ANALYZING THE EFFECTS OF TEMPORAL ACTIVATION OF JAGGED1-NOTCH IN DYSTROPHIC MUSCLE MYOGENESIS USING ZEBRAFISH (SAPJE) DMD MODEL

Synopsis of Ph.D. Thesis

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INTRODUCTION

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a severe, progressive, muscle degenerative disease that affects 1 in 3600-6000 live male births (Bushby *et al.,* 2010), for which, mutations in the Xp21DMD gene encoding dystrophin is the primary cause. The absence of dystrophin primarily affects skeletal muscle, heart and in some cases cognitive development of patients. The clinical symptoms of limb weakness, joint contractures, difficulty running, climbing, use of arms to get up from the floor, pseudohypertrophy etc. become visible by 4-5 years of age. The patients progressively become dependent on wheelchair assistance by ~10-12 years. The cardiac weakness becomes apparent in patients around 18-20 years, along with kyphoscoliosis (stiffness and curvature of the spine) due to loss of posture muscles. The advances in disease management practices and steroids have delayed disease progression and improved survival well into 3rd decade for patients. Though several factors are associated, respiratory failure was considered the leading cause of death before the invention of ventilators, now it is heart failure.

DMD locus is one of the largest, with 79 exons that extend 2.4 million bp encoding 21 kbp long pre-mRNA transcript (Monaco *et al.,* 1986; Hoffman *et al.,* 1987; Tennyson *et al.,* 1995), that is spliced at a developmental stage and in tissue specific manner (Doorenweerd *et al.,* 2017) but the absence of only full-length dystrophin is associated with DMD. Several internal promoters generate smaller dystrophin isoforms in various stages, functions of which are active areas of research. The full-length dystrophin mRNA is transcribed by three separate promoters with slightly different N-terminal, but same sized protein isoforms called Dp427. Two promoters active in the brain produce Dp427p and Dp427b, whereas a single promoter produces Dp427m expressed in skeletal muscle and heart.

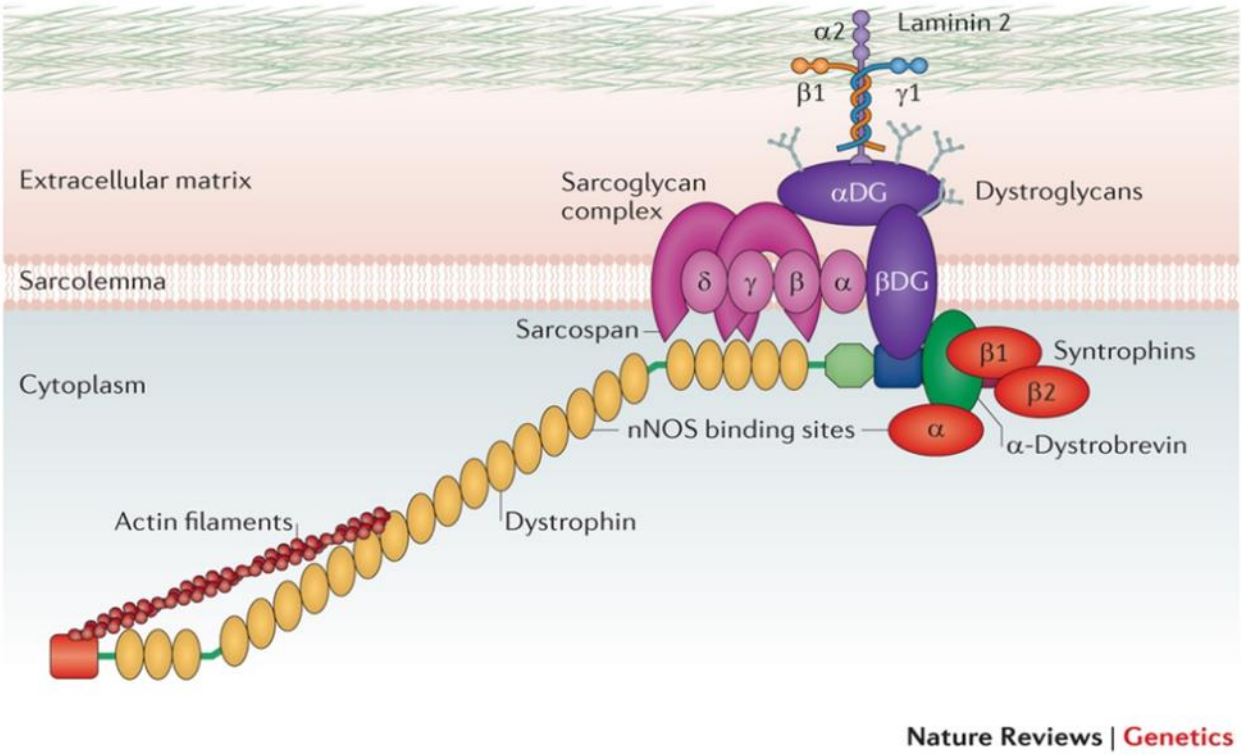

Fig 1: structure of Dystrophin and associated proteins.

In muscles, Dystrophin protein extends from sarcolemma associated Dystroglycan Complex (DGC) to the interior actin bundles, which provide mechanical support. It also acts as a scaffold for DAPC formation; hence, its loss leads to reduced DAPC formation (Ervasti *et al.,* 1990) and membrane fragility leading to muscle cell death in response to mechanical stress during contraction. A multimeric complex DGC also called DAPC (Dystrophin-associated Protein Complex) is involved in several signaling and scaffold functions (Ervasti, 2007). Mutations in other proteins of DAPC result in various forms of muscular dystrophies studied in mouse models. The Dp427 binds to several cytoskeletal, scaffolding, and signalling proteins in addition to membrane lipids, hence its absence deregulates several aspects of ECC, described best in skeletal muscles though it affects the heart and sometimes the brain too.

Several pathological processes are active in dystrophic muscles, yet there are no clear causal relationships between these. As a result, there are several contending theories with respect to the origin of pathology because of absence of dystrophin.

According to Rando (2001), the two hits model – the loss of Dystroglycan Complex (DGC) signal reduced NOS activity, which results in local ischemia and susceptibility to oxidative stress that ultimately leads to muscle loss and increased activity of mechanical stretch receptive calcium channels mediated X-ROS pathway – where microtubules serve as

mechanotransducers to activate NADPH oxidase2 (in complex called NOC) in the muscle cell membranes to produce ROS (Prosser *et al.,* 2012). The contrary studies suggest that stretch activable NOC stimulated by muscle contraction produces excess ROS that disrupts microtubule dynamics (Loehr *et al.,* 2018) in DMD. The excess ROS can lengthen calcium ingress and damage metabolic enzymes.

Another contributor to pathology is excess calcium and calcium activated proteases (Duncan, 1978; Bodensteiner *et al.,* 1978). Calcium is an important mediator of excitation-contraction coupling where neuronal stimulation opens ion channels causing extracellular calcium (ER) ingress that activates calcium induced calcium release from the endoplasmic reticulum via Ryr channels (ryanodine receptive – Ryr channels). To fine tune this process, several other types of calcium channels function together – that function aberrantly in DMD (reviewed by Gailly, 2012; Ohlendieck, 2000). Yet, therapeutic targeting of these channels has consistently failed, suggesting the failure of calcium reabsorption (reviewed by Duan *et al.,* 2021). The calcium reabsorption by calcium/ATPase channels located on ER called SERCA require energy from ATP. Muscle stores some energy in the form of phosphor-creatine (PCr) to regenerate ATP by Creatine Kinase (CK) which can carry out reversible phosphorylation between Cr and ADP. The CK activity and PCr levels remain low in DMD.

In addition to cytoplasmic ROS, the persistently high calcium reduces mitochondrial ATP production capacity (Rabylka *et al.,* 2014). The glycolysis derived pyruvate is low, which probably induces the pentose phosphate pathway that is used for supplying mitochondria with pyruvate (Ellis, 1980). It increases amino acid breakdown to provide mitochondrial TCA, resulting in low amino acids for protein synthesis and increased proteolysis thus, shifting the balance towards atrophic condition in DMD (Kumamoto *et al.,* 2000).

Part of the mystery is the metabolic dysfunction in DMD muscles, due to which it was considered a metabolic disease for a long time. From the first description of the disease (Meryon, 1852; Duchenne, 1861), mitochondrial failure, a 50% reduction in ATP levels, reduced glycolysis, increased fatty accumulation and abnormal glycogen aggregates in cytoplasm have been observed (Timpani *et al.,* 2015) in which mechanical role of dystrophin cannot be explained.

Dumont and co-workers (2015) found that Dystrophin interacts with Par1b/SerTherKinaseMark2 protein as a boundary element during asymmetric division, forming two stem cells and one that differentiates into muscle cell. Defect in this process can

explain exhaustion of myogenic potential (Sacco *et al.,* 2010), low multiplicity of myoblasts (Webster and Blau, 1990) small weak muscle fibers (Blau *et al.,* 1983) and increased fibrosis (D'Amore *et al.,* 1994), observed in patients and animal models.

Most advanced viral vector based mini dystrophin gene therapy and genome editing has been obstructed due to off target effects and immune response generated for both vector (Abdul-Razak *et al.,* 2016) and transgene (Mendell *et al.,* 2010) at clinically effective doses. Modified oligonucleotides with antisense sequence to mutated exon leads to skipping of the exon during splicing and translation of shorter Dystrophin mRNA from patients own genome. This method called AO mediated exon skipping, does not seem to evoke immune response but has low efficiency (Aartsma-Rus and Krieg, 2017) in terms of levels of dystrophin expressed and percent of fibers expressing dystrophin. Hence, it requires further refinement before it can show signs of clinical benefit. In addition to cost, other limitations of gene therapy are small percentage of patients it can be useful for. For example, the exon skipping, or nonsense read through therapies cannot be used for patients with exon deletions. Hence, therapy that can improve patient condition irrespective of type of genetic aberration that leads to dystrophin absence is still needed.

The disease modifiers are genetic changes outside/other than disease causing genes that can still change the outcome of the disease. Several such disease modifiers are identified for DMD (Reviewed by Quattrocelli *et al.,* 2017). The mutation in osteopontin encoding gene SPP1 was shown to increase fibrosis and interfere with glucocorticoid modulation increasing disease severity (Vianello *et al.,* 2017). The increase in another modifier TGFBP encoded by LTBP4 gene reduces TGF-β induced pathology in dystrophic muscles that delay disease progression by 1-2 years (Flanigan *et al.,* 2013). However, none of these modifiers showed as dramatic rescue as seen with Jag1 (Vieira *et al.,* 2015) in a severe Golden Retriever mode of DMD called GRMD. The loss of ambulation and early death around ~1-1.5 years in GRMD pups are shown by mouse model (mdx) of DMD. The single mutation created myogenin binding site in Jag1 gene which increased its expression only in muscles. The Jag1 acts as a ligand for evolutionarily conserved and developmentally important Notch receptors.

Notch Pathway

The notch receptor pathway is highly conserved in multicellular animals. In mammals, five ligands DLL1, 3, 4 and Jagged 1, 2 can activate 4 receptors viz., Notch 1, 2, 3, 4. It is cell - cell

contact dependent pathway where a cell expressing the ligand can activate notch receptors on the adjacent cell to induce events like deciding binary cell fate and boundary formation during early embryonic development. Once the pathway is activated, Notch Intracellular Domain is released into the cell. In nucleus, it activates transcription of target genes by forming a complex with CSL (an acronym for CBF-1/RBPJ-κ in *Homo sapiens/Mus musculus* respectively, Suppressor of Hairless in *Drosophila melanogaster*, Lag-1 in *Caenorhabditis elegans*) that recruits MAML (Mastermind-Like) and CBP/p300. Well-known targets of Notch include HEY and HES family of transcriptional repressors.

It is involved in Inner cell mass survival, neurulation, vascularization, smooth muscle development, and skeletal regeneration. Notch signaling has been implicated in various tissue-specific processes like maintenance and self-renewal of stem cells (Chiba, 2006), apoptosis or ablation and tumor formation or suppression (Craig and Radtke, 2017). This wide array of activities cannot be fully explained by non-redundant, combinatorial nature of all the pathway components and multiple layers of regulation.

In skeletal muscles, function of Notch is context dependent, too. In satellite cells notch signaling helps in the maintenance of a quiescent state primarily by upregulating proteins that bind ECM, the inhibitors of Metalloproteases (Philippos *et al.*, 2012). These metalloproteases digest ECM in case of injury. In activated satellite cells it blocks differentiation and promotes self-renewal. If Notch activity is blocked experimentally, satellite cells lose the ability to stay quiescent (Bjornson *et al.*, 2012), which in the long term leads to the depletion of the SC pool (Schuster-Gossler *et al.*, 2007). Both a high number of activated satellite cells and the downregulation of notch receptors and ligands have been observed in DMD patients (Church *et al.*, 2014).

Activating this pathway experimentally by co-culturing ligand and receptor expressing populations, using antibodies against receptor, injecting NICD, or overexpressing NICD by viral constructs gave ambiguous results- improving regeneration (Conboy and Rando, 2002) in some cases and causing poor regeneration (Church *et al.*, 2014) in others, in both, in vivo and in vitro models. On the other hand, blocking Notch activity stimulates premature differentiation, resulting in small and fragile muscles (Schuster-Gossler *et al.*, 2007).

It was found that Notch blocks MyoD and Myf5 expression along with MyoD activity, indirectly by occupying Myogenin and Mef2C regulatory regions and suppressing their expression (Buas *et al.*, 2010). Its target genes Hes1 and MyoR act in blocking myogenic

differentiation, but their knockdown does not affect delaying muscle degeneration (Buas *et al.*, 2009). The delay of myogenesis is also independent of CSL/RBPJκ (Shawber *et al.*, 1996), suggesting non - transcriptional function of the Notch pathway. Despite these observations, Notch mediated delay seems important for stable muscle formation. Clearly, this pathway plays multiple roles through various mechanisms, details of which can be important avenues for treatment for DMD.

Direct evidence comes from research (Vieira *et al.*, 2015) on the Golden Retriever model of Dystrophy (GRMD). The GRMD with this mutation were termed "Escapers" as they lived their whole life without loss of ambulation, albeit with slight muscle weakness. It was found that point mutation created a consensus binding site for Myogenin in the promoter region of Jagged1. This resulted in increased expression of Jagged1 in muscles as Myogenin is a muscle specific transcription factor. Further injections of Jagged1 mRNA in single cell embryos of DMD zebrafish -sapje reduced mortality by 70%. This is the only rescue of DMD reported in such a severe model till now. Though a reduction in dystrophic larval lethality was shown, the rescue mechanism remains unknown due to the complexity of Notch receptor signaling in muscles. The pathological processes mitigated by Jag1 overexpression also remain anonymous.

Zebrafish Model

The dystrophin null mdx mouse does not show the severity of DMD and resulting mortality, as seen in patients. This mild phenotype is attributed to long telomeres, overexpression of Utrophin (embryonic ortholog of dystrophin), small body size and protection from expansive contractions due to the posture and shape of the animal. It is also observed that number of revertant fibers expressing shorter forms of dystrophin increase with age causing greater regeneration capacity and contributing to almost normal ambulation and life span in mdx mouse (Partridge, 2013). Although, severity increases in the double knockout model, lacking both - dystrophin and Utrophin, other species-specific benefits remain. GRMD is similar in severity and mortality to human patients, but it is a more time consuming and costly model to work with, due to its longer life span.

Zebrafish generate many offspring quickly, show fast ex- utero development and the optical transparency of embryos and larvae offers easy genetic manipulations. The Zebrafish embryos absorb drugs readily, proving it to be a better model organism for studying human diseases. Entire genome detail of the Zebrafish is available for various genetic studies. It is considered a

better model for studying muscular dystrophy (Gibbs *et al.,* 2013) as all orthologues of human muscular dystrophy genes and components of the Human DGC are present. Sapje (Granato *et al.,* 1996) is a severe model of DMD, with larvae surviving only for a month.

Notch signaling pathway components are found to function similarly in muscle cell differentiation of Zebrafish (Pascoal *et al.,* 2013). Both isoforms Jagged1a and b were found to be efficient in rescuing sapje larvae, although Jagged1a slightly more potent for the same (Vieira *et al.,* 2015).

RATIONALE

The pathological aspects of DMD in cause-effect are unclear, making therapeutic targeting difficult. Interventions to reduce pathological characteristics described above, like – excess cytoplasmic or mitochondrial ROS, excess calcium, improving membrane repair, inhibitors of aberrant signaling pathways, stimulation of muscle growth via IGF1, stem cell therapies have consistently failed at clinical trials. The anti-inflammatory steroids extend the ambulatory span and improve several aspects of disease progression for ~2-4 years in patients, albeit with side effects like shortening stature, bone density changes etc. The new age anti-inflammatory treatments are showing promise at clinical trials in terms of side effects. But there is still no cure available or accessible for all the DMD. Muscle specific upregulation of notch ligand Jagged1 rescued GRMD. The mechanism of action behind this rescue is not clear. Also, aspects of pathology mitigated by Jag1 overexpression can inform about fruitful therapy targets. The original authors (Vieira *et al.,* 2015) hypothesized that Jag1 ligand stimulated Notch receptor mediated myoblast proliferation to rescue this disease. The dystrophic muscles have a much higher propensity for damage, hence only increasing myoblast proliferation during regeneration without reducing the rate of degeneration would lead to faster exhaustion of myogenic potential and exacerbation of disease – not the rescue seen in GRMD. Hence, we hypothesized that Jag1 overexpression reduces susceptibility to contraction mediated muscle damage to rescue DMD. We also compared pathological aspects mitigated by Jagged1 overexpression in the zebrafish and in vitro human muscle culture models.

PROPOSED STUDY OBJECTIVES

A) Comparative analysis of DMD pathology post Jagged1 upregulation: The survival rate of DMD larvae with or without Jag1 overexpression

Cell proliferation and apoptosis, oxidative stress

Investigation of structural changes in rescue with - dystrophin, dysferlin, desmin, vinculin, MHC, MLC, f-actin gene expression levels and their localization in control and Jagged1 upregulated larvae.

B) Analysis of differentially expressed genes (global) under Jagged1 upregulation in dystrophin null as compared to control.
C) Find receptor/s and known targets (HES and HEY family genes) involved in Jagged1 upregulated trunk muscles.
D) Substantiating newfound targets of this pathway by artificially blocking and upregulating these in cultured human muscles to validate the findings.

MATERIAL AND METHODS

To achieve the abovementioned goals, the following methodologies were employed.

Animal maintenance:

Zebrafish from local aquarium were procured and maintained under standard conditions according to protocols described in the Zebrafish book available at zfin.org.

Methodology

Embryos at the single cell stage were collected by breeding wild type zebrafish. Injection groups were - uninjected control, injected with only Cas9+ sgRNA (called DMD group), Cas9+sgRNA+empty Vector (called DMD+vector group), Cas9+sgRNA+CMV-Jagged1 (DMD+jagged1group), Only CMV-Jagged1 Injected (called Jag1 group).

1) Create dystrophic zebrafish.
2) Overexpress Jagged1 in the dystrophic background from single cell stage
3) Observing all the aspects of the disease at various time points (4,8,15 days post fertilization was chosen because the literature suggests that muscle damage does not initiate before 3 days in DMD zebra fish larvae; 20-30 days is the maximum survival expectancy.
4) Perform quantitative PCR of known Notch effector genes in all groups of larvae.

Zebrafish maintenance

Zebrafish bought from a local aquarium were kept in 24” ×12” ×12” L×W×H glass tanks 2/3 filled with ~40 L water. Less than 25 adult fishes are maintained in one tank to avoid overcrowding. Temperature of ~28°Cand 10 hours Light and 14 hours dark period maintained. The fishes were acclimatized for~ 1 month. Tap water is kept in the open for 24 hours to reduce Chlorine levels and used to replace half of the tank water by siphoning every week. Water Filters attached to small electric pumps are constantly on to clear the tank water of debris. Fish were fed three times in small quantities with a varied diet of micro worms, paramecia, cyclops, and aquarium bought fish food granules. Breeding Tanks are attached to the side of the tank and a single male and two females are kept overnight to obtain fertilized eggs. Viable eggs are collected by sieve from the breeding tank and cleaned of debris and fish excreta immediately. Eggs are transferred to E3 medium (details in the following section) with a drop of Methylene Blue. Every few hours checked under a microscope and dead eggs are removed. Eggs hatch between 24-48 hours under normal conditions and fresh E3 medium is added daily. Less than 30 eggs are kept in a single 6” diameter clean petri plate. After 4 days after fertilization the larvae are fed on paramecia, artemia hatchlings. Slowly after 7-8 days post fertilization diet of micro worms is introduced. Fish food granules are crushed and fed to larvae only after 25 days post-fertilization. Fertilized eggs were collected immediately from breeding tanks and injected with in 30 mins. The Femtojet microinjection apparatus from Eppendorf company. The glass capillaries were pulled using a microinjection puller from Sutter Instrument Company. The microinjections thus generated are always slightly variable in terms of diameter and stiffness hence slight adjustment in injection parameters is done in terms of time, Pi, Pc between experiments.

E3 Medium (From “The Zebrafish Book”- zfin.org)

1.0 ml Hank's Stock #1
Combine the following in order:
10.0 ml Solution #1
1.0 ml Solution #2
1.0 ml Solution #4
86.0 ml ddH_2O
1.0 ml Solution #5
0.1 ml Hank's Stock #2
1.0 ml Hank's Stock #4
95.9 ml dd H_2O
1.0 ml Hank's Stock #5
1.0 ml fresh Hank's Stock #6
Use about 10 drops 1 M NaOH to pH 7.2

Store Hank's Premix in the refrigerator along with the Hank’s solutions.

Hank’s Stock Solutions:

Stock #1	8.0 g NaCl,0.4 g KCl, in 100 ml dd H_2O

Stock#2	0.358 g Na_2HPO_4 Anhydrous,0.60 g KH_2PO_4, in 100 ml ddH_2O
Stock#4	0.72 g $CaCl_2$, in 50 ml ddH_2O
Stock#5	1.23 g $MgSO_4x7H_2O$, in 50 ml dd H_2O
Stock#6	0.35 g NaHCO3, 10.0 ml dd H2O

Zebrafish model of DMD

Danio rerio (sapje) with stop codon in the 4th Exon of the Dystrophin gene, an accepted model of DMD, is available from Germany. We found it would cost ~1900 Euros-minimum1,60,000/-INR and would take at least 8-9 months to procure the fish. It guarantees replacement if fishes are dead upon arrival, but it would take an additional 8-9 months. Hence, in-house Knock out of DMD was generated as a model of disease.

CRISPR/Cas9 to create Knockout of DMD

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats which are part of the bacterial defense system against viral pathogens. These sequences have "PAM" motifs that are identified and bound by Cas9 enzyme which can induce double strand breaks to degrade viral genes. The enzyme requires a guide RNA that is complementary to the 22-25 nucleotides immediately before "PAM" sequence. The resulting double strand breaks are mostly repaired via Non-Homologous End Joining (NHEJ) pathway. NHEJ pathway is not as error-free as Recombination Repair, leading to addition or deletion of few or long stretch of nucleotides during repair which can disrupt the reading frame of the target gene. system is used to create double strand breaks in the gene of interest to create stable knockouts.

As there are several shorter dystrophin isoforms which might be important for viability or development, we chose only initial exons for knockout. The loss of initial N terminal exons gives rise to more severe dystrophy in both human patients and animal models. Thus, our strategy creates a more severe dystrophic model for better assessment without affecting functions of other isoforms of dystrophin.

Guide RNA

Cas9 activity needs "Guide" RNAs which are single stranded RNAs that requires at least "SPACER" region that is complementary to the target region hence will bind it by Watson-Crick base pairing this usually ends with "PAM" sequence on the template DNA. The

sequence complementary to “PAM” is not included in “guideRNA” hence this region does not take part in RNA binding, but this is required for Cas9 enzyme activation. There are slight differences in the number of nucleotides from “PAM” sequence that a given Cas9 will introduce single or double strand cut in the strand binding gRNA or the opposite strand. Different organisms have different Cas9 enzymes, artificially mutated enzymes are also available based on need from “Addgene.org”.

The efficiency and off target effects of this technique depend on efficiency and specificity of guide RNA sequence hence several online tools like “CHOP CHOP” and “Cas Designer” were used to identify the best fit. The genomic sequence for Zebrafish Dystrophin gene (also called DMD gene) was downloaded from NCBI database and Ensembl database to find annotated exon-intron sequences. Exonic sequences of the first 10 exons were copied and used to generate possible sgRNAs containing “NGG” PAM motifs as wild type Cas9 is used.

The sgRNA sequences were also checked for possible off target by doing NCBI- BLAST. 66 base pair long oligonucleotide with 20 bp gene specific region in the middle were pre-synthesized by order as oligos given in the table below. The region in red colour is target sequence identified by software tool that end with “NGG” which is not made to be part of sgRNA. The constitutive sequence at the start of oligonucleotide is complementary T7 sequence. The constitutive sequence at the end is scaffold region of guide RNA that is necessary for its successful folding and binding to Cas9.

The gRNAs target sequences in 6th and 7th exons in zebrafish DMD gene. The possible guide RNAs in other exons were too far apart due to longer intronic regions. It would have been difficult to run genomic PCRs of such lengths to confirm knockouts. Hence region from 6th exon to the end of 7th exon was used as it is composed of 2500 bp including intron. There were multiple “PAM” containing possible target sites present. Total 5 guide RNA primers were designed. Initially, the resulting sgRNAs using these primers were used in combination of 3, 4 and all 5 sgRNAs. It was found that a combination of 3 guide RNAs – numbers 2, 3 and 5 were most efficient for knockout hence for experiments only these 3 were used. The guide RNAs can have toxic side effects hence use was optimized to reduce mortality and increase knockout generation.

The in vitro gRNAs were produced using ABM sgRNA synthesis kit (G952: GeneCraft-R Classic CRISPR sgRNA Synthesis Kit (S. pyogenes)). The primers described in the above

table were first converted to double stranded DNA with help of First Strand Synthesis primers from Kit. The resulting dsDNA has T7 promoter site just before transcribed region shown in red. When this dsDNA is used for invitro transcription, it produces RNA from T7 promoter on plus strand. The concentration is confirmed using Nanodrop and product is run on 2% agarose gel to check for possible degradation.

Following is the snapshot of the computer screen during sequence alignment and features highlighted using Snapgene viewer software. The sequences in RED color are "SPACERs" sequences used in generation of single stranded guide RNA (sgRNA).

1	TTCTAATACGACTCACTATAGGGAGCGTTGAAAGCGAAACCGTGTTTTA GAGCTAGAAATAGCAAG
2	TTCTAATACGACTCACTATAGGGAAACTACCAAGACGTCAACGGTTTTA GAGCTAGAAATAGCAAG
3	TTCTAATACGACTCACTATAGGGCGAAAAGATCCTGTTAAGCTGTTTTAG AGCTAGAAATAGCAAG
4	TTCTAATACGACTCACTATAGGGACTCTTCTCCGCAACACCGAGTTTTAG AGCTAGAAATAGCAAG
5	TTCTAATACGACTCACTATAGGGAGAAGAGTTTAGGAATCGACGTTTTA GAGCTAGAAATAGCAAG

The wild type Cas9 plasmid available from addgene.org. pCS2Cas9 plasmid containing CMV promoter was acquired from addgene to express Cas9 in vivo. Plasmid was cloned in transformed DH5a *E. coli* strain. Single colonies were picked up from Ampicillin- Plates and grown overnight in liquid LB media. Plasmid was isolated using Qiagen Plasmid isolation using supplier protocol.

Genomic PCR

Primers were pre-synthesized by order covering 6th and 7th exon that gives ~700 basepair PCR product to identify deletion in CRISPR/Cas9 knockout Zebra fish. Wildtype and DMD knockout zebrafish larvae were quickly euthanized in cold water. Genomic DNA was isolated from single larva from both groups using Qiagen Genomic DNA isolation kit as per manufacturer protocol. PCR cycling parameters were standardized for amplification of region of interest.

Isolated genomic DNA was quantified using Nano Drop. 1ng of gDNA was used in the 20ul PCR amplification with Taq DNA Polymerase and buffer from Thermofisher. The following primers were used in final concentration of 0.5uM in reaction. The Eppendorf thermal cycler was used with the following parameters: initial denaturation at 95°C for 3

minutes, denaturation at 95°C for 15 seconds, Annealing at 54°C, 30 seconds, extension at 72°C for 2minutes for 35 cycles and final extension at 72°C for 5 minutes. The amplification product was run on 1.6% agarose gel with Ethidium Bromide, visualized under UV light, and imaged.

Primer sequence for genomic PCR of DMD

Forward Primer (g_ZF_DMD)	ACTGTATGTGCATCCTCTCC
Reverse Primer (g_ZF_ DMD)	TCTGTATCGCTCAGAACAG

Overexpression of jagged1

DH5-α Transformation

DH5α strain of *E. coli* was first made competent for transformation using calcium chloride method. LB-Agar plates without antibiotic/s were poured in aseptic conditions and used to grow colonies by streaking with the use of a cooled glass rod dipped in 100% ethanol and held in flame to sterilize it first. Individual colonies were picked with autoclaved toothpicks and grown into liquid LB media overnight. These individual overnight cultures were made to 1% cultures by adding fresh LB media and grown for 2-3 hours till OD reached between 0.6-0.8. This represents the Log phase of growth at which they are maximally sensitive. The cells were pelleted sand resuspended in 150mM CaCl2 containing 15% Glycerol. This step was repeated two times and preserved in a $CaCl_2$ mixture at -80℃. In this condition they remain viable and transformation competent for six months.

The transformation Protocol was standardized for optimum temperature and time for the heat shock method.

Competent cells and DNA are thawed on ice for 20-30 minutes. 2-5 microliters of DNA depending on concentration is added to competent cells. Incubated on ice for 20-30 minutes. Then heat shock at 42℃ is given for 90 seconds and vials are put back on ice. Volume is made to 1ml by adding fresh liquid LB media. Cells are grown at 37℃ for one hour, pelleted down and resuspended on antibiotic agar plates by streaking 20-50 microliters. The next day colonies are counted for different concentrations of DNA used and transformation efficiency is calculated. The plasmid containing gene of interest is isolated from the individual colonies grown in liquid LB media.

Cloning of Human Jagged1: Jagged1 and its receptors -Notch are developmentally important hence in vivo Jagged1 expression needed to be undisturbed in zebrafish. Identification of overexpression was also needed, hence human CDS sequence was cloned into the expression vector.

The pET28 vector with full length human Jagged1 inserts was a kind gift from Prof. Rajan Dighe and Prof. Annapoorni Rangarajan, Dept. of MRDG, IISc, Bangalore. The specific primers were designed to clone the full-length Jagged1 mRNA sequence using the NCBI primer blast tool. Restriction enzymes XbaI and HindIII were used to clone full length human Jagged1 into PCDNA3.1 plasmid which contains CMV promoter which gives constitutive expression in mammalian cells as well zebrafish. The plasmid was transfected in a transformed DH5α bacteria which lack recombination proteins hence mutations in insert sequences are very rare.

Primer sequence for human Jagged1 full length cloning

XbaI_jag1 Forward Primer	ACCGAATTCATGCGTTCCCCACGGAC
HindIII_jag1 Reverse Primer	TTCTCGAGTACGATGTACTCCATTCGGTTTAAG

Colony PCR was used to confirm product length with same primers. Selected colonies were used to isolate plasmid and sequence was confirmed using Blast alignment tool.

Full length JAG1 in Expression Plasmid: pcDNA3.1 Plasmid was sequenced and confirmed using NCBI align tool.

PCDNA31_PR ATGCGTTCCC CACGGACGCG CGGCCGGTCC GGGCGCCCCC TAAGCCTCCT

humanJAG1 ATGCGTTCCC CACGGACGCG CGGCCGGTCC GGGCGCCCCC TAAGCCTCCT

PCDNA31_PR GCTCGCCCTG CTCTGTGCCC TGCGAGCCAA GGTGTGTGGG GCCTCGGGTC

humanJAG1 GCTCGCCCTG CTCTGTGCCC TGCGAGCCAA GGTGTGTGGG GCCTCGGGTC

PCDNA31_PR AGTTCGAGTT GGAGATCCTG TCCATGCAGA ACGTGAACGG GGAGCTGCAG

humanJAG1 AGTTCGAGTT GGAGATCCTG TCCATGCAGA ACGTGAACGG GGAGCTGCAG

- Muscle Structure quantification by bipolar microscopy:

  The regular - tandem arrangement of myotomes in zebra fish muscles acts like fixed shaped crystal. When these muscles are cleared of cellular content, they diffract light in a very specific manner called birefringence which can be visualized under polarized microscope. The wild type muscles show bright under polarized microscope while damages can be seen as darker lesions or overall reduction in birefringence. The 4- and 8-day old larvae in all groups were fixed in 100% Methanol overnight and then transferred to Methyl Salicylate solution for clearing it. They were visualized under light microscope between two polarizer-filters which filter out light of undefined or mixed polarization allowing only even or single polarized light. The number of areas with low birefringence or lesions were counted irrespective of size of the area. The percentage of birefringence intensity was also measured with the help of ImageJ software.

- Spontaneous Swimming and Evoked Escape Assay for Muscle Function quantification:

  The dystrophic larvae are weak and fragile. The disease is progressive in nature. The changes in the locomotory behavior of dystrophic larvae are already known. In the original "Escaped" GRMD the muscle weakness has already been reported. To find out if such weakness exists in zebrafish larvae injected with Jagged1 plasmid swimming capacity was assessed at 4 dpf and 8 dpf by two methods spontaneous swimming for 2 minutes in groups of 5 larvae 2 touch evoked escape behavior where single larvae are kept in a petri-plate and soft and fine paintbrush is used to evoke the escape behavior which is recorded for 1 minute. The circular white paper with 1 mm concentric circles was placed at the bottom of the transparent petri-plate from outside. The videos are converted to .AVI format and analyzed in ImageJ software. Total distance travelled by larvae in a given time is measured.

- Cytoplasmic ROS quantification

  The cell permeable reagent 2',7' dichlorofluorescein diacetate (DCFDA, also known as H2DCFDA, DCFH-DA, and DCFH) is useful to quantitatively assess reactive oxygen species in live cell samples. It measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell. After absorption in cells H2-DCFDA is then deacetylated by cellular esterases to a non-fluorescent compound, which ROS can

oxidize into 2', 7' dichlorofluorescein (DCF). DCF is highly fluorescent and is detected by fluorescence spectroscopy with excitation/emission at 485 nm / 535 nm. Five larvae from each injection group were taken. The H2-DCFDA dye was added at a final concentration of 1.8uM and incubated in the dark for 20 mins. Then washed with PBS for 3 times in dark and imaged under florescent confocal microscope.

- Mitochondrial ROS quantification

  The JC1 is lipophilic cationic carbocyanine dye that accumulates in mitochondria. It emits a red fluorescence at 590nm when it is in dimeric form. In the monomeric form it emits green fluorescence at 488nm. When oxidative stress is high in mitochondria it reacts with this dye causing the monomerization of it increasing green emission. The ratio of Red/Green emission to assess mitochondrial membrane potential and thus health in any disease condition. The absorption greatly reduces after 4 dpf in larvae so in vivo mitochondrial potential measurement from muscles directly under a confocal microscope were unsuccessful. Hence, Red and Green emissions were measured on larval muscle suspensions by FACS (Fluorescence Activated Cell Sorter). 10 larvae from each injection group were quickly euthanized in cold water. The head till pouch was removed, so major tissue remains in muscles. These were digested in trypsin EDTA (1X) and Collagenase IV (1mg/ml) for 5 mins. The CO_2 free complete media was used to neutralize the enzymes (trypsin and collagenase IV) and reduce the stress of cells in single cell suspension. The JC1 dye was added to this media at 5uM final concentration for 10 minutes and emission was measured in the BD Accuri machine. The data acquired were analyzed using BD Accuri software and Prizm software to generate graphs.

- Cell Cycle Analysis by Propidium Iodide (PI) staining

  Propidium Iodide is not permeable to live cells. It is absorbed only by dead cells. Once inside cells it binds to DNA by intercalating between the nitrogen bases. Once it binds to DNA it is stable and emits red fluorescence which can be assessed by fluorescent microscopy or FACS. In FACS the inherent properties of cells and nucleus to diffract the light captured as forward scatter and side scatter depending on size and content of cytoplasm and nucleus. Based on PI staining in fixed cells the cell cycle analysis was carried out on BD Accuri - FACS machine. Five larvae from each group were fixed in

4% PFA overnight at 4 ℃. The head and fat pouches were cut off. Then larvae tails were digested with trypsin and collagenase-IV for 5 mins at 37℃. The digestion was stopped by washing 3 times with PBS. The pipetting was done to further disrupt the tissue into suspension. This suspension was passed through 45um membrane filter to remove debris. The single cell suspension was treated with 30-40 uM PI final concentration for 20 mins in dark and used for cell cycle analysis.

- Immunohistochemistry of Whole Larval Muscles

  Larvae at 4 dpf and 8 dpf were fixed in 4% Paraformaldehyde at 4℃ overnight. Then transferred to 100% Methanol for 24 hours at -20℃. The larvae were gradually rehydrated with 75%, 50% and 20% Methanol for 10-15 mins. Twice washed with PBS. Incubated with blocking buffer (made in PDT) for 1.5 hours. Then primary anti-dystrophin antibody (MANDRA-11 from DSHB) and anti-β-Dystroglycan (Mandag1 from DSHB, Cardiac Myosin Heavy Chain (MYH-7) (BA-F8 from DSHB), Notch 1 (b-TAN 20 from DSHB), Notch 2 (C651.6DbHNFrom DSHB), Notch-3 (PAL147Hu01 from Cloud Clone), Notch 4 (PAL149Hu01 from Cloud Clone), Jagged1 (TS-1.15H from DSHB) was incubated overnight at 4℃. Washed 3 times with PBST 15-20 mins each. The secondary anti-mouse, anti-rat conjugated with fluorophores (in blocking buffer) was incubated for 2 hours. Nuclei were counterstained with Hoechst for 40 mins, washed before mounting on slide with Vectashield as anti-fade reagent, and imaged with SP8 confocal microscope.

- Protein Isolation

  Adult zebrafish were anesthetized and euthanized by immersing them in ice water. Trunk muscles were dissected out and immediately frozen in liquid N_2. Embryos at various stages of development were dechorionated and immediately frozen in liquid N_2. They were crushed mechanically in lysis buffer and immersed them liquid N_2 a few times and incubated at 4℃ for 45 minutes. It was centrifuged at 12000 rpm for 10 minutes. The supernatant was collected in fresh tube and pellet was discarded. Lysis buffer composed of 20mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1mM MgCl2, 1% Triton X-100, 10% glycerol (W/V), 1mM EDTA, 1mM DTT and cocktail of protease inhibitors. Protein was quantified by BCA method.

- Western Blot

  This is a sensitive quantitative or semi-quantitative measurement method for the amount of protein present in the sample. The protein sample is prepared in loading buffer, a mixture of beta-mercapto ethanol to reduce disulfide bonds in proteins, glycerol to make sample solution sink in the well and bromophenol blue to visualize the sample. Protein in loading buffer is heated at 95℃ for 5minutes and loaded onto Polyacrylamide Gels. This method is called SDS- PAGE as proteins are denatured and become negatively charged because of Sodium Dodecyl Sulphate (SDS). As proteins are electrophoresed movement from anode to cathode they move according to their molecular weights as their electric charges are similar due to SDS. The speed of protein moving also depends on the size of the pores made by percentage of Polyacrylamide which APS and TEMED crosslink. Our proteins of interest – Dystrophin (427KDa), Notch receptors range from 206 to 270 KDa, Jagged1- 135 KDa in zebrafish and 133KDa Human which is injected into zebrafish. We have used 12% and 10% polyacrylamide gels to standardize alfa-tubulin, MHC, Notch1-4 and 7.5% gel for Dystrophin WB.

  For blotting- the proteins separated on the polyacrylamide gel are transferred to PVDF membrane. The PVDF membranes are kept in Blocking Buffer (made of 2-5% BSA dissolved in TBST) for 2 hours. Incubated overnight with antibody for the protein of interest at 4℃, washed 3 times, 15 minutes each with TBST. Membrane is incubated in Blocking Buffer for 2 hours. Incubated with secondary – HRP conjugated antibody for 2-3 hours and washed again. Blot is developed by incubating it with mixture of substrates-peroxide and luminol. Secondary antibody bound Horse Radish Peroxidase converts Luminol to 3-aminopthalate in the presence of peroxide which emits light that can be captured on either X-ray film or as a picture in a Chemi-Dock machine.

- RNA isolation from larval tail muscles

  RNA was isolated with TRIZOL reagent from a pool of 5-10 larvae after head and fat pouch removal. 300ul of TRI reagent was added to larvae after water was completely removed from 1.5 ml centrifuge tubes and immediately immersed in liquid Nitrogen and crushed. After that incubated at 4 C for 15 mins. 100ul of chloroform was added and mixed by inversion and stood at room temperature for 10 mins. Next tubes were centrifuged at 12000g for 15 mins at 4 C. The aqueous phase was carefully removed

without touching sides or interphase into a fresh 1.5 ml centrifuge tube. 100ul of Isopropanol was added and mixed by inversion; incubated 10 mins at room temperature. Centrifuged 12000g at 4 C for 10 mins to pellet RNA. The pellet was washed with 70% ethanol and centrifuged at 8000g at 4 C for 8-10 mins. The ethanol was removed gently; tubes were inverted to dry on tissue paper for 3-4 mins. RNAase free water was added and mixed by tapping for 1min. Concentration was measured in Nanodrop machine along with A260/280 and A 230/260 ratios to determine the quality of purification. 100ng of RNA was run on 2% agarose gel to check for degradation and genomic DNA contamination. DNAse-I treatment was given whenever genomic DNA was seen on agarose gel.

- cDNA synthesis oligoDT method

  Most of the eukaryotic mRNAs have poly-A tails to which Oligo nucleotide of Ts can only bind and act as primer for 1st strand synthesis. mRNA is converted to cDNA by enzyme Reverse Transcriptase in the presence of dNTPs when mixture is incubated at 42°C. Riblock is added to avoid RNA degradation. Both mixture-enzyme and mixture-template are used as control. Enzyme is heat inactivated by incubating mixture at 70°C for 10 minutes. cDNA is quantified. PCR for GAPDH is carried out using cDNA as template for quality check.

- Quantitative PCR

  During process of dystrophic changes expression of a lot of genes change including GAPDH hence β-actin and microβ-globulin were used as housekeeping genes based on literature and running real time PCR (RT-PCR) with different concentrations of RNA from controls and DMD conditions to confirm their expression levels correlate with RNA concentration and not dystrophic condition. 10 larvae from each injection group were randomly collected and frozen in TRI solution. RNA was isolated by standard TRIZOL method. The RNA integrity was checked on 2% agarose gel electrophoresis. 1ug of RNA was converted to cDNA with BioRAD cDNA synthesis kit by following the manufacturer's instructions. The primers specific to Hes1, HeyL, Hey1, Her6, Her9, Her12 using NCBI blast against their CDS specific to zebrafish were used. The quantitative PCR was done using SYBR Green method on One Step Real Time machine using standard protocol.

Primer sequences of zebrafish Notch target genes

βActin Forward Primer	CCAGCCATCCTTCTTGGGTAT
βActin Reverse Primer	CTTCATTGTGCTAGGGGCCA
Microβglobulin Forward Primer	TGGCTAAAGAAACTCCCCCA
Microβglobulin Reverse Primer	TGGTGATGTCAGGAGGGTGA
HeyL Forward Primer	TGGGTGGCAAAGGCTACTT
HeyL Reverse Primer	CACCGATGGGGTCTGATGAT
Hey1 Forward Primer	AAAACGTCGCAGAGGGATCA
Hey1 Reverse Primer	CCTGTTTCTCAAAGGCGCTG
Hey2 Forward Primer	GGCCACAGGAGGAAAAGGATATT
Hey2 Reverse Primer	GCTGCTGAGGTGAGAAACCA
Her6 Forward Primer	ACAAATGACCGCTGCCCTAA
Her6 Reverse Primer	GGCGTTGATCTGTGTCATGC
Her9 Forward Primer	ATCACCTATTGCTGGTGCCC
Her9 Reverse Primer	GCGCTTTTCCATGATTGGCT
Her12 Forward Primer	ACAACATAAAGCAAGCTGTTCG
Her12 Reverse Primer	CTATCGGCTTCCTCAGCTTTATT
Human Jagged1 Forward Primer	AGTGCCTCTGTGAGACCAAC
Human Jagged1 Reverse Primer	TTGCTACAAGTTCCCCCGTT

- Statistical analysis:

  Prism7 software was used to carry out analysis and generating graphs. Using Image J software, the fluorescence intensity of IHC images were measured.

- Human DMD cell line as invitro disease model

  The DMD patient derived immortalized myoblast cell lines were kindly shared by Dr. Vincent Mouly from Institute de Myologia, France under MTA with the Dean, Faculty of Science, The M S University of Baroda, Vadodara, along with age matched healthy control lines for comparison. The myoblasts were counted with a cytometer in 5 squares. The formula “mean number of cells*dilution factor/1000 was used to calculate cells/ml and accordingly 2*105cells were plated for each experiment. When cells reach 70-80% confluence, medium is removed, and dishes rinsed with 10ml F10 (or PBS). The cells were incubated with 2 ml of trypsin for 5-10min at 37°C, 5% CO2, checked under microscope for cell detachment.

When cells are floating, 3ml of proliferation medium is added and collected in a tube. The dishes were rinsed with 5ml of proliferation medium and mixed with the previously collected cells.

To determine the number of divisions since the last passage using the formula:

Division nb = log (counted number of cells/ number of seeded cells)/log 2

The growth media (GM) protocol as shared by Dr. Mouly consists of

1Vol medium 199 (Invitrogen, 41150020) + 4Vol DMEM (Invitrogen, 61965-026) + 20% FBS

supplemented by:

•Fétuin: 25µg/ml 10344026 Life Technologies

•hEGF: 5ng/ml PHG0311 Life Technologies

•bFGF: 0,5ng/ml PHG0026 Life Technologies

•Insuline: 5 µg/ml Sigma, 91077C-1G

•Dex: 0,2µg/ml sigma D4902-100mg

The 6 well plates were coated with Matrigel-GFR diluted 1:20 in DMEM and incubated for 2 hours at 37℃ to be used for differentiation. The cells were induced to differentiate when confluence reached ~70% by changing media containing- DMEM with 10ug/ml Insulin and 50ug/ml Gentamycin (DM). The cells were left to differentiate for 5-6 days in DM, changing media if necessary. The myoblast fused and differentiated into multinucleated myotubes.

- Calcimycine to recapitulate contraction mediated damage in myotubes

  The ionophores interfere with channels and cause continuous calcium ingress in myotubes that simulates the effects of sustained muscle contraction. Based on previous studies 5uM final concentration was used on 6-day post differentiation control and DMD myotubes. The cell death, mitochondrial ROS, etc., were measured after 24 and 48 hours of treatment.

- The Jag1 mini peptide treatment of myotubes

  The transfection of immortalized cell lines is very difficult with conventional chemical methods. The viral transduction requires antibiotic selectable markers many of which interfere with the antibiotics used for the immortalization of myoblasts. Hence, we used 17 amino acids length peptide fraction of Jag1 that can act as ligand to study the effect of Jag1 during differentiation window that best corresponds to myogenin expression in

in vitro cultures. This allowed the examination of possible effect of myogenin mediated Jag1 upregulation which was not possible in in vivo zebrafish model.

- Mitochondrial ROS with JC1 dye

  JC1 dye with final concentration of 10uM was added to cultured myotubes for 20 mins in the dark, washed with 1X PBS and imaged with two photon Zeiss Lsm 880 confocal microscope. The average image intensity of minimum 300 fibers was quantified for each replicate. Red/Green intensity ratio was used as an indicator of mitochondrial oxidative stress.
- Propidium Iodide staining as a measure of apoptosis induction

  The myotubes were incubated for 30 mins in dark with 50uM final concentration of PI, along with Hoechst (1: 10,000 dilution) washed with 1X PBS and imaged under Zeiss LSM 880 confocal microscope. The number of myonuclei stained with Hoechst and PI were counted.

RESULTS AND DISCUSSION

The Knockout of DMD:

The CRISPR/Cas9 mediated knockout (KO) of DMD was performed by injecting cas9 expressing plasmid and mixing 3 guide RNAs into a single cell embryo. The final concentration injected was about 15-20 ng per larva. The exons 6 and 7 form a domain of N-terminal of dystrophin hence it gives rise to a more severe phenotype. The DMD knockout being larval lethal, all successful knockouts die in 15 days. Some of the surviving larvae were checked for heterozygous for DMD $KO^{+/-}$ to be bred for next generation but none of the surviving larva were heterozygous, hence were not used further. Every clutch injected with CRISPR/Cas9, 3 to 4 larvae were randomly selected for isolation of genomic PCR of locus and 3-4 larvae were randomly selected for immunostaining for dystrophin to validate the absence of protein and calculate the percentage of KO efficiency.

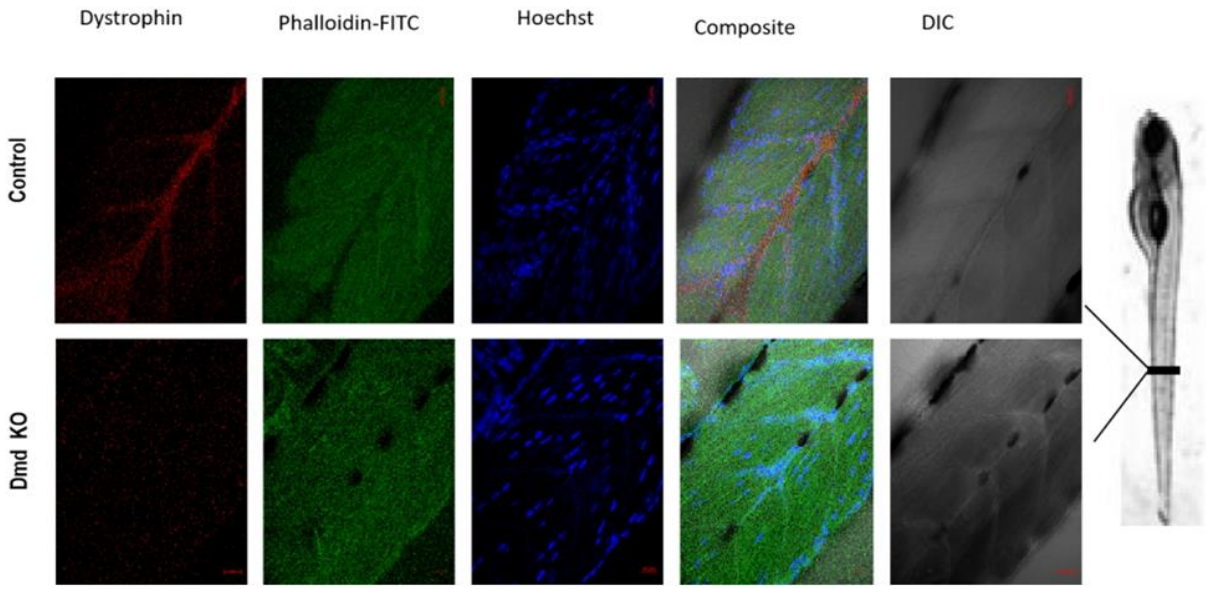

Fig 2: Immunolocalisation of dystrophin in DMD knockout and control larve

The protein dystrophin is a part of a large complex at the muscle membranes called DAPC (Dystrophin Associated Protein Complex) or DGC (Dystroglycan complex) because most proteins are glycosylated. To confirm that the knockout is specific to dystrophin- another protein called β-Dystroglycan (Dgl1) was also immunolocalized. β-Dystroglycan is a transmembrane protein of DAPC which on outer side of membrane binds to α-Dystroglycan and on cytoplasmic side binds dystrophin. This protein is present in DMD KO muscles as shown in the IHC images. The disruption of proper organization of muscles is evident which has been reported to reduce expression of all DAPC components. We found non-significant, slight reduction of fluorescence intensity of both α and β-Dystroglycan by IHC. (Florescence Intensity graph not shown here). This slight reduction could be due to removal of background due to mislocalization due to disruption of muscle structure.

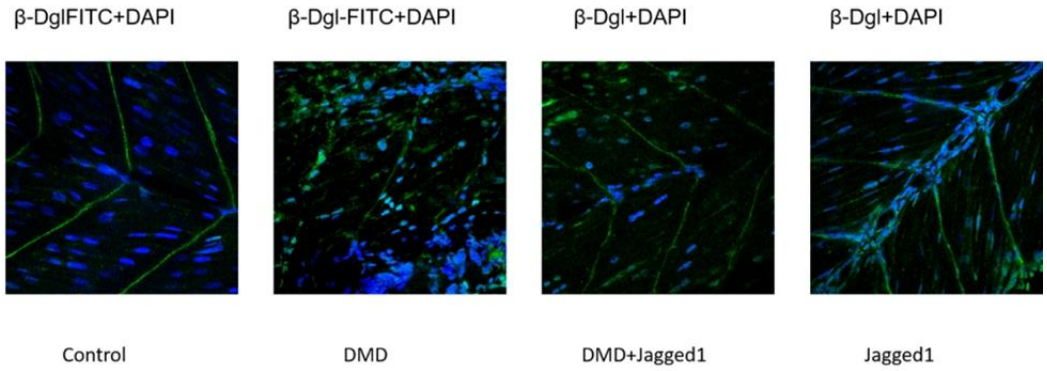

Fig 3: β-Dystroglycan localization in different experimental group of larve

These images also validate the other finding that Jagged1 overexpression restores the muscle structure in dystrophic conditions. This could be due to translational function of Notch receptor. Study (Pascoal *et al.*, 2013) showed that Notch signaling is required for localization of desmin and vinculin mRNA to myotendinous junctions for their successful translation. The study found zebra fish larvae were fragile and muscle structure was disorganized due to abrogation of Notch signaling. However, we have not found an increase in desmin or vinculin expression (data not shown) in jagged1 overexpression. The continued Jagged1 expression could have improved turnover of desmin and vinculin during dystrophic damage to keep muscle structure intact. Which in turn can slow down exercise induced damage and disease progression.

The knockout was also confirmed by genomic PCR. Below is an image of PCR gel electrophoresis on 1.6% agarose gel.

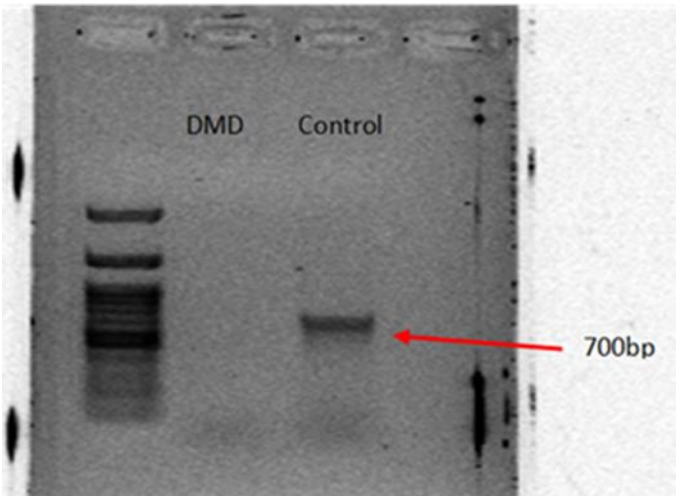

Fig 4: Genomic PCR product of DMD on 1.2% Agarose gel

Jagged1 overexpression preserves muscle structure:

The phenotype of dystrophic larvae is very evident under simple light microscope. The larvae are bent backed, curved tailed. The embryonic fat pouches are small. All such phenotypes are improved as seen in the images placed overleaf.

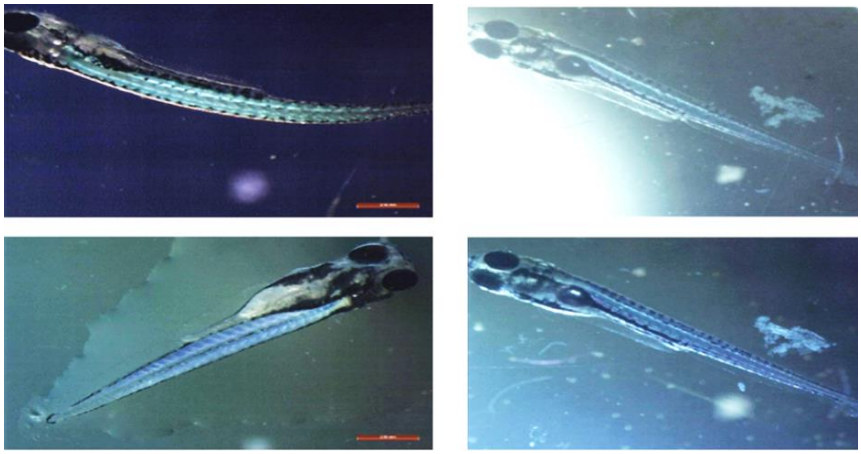

Fig 5: 4dpf control larve

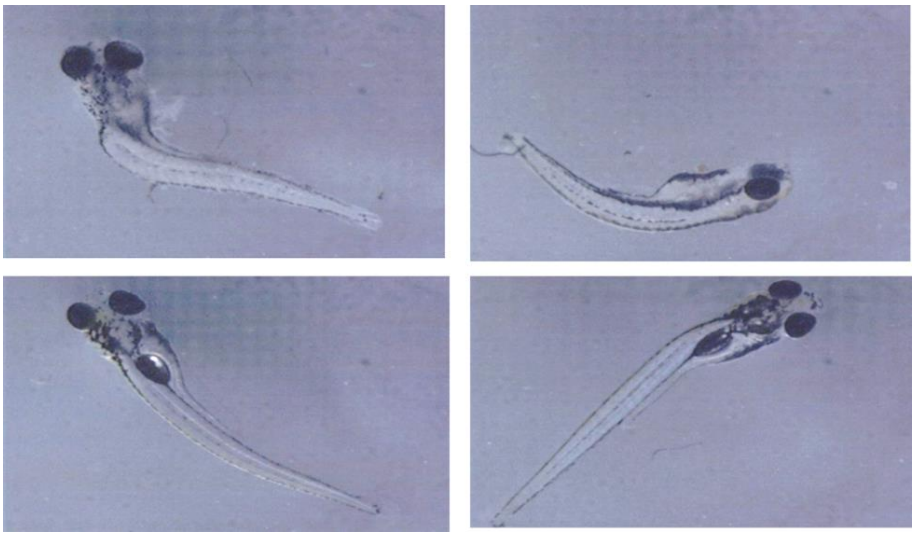

Fig 6: DMD knockout 4dpf larve

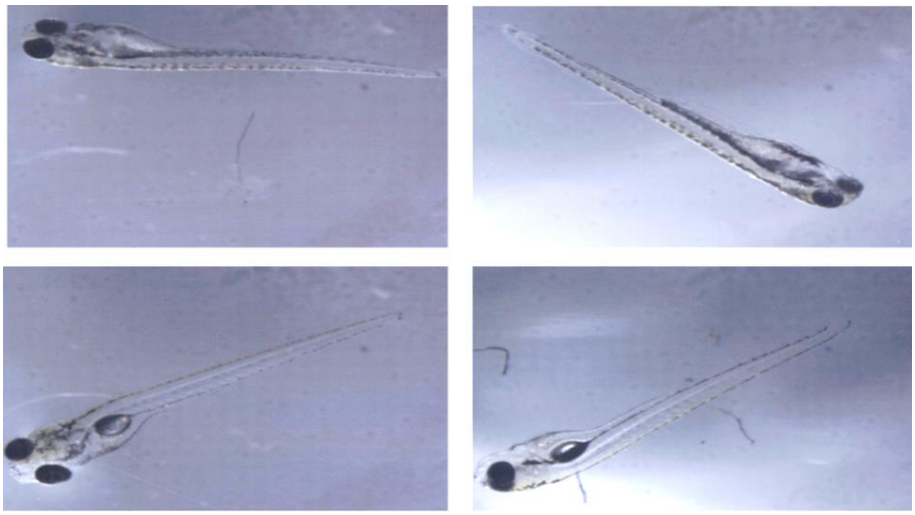

Fig 7: DMD+Jagged1 4dpf larve

The regular - tandem arrangement of myotomes in zebra fish muscles acts like fixed shaped crystals. When these muscles are cleared of cellular content, they diffract light in a very specific manner called birefringence which can be visualized under a polarized microscope. The wild type muscles show bright under polarized microscope while damages can be seen as darker lesions or an overall reduction in birefringence. The larvae from control, DMD and DMD+ Jagged were visualized under light microscope between two polarizer-filters - which filter out light of undefined or mixed polarization allowing only even or single polarized light. The percentage of birefringence intensity was also measured with the help of Image J software. Below are representative images and graphs showing the intensity of birefringence percentage and number of areas with low birefringence in control, DMD and DMD injected with Jagged1 plasmid.

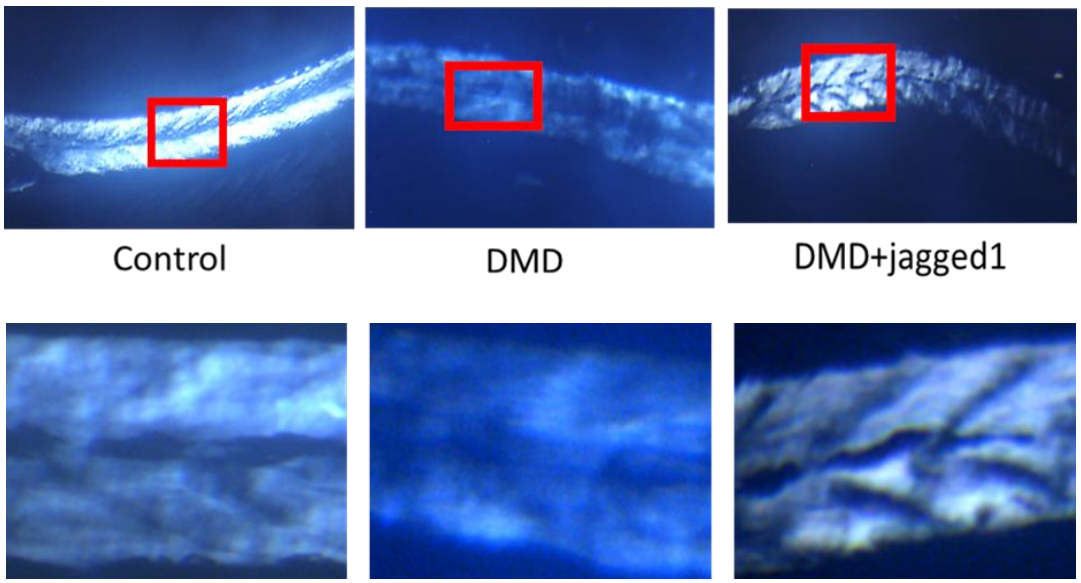

Fig 8: The representative images of Birefringence Assay by Bipolar Microscopy

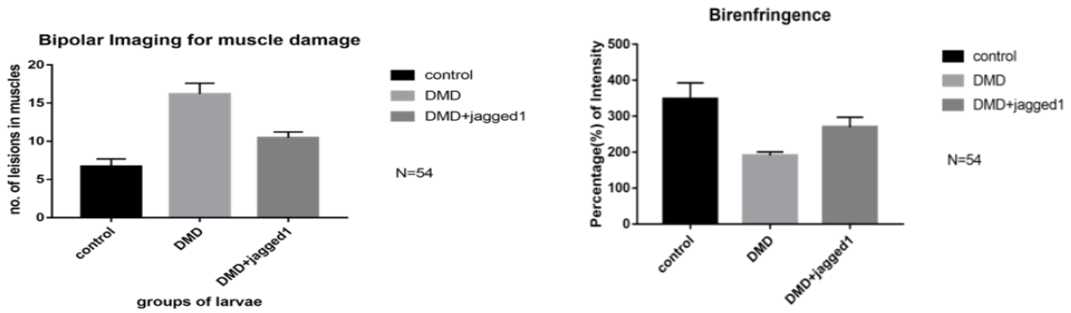

Fig 9: Quantification of Grey Intensity and Lesions

Jagged1 mediated rescue is independent of muscle type switch:

It is reported in the literature that slow oxidative type muscles are preserved longer than fast glycolytic type muscles. The most oxidative muscles - cardiac - take 20 years to show pathology when functional dystrophin is absent in heart. For this reason, therapeutic approaches to increase oxidative type muscle programming in DMD have been underway. One of the major targets has been calcineurin signaling activity. It has been found that Calcineurin - a calcium, calmodulin dependent phosphatase changes transcriptomic profile in muscles from glycolytic to oxidative type. Hence, some clinical trials involved chemical activation of calcineurin or aimed at increasing its expression levels. Hence, we sought to check if Jagged1 mediated rescue involves increasing slow oxidative type muscles or increase in slow cardiac type Myosin Heavy Chain in the existing muscles that might reduce the susceptibility of these larval muscles to exercise mediated damage.

The whole larval muscles were immuno-stained for cardiac slow myosin heavy chain and imaged in confocal microscope to assess the expression levels. The fluorescence intensity data were analyzed with the help of ImageJ software.

Larval gro	Area	Mean	min	max
Control	7.949	13.269	0	82
DMD	7.786	24.696	0	84
DMD+jagg	7.913	21.724	0	85

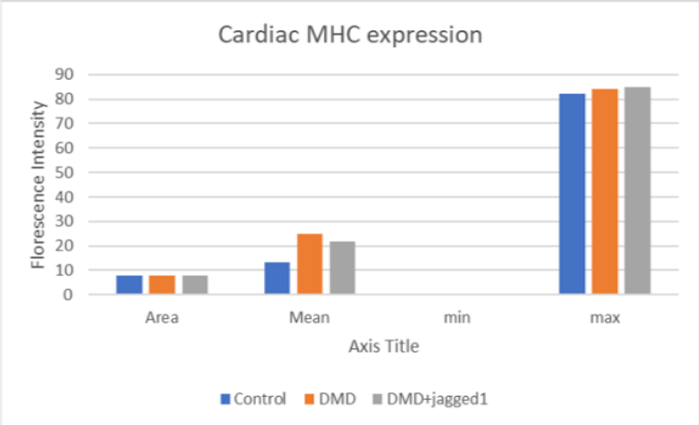

Fig 10: The graph showing Florescence Intensity of cardiac MHC expression.

Jagged 1 rescues muscle function in dystrophic larvae

The dystrophic larvae are weak and fragile. The disease is progressive in nature. The changes in the locomotory behavior of dystrophic larvae are already known. In the original "Escaped" GRMD the muscle weakness has already been reported. To find out if such weakness exists in zebrafish larvae injected with Jagged1 plasmid swimming capacity was assessed at 4 dpf and 8 dpf by two methods (1) spontaneous swimming for 2 minutes in groups (2) touch evoked escape behavior where single larva is kept in a petri-plate and soft

and fine paintbrush is used to evoke the escape behavior which is recorded for 1 minute. Distance travelled by larvae is measured. The variability of spontaneous swimming in 4 dpf larvae is too high in all groups.

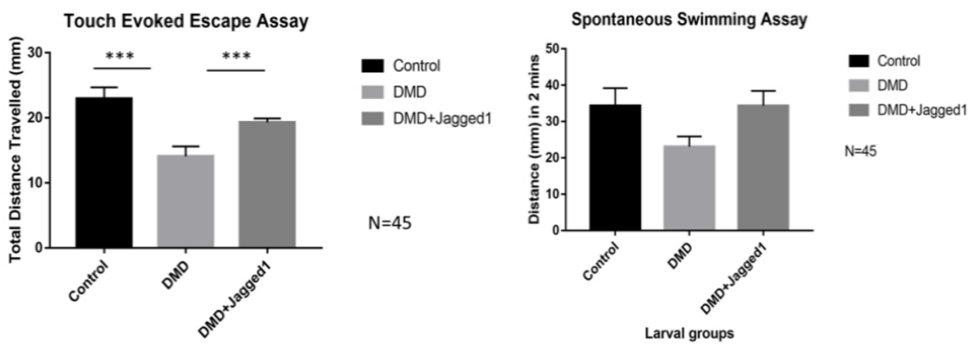

Fig 11: The representative graphs of assay carried out at 8 dpf.

Jagged 1 reduces cytoplasmic ROS in dystrophic muscles:

Oxidative stress is one of the major contributors to pathology in DMD. The literature describes that both cytoplasmic ROS and mitochondrial ROS are high in dystrophic muscles. The increased cytoplasmic ROS leads to increased Ca^{2+} mediated pathology which increases the Ca^{2+} mediated complex1 ROS generation from mitochondria. On the contrary, the increased mitochondrial ROS from complex1 affects the energy generation required for muscle function. The disturbed mitochondrial redox balance is also known to activate cell death pathway. Both these mechanisms are active in dystrophic pathology and which oxidative stress reduction is more important is not yet clear. So, we did both cytoplasmic oxidative stress quantification with H2DCFDA staining in larval muscles at 4 dpf and 8 dpf.

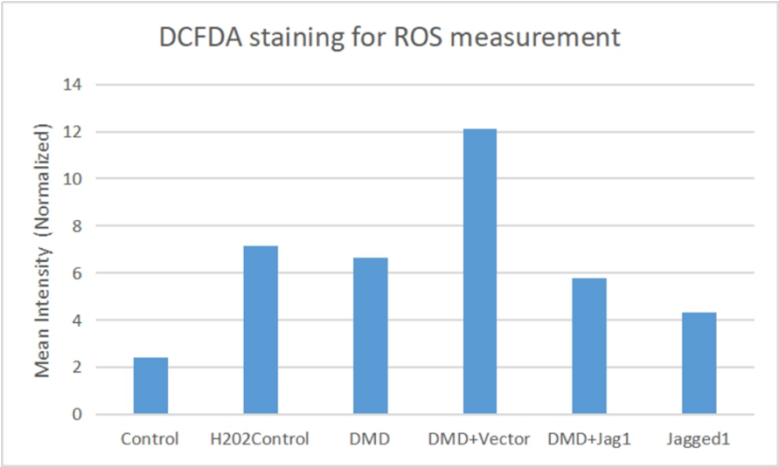

Fig 12: Representative graph of ROS quantification at 8 dpf

The fluorescence intensities were first normalized to area. The control larvae were treated with H_2O_2 as positive control of oxidative stress. It was seen that oxidative stress is high in dystrophic larvae, especially in empty vector added dystrophic larvae. Jagged1 over expression reduces oxidative stress and dystrophic condition significantly. The cytoplasmic oxidative stress has been shown to exacerbate calcium ion dysregulation and increase mitochondrial oxidative stress. Thus, reducing cytoplasmic oxidative stress can reduce pathology though treatment with antioxidant therapies has not worked. The overactivity of membrane-bound NADPH Oxidase complex (NOC) has been implicated in dystrophic condition. There are several positive and negative regulators of NOC e.g., p38MAPK, ERK1/2, NHE1 of which positive regulator p38 MAPK is overactive in DMD. NHE1 is overactive in DMD which keeps membrane pH basic. Inactivation of NHE1 can acidify local pH and inactivate NOC. The antioxidants nullify reactive oxygen and nitrogen species, but they do not affect activity of NOC hence have limited use. We hypothesize that Jagged1 overexpression either reduces activities positive regulators of NOC or activates negative regulators to reduce cytoplasmic oxidative stress in dystrophy; the investigation of which is important for future but beyond the scope of this study at present.

Jagged1 over expression increases mitochondrial oxidative stress:

JC1 dye accumulates in mitochondria. In dimeric form it emits red fluorescence and green fluorescence as monomer. The reactive species reacts with dimer to convert them into monomers hence ratio of red to green is used to infer mitochondrial oxidative stress. Dystrophic mitochondria are known to produce more H2O2 which contributes to mitochondrial energy production. Energy starvation and low mitochondrial ATP production in DMD have been reported.

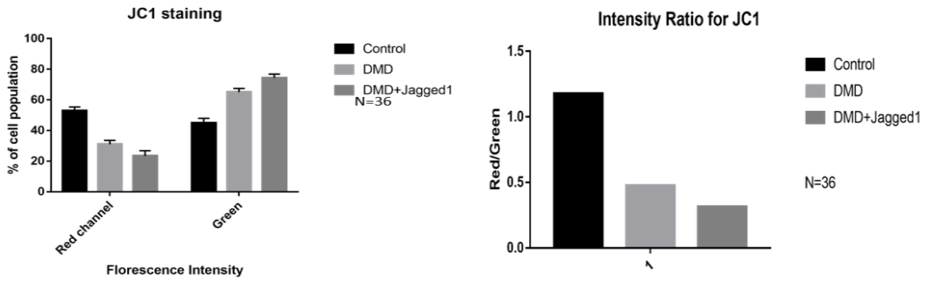

Fig 13: Representative Graph based on observed fluorescence intensities of ATP production

Hence, we expected that rescue might reduce the mitochondrial oxidative stress too. On the contrary, mitochondrial oxidative stress increases by jagged1 overexpression in dystrophic larvae, as is evident from the graph shown above.

This could be due to several reasons. Increased mitochondrial H_2O_2 production or increased electron transport chain activity, especially complex1 activity. An increase in fatty acid oxidation can induce higher ROS production from mitochondria. This possibility is more likely as fatty acid oxidation produces more energy than glucose oxidation, both repressed in DMD. Hence in energy starved muscles increasing fatty acid oxidation might be beneficial. The mitochondrial ROS has been seen as pathological and several attempts have been underway to reduce it. On the other hand, approaches to increase fatty acid oxidation or glycolysis as therapy have been very limited. This finding changes the therapeutic approach to this disease, hence is most important of the findings.

Jagged1 over expression reduces cell death in dystrophic muscles.

The ultimate result of all ROS, Calcium and mitochondrial ROS mediated pathology is muscle death that drives the disease progression. Hence, we next quantified the percentage of cells in various cell cycle stages and those undergoing programmed cell death. Propidium Iodide (PI) is not permeable to live cells. It is absorbed only by dead cells. Once inside cells it binds to DNA by intercalating between the nitrogen bases. Once it binds to DNA it is stable and emits red fluorescence which can be assessed by fluorescent microscopy or FACS. In FACS the inherent properties of cells and nucleus are to diffract the light that is captured as forward scatter and side scatter depending on size and content of cytoplasm and nucleus. The cell cycle analysis was carried out based on PI staining in fixed cells.

The first thing evident in the data above is that the total number of cells is drastically decreasing in dystrophic conditions. The cells in S-phase and G2/M phase are still present, while G0/G1 which represent the terminally differentiated cells are most affected. It is known that most cells in larval tails are muscles composed of terminally differentiated myofibers. These myofibers are fragile in dystrophic conditions, leading to frequent tearing upon slight mechanical disruption. The process of making single cell suspension leads to excessive loss of cells only in dystrophic group which agrees with literature. Hence, percentage of S phase, G2/M were compared only in DMD+Jagged1 with Control as dystrophic percentage are distorted except for apoptotic cells. The percentage of S phase

and G2/M phase were highly variable but comparable to control in DMD+Jagged1. On the other hand, percentage of G0/G1 is slightly but significantly higher than control in jagged1 rescue group which indicates differentiated muscles are more in the rescued dystrophic condition. The higher variability seen in S and G2/M phases in DMD+Jagged1 group could be due to random distribution of plasmid as this pathway is known to be involved in self-renewal of myoblast.

The original research group also claimed that the rescue by Jagged1 could be due to increased self-renewal of myogenic cells. However, the increase in mechanically strong terminally differentiated muscles is evident here. The percentage of apoptotic cells normalized to total cell number also decreased with the Jagged1 rescue as seen in the representative graph below.

Effectors of Jagged1-Notch pathway involved in the rescue.

The Jagged1-Notch pathway overexpression or higher activity has been associated with cancers. This has been the prime reason for not using it directly as a therapy despite the most dramatic rescue of DMD seen in the original "Escaper" GRMD. Hence it is important to find out effectors of this pathway which may not be oncogenic. The Notch receptors 1-4 can function as homodimers or heterodimers with variable outcomes increasing difficulty of discernment. The Notch 1-4 were quantified during various stages of development. The antibody against human Notch4 turned out to have epitope that is absent in zebra fish Notch4, instead bound to Notch1b in zebrafish hence no information could be generated with respect to Notch4. While Notch 1a and Notch1b were most abundant, no significant differences were found when Jagged1 was over-expressed in Notch 2 or 3 either. (Data not shown). It is also known that Notch receptor activity is transient and dependent on NICD cleavage rather than protein levels of Notch receptors present on membrane.

Hence, bonafide canonical effectors associated with all Notch receptors were checked in all experimental groups. Hes and Her family of transcriptional repressors are direct targets of Notch signaling. These effectors have redundant and overlapping functions in various tissues. Specific primers were designed with the help of NCBI primer blast, annealing temperature standardized, and real time PCR was carried out for expression level comparison. Following is the representative graph of log2 fold change in transcript levels.

As expected, injection of Jagged1 in control embryos leads to much higher expression of Notch effectors when compared with DMD+Jagged1. This could be due to several reasons. One could be that the energy required for high transcription activity is lower in dystrophic larvae than control larvae. The transcriptional changes are already present in dystrophic condition including altered Notch components. The Notch receptor involved in signaling could be different in control than in dystrophic condition. As none of the effectors of Notch receptors are differentially upregulated significantly in only DMD+Jagged1 condition; there is distinct possibility of receptor independent activity of Jagged 1 involved in the rescue. Jagged1 has been shown to activate JAK/STAT3 pathway non-canonically. STAT3 is overactive in DMD and has been shown to be involved in the pathology. Due to the extent of transcriptional and non-transcriptional, canonical, non-canonical activities of any Notch receptors which might be directly or indirectly involved, it is beyond the scope of this study to find more effectors.

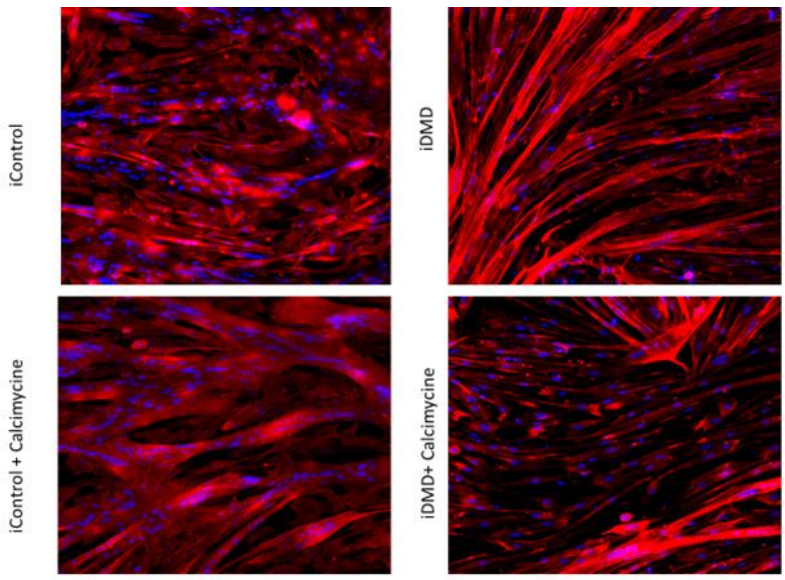

Fig 14: DMD myotubes with Calcimycine Treatment

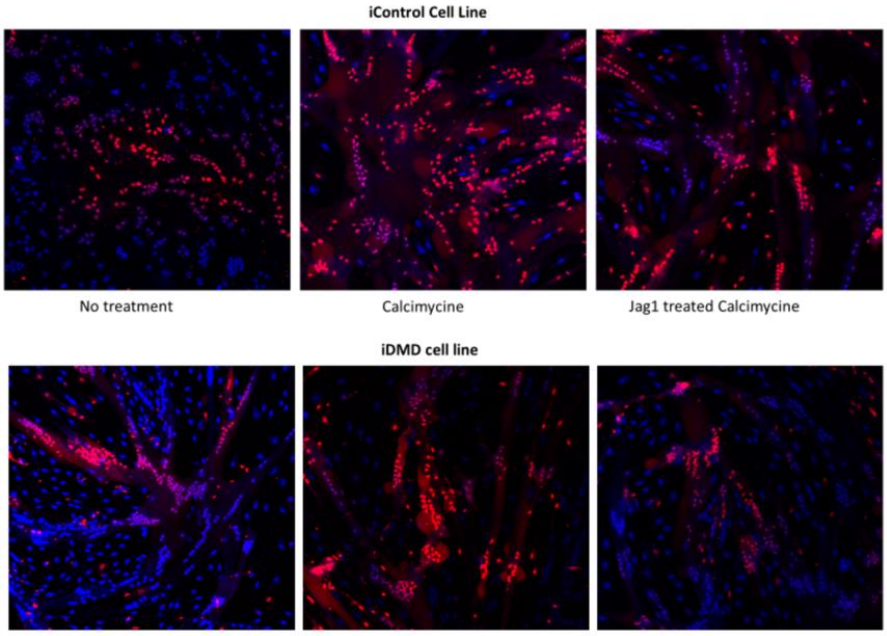

Fig 15: The Jag1 peptide treatment against Calcimycine

CONCLUSION

The complete rescue of DMD by Jagged1 has already been proven beyond doubt but its application has been limited due to lack of mechanism. Due to association of Jagged1with cancers, it is not feasible to use it as therapy directly. Hence this work is very important from the therapy development as it focuses on pathological aspects modulated by Jagged1 overexpression in dystrophic condition. This work is first report of The Jagged1 overexpression modulating several aspects of dystrophic pathology. The study found Jagged1 overexpression is increasing mechanical stability of dystrophic muscles hence reducing rate of pathogenesis at the source. The muscle cell damage and resulting cell death are reduced early in the disease progression. It preserves muscle function in dystrophic zebrafish larvae. It is already known in literature, function of Notch signaling in mRNA localization dependent translation of desmin and vinculin in muscle-tendon junctions, which are very important for load bearing during exercise. The therapeutic approaches to increase the expression of desmin, vinculin or other myotendinous proteins for DMD has been missing. This study points to a new direction for therapy development. The rescue involves the reduction of cytoplasmic ROS but increases in mitochondrial ROS which could lead to increased ATP production from mitochondria -which needs further validation. The increased mitochondrial ROS production under Jagged1 overexpression is one of the

most important findings as it highlights the critical assessment of causal factors and adaptation to disease. There are several trials ongoing to target mitochondrial pathology – which includes reducing oxidative stress which needs to be assessed more carefully. This finding also points to one more therapeutic approach – increasing fatty acid oxidation in DMD, which has not been explored. The rescue could be via non-canonical and independent of receptor signaling.

This study highlights careful discernment of pathological versus adaptive changes in DMD. It points out which aspects of pathology needs more attention than others. It points to couple of new and unexplored avenues of therapy for this debilitating disease condition.

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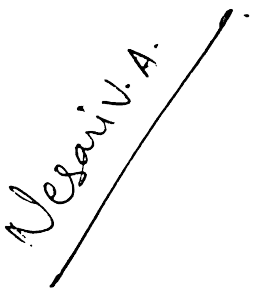

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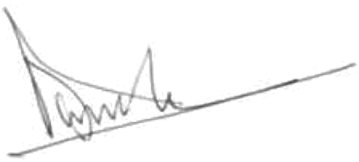

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REVIEW ARTICLE

Is the fundamental pathology in Duchenne's muscular dystrophy caused by a failure of glycogenolysis–glycolysis in costameres?

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Abstract. Duchenne muscular dystrophy (DMD) is the most common form of progressive childhood muscular dystrophy associated with weakness of limbs, loss of ambulation, heart weakness and early death. The mutations causing either loss-of-expression or function of the full-length protein dystrophin (Dp427) from the *DMD* gene are responsible for the disease pathology. Dp427 forms a part of the large dystroglycan complex, called DAPC, in the sarcolemma, and its absence derails muscle contraction. Muscle biopsies from DMD patients show an overactivation of excitation-contraction-coupling (ECC) activable calcium incursion, sarcolemmal ROS production, NHE1 activation, IL6 secretion, etc. The signalling pathways, like Akt/PBK, STAT3, p38MAPK, and ERK1/2, are also hyperactive in DMD. These pathways are responsible for post-mitotic trophic growth and metabolic adaptation, in response to exercise in healthy muscles, but cause atrophy and cell death in dystrophic muscles. We hypothesize that the metabolic background of repressed glycolysis in DMD, as opposed to excess glycolysis seen in cancers or healthy contracting muscles, changes the outcome of these 'growth pathways'. The reduced glycolysis has been considered a secondary outcome of the cytoskeletal disruptions seen in DMD. Given the cytoskeleton-crosslinking ability of the glycolytic enzymes, we hypothesize that the failure of glycogenolytic and glycolytic enzymes to congregate is the primary pathology, which then affects the subsarcolemmal cytoskeletal organization in costameres and initiates the pathophysiology associated with DMD, giving rise to the tissue-specific differences in disease progression between muscle, heart and brain. The lacunae in the regulation of the key components of the hypothesized metabolome, and the limitations of this theory are deliberated. The considerations for developing future therapies based on known pathological processes are also discussed.

Keywords. Duchenne muscular dystrophy; muscle; metabolism; glycolysis; therapeutic interventions.

The *DMD* locus, the protein complex of dystrophin and the disease aetiology

Duchenne muscular dystrophy (DMD) is a severe, childhood-onset, progressive muscle-degenerative disease. Clinical symptoms typically include progressive muscle weakness apparent by around 3–5 years of age, pseudo-hypertrophy of calf muscles, Growers' sign, joint contractures, and loss of ambulation by 10–12 years of age. Patients surviving beyond 20 years often develop heart weakness, kyphoscoliosis, and breathing difficulties due to the diminishing capacity of the respective muscles. Some patients suffer from nonprogressive cognitive delay or disability. Lung failure was the primary cause of death before ventilators became available; now, it is majorly attributed to heart failure (Wittlieb-Weber *et al*. 2020). A whole range of severity and disease progression is seen in patients with several genetic aberrations in the causal gene *Dystrophin* (*DMD/DYS)* (Bladen *et al*. 2015; Juan-Mateu *et al*. 2015).

The human *DMD* locus on chromosome Xp2.1 forms one of the largest genes, with 79 exons spanning 2.4 Mbp, coding for a ~14 kb mRNA (Koenig *et al*. 1987), which produces the protein dystrophin (Hoffman *et al*. 1987). The *DMD* gene transcribes several minor isoforms of dystrophin, abbreviated as Dp40, Dp71, Dp116, and Dp260, from internal promoters, but the absence of only the functional full-length isoform Dp427 is associated with DMD. The full-length isoform is expressed in skeletal muscle, heart, and brain from three different promoters. The two separate promoters produce Dp427b and Dp427p

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