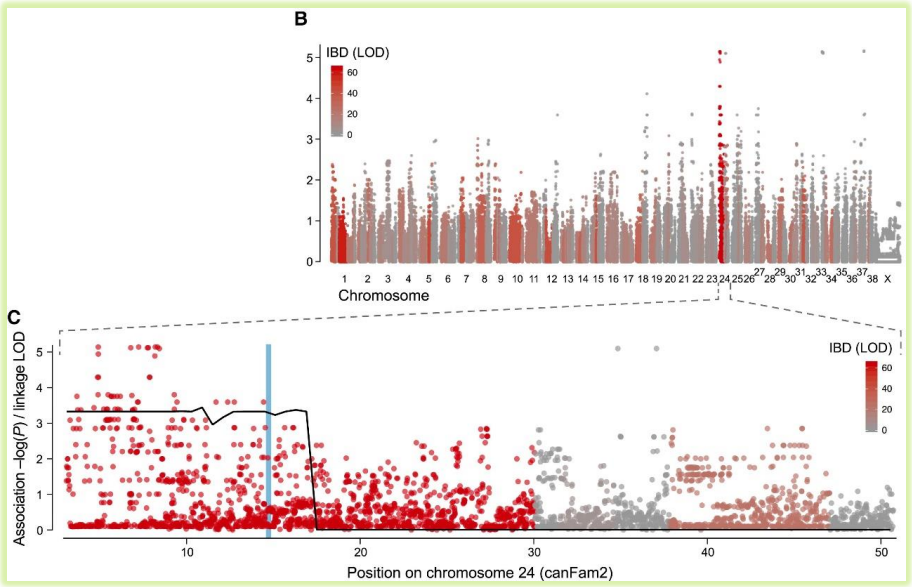

Figura 1b-c. Evidenza di IBD lungo il genoma testato. La linea nera in figura c rappresenta i valori di LOD score all'interno del cromosoma 24. In azzurro la posizione del locus Jagged1 successivamente identificato.

3.1.2 Analisi di linkage

Il picco di linkage significativo è individuato all'interno di una regione che si estende per 27 Mb a partire dall'inizio del cromosoma 24. Il LOD score massimo è pari a 3.31, valore al di sopra della soglia per accettare l'ipotesi di linkage. Questo segmento genomico che comprende approssimativamente 350 geni codificanti proteine mostrava sia IBD sia associazione con il fenotipo dei cani escapers. I dati di IBD, studio di associazione e analisi di linkage convergono tutti sulla stessa regione di 27Mb all'interno del cromosoma 24.

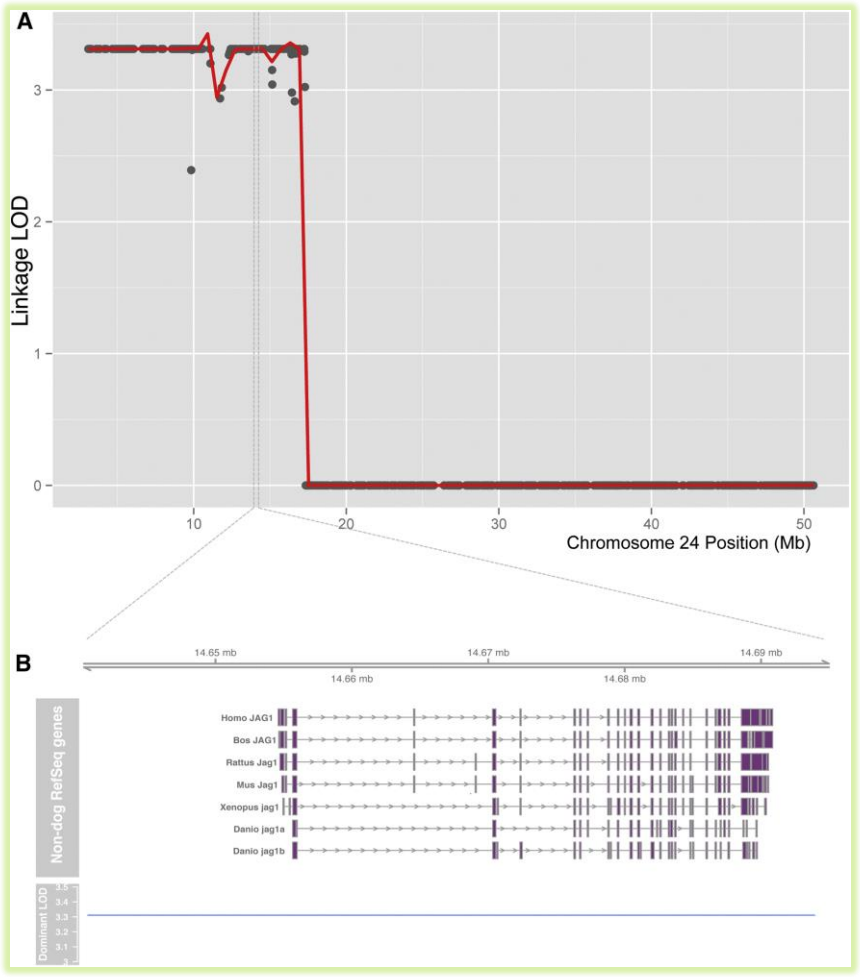

Figura S1a-b. Valori di LOD score all'interno del cromosoma 24 e ingrandimento del locus Jagged1 successivamente identificato.

3.2 Profilo di espressione genica muscolare di cani escapers e affetti

I profili di espressione dei due esemplari escapers sono molto simili tra loro e più simili ai wild-type che non agli affetti. I geni differenzialmente espressi tra escapers e affetti sono 114; di questi, 65 sono espressi in modo differenziale anche tra escapers e wild-type, implicandoli in un possibile meccanismo compensatorio presente solo negli escapers. Solo uno di questi 65 geni è situato all'interno del picco di associazione nel cromosoma 24, il gene *Jagged1*.

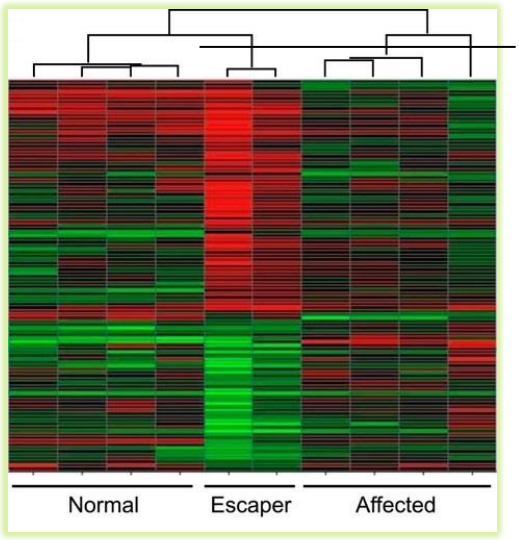

Figura 2a. Heat Map che mette a confronto l'espressione genica nel muscolo di due cani GRMD escapers, quattro wild-type e quattro affetti.

I livelli del trascritto *Jagged1* sono due volte più elevati negli individui escapers in confronto sia ai wild-type sia agli affetti.

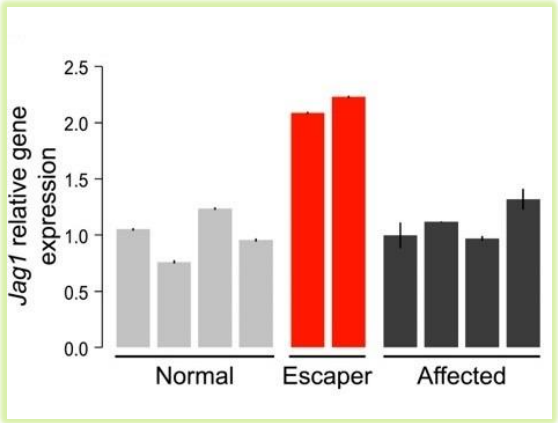

Figura 2b. Espressione genica relativa di Jagged1 in campioni muscolari di cani GRMD escapers a confronto con affetti e wild-type.

Per di più, l'analisi dei livelli di proteina conferma i risultati ottenuti esaminando i trascritti.

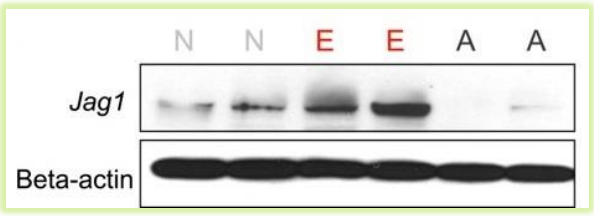

Figura 2c. Livelli di proteina Jagged1 nel muscolo di cani GRMD a confronto con affetti e wild-type.

3.3 Sequenziamento whole-genome dei cani escapers

La mutazione puntiforme G>T all'interno della regione del promotore di *Jagged1* è presente solo negli escapers. La variante è specifica del pedigree degli escapers ed è stata introdotta nell'incrocio singolo dove l'esemplare femmina B1F3 è il portatore. La variante di *Jagged1* è assente in tutti i cani affetti da distrofia, mentre entrambi gli escapers sono eterozigoti nel locus. Pertanto si può dire che la mutazione segreghi con il fenotipo dei cani escapers.

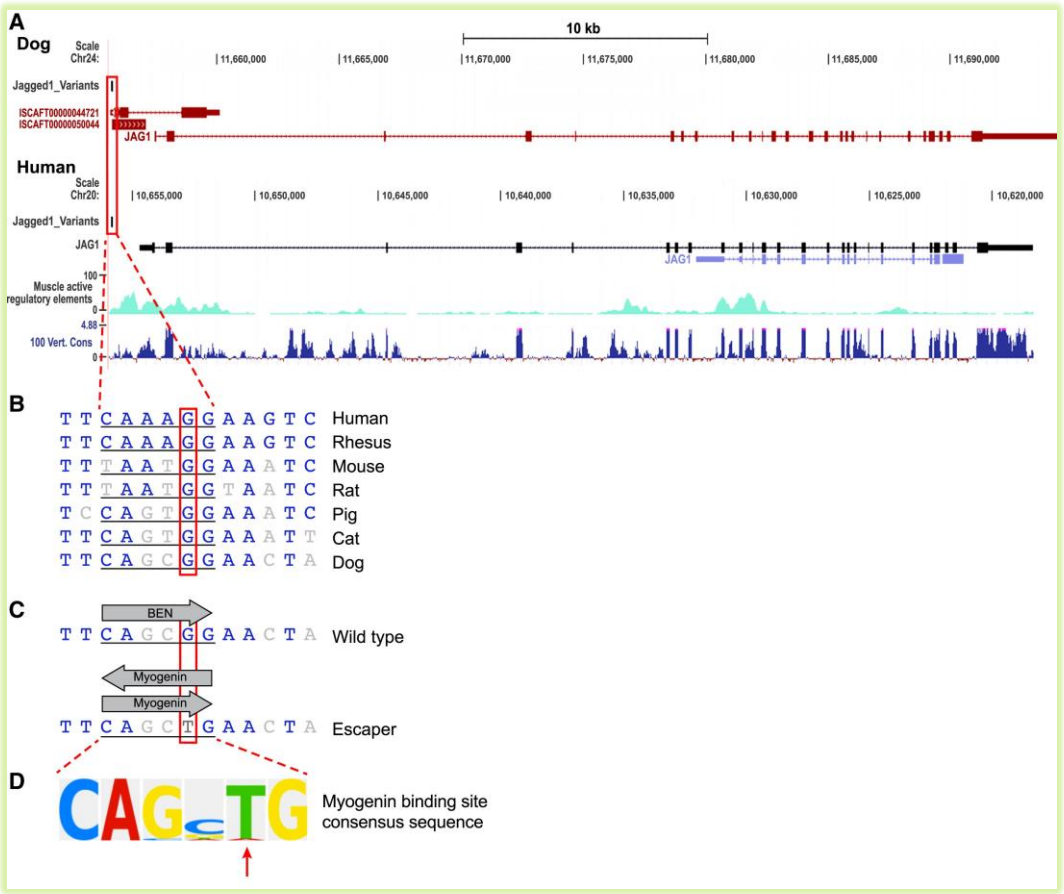

Figure 3a-b-c-d. Locus di Jagged1 in cane e uomo, che evidenzia la posizione della variante conservata tra specie.

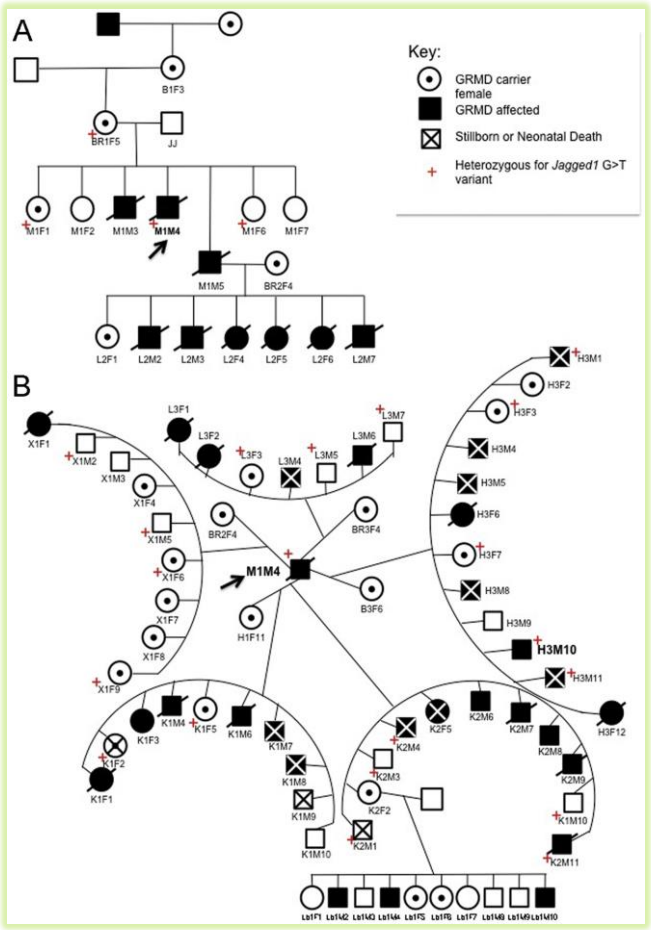

Figure S3a-b. Albero genealogico dell'escaper Ringo, M1M4. Discendenza di Ringo, tra cui Sulflair, H3M10.

3.4 Analisi funzionale della variante di *Jagged1*

3.4.1 Analisi dei siti di legame per fattori di trascrizione

L'analisi informatica rivela che la mutazione G>T crea un E-box specifico per il fattore di trascrizione miogenina, con la sequenza 5'-CANNTG-3'. La base nucleotidica timina è presente in quinta posizione dell'E-box in ben 29 mammiferi euteri, indicando che questa posizione è altamente conservata nel corso dell'evoluzione.

3.4.2 Saggio EMSA

La banda indicativa della formazione del complesso con la miogenina è presente solo nella corsia con l'oligonucleotide corrispondente alla sequenza degli escapers, pertanto la miogenina si lega in maniera robusta a questa regione. Per dimostrare la specificità del legame si fanno due esperimenti di competizione.

Se è presente sonda escaper non marcata in eccesso il segnale della banda scompare, indicando che la sonda non marcata compete efficientemente con il legame della sonda marcata. La presenza di sonda wild-type non marcata in eccesso non ha effetto sul legame della sonda escaper marcata. L'anticorpo anti-miogenina provoca supershift (rallentamento della corsa elettroforetica legandosi alla proteina legata all'oligonucleotide. Diversamente un anticorpo non specifico non causa alcun effetto.

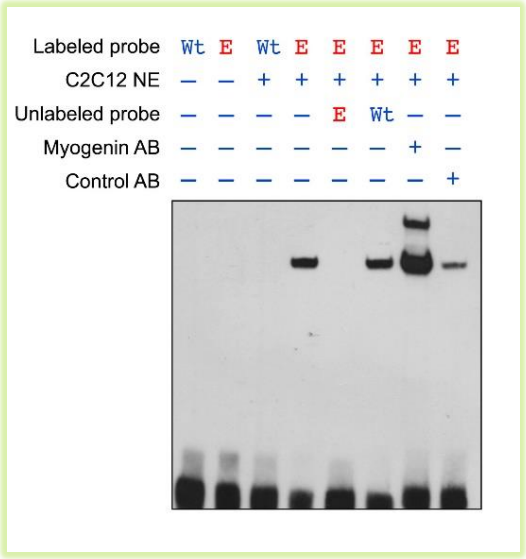

Figura 3e. Saggio EMSA che mostra come la miogenina si leghi in maniera specifica alla sequenza di DNA caratteristica dei cani escapers.

3.4.3 Saggio della luciferasi

Nelle cellule muscolari C2C12 trasfettate con la sequenza escaper si nota attivazione del reporter tre volte maggiore rispetto a quelle trasfettate con la sequenza wild-type, indipendentemente o meno dalla presenza del costrutto sovraesprimente miogenina. Difatti le cellule C2C12 esprimono intrinsecamente tale fattore di trascrizione. Nelle cellule renali HEK293T solo la sovraespressione di miogenina attiva l'espressione del reporter con a monte il promotore escaper, senza effetti se è presente il promotore wild-type, facendo rilevare livelli di luciferasi simili a quelli riscontrati nelle cellule muscolari di cui sopra. La creazione di un nuovo sito di legame per la miogenina a livello del promotore del gene *Jagged1* è quindi essenziale per guidare l'aumento dell'espressione di *Jagged1* nel muscolo scheletrico dei cani escapers.

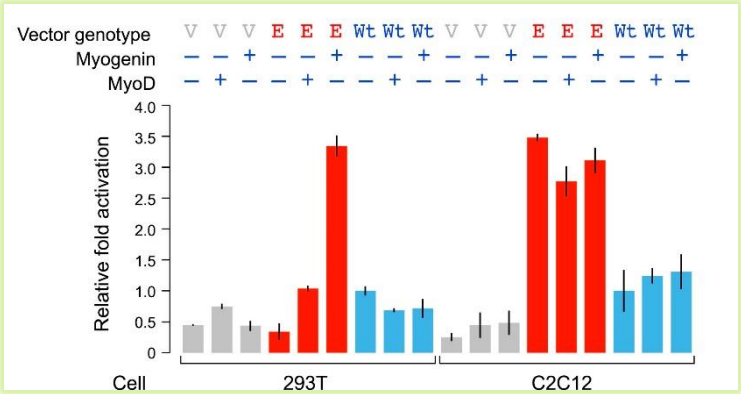

Figura 3f. Saggio della luciferasi che mostra l'attività di sequenze escaper e wild-type in cellule muscolari e non, con o senza espressione di miogenina.

3.5 La sovraespressione in vivo di *Jagged1* recupera il fenotipo muscolare di *sapje*

3.5.1 Analisi del fenotipo mediante saggio di birifrangenza

In tutti gli esperimenti una media del 24% degli esemplari non iniettati presenta il fenotipo distrofico, percentuale di omozigoti che ci si aspetta per un incrocio tra eterozigoti. Tra gli iniettati vi è una percentuale minore e statisticamente significativa di pesci con fenotipo distrofico.

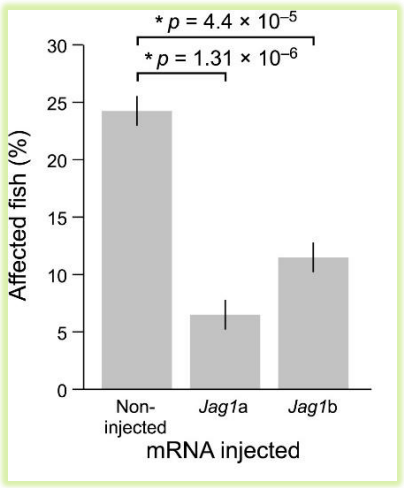

Figura 4a. Percentuale di zebrafish affetti iniettati e non secondo l'analisi del fenotipo a 4dpf.

3.5.2 Analisi del genotipo

L'analisi del genotipo rivela che una grande percentuale di zebrafish con mancanza di distrofina iniettati con le isoforme *jagged1a* e *jagged1b* (rispettivamente il 75% e il 60%) esibisce una birifrangenza normale, dimostrando il recupero del fenotipo distrofico.

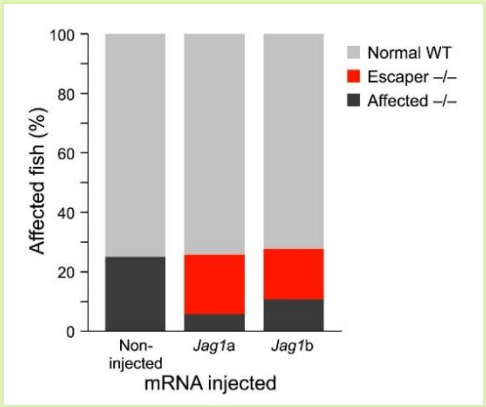

Figura 4b. Genotipo di zebrafish iniettati e non. In rosso gli esemplari in cui è avvenuto recupero del fenotipo distrofico.

3.5.3 Immunofluorescenza

Nonostante la distrofina sia presente solo negli esemplari wild-type, la struttura delle fibre è ben organizzata anche negli iniettati, mentre negli affetti vi sono evidenti anomalie.

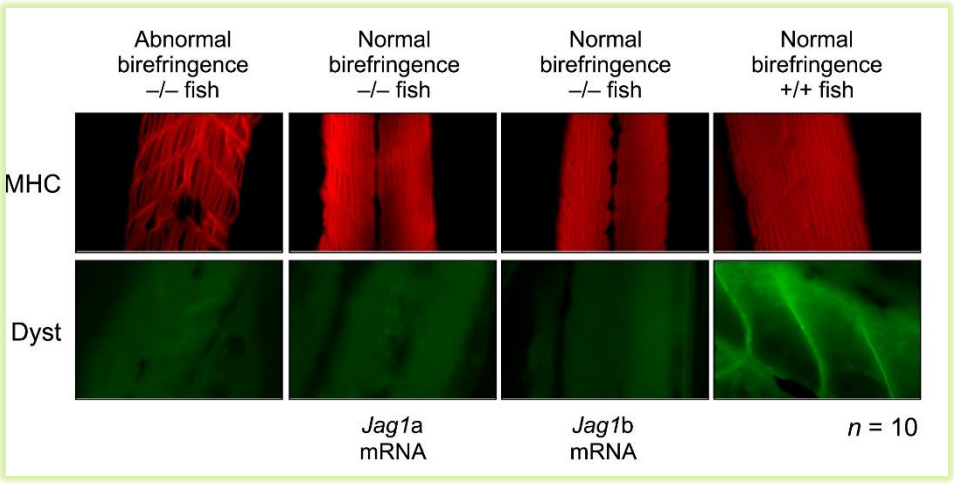

Figura 4c. Immunofluorescenza in zebrafish affetti, inettati con conseguente recupero del fenotipo e wild-type.

3.6 Espressione di *Jagged1* durante rigenerazione muscolare e proliferazione cellulare in topi e cani

3.6.1 Danno da cardiotossina

La cardiotossina è un noto agente mionecrotico e la necrosi delle miofibre è in grado di attivare le cellule satelliti per permettere la rigenerazione del tessuto. La quantità massima di *Jagged1* espressa è presente dopo quattro giorni dal danno indotto da cardiotossina.

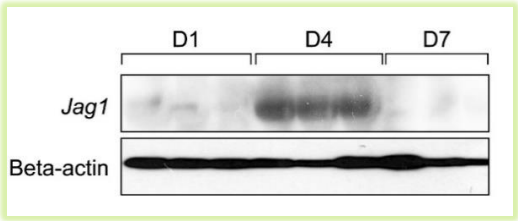

Figura 4d. Livelli di proteina Jagged1 nel muscolo di topo con danno indotto da cardiotossina, misurati 1, 4, 7 giorni dopo il danno. La beta-actina è utilizzata come controllo.

3.6.2 Saggio di differenziamento muscolare

I livelli di proteina *Jagged1* risultano rilevabili a partire dal momento in cui la confluenza è pari al 90%, aumentando durante il differenziamento con un picco al terzo giorno dopo il cambio di terreno.

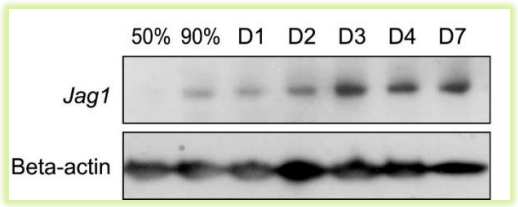

Figura 4e. Livelli di Jagged1 durante differenziamento muscolare. La beta-actina è utilizzata come controllo.

3.6.3 Saggio di proliferazione cellulare

La velocità di proliferazione delle cellule dei cani escapers è significativamente più alta di quella degli esemplari affetti, mentre non differisce significativamente da quella dei wild-type. Presumibilmente è la sovraespressione di *Jagged1* la responsabile dell'aumento della velocità proliferativa caratteristico di cellule muscolari di cani escapers.

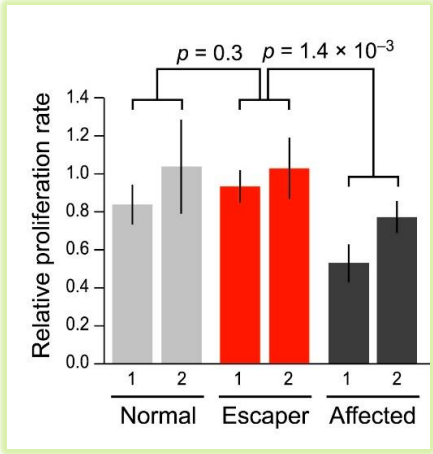

Figura 4f. Saggio di proliferazione di cellule muscolari di due cani wild-type, due escapers e due affetti.

4 DISCUSSIONE

Jagged1 è uno dei cinque ligandi di superficie cellulare che funzionano primariamente nella via di segnalazione altamente conservata Notch. Essa gioca un ruolo chiave nella determinazione del destino cellulare ed è attiva durante lo sviluppo di molti sistemi d'organo. Al momento restano ancora molti aspetti da chiarire riguardo il percorso Notch attivo a livello muscolare. L'interazione tra *Jagged1* e Notch porta ad una cascata di tagli proteolitici che culminano nella traslocazione al nucleo del dominio intracellulare di Notch (NICD), il quale interagisce con altri fattori già legati al DNA richiamando altre proteine, con effetto finale l'attivazione della trascrizione di geni bersaglio a valle. Il locus *Jagged1* nel genoma umano è localizzato a livello del braccio corto del cromosoma 20, in posizione 20p12.3. Il trascritto composto di 26 esoni e lungo 36kb produce una proteina di 1218 amminoacidi. Essa possiede un piccolo dominio intracellulare, un dominio transmembrana e una più grande componente extracellulare, responsabile del legame ai recettori Notch. Mutazioni nel locus *Jagged1* sono associate a diverse patologie, compresa la sindrome di Alagille, alcuni casi di tetralogia di Fallot, oltre a svariati tipi di cancro. Nei mammiferi *Jagged1* è ampiamente espresso durante tutte le fasi dello sviluppo, in molti tessuti e sistemi. A livello muscolare un'iniziale aumento della segnalazione Notch è cruciale per l'attivazione e la proliferazione delle cellule satelliti negli stadi precoci della rigenerazione del tessuto. Tuttavia se la via Notch rimane costitutivamente attiva viene ritardata l'espressione dei fattori MyoD e miogenina, quindi il segnale necessita di spegnimento, in modo da permettere l'uscita dal ciclo cellulare, l'espressione di MRFs e il differenziamento in senso miogenico. Il responsabile è l'antagonista di Notch chiamato Numb, il quale interagisce con la porzione intracellulare del recettore prevenendo la sua traslocazione nucleare. Numb è localizzato asimmetricamente nelle cellule satelliti in mitosi, dando origine a due cellule figlie con caratteri eterogenei: una è destinata a differenziarsi in mioblasto e successivamente in miotubo e miofibra, mentre l'altra permette il mantenimento del pool di cellule a carattere staminale. L'autorinnovamento di cellule satelliti attivate è reso possibile in particolare dalla sovraespressione di Pax7, fattore trascrizionale importante per mantenere lo stato indifferenziato di queste cellule, indotta dal dominio intracellulare di Notch. Le cellule satelliti del muscolo distrofico non sono in grado di mantenere un corretto equilibrio tra proliferazione, autorinnovamento e differenziamento, perdendo in particolare la capacità di autorinnovarsi e quindi di sostenere i cicli di degenerazione e rigenerazione a cui va incontro il muscolo distrofico. La via di segnalazione Notch regola questi eventi cellulari e potrebbe avere potenziale terapeutico per pazienti umani colpiti da distrofia muscolare. È comunque necessaria una regolazione dinamica di Notch per

bilanciare autorinnovamento e differenziamento di cellule satelliti, in modo da portare benefici ai muscoli distrofici.

La sovraespressione di *Jagged1* in cani GRMD escapers è responsabile di un fenotipo distrofico attenuato, il quale è possibilmente dovuto ad aumentata proliferazione delle cellule satelliti. *Jagged1* agisce pertanto come mediatore del processo rigenerativo che nei muscoli distrofici risulta alterato. Sequenziando i genomi di pazienti umani affetti da distrofia muscolare di Duchenne si potrebbe verificare la presenza di varianti all'interno dei loci genici di *Jagged1* o di altri fattori coinvolti nella via Notch. Mutazioni in queste regioni potrebbero essere implicate nel modulare il fenotipo della patologia, rendendolo più o meno severo, similmente a quanto osservato in cani GRMD escapers. Si può pensare dunque di trasferire le conoscenze acquisite nei cani ai pazienti umani distrofici. Allo scopo di ripristinare la segnalazione Notch sregolata non è conveniente la diretta iniezione di *Jagged1* esogeno in forma solubile con vettori adeno-associati, poiché questa proteina interferisce con il legame tra Notch e il suo ligando, inibendo la trasduzione del segnale a valle. L'alternativa può essere rappresentata da una piccola molecola o un fattore di trascrizione che aumenti l'espressione di *Jagged1* nel muscolo scheletrico distrofico. Un trattamento ottimale della patologia potrebbe combinare approcci terapeutici diversi finalizzati ad un unico obiettivo: ridurre i sintomi della distrofia muscolare e portare beneficio alla vita dei pazienti affetti.

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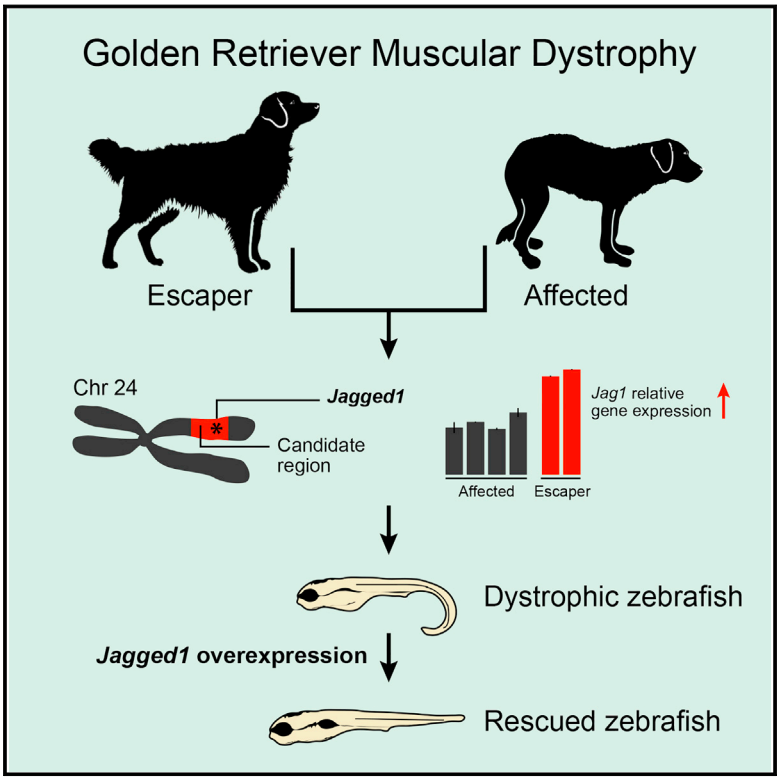
APPENDICE

Article

Cell

Jagged 1 Rescues the Duchenne Muscular Dystrophy Phenotype

Graphical Abstract



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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.

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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Cell

Article

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,

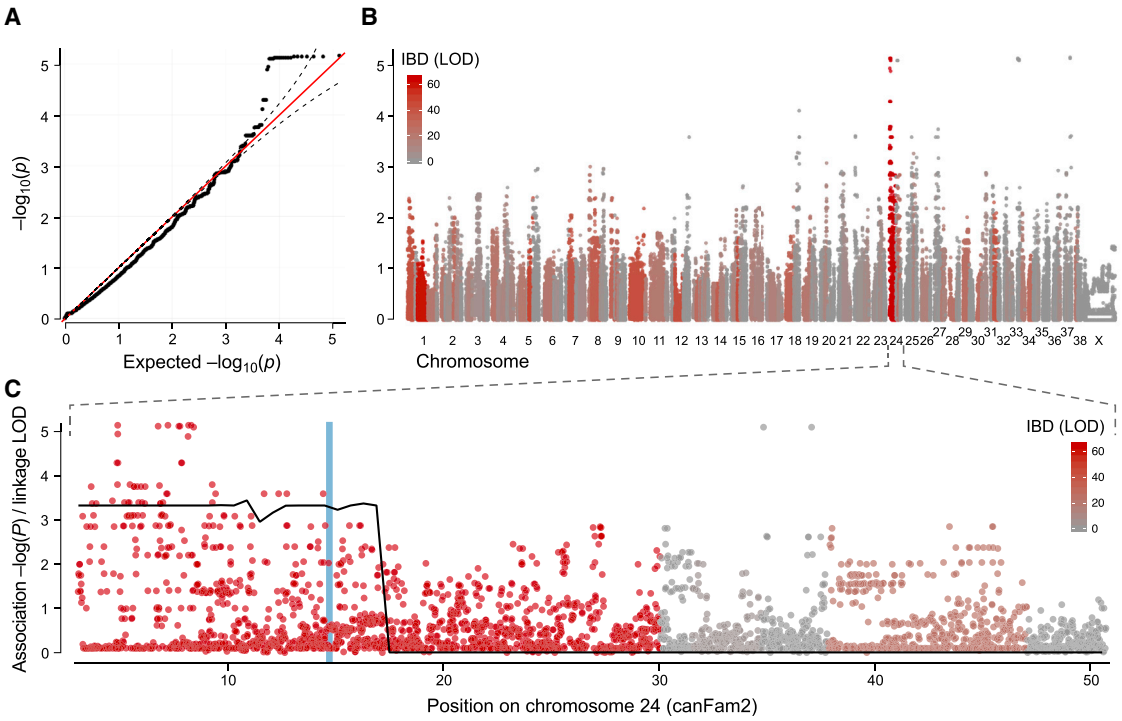

Figure 1. Combining Association, Linkage, and Identity-By-Descent Analysis Identifies a 30 Mb Candidate Region on Chromosome 24

(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 2 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 (gray) and are most likely false positives due to the small sample size. (C) The mapped region extends 27 Mb 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 and association peak that includes the putative driver gene *jagged1* (blue line) identified through gene expression profiling. See also Figures S1 and S2.

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, *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 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 ~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 *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) (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 *Jagged1* variant, while both escapers were heterozygous. Thus, the novel *Jagged1* mutation segregates with the escaper phenotype in this family. Four additional individuals

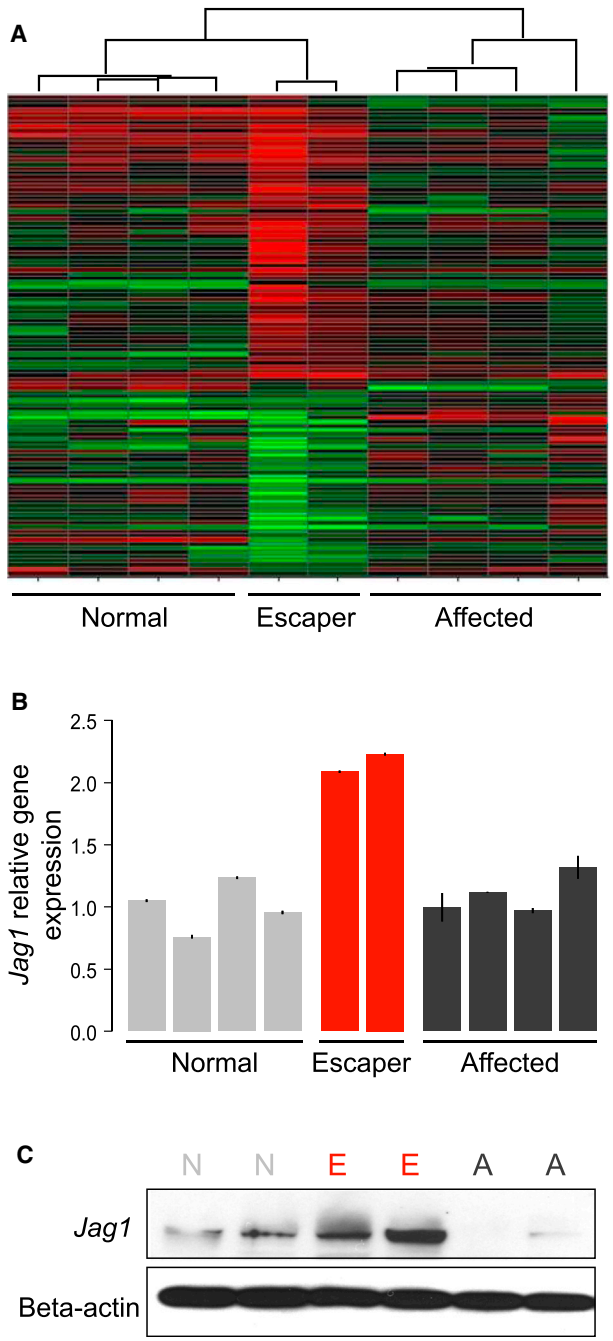

Figure 2. Altered *Jagged1* Expression in Escaper GRMD Dogs

(A) mRNA microarray comparing muscle gene expression of escaper GRMD dogs with related severely affected and WT dogs.

(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 WT dogs; bars indicate SD from the mean.

(C) *Jagged1* protein levels in the muscle of escaper GRMD dogs (E) as compared to severely affected (A) and WT dog muscle (N); Beta-actin is the loading control. See also Table S1.

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.

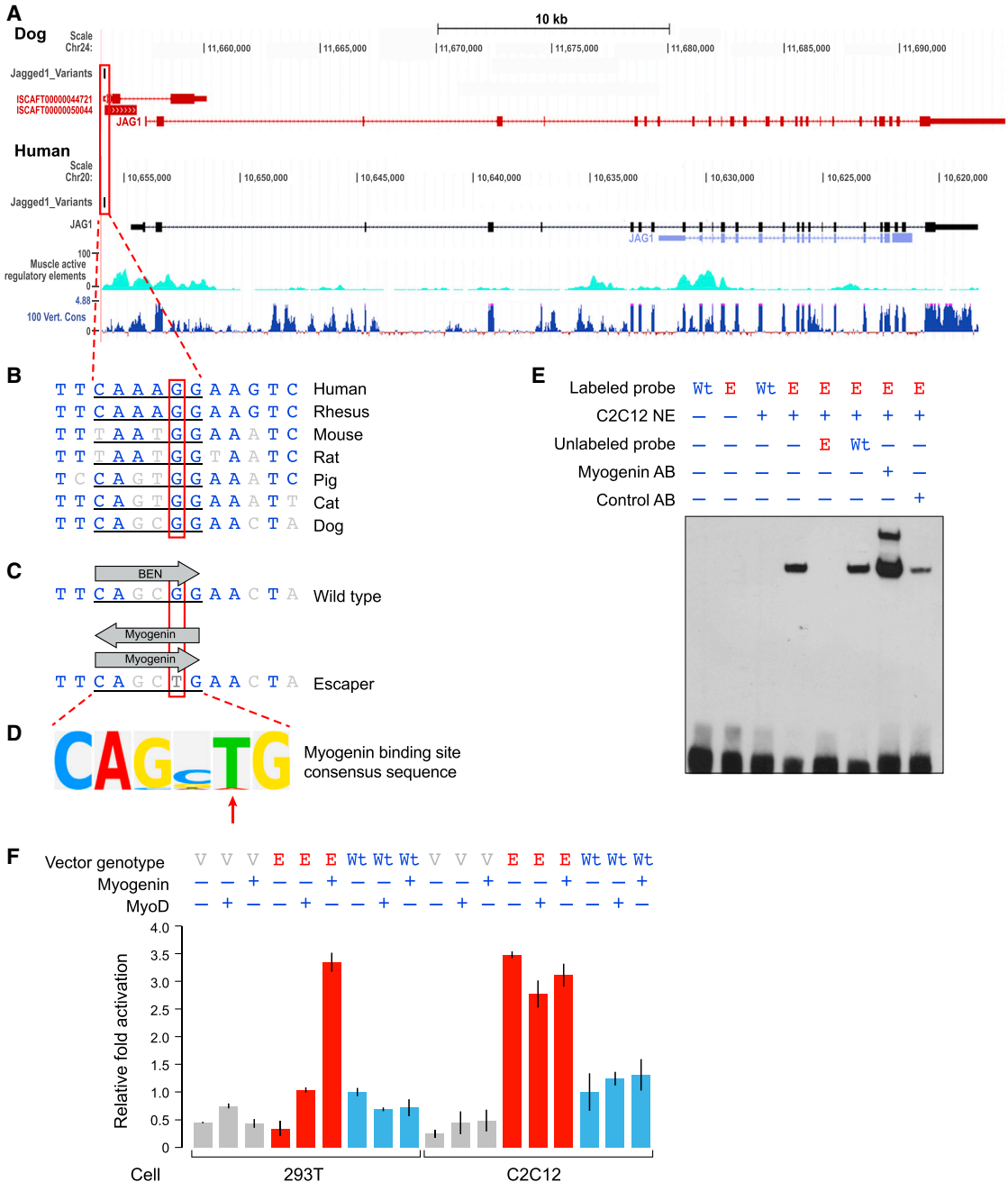

Figure 3. Variant Located in the *Jagged1* Promoter of Escaper GRMD Dogs

(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 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

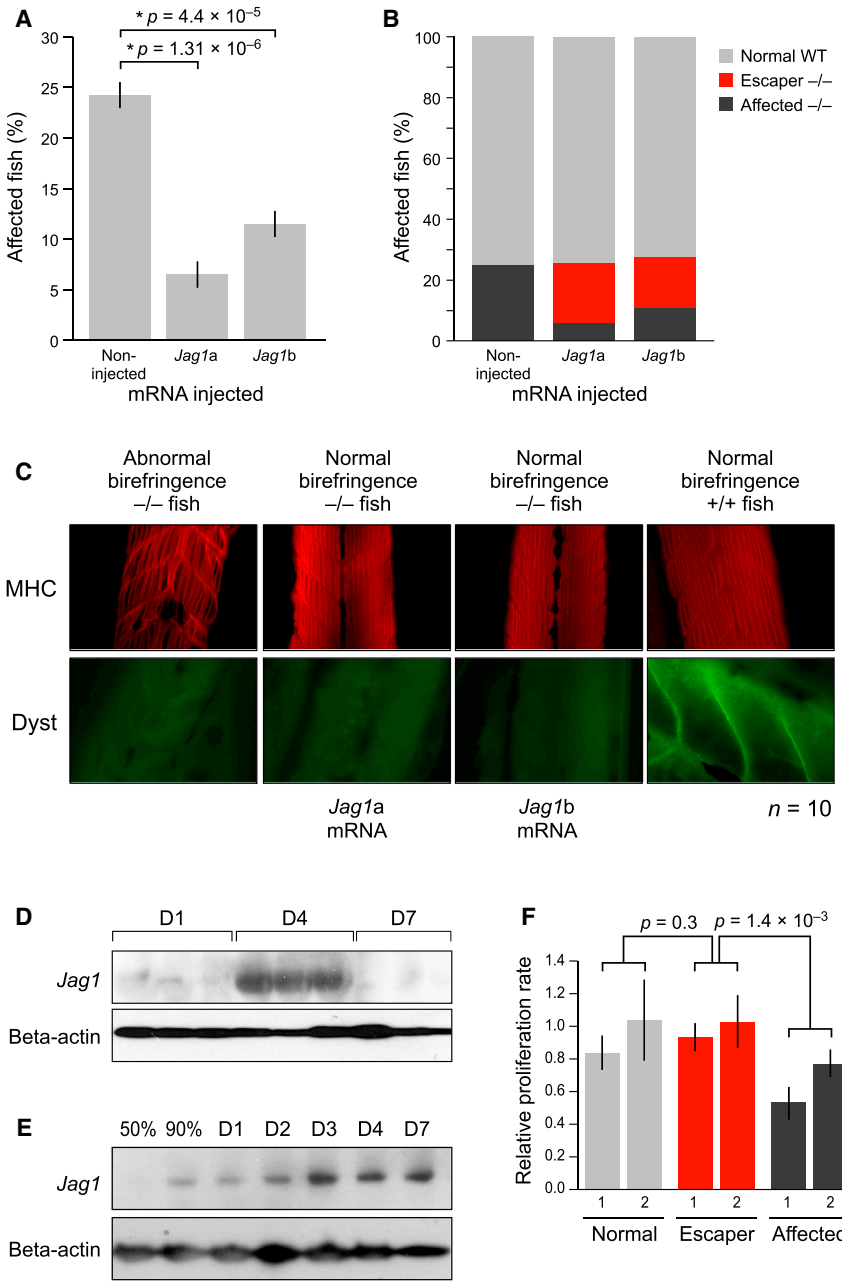

Figure 4. Functional Analysis of *jagged1* Expression

(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 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).

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).

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 ($p = 1.31 \times 10^{-6}$ for *jagged1a*, $p = 4.4 \times 10^{-5}$ for *jagged1b*, Figure 4A). Genotypic analysis revealed that about 75% of dystrophin-null fish injected with *jagged1a* and 60% of of those injected with *jagged1b* had normal birefringence, which demonstrated a common rescue

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

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 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 *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 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 *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.

EXPERIMENTAL 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 escaper 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.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 WT, 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 30x depth of three dogs (two escapers and one affected dog). Samples were sequenced on an Illumina HiSeq 2000, and sequencing reads were aligned to the CanFam 3.1 reference sequence using BWA. Following GATK base quality score recalibration, indel realignment, and 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 WT 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,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 from either one of the zebrafish *jagged1* gene copies: *jagged1a* or *jagged1b*. Zebrafish injected with either mRNA or 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 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: GSE69040.

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

N.M.V, I.E., M.S.A., Y.B.M., S.V.-A., L.M.K., K.L.T., and M.Z. designed the study. N.M.V., Y.B.M., M.S.A., and J.L.M. performed experiments. N.M.V., I.E., Y.B.M., E.K.K., A.E., and K.L.T. performed data analysis and interpretation. N.M.V., I.E., K.L.T., L.M.K, and M.Z. wrote the paper with input from the other authors.

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Cell

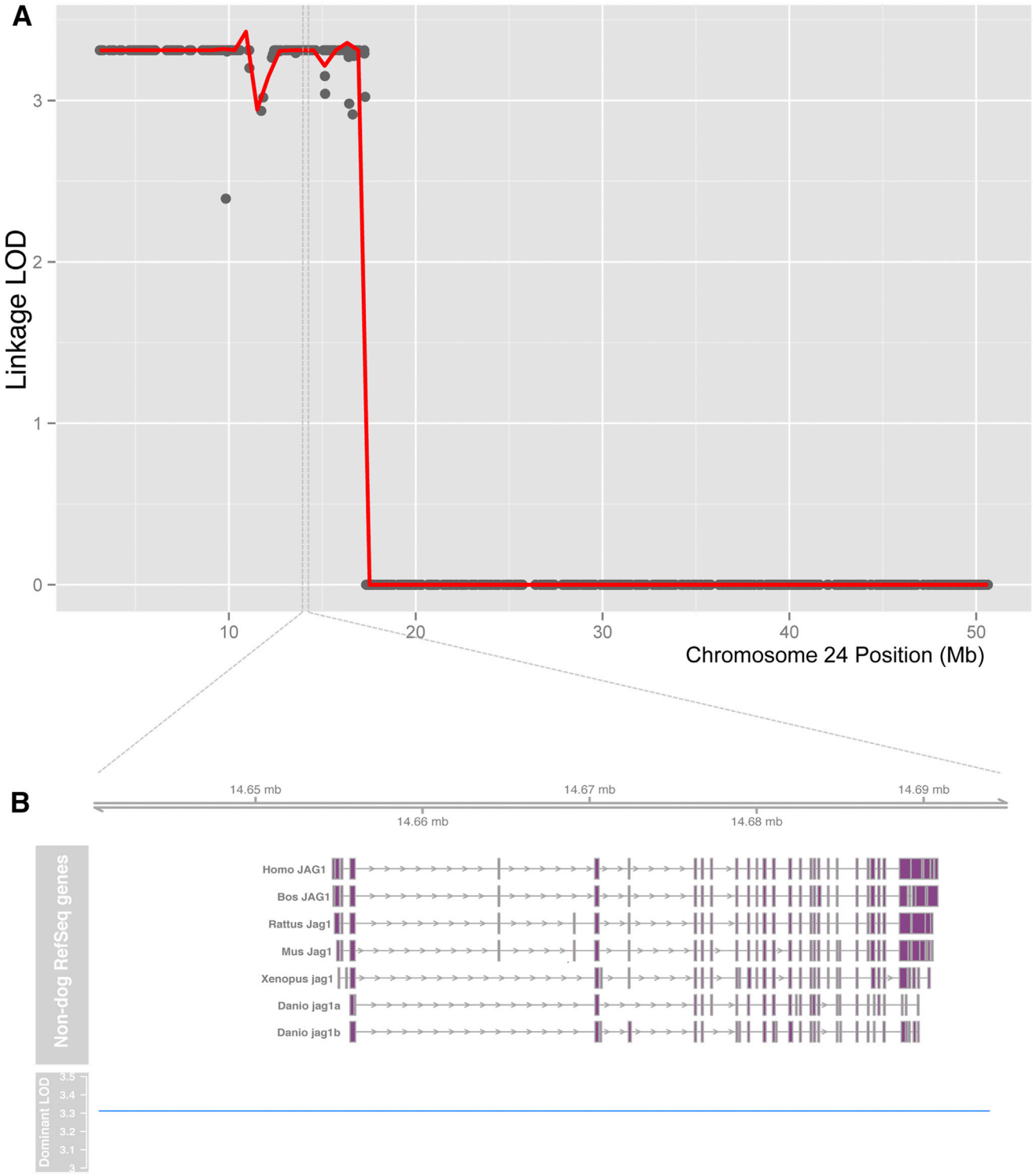

Figure S1. Chromosome 24 Linkage Results, Related to Figure 1

(A) Linkage analysis of the escapers' pedigree identified a 27Mb 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.

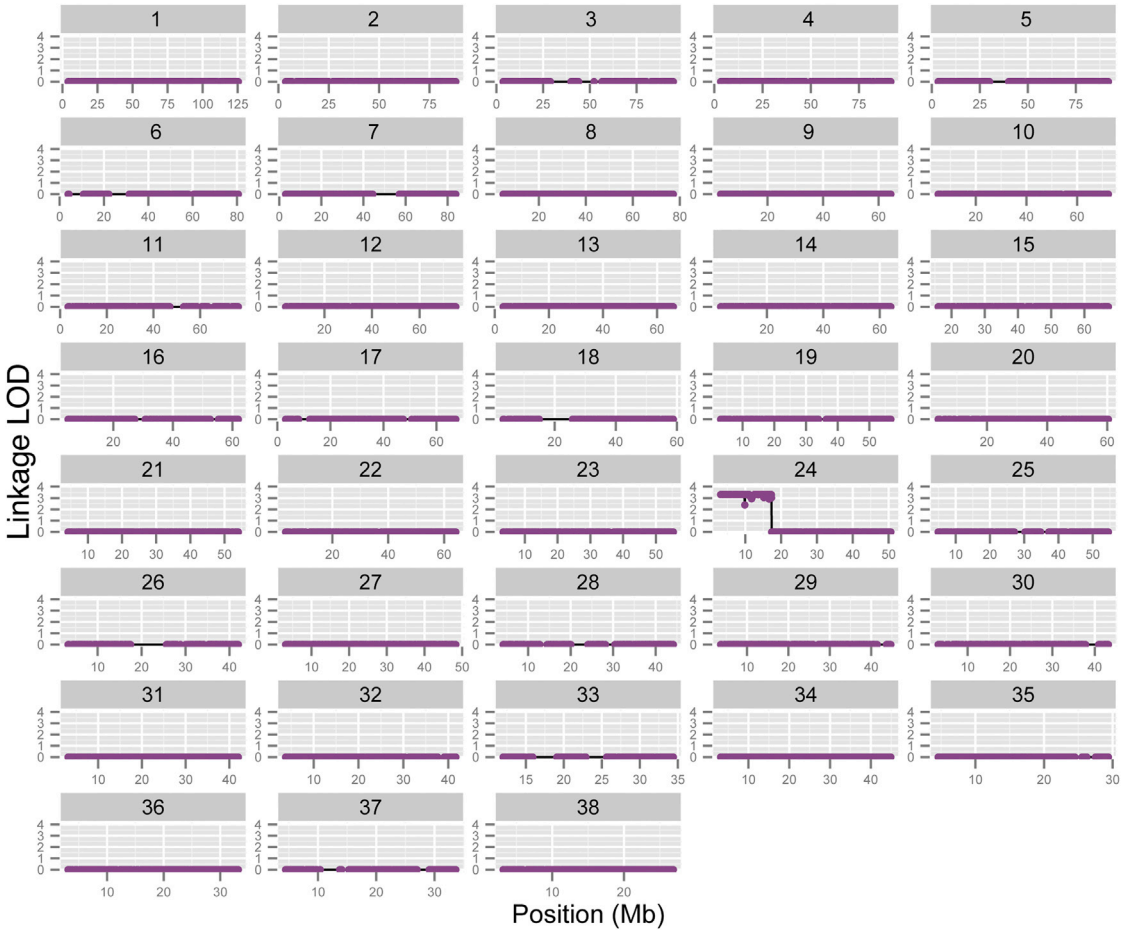

Figure S2. Genome-wide Linkage Analysis Results, Related to Figure 1

A single linkage peak was identified in the escapers' pedigree, mapping to chromosome cfa24:3,073,196-30,066,497 (CanFam2).

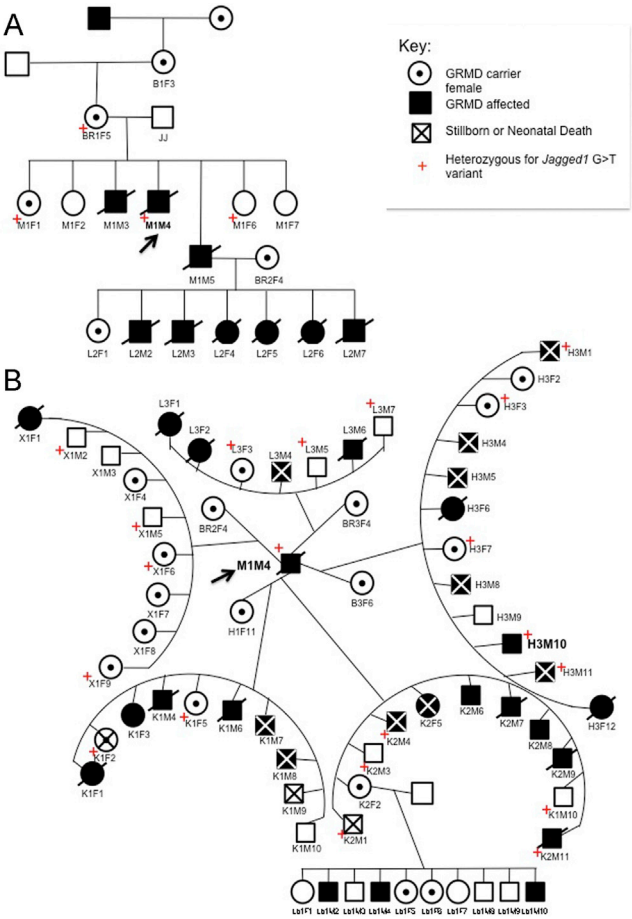

Figure S3. GRMD Dogs' Pedigree and Genotype, Related to Figure 3

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.

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Supplemental Information

Jagged 1 Rescues the Duchenne Muscular Dystrophy Phenotype

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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'-ACCCAACCTTTTCTGCACTC-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: CTCCTTTATTTCAGCGGAACTAAAGAAGTCTC and for the variant CTCCTTTATTTCAGCTGAACTAAAGAAGTCTC. 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^OC for 1 hour at 380 mA. Membrane crosslink was performed at 120mJ/cm^2. 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′-AGCCAGCTGAACCAATTAACTCAC-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 Not1 restriction digestion. mRNA was synthesized with SP6 message machine kit (AMBION) 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^{O}C and 5% CO_2 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.

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