Chapter 2

Materials and Methods

Ethical Approval: Protocols followed in the current study were approved by the Institutional Animal Ethics Committee (IAEC No. Z/IAEC-3/10-2019) of the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda. The IAEC is constituted by Committee for Control and Supervision of Experiments on Animals (CCSEA), Department of Animal Husbandry and Dairying. Ministry of Fisheries, Animal Husbandry, and Dairying, New Delhi. All the methods are reported in accordance with Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines.

1. CREATING IN-HOUSE ZEBRAFISH DMD MODEL

A. ZEBRAFISH MAINTENANCE

Zebrafish bought from the local aquarium were kept in 24" ×12" ×12" L×W×H glass tanks 2/3 filled with ~40 L water. Less than 25 adult fish were maintained in one tank to avoid overcrowding, and a temperature of ~28°C and 10 hours light -14 hours dark period was maintained. Fish were acclimatized for~ 1 month. Tap water was kept 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 were constantly on to clear the tank water of debris. Fish were fed three times in small quantities with a varied diet of micro-worms, brine shrimp, paramecia, cyclops, and aquarium fish food granules. Breeding tanks were attached to the side of the tank, and a single male and two females were kept overnight to obtain fertilized eggs. Viable eggs were collected by sieve from the breeding tank and cleaned of debris and fish excreta immediately. Eggs were transferred to E3 medium (Table 2.1) with drops of methylene blue. They were checked under a microscope every few hours, and dead eggs were removed. Eggs hatch between 24-48 hours under normal conditions, and fresh E3 medium was added daily. Less than 30 eggs were kept in a 6" diameter clean Petri plate.

After 4 days post-fertilization (dpf), the larvae were fed on paramecia, artemia hatchlings. Slowly after 7-8 dpf diet of micro-worms was introduced. Fish food granules were crushed and fed to larvae only after 25 days post-fertilization. Fertilized eggs were collected immediately from breeding tanks and injected within 30 minutes using the FemtoJet micro injector (Eppendorf™, Germany). The glass capillaries were pulled using a P-97

micropipette puller (Sutter Instrument Company, USA). The microinjections thus generated are always slightly variable in terms of diameter and stiffness. Hence, slight adjustment in injection parameters was made in terms of time, injection pressure, and compensation pressure between experiments.

Table 2.1: Composition of E3 Medium (Source: "The Zebrafish Book"- zfin.org)

Sr. No.	Components added in the order	Volume (ml)
1.	Hank's Stock #1 (8.0 g NaCl,0.4 g KCl, in 100 ml dd H_2O)	1.0
2.	Hank's Stock #2 (0.358 g Na_2HPO_4 Anhydrous,0.60 g KH_2PO_4, in 100 ml ddH_2O)	0.1
3.	Hank's Stock #3 (0.72 g $CaCl_2$, in 50 ml ddH_2O)	1.0
4.	Double distilled water (ddH_2O)	95.9
5.	Hank's Stock #4 (1.23 g $MgSO_4$ x $7H_2O$, in 50 ml dd H2O)	1.0
6.	Freshly prepared Hank's Stock #5 (0.35 g $NaHCO_3$, 10.0 ml dd H_2O)	1.0
7.	Use about 10 drops of 1 M NaOH to pH 7.2	
Store Hank's Premix (Stock 1-4) in the refrigerator along with Hank's solutions. Add stock 5 freshly.		

B. ZEBRAFISH MODEL OF DMD

An accepted DMD model is *Danio rerio (sapje),* with a stop codon in the fourth exon of the Dystrophin gene. The lack of dystrophin in zebrafish larvae 2 days post-fertilization shows a visible phenotype of the bent tail and smaller dysmorphic swim bladders. Under the bipolar microscope, the damage/lesion in tail muscles can be visualized as dark areas compared to the bright birefringence of normal muscles (Guyon et al., 2003). The zebrafish embryo is amenable to CRISPR/Cas9-mediated knockout. Since *DMD* is not duplicated in zebrafish, its knockout could be easily generated as a model of disease using CRISPR/Cas9 tool.

C. CRISPR/CAS9 TO CREATE KNOCKOUT OF DMD IN-HOUSE

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, part of the bacterial defense system against viral pathogens. These sequences have "PAM" motifs

identified and bound by the Cas9 enzyme, which can induce double-strand breaks to degrade viral genes. The enzyme requires a guide RNA complementary to the 22-25 nucleotides immediately before the "PAM" sequence. The resulting double-strand breaks are mostly repaired via Non-Homologous End Joining (NHEJ) pathway. The NHEJ pathway is not as error-free as recombination repair, leading to the addition or deletion of a few or long stretches of nucleotides during repair, which can disrupt the reading frame of the target gene. The system creates double-strand breaks in the gene of interest to create stable knockouts (Hwang et al., 2013).

Several shorter dystrophin isoforms might be essential for viability or development, so we chose only initial exons for the knockout. The loss of initial -N terminal exons gives rise to more severe dystrophy in both human patients and animal models. Thus, our strategy was to create a more severe dystrophic model for better assessment without affecting the functions of other isoforms of dystrophin.

D. Guide RNA

Cas9 activity needs "Guide" RNAs which are single-stranded RNAs that require at least a "SPACER" region that is complementary to the target region and hence will bind it by Watson-Crick base pairing. This usually ends with a "PAM" sequence on the template DNA. The sequence complementary to "PAM" is not included in "guide RNA"; 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 binding sgRNA or the opposite strand. Different organisms have different Cas9 enzymes; artificially mutated enzymes are also available based on need from "Addgene.org".

This technique's efficiency and off-target effects depend on the efficiency and specificity of the guide RNA sequence. Hence, several online tools like "CHOP CHOP" and "Cas Designer" were used to identify the best fit (Labun et al., 2019). Genomic sequence for the zebrafish Dystrophin gene (also called DMD gene) was downloaded from NCBI and Ensembl databases 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 wildtype Cas9 is used.

The sgRNA sequences were also checked for possible off-target by doing NCBI-BLAST. 66 nt long oligonucleotide with 20bp gene-specific region in the middle were pre-synthesized as primers by order as oligos given below (Table 2.2). The region in red color is the target sequence identified by a software tool that ends with "NGG," which is not made to be part of sgRNA. The constitutive sequence at the start of the oligonucleotide is a complementary T7 sequence. The constitutive sequence at the end is the scaffold region of guide RNA necessary for its successful folding and binding to Cas9.

The sgRNAs target sequences in the 6th and 7th exons in the zebrafish DMD gene. The possible guide RNAs in other exons were too far apart due to more extended intronic regions. Running genomic PCRs of such lengths to confirm knockouts would have been challenging. Hence the 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. A total of 5 guide RNA primers were designed. Initially, the resulting sgRNAs using these primers were combined with 3,4 and all 5 sgRNAs. It was found that a combination of 3 guide RNAs - numbers 2,3 and 5 was most efficient for knockout; hence, only these 3 were used for experiments. The guide RNAs can have toxic side effects; hence use was optimized to reduce mortality and increase knockout generation.

Table 2.2: The table shows complete-length primer oligonucleotides synthesized to be used for guide RNA synthesis.

1.	TTCTAATACGACTCACTATAGGGAGCGTTGAAAGCGAAACCGTGTTT TAGAGCTAGAAATAGCAAG
2.	TTCTAATACGACTCACTATAGGGAAACTACCAAGACGTCAACGGTTT TAGAGCTAGAAATAGCAAG
3.	TTCTAATACGACTCACTATAGGGCGAAAAGATCCTGTTAAGCTGTTT TAGAGCTAGAAATAGCAAG
4.	TTCTAATACGACTCACTATAGGGACTCTTCTCCGCAACACCGAGTTTT AGAGCTAGAAATAGCAAG
5.	TTCTAATACGACTCACTATAGGGAGAAGAGTTTAGGAATCGACGTTT TAGAGCTAGAAATAGCAAG
	The T7 promoter sequence, marked in red, is crRNA (target specific), followed by tracrRNA, which serves as a scaffold.

The *in vitro* sgRNAs were produced using the ABM sgRNA synthesis kit [G952: GeneCraft-R Classic CRISPR sgRNA Synthesis Kit (*S. pyogenes*)]. The primers described in the above table (Table 2.2) were first converted to double-stranded DNA with the help of First Strand Synthesis primers from Kit. The resulting dsDNA has a T7 promoter site

just before the transcribed region shown in red. When this dsDNA is used for invitro transcription, it produces RNA from the T7 promoter on the plus strand. The concentration was confirmed using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™, USA), and the product is run on 2% agarose gel to check for possible degradation.

Following is the computer screen snapshot during sequence alignment and features highlighted using Snapgene viewer software (Figure 2.1). The sequences in red color are "SPACERs" sequences used to generate single-stranded guide RNA (sgRNA).

Figure 2.1: The targeted genomic region of DMD in zebrafish showing guideRNA highlighted in dark green, exons 6 and 7 regions highlighted in grey, genomic PCR primers that would generate 700 bp product in light blue and bracketing longer 1200 bp product generating primers in magenta. Image made in the Snapgene tool.

The wildtype Cas9 was expressed with pCS2Cas9 plasmid containing CMV promoter, acquired from addgene.org to express Cas9 *in vivo* (Figure 2.2). Plasmid was cloned in transformed DH5-α *E. coli* strain. Single colonies were picked up from Ampicillin - Plates and grown overnight in liquid LB media. Plasmid was isolated using QIAGEN Plasmid Kit (QIAGEN, USA) using supplier protocol.

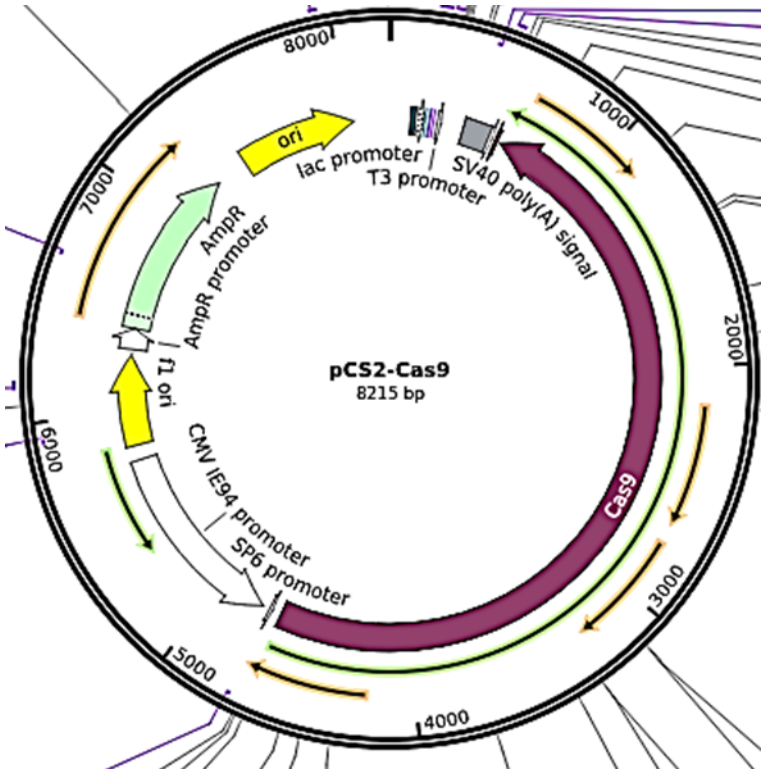

Figure 2.2: Map of the Cas9 expression plasmid (Source: Gagnon et al., 2014).

E. GENOMIC PCR

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

Isolated genomic DNA was quantified using NanoDrop™ spectrophotometer. 1ng of gDNA was used in the 20 µl PCR amplification with Taq DNA Polymerase and buffer from ThermoFisher Scientific™ (USA). The primers that were used in the reaction, had a final concentration of 0.5uM and the list of primer is appended below (Table 2.3). The Eppendorf thermal cycler (Eppendorf India Private Limited) was used with the following parameters: initial denaturation at 95ºC for 3 minutes -1 cycle, denaturation at 95ºC for 15 seconds, Annealing at 54ºC, 30 seconds, extension at 72ºC for 2 minutes -35 cycles and final extension at 72ºC for 5 minutes. The amplification product was run on 1.6% agarose gel with Ethidium Bromide, visualized under UV light (Gel doc, BioRad, USA), and imaged.

Table 2.3: Primer sequence for genomic PCR of DMD

Sr. No.	Name of Primer	Primer sequence	Size (bp)
1	Forward Primer DMD 6^{th} & 7^{th} exon	ACTGTATGTGCATCCTCTCC	700
2	Reverse Primer DMD 6^{th} & 7^{th} exon	TCTGTATCGCTCAGAACAG	700
3	Forward Primer DMD wildtype	GGTCAAAACCATGCTGTCATTACT	1200
4	Reverse Primer DMD wildtype	ACCCCCAAATCGAGCATCAAC	1200
5	Forward Primer 79exonDMD	TGTCAACAACTGGACCGGA	320
6	Reverse Primer 79exonDMD	TGAGTTGATAAAGCACCCCTGT	320

2. FOR OVEREXPRESSION OF JAGGED1 IN DMD MODEL

A. DH5-α TRANSFORMATION

DH5-α strain of *E. coli* was first made competent for transformation using the Calcium Chloride method. LB-Agar plates without antibiotics were poured in aseptic conditions and used to grow colonies by streaking with a cooled glass rod dipped in 100% ethanol and held in a 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 until OD reached 0.6-0.8. This represents the Log phase of growth at which they are maximally sensitive. The cells were pelleted and resuspended in 150mM $CaCl_2$ containing 15% glycerol. This step was repeated two times and preserved in a $CaCl_2$ mixture at -80°C. In this condition, they remain viable and transformation competent for six months.

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

Competent cells and DNA were thawed on ice for 20-30 minutes. 2-5 microliters of DNA, depending on concentration, were added to competent cells and incubated on ice for 20-30 minutes. Then heat shock at 42°C was given for 90 seconds, and vials were put back on ice. Volume was made to 1ml by adding fresh liquid LB media. Cells were grown at 37°C for one hour, pelleted down and resuspended on antibiotic-Agar plates by streaking 20-50 microliters. The following day colonies were counted for different concentrations of DNA

used, and transformation efficiency was calculated. A plasmid containing the gene of interest was isolated from the individual colonies grown in liquid LB media.

B. CLONING OF HUMAN JAGGED1

Jagged1 and its receptors - Notch are developmentally important; hence *in-vivo* Jagged1 expression must be undisturbed in zebrafish. Identification of overexpression was also needed. Therefore, human CDS sequence was cloned into an expression vector.

The pET28 vector with full-length human Jagged1 CDS was a kind gift from Prof. Rajan Dighe and Prof. Annapoorni Rangrajan, Department of MRDG, IISc, Bangalore. The specific primers were designed to clone the full-length Jagged1 mRNA sequence using the NCBI primer blast tool (Table 2.4). Restriction enzymes XbaI and HindIII were used to clone full-length human Jagged1 into PCDNA3.1 plasmid containing CMV promoter, which gives constitutive expression in mammalian cells and zebrafish. The plasmid was transfected in a transformed DH5α bacteria, which lacks recombination proteins; hence mutations in insert sequences are sporadic.

Table 2.4: Primer sequence for human Jagged1 full-length cloning

Sr. No.	Restriction enzyme used in primer	Primer sequence
1	XbaI_jag1 Forward Primer	ACCGAATTCATGCGTTCCCCACGGAC
2	HindIII_jag1 Reverse Primer	TTCTCGAGTACGATGTACTCCATTCGGTTTAAG

Colony PCR was used to confirm product length with the same primers. Selected colonies were used to isolate the plasmid, and the Blast alignment tool confirmed the sequence (Table 2.5).

Table 2.5: Full-length JAG1 in Expression Plasmid: pcDNA3.1 plasmid was sequenced and confirmed using NCBI align tool.

Sr No.	Gene	Sequence
1.	PCDNA31_PR	ATGCGTTCCC CACGGACGCG CGGCCGGTCC GGGCGCCCCC TAAGCCTCCT
2.	humanJAG1	ATGCGTTCCC CACGGACGCG CGGCCGGTCC GGGCGCCCCC TAAGCCTCCT
3.	PCDNA31_PR	GCTCGCCCTG CTCTGTGCCC TGCGAGCCAA GGTGTGTGGG GCCTCGGGTC

4.	humanJAG1	GCTCGCCCTG CTCTGTGCCC TGCGAGCCAA GGTGTGTGGG GCCTCGGGTC
5.	PCDNA31_PR	AGTTCGAGTT GGAGATCCTG TCCATGCAGA ACGTGAACGG GGAGCTGCAG
6.	humanJAG1	AGTTCGAGTT GGAGATCCTG TCCATGCAGA ACGTGAACGG GGAGCTGCAG

The dystrophic larvae showed an evident phenotype of bent tails, smaller or bent swim bladder, and reduced swimming. To test the rescue of dystrophic phenotype, muscle structure by bipolar imaging and muscle-specific immunostaining was done. For muscle function assessment in larvae, well-known methods like voluntary swimming and evoked escape assays were performed. In addition, cytoplasmic and mitochondrial ROS were compared with dye-based imaging methods. The known targets of notch signaling were quantified to identify possible downstream effectors involved in Jag1-mediated rescue.

3. FOR SURVIVAL, PATHOLOGICAL AND BEHAVIORAL ASPECTS

A. IMMUNOHISTOCHEMISTRY OF WHOLE LARVAL MUSCLES

Larvae of 4 dpf and 10 dpf were fixed in 4% Paraformaldehyde at 4°C overnight and then transferred to 100% Methanol for 24 hours at -20°C. The larvae were gradually rehydrated with 75%, 50%, and 20% methanol for 10-15 minutes and twice washed with PBS. They were incubated with a blocking buffer (made in PDT) for 1.5 hours. Then primary anti-dystrophin antibody (MANDRA-11 from DSHB, Iowa, USA) and anti-β-Dystroglycan (Mandag1 from DSHB, Cardiac Myosin Heavy Chain (MYH-7) (BA-F8 from DSHB, Iowa, USA), Notch 1 (b-TAN 20 from DSHB, Iowa, USA), Notch 2 (C651.6DbHNFrom DSHB), Notch-3 (PAL147Hu01 from Cloud Clone, USA), Notch 4 (PAL149Hu01 from Cloud Clone, USA) and Jagged1 (TS-1.15H from DSHB, Iowa, USA) were incubated overnight at 4°C and washed 3 times with PBST 15-20 minutes each. The secondary anti-mouse, anti-rat conjugated with fluorophores (in blocking buffer) was incubated for 2 hours. Nuclei were counter-stained with Hoechst for 40 minutes, washed before mounting on the slide with VECTASHIELD (Vector Laboratories, USA) as an anti-fade reagent, and imaged with Leica SP8 confocal microscope (Leica Microsystems, Germany).

B. PROTEIN ISOLATION

Adult zebrafish were anesthetized and euthanized by immersing them in ice water. Trunk muscles were dissected out and immediately frozen in liquid nitrogen. Embryos at various stages of development were dechorionated and immediately frozen in liquid nitrogen. They were crushed mechanically in lysis buffer, immersed in liquid N_2 a few times, and incubated at 4°C for 45 minutes. It was centrifuged at 12000 rpm for 10 minutes. The supernatant was collected in a fresh tube, and the pellet was discarded. Lysis buffer comprises 20mM Tris (pH7.8), 137 mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 1% Triton X-100, 10% glycerol (W/V), 1mM EDTA, 1mMDTT and a cocktail of protease inhibitors. Protein was quantified by the BCA method (ab102536; Abcam, USA).

C. WESTERN BLOT

This is a sensitive method for quantitative or semi-quantitative measurement of the amount of protein present in the sample. The protein sample was prepared in loading buffer, a mixture of beta-mercaptoethanol to reduce disulfide bonds in proteins, glycerol to make the sample solution sink in the well, and bromophenol blue to visualize the sample. Equal amounts of protein sample (20-50ug) in loading buffer were heated at 95°C for 5 minutes 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 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 the percentage of Polyacrylamide, which APS and TEMED crosslink. The proteins we are studying include Dystrophin (427KDa), Notch (1,2,3,4) receptors with a range of 206 to 270KDa, Jagged1 with a size of 135 KDa in zebrafish and 133KDa in humans. 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 were transferred to the PVDF membrane. The PVDF membranes were kept in a blocking buffer (2-5% BSA dissolved in TBST) for 2 hours. Incubated overnight with antibody for the protein of interest at 4°C, washed 3 times, 15 minutes each with TBST. The membrane was incubated in Blocking Buffer for 2 hours. Incubated with secondary – HRP conjugated antibody (Thermo Scientific™, USA) for 2-3 hours and rewashed. Blot was developed by incubating it with a 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 was captured in a ChemiDoc Imaging Systems (BioRad, India).

C. 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. 300 µl of TRIzol reagent was added to larvae after water was removed entirely from 1.5 ml centrifuge tubes and immediately immersed in liquid nitrogen and crushed, after that, incubated at 4°C for 15 minutes. 100ul of chloroform was added, mixed by inversion, and kept at room temperature for 10 minutes. Next, tubes were centrifuged at 12000g for 15 minutes at 4°C. The aqueous phase was carefully removed without touching sides or interphase into a fresh 1.5 ml centrifuge tube. 100 µl of Isopropanol was mixed by inversion, incubated for 10 minutes at room temperature, and centrifuged 12000g at 4°C for 10 minutes to pellet RNA. The pellet was washed with 70% ethanol and centrifuged at 8000g at 4°C for 8-10 minutes. The ethanol was removed gently, and tubes were inverted to dry on tissue paper for 3-4 minutes. RNAase-free water was added and mixed by tapping for 1min. Concentration was measured in the NanoDrop™ 2000 Spectrophotometer 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 an agarose gel.

D. cDNA SYNTHESIS OLIGODT METHOD

Most eukaryotic mRNAs have poly-A tails to which the Oligonucleotide of Ts can only bind and act as a primer for first-strand synthesis. mRNA was converted to cDNA by the enzyme Reverse Transcriptase in the presence of dNTPs, and the mixture was incubated at 42°C. RiboLock RNase inhibitor was added to avoid RNA degradation. Both mixture-Enzyme and mixture-template were used as control. The enzyme was inactivated by incubating at 70°C for 10 minutes. cDNA was quantified. PCR for GAPDH was carried out using cDNA as a template for quality check.

E. QUANTITATIVE PCR

To assess the expression levels of housekeeping genes in the context of dystrophic changes, we focused on the housekeeping genes β-actin and microβ-globulin instead of GAPDH which is known to differentially express during DMD pathogenesis, making it unsuitable as reference (Hildyard et al., 2019). Real-time PCR (RT-PCR) was performed using RNA samples from both control and DMD conditions at different concentrations. Ten larvae from each injection group were randomly collected and frozen in TRIzol solution. RNA was isolated by the standard TRIzol method. The RNA integrity was checked on 2% agarose gel electrophoresis. 1µg of RNA was converted to cDNA with a BioRAD cDNA synthesis kit (cat no. 1708890) following the manufacturer's instructions. The primers specific to Hes1, HeyL, Hey1, Her6, Her9, and Her12 using NCBI BLAST against their CDS specific to zebrafish were used (Table 2.6). The quantitative PCR was done using the SYBR Green method on the StepOnePlus™ Real-Time PCR System (Thermo Scientific™ USA).

Table 2.6: Primer sequences of zebrafish Notch target genes used for Real-time PCR

Sr. No.	Gene	Sequence
1.	βActin Forward Primer	CCAGCCATCCTTCTTGGGTAT
2.	βActin Reverse Primer	CTTCATTGTGCTAGGGGCCA
3.	Microβglobulin Forward Primer	TGGCTAAAGAAACTCCCCCA
4.	Microβglobulin Reverse Primer	TGGTGATGTCAGGAGGGTGA
5.	HeyL Forward Primer	TGGGTGGCAAAGGCTACTT
6.	HeyL Reverse Primer	CACCGATGGGGTCTGATGAT
7.	Hey1 Forward Primer	AAAACGTCGCAGAGGGATCA
8.	Hey1 Reverse Primer	CCTGTTTCTCAAAGGCGCTG
9.	Hey2 Forward Primer	GGCCACAGGAGGAAAAGGATATT
10.	Hey2 Reverse Primer	GCTGCTGAGGTGAGAAACCA
11.	Her6 Forward Primer	ACAAATGACCGCTGCCCTAA
12.	Her6 Reverse Primer	GGCGTTGATCTGTGTCATGC
13.	Her9 Forward Primer	ATCACCTATTGCTGGTGCCC
14.	Her9 Reverse Primer	GCGCTTTTCCATGATTGGCT
15.	Her12 Forward Primer	ACAACATAAAGCAAGCTGTTCG

16.	Her12 Reverse Primer	CTATCGGCTTCCTCAGCTTTATT
17.	Human Jagged1 Forward Primer	AGTGCCTCTGTGAGACCAAC
18.	Human Jagged1 Reverse Primer	TTGCTACAAGTTCCCCCGTT

4. VALIDATION IN HUMAN MYOBLASTS

A. HUMAN DMD CELL LINE AS AN *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. Based on the formula given below, the cell counting per ml was done, and accordingly $2*10^5$ were plated for each experiment:

(Mean number of cells × Dilution factor)÷1000

When cells reached 70-80% confluence, the medium was removed, and dishes were rinsed with 10ml F10 (or PBS). The cells were incubated with 2 ml of trypsin for 5-10min at 37°C, 5% CO_2, and checked under an inverted microscope for cell detachment.

When cells floated, 3ml of proliferation medium was 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, used the following formula:

Division number= log(counted number of cells ÷ number of seeded cells) ÷log2

The growth media (GM) protocol, as shared by Vincent Mouly from Institute de Myologia, France, consists of the following:

Table 2.7: List of antibodies used in immunolocalisation.

1	1Vol medium 199	41150020	Invitrogen
	4Vol DMEM	61965-026	Invitrogen
2	20% FBS	26140079	Gibco®
Supplemented by			
3	Fétuin: 25µg/ml	10344026	Life Technologies

4	hEGF: 5ng/ml	PHG0311	Life Technologies
5	Bfgf: 0.5ng/ml	PHG0026	Life Technologies
6	Insulin: 5 µg/ml	91077C-1G	Sigma
7	Dex: 0,2µg/ml	D4902-100mg	Sigma

The six healthy plates were coated with Matrigel-GFR diluted 1:20 in DMEM and incubated for 2 hours at 37°C. The cells were induced to differentiate when confluence reached ~70% by changing media containing DMEM with 10 µg/ml Insulin and 50 µg/ml Gentamicin (DM). The cells were left to differentiate in DM for 5-6 days, changed the media as and when required. The myoblasts fused and differentiated into multinucleated myotubes.

B. CALCIMYCINE TO RECAPITULATE CONTRACTION-MEDIATED DAMAGE IN MYOTUBES

The ionophores interfere with channels and cause continuous calcium ingress in myotubes, simulating sustained muscle contraction's effects. Based on previous studies, 5µM 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.

C. 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 that have been used to immortalize myoblasts. Hence, we used 17 amino acids length peptide fraction of Jag1 that can act as a ligand (Nickoloff et al., 2002) to study the effect of Jag1 during the differentiation window that best corresponds to myogenin expression in *in-vitro* cultures. This allowed the examination of the possible effect of myogenin-mediated Jag1 upregulation, which was not possible in the *in vivo* zebrafish model.

Table 2.8: Primer sequences of human Notch target and myogenesis genes used for Real-time PCR

Sr. No.	Gene Name	Sequence
1.	QP_hu_Hes1_FP	ACACGACACCGGATAAACCAA
2.	QP_hu_Hes1_RP	AATGCCGCGAGCTATCTTTCT
3.	QP_hu_Hes4_FP	GAGCCTCGCTCAGCTCAAA
4.	QP_hu_Hes4_RP	CAGGTGTCTCACGGTCATCT
5.	QP_hu_Hes5_FP	AGAGAAAAACCGACTGCGGA
6.	QP_hu_Hes5_RP	GACGAAGGCTTTGCTGTGCT
7.	QP_hu_HeyL_FP	CGTTCGCCATGAAGCGAC
8.	QP_hu_HeyL_RP	CCGTTTCTCTATGATCCCTCTGT
9.	QP_hu_Hey1_FP	TGCGGACGAGAATGGAAACTTG
10.	QP_hu_Hey1_RP	TTGCTCCATTACCTGCTTCTCAA
11.	QP_hu_Hey2_FP	CCGCTAGGAGCAGACCGC
12.	QP_hu_Hey2_RP	GGTCTCGTCCATGTCGCTCT
13.	QP_hu_Hes3_FP	GAAACACTACTCGCACCAGATCC
14.	QP_hu_Hes3_RP	TTGCAAGGAGTTCTGAAGGCT
15.	QP_hu_Bactin_FP	CTTCGCGGGCGACGAT
16.	QP_hu_Bactin_RP	ACATAGGAATCCTTCTGACCCA
17.	QP_hu_B2M_FP	AGCAGCATCATGGAGGTTTG
18.	QP_hu_B2M_RP	CAAACATGGAGACAGCACTCA
19.	QP_hJAG1FP	ACTAAGCTTAGTATATTAGAGCCGGGACG
20.	QP_hJAG1RP	CAGGATCCTTTACGATGTACTCCATTCG
21.	QP_hMYOgFP	ATCCAGTACATCGAGCGCCT
22.	QP_hMYOgRP	GAGCAGATGATCCCCTGGGTTG
23.	QP_hMyoD1FP	CACGTCGAGCAATCCAAACC
24.	QP_hMyoD1RP	TGTAGTCCATCATGCCGTCG

Statistical analysis

GraphPad Prism 7.0 software (GraphPad Software Inc., USA) was used to analyze and generate graphs. Image J software measured the fluorescence intensity for Images of IHC. The specific methods are described in detail as a part of the chapters as required.