Chapter1

Introduction

ETIOLOGY OF DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is one of the most common inheritable childhood-onset, severe, progressive muscle degenerative diseases. The progressive decline of skeletal and heart muscle function results in early death from lung or heart failure. Despite its X linkage, a recent study found 48% of *de novo* mutations in patients when sequenced three generations of patients in a cohort of 20 families (Shasthri et al., 2021). The *de novo* mutation rate is reported to be as high as 71% in a study that collected data from 110 unrelated families (Murugan et al., 2013). This suggests that the associated gene locus is more susceptible to mutations than other significant genes, though its reasons are poorly understood. The incidence rate has been reported as 1: 3000 to 1: 6000 live male childbirths globally (Bushby et al., 2010). In India, the national occurrence rate has yet to be reported. However, some hot spots of DMD have been suggested where regional studies have found higher rates than 1:3000. Epidemiologically, DMD is considered the most common form of childhood dystrophy (Nalini et al., 2017). This disease has been known for more than 150 years- the first description of the condition was given by Meryon (1852) and Duchenne (1861), after whom the disease was named. Clinical symptoms typically include progressive muscle weakness that becomes apparent around 5 years of age, pseudo-hypertrophy of calf muscles, Grower's sign - use of arms to get up from the floor (Gowers,1879), joint contractures and loss of ambulation by 12 years of age. The average life expectancy of these patients is about 25 years. Those patients surviving beyond 20 years often develop heart weakness or complication, the curvature of the spinal cord (kyphoscoliosis), difficulty in maintaining posture and difficulty in breathing due to loss of respective muscles. Many patients also suffer from cognitive delays or disabilities. A whole range of severity and disease progression is seen in patients, still the quality of life for the patients and their families decreases drastically.

DMD LOCUS

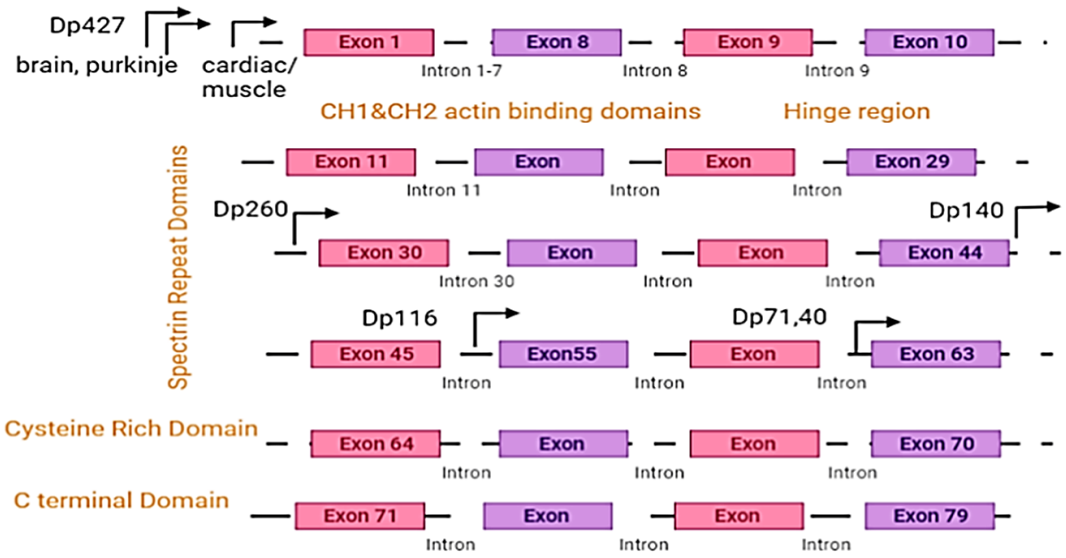

Figure 1.1: The DMD/DYS locus map: The black arrows mark 3 promotors encoding full-length transcripts from exon1 -Purkinje, brain, heart/skeletal muscle. The internal promoters encode smaller isoforms Dp260, 116,140,71 and 40. Exons 1-12 encode actin binding and hinge domains, exons 11-63 encode spectrin repeat domains, exons 64-70 encode cysteine-rich domain, and exons 71-79 encode C-terminal domain.

Mutations in the Xp21located DMD gene encoding dystrophin are the primary cause of this disease. Due to X linkage, it is inheritable from the mother to 50% of male progeny. It is one of the largest genes with 79 exons, 2100 Kbp long pre-mRNA transcript (Monaco et al., 1986; Hoffman et al., 1987; Tennyson et al., 1995), that is spliced in a developmental stage as well as tissue-specific manner (Feener et al., 1989; Bies et al., 1992; Doorenweerd et al., 2017). This locus has 7 verified promoters (Blake et al., 2002; Muntoni et al., 2003), 15 shorter alternate transcripts that give rise to shorter isoforms truncated from N-terminal and C-terminal expressed in many tissues, and their functions are currently an active area of research. Only the brain, heart, and skeletal muscles express full-length transcripts, though slight expression has also been reported from smooth muscles of the gastrointestinal tract (Koeing et al., 1987). The full-length transcript translates to 427KDa sized dystrophin isoform (Dp427), which contains two-actin binding domains, a string of spectrin-like domain repeats followed by a C-terminal domain. Dp427 is expressed in the brain in the hippocampus, cerebellum, cerebral cortex, and amygdala (Lidov et al., 1990; Bies et al., 1992; Knuesel et al., 2000; Sekiguchi et al., 2009). Two promoters, one in Purkinje cells and another in the cortex, give rise to slightly different N-terminal domains of full-length dystrophin isoforms in the brain. In some DMD patients,

cognitive delay is observed, though neurodegeneration or progressive decline in cognitive function is not seen. Only cerebral and cerebellar hypometabolism is reported in DMD patients (Bresolin et al., 1994). The significance of the Dp427 isoform in the brain is unclear, as the shorter Dp140 is predominant in the brain and has been shown to affect cognition without muscle dystrophy (de Brouwer et al., 2013). The dystrophin expression from a common promoter between heart and skeletal muscle explains cardiac phenotype in the heart of DMD patients. When this skeletal or heart muscle promoter is mutated, X-linked Dilated Cardiomyopathy arises without skeletal muscle symptoms due to the absence of dystrophin in the heart (Muntoni et al., 1993). The reasons behind this are poorly understood (reviewed by Nakamura, 2014) but are suggested to be due to differential brain promoter activity between skeletal muscle and heart (Muntoni et al., 1993). Despite the same promoter, transcript and protein required in both tissues, it is unclear why cardiac weakness (in DMD or XLDCM) takes a decade longer to manifest than skeletal muscle.

Databases now have extensive information on mutation and patient life history. However, there is no clear correlation between genotype-phenotype for DMD. The only correlation apparent is that N and/or C-terminal loss increases the severity of the disease more than loss of internal exons, which code for spectrin-like repeat domains.

The same gene locus is also responsible for Becker's muscular dystrophy, where disease onset is late, life expectancy is average, muscle loss is less severe, and ambulation is mostly preserved till old age. In BMD, mutations lead to smaller and partially functional form – mini dystrophin expression owing to retention of the translational reading frame. In DMD, mutations cause changes in the reading frame (Monaco et al. 1986), leading to loss of functional dystrophin expression. The reading frame rule is widely accepted (Aartsma-Rus et al., 2006) and found to be true for about 90% of mutations in the meta-analysis on various DMD sequence databases (Bladen et al., 2015; Juan-Mateu et al., 2015) to distinguish BMD and DMD however, there is some disagreement among experts (Kesari et al., 2008).

DYSTROPHIN PROTEIN

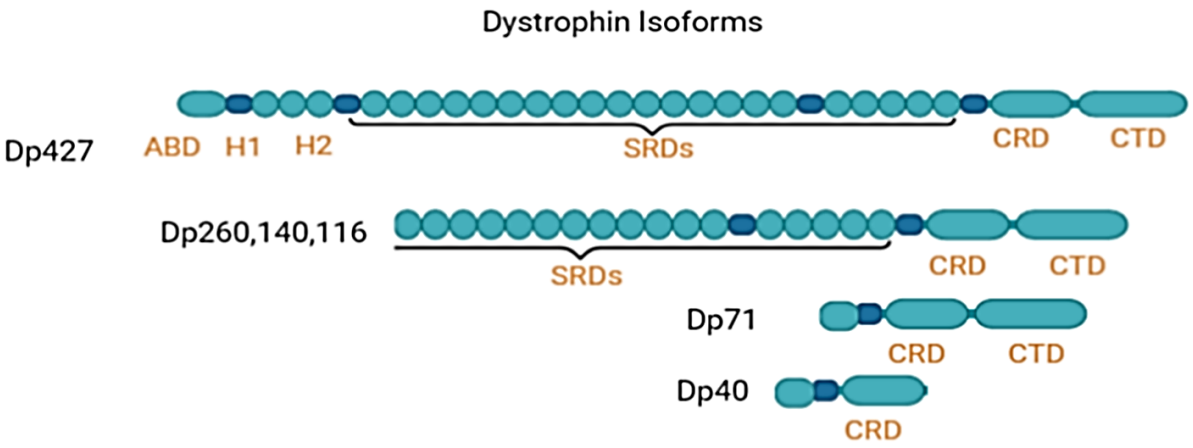

Figure1.2: Structure of dystrophin isoforms: Dp427 is a full-length isoform with Actin Binding Domain (ABD) in N-terminal, small Hinge domains (H1 andH2), a string of spectrin-like repeat domains (SRDs), Cysteine Rich Domain (CRD) and C-terminal Domain (CTD). The shorter isoforms Dp260,140,116 etc., have alternate N-terminal, lesser number of SRDs and similar CRD, and CTD. The shortest isoforms Dp71 and 40 with alternate N-terminal and later isoforms lacking CRD.

Full-length protein dystrophin expressed in the brain, heart and skeletal muscle is composed of (A) 2 actin binding (Calponin Homology- CH1 and CH2) domains in the N-terminal region, (B) 24 spectrin-like domains forming flexible middle rod region, (C) cysteine-rich domain and (D) protein binding C-terminal region.

The C-terminal part is thought to be responsible for the formation of Dystrophin Associated Protein Complex (DAPC) through binding β-Dystroglycan at the plasma membrane (Campbell and Kahl., 1989; Suzuki et al., 1992; Jung et al., 1995; Ishikawa-Sakurai et al., 2004; Hnia et al., 2007). The shorter isoform composed mainly of the C-terminal region called Dp71 is anticipated to function as a tumour suppressor (Suárez-Sánchez et al., 2016) via its ability to form DAPC in nuclear membranes of mouse neurons (Rodríguez-Muñoz et al., 2015) and bind to nuclear lamins (Villarreal et al., 2011; Suárez-Sánchez et al., 2014).

The spectrin-like domain (SRD) repeats forming the flexible middle rod- were initially thought to be of little importance as large deletions result in mild DMD phenotype or BMD (England et al., 1990; Ascadi et al., 2012). However, more recent genetic approaches to express dystrophin revealed that a minimum of 7-8 of these domain repeats are required to rescue the *mdx* model, which is not a severe model of DMD compared to human patients. Zhao and co-

workers (2016) showed the presence of many sarcolemma-binding domains in the dystrophin SRD region, which is thought to function towards shock absorption during muscle contraction.

N-terminal has 2 CH_2 domains that can bind actin filaments and a very small flexible region that is not big enough to be a designated domain. This very small flexible region composed of a few residues differs between all three full-length dystrophin isoforms – two in the brain and one typical between skeletal and heart muscles. Utrophin-a paralogue of dystrophin, has a similar structure and carries out the same function in prenatal muscles. Utrophin also has N-terminal CH_2 domains that bind to actin filaments with higher affinity than dystrophin and is shown to be capable of forming functional DAPC at the sarcolemma, thus rescuing muscle pathology in *mdx* mouse model of DMD (Tinsley et al., 1996, 2011; Peladeau et al., 2018). It should be noted that utrophin protein levels are already high in patients (Nguyen et al., 1991) nevertheless, its replacement with dystrophin at Dystroglycan Complexes (DGCs) does not occur (Nguyen et al., 1991; Gramoloni and Jasmin, 1999).

DMD PATHOLOGY

DMD was first characterized as a metabolic disease. However, lack of mechanical integrity was considered the primary cause following the identification of the DMD gene locus and its product dystrophin based on its structure and binding partners. Though, oncogenic and tumour suppressor functions in cancers of myogenic and non-myogenic origins have been suggested (Jones et al., 2021; Wang et al., 2014). The function of full-length dystrophin is still unclear in brain or muscle tissues. In muscles, though, mechanical function is widely accepted for Dp427 by connecting extracellular matrix components to actin filament bundles inside the cell through the DAPC, also called DGC, because most proteins of this complex are glycosylated. In the submembrane regions, dystrophin with other intermediate filaments like desmin and vinculin form an active cytoskeleton with F-actins, microtubules termed “costameres”. Hence, dystrophic muscles are ineffective in maintaining or transmitting mechanical forces during muscle contraction, leading to increased damage. The merits and lacunae of this hypothesis are discussed in the following sections in detail. The irrefutable fact is that exercise leads to damage (Mokhtarian et al., 1999) as well as atrophy in dystrophic muscles instead of growth, as seen in healthy muscles. The increased incidences of damage induce the inflammatory response required for tissue regeneration.

Nevertheless, continuous damage generates areas in different stages of inflammation-repair, thus leads to asynchronous regeneration (Dadgar et al., 2014). Additionally, the presence of Dp427 in replicating muscle-stem cells called satellite cells (Dumont et al., 2015; Chang et al., 2016) suggests that poor regeneration could directly result from absence of dystrophin. The degeneration-regeneration cycles and associated inflammation cause exhaustion of myogenic potential (Blau et al., 1983), fibrosis and ultimately, loss of muscle function. Despite extensive research that derived mechanistic details of these pathological processes, the causal relationship between pathological processes in dystrophic muscles has remained unclear.

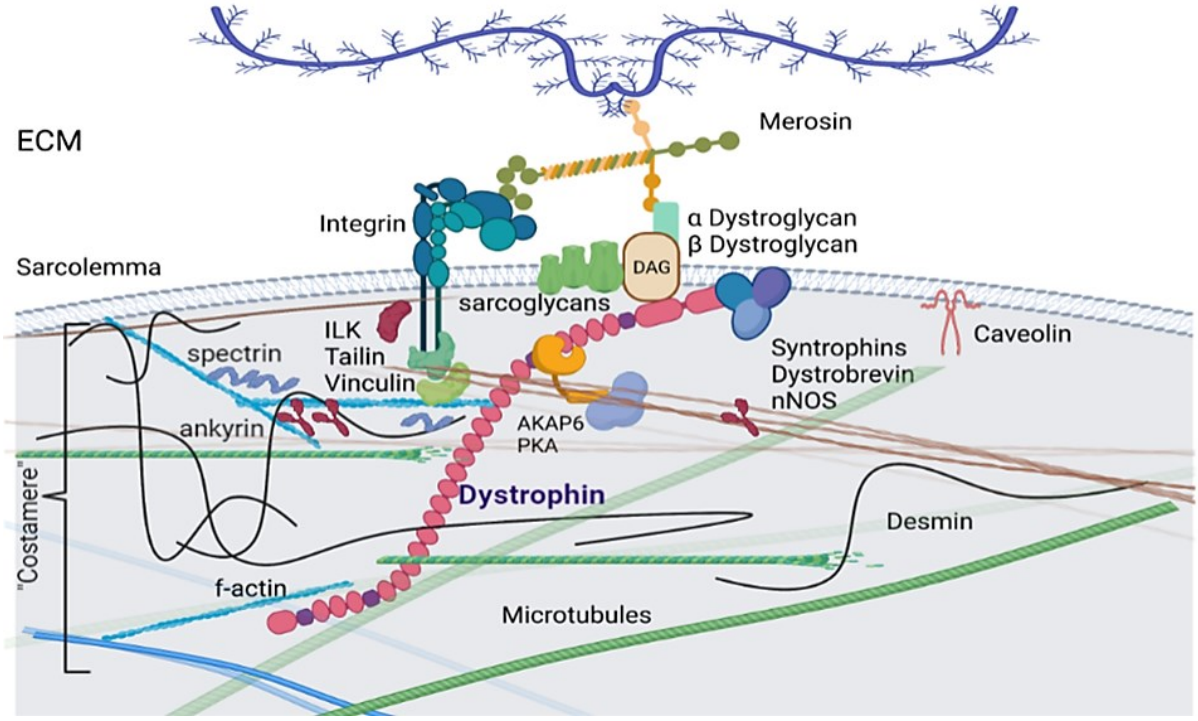

Figure1.3 The DAPC and Costameres: The Dystroglycans, Sarcoglycans, Syntrophins, Dystrobrevins, and Integrins form two major signaling complexes in the membrane, which are connected to the ECM on the outer side and cytoskeleton called “Costameres” inside the cell. The costameres comprise non-sarcomeric actin, actinin, ankyrins, spectrin, intermediate filaments like desmin, vinculin, dystrophin and microtubules (Adapted from Fairclough et al., 2013; Mukund & Subramanium, 2020).

MOLECULAR MECHANISMS OF DMD PATHOPHYSIOLOGY

The Dp427 expresses during satellite cell proliferation, but myoblast stages do not require dystrophin during self-renewal or myotube formation (Dumont et al., 2015). The dystrophin expression and myogenin mark the completion of muscle fiber maturation (Yoshimoto et al., 2020). Accordingly, muscle formation in the absence of dystrophin remained normal (Blau et al., 1983; Sacco et al., 2010).

The vertebrate muscle is formed by myoblast cell fusion and elongation to form multinucleated myofibril covered with a single sarcolemma. Myofibrils are collected in additional extracellular matrix- coverings called hypophysis and epiphysis. The single motor unit comprises all the muscle fibers of different types innervated by a single neuron and simultaneously excited. The primary function of skeletal muscle is to convert the chemical energy of ATP into mechanical force carried out in the tandemly repeated units of an actin-myosin assembly called sarcomeres. The fast glycolytic type, slow oxidative type and mixed type fibers are classified based on (as their names suggest) metabolism and rates of ATP lysis/use by myosin heads for force production. The composition of fiber types varies in a given muscle in different locations in the body. This metabolic and functional segregation of fiber types is functionally significant and extraordinarily malleable in adaptation to nutritional or hormonal changes and intensity-duration of exercise and training. The elemental fiber composition of a given muscle in the body is not known to be affected in DMD. The fast glycolytic type fibers from a given muscle are known to be more susceptible to activity-induced damage in DMD (Webster et al., 1988).

The motor neurons release acetylcholine at NMJ, which binds to its nicotinic receptors on sarcolemma opening ion channels, causing ingress of Na^{+} ions, thus membrane depolarization that is carried throughout muscle via T tubules. Membrane depolarization further activates voltage-gated ion channels – most notably the voltage-gated Ca^{+2} channels so that extracellular Ca^{+2} enters sarcoplasm near the Ryanodine Receptor (Ryr) channels in the sarcoplasmic reticulum (SR), which are Ca^{+2} activated Ca^{+2} release channels. The resulting Ca^{+2} spike seen in stimulated muscles is an integral part of Excitation Contraction Coupling (ECC). The excess calcium then changes troponin/tropomodulin conformations to allow myosin heads from the thick filament to bind thin (actin) filaments of the sarcomere to initiate contraction. Several calcium-dependent proteins – the calmodulins, CaM Kinases, Phosphatases etc. also get activated, which are essential in supporting muscle contraction indirectly. The calcium also augments ROS production required for signalling during muscle contraction. The mechanical

stretch-mediated ROS production also occurs in the sarcolemma region via NADPH oxidase complex (NOC), which can, in turn, augments calcium and sodium ion channel activities due to the activation of sodium-hydrogen exchanger (NHE). Calcium also activates pyruvate dehydrogenase phosphatase (PDP) – which activates the PDH complex to increase mitochondrial metabolism to meet the increased ATP need of muscle contraction. In addition to sarcomere-associated signaling complexes directly activated by contraction (Mukund and Subramaniam, 2020), p38MAPK, ERK1/2, JNK signaling pathways etc., also get activated and contribute to exercise-mediated growth in healthy muscles.

It is important to note that ECC is typical in DMD (Mancinelli et al., 1989). The ECC-associated calcium ingression (Blake et al., 2002), stimulated glucose uptake (Schneider et al., 2018), ROS from NADPH oxidase complex (Xia et al., 2003), NHE activation (Fliegel, 2019), p38MAPK (Brenan et al., 2021), as well as ERK1/2 (Taylor et al., 2012) activation occur in healthy exercising muscles too. In this situation, these responses occur either in excess, or outcomes turn pathological in the absence of functional Dp427, reasons for which are not clearly understood, as discussed in the review published by our group (Nesari et al., 2023). Additionally, nutritional pathways like insulin and IGF Akt/PKB are also active in dystrophic conditions. The prevalent theories of initiation of pathology, like mechanical stress, membrane fragility, increased ROS, $Ca^{+2,}$ and altered metabolism, are described here. Our alternate hypothesis of pathology initiation is in the last chapter.

THEORY OF MECHANICAL FRAGILITY IN PATHOLOGY

It is observed that in the absence of dystrophin, DAPC formation is significantly reduced (Ervasti et al.,1991; Ohlendieck et al.,1991). On the contrary, studies (Johnson et al., 2013) have found that only a small fraction of DGC contains dystrophin, and DGC is formed at the membrane without both utrophin and dystrophin, respectively, thus casting doubts on such hypotheses. Though functions of the DAPC complex are affected in DMD, mutations of most DAPC components do not give rise to a DMD-like phenotype. Out of about 28 DAPC proteins, only 2 are known to cause very severe muscular dystrophies (Ehmsen et al., 2002). Absence of Laminin α-2 is known to cause one form of severe congenital muscular dystrophy termed MDC1A and the other form caveolin3 involved in clinically heterogenous groups of Limb Girdle Muscular Dystrophies (LGMDs)-none of these involves membrane damage. The rest of the DAPC proteins are associated with different milder diseases (Shin et al., 2013).

The concept of costameres emerged during the 1980s (Pardo et al., 1983), which is cytoskeletal arrangement below the muscle membrane connected to the Z lines of sarcomeres and the protein complexes in sarcolemma which are in turn attached to the ECM (Peter et al., 2011). It has been suggested that the Z lines from the interior of muscles transmit the force horizontally to the plasma membrane via costameres. The DAPC and integrin complex are two major signalling hubs in the sarcolemma. On the outer membrane, they are connected via laminins in ECM which can bind both α-Dystroglycan and integrin. On the cytoplasmic side, these are connected via costameric components, which are thought to keep these complexes aligned with Z lines during muscle contraction. These comprise non-sarcomeric Actins, Actinins, Spectrins, Desmin, Vinculin, Talin etc. The costameric F-Actin and microtubules undergo increased dynamics of de-polymerization and reformation during muscle contraction, which requires ATP, known to be low in DMD. Hence, we argue that a lack of glycolysis-derived energy is more likely to affect the costameric cytoskeletal formation and result in fragility.

THE ROLE OF OXIDATIVE STRESS

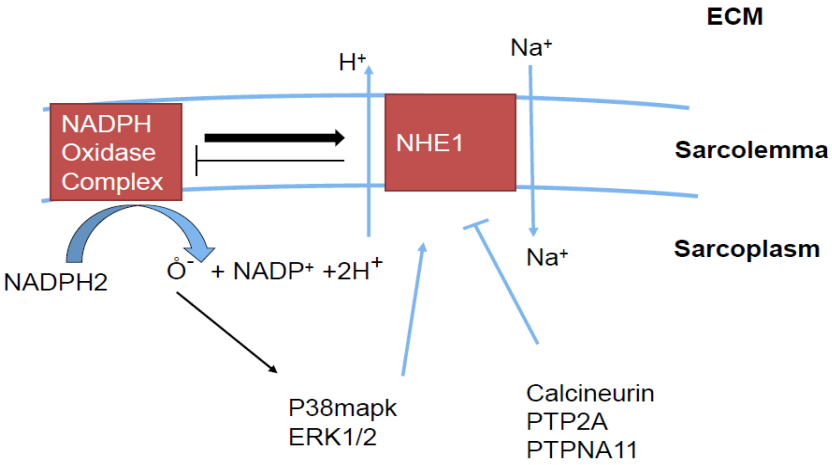

Figure 1.4 Pathogenic ROS production in DMD: The NADPH oxidase complex produces hydrogen ions that can activate the NHE1 exchanger, which results in the augmentation of sodium influx and the continuation of NOC activity. Several factors like kinases, phosphatases, and microtubules regulate the NHE1 activity.

According to Rando's (2001) two-hits model – the loss of 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 NOC in the muscle cell membranes to produce ROS (Prosser et al., 2012). It is now known that during muscle contraction, the NADPH oxidase in the sarcolemma and SR generates ROS from O_2 by oxidizing NADPH2 to $NADP^+$ and $2H^+$ (Xia et al., 2003). The ROS thus generated is known to

activate signalling and transcriptional programs required for adaptation to exercise (Powers et al., 2010). It also augments membrane depolarization during ECC and force production in healthy muscles (Roy et al., 2017). Hence, knowing how or why it becomes pathogenic in DMD is crucial.

Five different Nox genes code for the catalytic subunit of NADPH Oxidase complex. It has been shown that Nox2 and Nox4 are active in muscles. Nox4 is constitutively active in the endoplasmic reticulum, while Nox2, present in the cell membrane complex, is responsible for the pathogenic levels of ROS generated in *mdx* mouse muscles (Whitehead et al., 2010). However, other sources of ROS are mitochondria (Huges et al., 2019) and Xanthine Oxidase (Lindsay et al., 2018). The details of XO regulation are not as well discerned, but clinical trials with XO inhibitors were stopped due to lack of benefit. The continued NADPH supply from the pentose phosphate pathway can fuel its activity (Gupte et al., 2006) and is known to be high in DMD (Kar & Pearson,1972; Mizuno, 1985). The NADPH oxidase is pH sensitive; where acidic pH is known to reduces its activity significantly (Brennan-Minnella et al., 2015). Extracellular acidification in response to exercise does not occur in DMD (Ellis, 1980), adding to its continued action. The pH also depends on the sarcolemmal Sodium/Hydrogen ion exchanger.

The NHE1 maintains pH in many tissues, including the heart and skeletal muscles. NHE1 is a proton/sodium exchanger that can explain why oxidative burst augments ECC coupling. Studies conducted by Lacroix and colleagues (2004) found that intracellular proton levels can directly activate this antiporter so it can get activated by protons generated by the NADPH oxidase complex. The higher activity of NHE1 has been found in *mdx* muscles though it has been attributed to the higher activity of ATP receptor P2A (Iwata et al., 2007). It could be the reason for the continued activity of NADPH oxidase in turn as NHE1 activity - export of protons keeps local pH high; thus, the positive loop continues. It has also been shown to be responsible for sodium-mediated hypercalcemia with increased volume in the heart (Burr et al., 2014). The NHE1 has a long intracellular C-terminal chain having sites for various signaling molecules like p38 MAPK, ERK1/2, Calcineurin, PTP2A, PTPN11 etc. The p38 MAPK activates NHE1- also known to be overactive in DMD in animal models and patient samples alike (Smythe & Forwood, 2012).

Overexpressing calcineurin has been shown to ameliorate *mdx* pathology (Chakkalakal et al., 2004) by utrophin upregulation and glycolytic to slow oxidative type switch (Fajardo et al.,

2018). One study showed that Myospryn/AKAP6 - (an inhibitor of calcineurin mediated slow type transcription program, binds dystrophin dp70 via the TRIM domain (Reynolds et al., 2008). It also showed that in the absence of dystrophin, the positive loop of myospryn localization that activates PKA signal to increase myospryn transcription is disrupted. Mutations in the myospryn gene have not been associated with any skeletal muscle disease - it only causes hypertrophic cardiomyopathy, while dystrophin causes a dilated type of cardiomyopathy. In addition, studies have suggested that calcineurin is activated at higher calcium-calmodulin levels, making high calcineurin activity the result of underlying Ca^{+2} accumulation in DMD. NHE1 mutations are associated with dilated cardiomyopathy, hence more likely to be part of the dystrophin signalling cascade during contraction. To what extent the NHE1 inactivation depends on dystrophin is not clear yet, due to the complexities of NHE1 regulation. NHE1 inhibitors as therapy for DMD are in the initial phases of clinical trials and can potentially target skeletal muscle and heart pathology simultaneously.

THE ROLE OF CALCIUM

The membrane depolarization in response to neuronal stimulation leads to the ingress of extracellular Ca^{+2} that activates Ca^{+2} channels in the SR. This is the most important event for the initiation of muscle contraction. Several Ca^{+2} channels are present in a muscle cell to maintain calcium homeostasis.

Increased intracellular Ca^{+2} levels and the consequently higher activity of Ca^{+2} - dependent proteases have played a significant role in the histopathology of DMD even before identifying its gene locus (Bodensteiner et al., 1978; Duncan, 1978). Similar effects were studied, in detail, using *mdx* mouse model (Ohlendieck, 2000).

The skeletal muscles express three types of TRP (Transient Receptor Potential) channels- the TRPVs (Vanilloid), TRPCs (Canonical) and TRPM (Melastatin) that differ in their activation mechanism- e.g. TRPC are stretch sensitive. In contrast, TRPV is shown to act as Store Operated Calcium Entry channel (SOEC) (Choi et al., 2020). The altered activity of these calcium channels has been implicated in DMD by Gailly (2012) in addition to calcium leak channels (Fong et al., 1990; Alderton and Steinhardt, 2000), Ryanodine Receptors (Ryr) and IP_3R (Inositol 1,4,5 Triphosphate Receptor) Ca^{+2} release channels located at SR (Deval et al., 2002). The TRPVs and TRPCs were shown to be regulated by α-syntrophin (Vandebrouck et al., 2007; Sabourin et al., 2009; Harisseh et al., 2013) and Src Kinase via caveolin3-binding

(Gervásio et al., 2008) this could be an indirect effect of absence of systrophin than direct. The higher PLC (PhosphoLipase-C) activity leads to high IP_3 levels; hence higher IP_3R-mediated calcium release has been reported in DMD (Liberona et al., 1998; Mondin et al., 2009). The PLC was shown to be regulated by syntrophin1-α/dystrophin (Sabourin et al., 2012), this again, could be an indirect effect than the primary cause. The Ryr channels in SR, which are required for the amplification of excitation-contraction coupling, are activated by extracellular Ca^{+2} influx to release calcium from the SR. Therefore, sustained cytoplasmic Ca^{+2} itself could be responsible for the higher activity of these channels. In normal conditions, to regulate this cascade of Ca^{+2} release in the sarcoplasm, Ca^{+2}/ATPses (termed SERCA) in SR reabsorb Ca^{+2} by expending ATP. The activity of these channels was reported to be low in DMD (Landi et al., 1986; Kargacin & Kargacin, 1996). Sarcolipin - an inhibitor of SERCA, overexpressed in *mdx* mice (Schneider et al., 2013), the knockdown of which ameliorated pathology in *mdx* (Voit et al., 2017). The SERCA-1 overexpression also ameliorated the pathology of more severe dmd/utrn double knockout mice (Mázala et al., 2015). The Ca^{+2}/ATPses depend on Creatine Kinase to generate ATP from ADP and P-Cr (Maria & Wallimanns,1990). Creatine Kinase activity is also shown to be low in DMD patients (Landi et al., 1986), which in turn depends on the cellular P-Cr levels and ADP/ATP ratios. P-Cr levels are known to be low in dystrophic muscles and fail to regenerate during recovery after exercise (Percival, 2013; Rybalka et al., 2014). Creatine supplementation has been shown to amend DMD pathology in *mdx* mice by improving Ca^{+2} handling via Ca^{+2}/ATPase activity (Pulido et al., 1998), suggesting that chronic calcium is more likely a result of energy deficiency in DMD, not primary pathology.

THE ROLE OF MEMBRANE STABILITY

Through the above-described narrative of calcium-mediated pathology, the underlying reason cited has been the same - the mechanical fragility leads to increased calcium ingression, which has been disputed (Collet et al., 1999). The initial ingress of calcium was found to be expected in the muscles of patients and *mdx* (Pressmar et al., 1994). However, the hypotonic stress-affected dystrophic muscle membranes were reportedly more fragile than usual, making them susceptible to higher calcium spikes than the latter (Menke & Jockusch, 1995). The membrane fragility hypothesis was based on observations of lesions found in patient biopsies (Mokri & Engel, 1975). However, subsequent patch clamp studies have found little difference in the mechanical properties of sarcolemma to support such a hypothesis (Hutter, 1992). Studies have found that membrane lesions also appear during exercise in normal muscles that are repaired in

a Ca^{+2}, ATP-dependent manner (Barthélémy et al., 2018). The studies in *mdx* showed integrin-α7 or β1 over-expression increased survival, hypertrophy, and ameliorated membrane damage completely (Boppart et al., 2011; Liu et al., 2012). Integrins were reported to be upregulated in animal models and patients of DMD (Hodges et al., 1997). In addition, the fact that in MDC1A (where integrin ligand Laminin is absent) - more severe muscle death happens in the absence of membrane pathology (Patton, 2000) suggests membrane damage may not be the primary pathology in the case of DMD. It indicates that the integrins, dysferlin, and myoferlin upregulation in DMD are compensatory pathways to improve metabolism and membrane repair. Thus, like excess calcium, the failure to repair membrane damage could also result from underlying energy deficiency in DMD.

THE ROLE OF METABOLISM

This low ATP production and general metabolic dysfunction observed since the first disease description is another mystery about DMD. The mitochondrial failure (Baron et al., 2011)- 50 % reduction in ATP levels (Percival, 2013; Rybalka et al., 2014), reduced glycolysis (Hess, 1965), abnormal lipid metabolism (Srivastava et al., 2017) increased fatty accumulation (Bonsett et al., 1979,1994) and abnormal glycogen aggregates (Stapleton, 2014) in cytoplasm have been observed in muscle biopsies of DMD patients (Timpani, 2015).

The abnormality of glycogen aggregates is not amount but length and branching - it is very long with negligible branching from DMD muscle biopsies. Higher activity of debranching enzyme and lower activities of glycogen phosphorylase as well as branching enzyme activities are reported in DMD; whereas glycogen synthase activity is reported to be slightly high (Stapleton, 2014). Additionally, insulin sensitivity is normal in patients, which secondarily reduces the later stages of the disease. This is likely because functional muscles are not left to respond to insulin. Glycogen synthesis results from excess glucose or insulin stimulation, whereas energy-need sensing mechanisms stimulate glycogen debranching and degradation. Thus, the glycogen abnormality indicates a disconnect between energy need sensing and energy production or possibly energy production fails to increase in response to the need in muscles of DMD patients. The latter seems more possible as glycolysis and mitochondrial ATP production are consistently low in many studies. Acidification due to anaerobic glycolysis leading to the production of lactic acid during contraction is lacking in BMD also, though mitochondrial ATP production is average in BMD (Lodi et al., 1999). The uncoupling protein (UCP2) on the inner mitochondrial membrane is responsible for converting energy into heat instead of ATP from ETC.

Upregulation of UCP2 and downregulation of proteins of ETC and Glycolysis can be partly responsible for lower mitochondrial ATP production in DMD, as found in GRMD (Markham et al., 2017). There are abnormally higher lipid drops observed in muscle biopsies of DMD patients. Normally in skeletal muscles - especially glycolytic fiber type - fatty acid oxidation is repressed under high glucose, which results in higher acetyl-CoA converted to malonyl-CoA and used for fatty acid synthesis in the sarcoplasm.

Malonyl-CoA is an inhibitor of Carnitine-Palmitoyl Transferase1 on the outer mitochondrial membrane responsible for entering fatty acids in mitochondria for oxidation. There are multiple mechanisms to regulate fatty acid synthesis and oxidation, including glucose metabolism, but, in this case, how much dysregulation can be attributed to it is unclear. The same is true for mitochondrial ATP production - which is repressed in DMD. Studies indicate that lower glycolysis results in lower mitochondrial ATP production in DMD (Hintz et al., 1987; Sharma et al., 2003), while others have argued that mitochondrial deficiency is primary pathology based on the inability of octanoate (glycolysis independent) catabolism is affected in DMD (Chinet et al., 1994; Timpani et al., 2015). A study on the *mdx* mice showed partial dystrophin restoration by exon skipping improved membrane repair via mitochondrial energy production (Vila et al., 2016), favouring such supposition. Other known regulators of energy homeostasis like AMPK, PGC1α, and AKT pathways are also downregulated, and here forced stimulation seems to rescue pathology in *mdx* mice (Garbincius et al., 2015), it has been disputed though (Ljubicic et al., 2012) and hence not resulted in viable therapy yet. The critical unanswered question is whether dystrophin function is associated with regulating energy production or whether this hypometabolism is a mitigation measure like the upregulation of dysferlin by cells to survive in the absence of dystrophin.

Though the details of pathophysiological processes are well discerned for this disease, unresolved cause-effect relationships are responsible for failing therapy approaches.

THERAPY OPTIONS FOR DMD

In the mouse model of DMD (*mdx* mouse), treating individual aspects of the disease with antioxidants (Hnia et al., 2007; Whitehead et al., 2008), inhibitors of $NADPH_2$ oxidase (Gonzalez et al., 2014) or microtubules (Khairallah, 2012), proteasome inhibitors (Bonuccelli, 2003), inhibitors of HDACs (Consalvi, 2013), inhibitors of Proton pumps (Sali et al., 2013), inducing autophagy (Pauly et al., 2012), increasing NOS (Wehling, 2001) shows amelioration

of symptoms. However, none of these has translated into a cure for this debilitating disorder, partly because the *mdx* mouse is not similar in severity or progression to the human condition. There is a possibility that these pathophysiological manifestations are not causal factors but a result of the underlying defect.

Most advanced viral vector-based mini dystrophin gene therapy and genome editing have been obstructed due to off-target effects and immune response to both vector (Ramos and Chamberlain, 2015; Abdul-Razak, 2016) and transgene (Mendell et al., 2010) at clinically effective doses. Modified oligonucleotides with antisense sequence to mutated exon lead to skipping of the exon during splicing and translation of shorter dystrophin mRNA from patients' genome. This method is called Antisense Oligo (AO) mediated exon skipping. It does not seem to evoke an immune response but has low efficiency (Lu, 2014; Aartsma-Rus & Krieg, 2017) regarding levels of dystrophin expressed and per cent of fibres expressing dystrophin, thus requires further refinement before it can show signs of clinical benefit. Though few drugs are now clearing clinical trials for exon skipping, it will be a few years before their effects become apparent. These therapies are costly, and not all exons are amenable to skipping. In fact, there are drugs (e.g., Altalauren) to inhibit nonsense-mediated decay (NMD) for treatment, but these will be selective only for patients having nonsense mutations.

Stem cell (SC) therapy via allogenic transplant (Mendell & Clark, 2006) from regular donors and autologous transplant of genetically dystrophin-restored stem cells in DMD patients (Partridge, 2004) have been tried. There are cells like pericytes outside the basal lamina with myogenic potential, hence useful for cell transplant therapy. Since the 1980s, SC transplants have been under clinical trials, and results are encouraging. However, there are limitations: - i) the number of injections required is large, ii) the immune response towards injected SCs and iii) the rapid death of injected SCs (Sienkiewicz et al., 2015). Some studies have shown the injected SCs die within 72 hours (Fan et al., 1996), while others have shown that 90% of injected SCs are cleared within an hour by immune cells (Sku & Tremblay, 2013; Maffioletti et al., 2014).

To date, only steroids have consistently proven extension of ambulation by 1.5-3 years, delay in scoliosis and improvement in many aspects of DMD in patients (Markham et al., 2008; Parreira et al., 2010; Pardo et al., 2011; Schram et al., 2013; Kim et al., 2014; Gloss et al., 2016).

GENETIC MODIFIERS OF DMD

Genetic modifiers are secondary mutations in other genes that can affect the pathophysiological processes, thus, leading to disease progression. Based on large-scale genetic screens, various groups have identified gene spp1 encoding Osteopontin, LTBP4 and notch receptor ligand Jagged1 as genetic modifiers of DMD (Quattrocelli et al., 2017). Osteopontin is highly expressed in severe cases of DMD. It is assumed to promote fibrosis, influencing pro-inflammatory macrophages and increasing myoblast number at the expense of myoblast migration and fusion. LTBP4 is a Latent TGF-β Binding Protein and, as the name suggests, binds TGF-β in tissues reducing its availability. Due to this, it is also involved in regulating fibrosis, inflammation, and muscle regeneration in DMD. Higher levels of LTBP4 and lower levels of osteopontin correlate with a delay in loss of ambulation by 1.5-2 years and slower overall disease progression based on DMD patients and animal models data (Quattrocelli et al., 2017).

On the other hand, notch receptor Jagged1 upregulation in the Golden Retriever model of DMD had shown stark changes in two significant outcomes of the disease - loss of ambulation and early death (Vieira et al., 2015). The GRMD is a severe DMD model with rapid ambulation loss and death of dystrophic pups within a year or two. The "escapers" found in the study lived an everyday life with mild muscle weakness and their subsequent generations showed the same escaped phenotype. The same study showed increased survival at one month in dystrophic zebrafish with Jag1 mRNA injection. Hence, we chose to focus on Jagged1 upregulation in DMD for our study.

ANIMAL MODELS OF DMD

The animal models of DMD range from invertebrate animals like *C. elegans* and *D. melanogaster* to vertebrates like monkeys (Egorova et al., 2021). The most used models are mouse (*mdx*), golden retriever dog, zebrafish (Sapje) and recently developed rat model (Larcher et al., 2014; Nakamura et al., 2014). The dystrophin null *mdx* mouse does not show severe pathology or mortality as seen in patients. This mild phenotype is attributed to long telomeres, thus more significant regeneration potential, overexpression of utrophin, small body size and protection from expansive contractions due to the posture and shape of an animal. It is also observed that several revertant fibers expressing shorter isoforms of dystrophin increase with age, causing better regeneration and contributing to almost normal ambulation and life span in

mdx mice (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 challenging to maintain the mutants in a colony. Additionally, GRMD is a more time-consuming and costly model.

Zebrafish have a short generation time, produce many offspring, and show fast external development. The eggs and early larval stages are transparent, thus offering ease of genetic manipulation and observation of dystrophic phenotype. The zebrafish embryos absorb drugs quickly, proving it to be a better model organism for studying human diseases. The 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 is a severe model of DMD, with larvae surviving only for a month (Granato et al., 1996).

Notch signaling pathway components are found to function similarly in the muscles of zebrafish (Pascoal et al., 2013) during damage and regeneration (Sultan et al., 2021). Both isoforms Jagged1a and b were found to be efficient in rescuing Sapje larvae, although Jagged1a mRNA injection showed a slightly more survival percentage in Sapje larvae at one month (Vieira et al., 2015). For this study, we have used human Jagged 1 instead of zebrafish to avoid dysregulation of inherent Jagged1 ligands, which are involved in normal embryonic development, disruption of which might make results challenging to comprehend.

JAGGED1-NOTCH SIGNALLING

Jag1/Jagged1 is one of the five cell surface ligands that can bind to membrane Notch receptors, primarily working in a cell-cell contact-dependent manner. Jagged1 protein structure comprises a small intracellular domain, transmembrane domain and a sizeable extracellular domain containing highly conserved DSL (Delta, Serrate and LAG1) motif, 16 EGF-like repeats and cysteine-rich region. The extracellular domains bind to the Notch receptors to activate signalling in the neighboring cell.

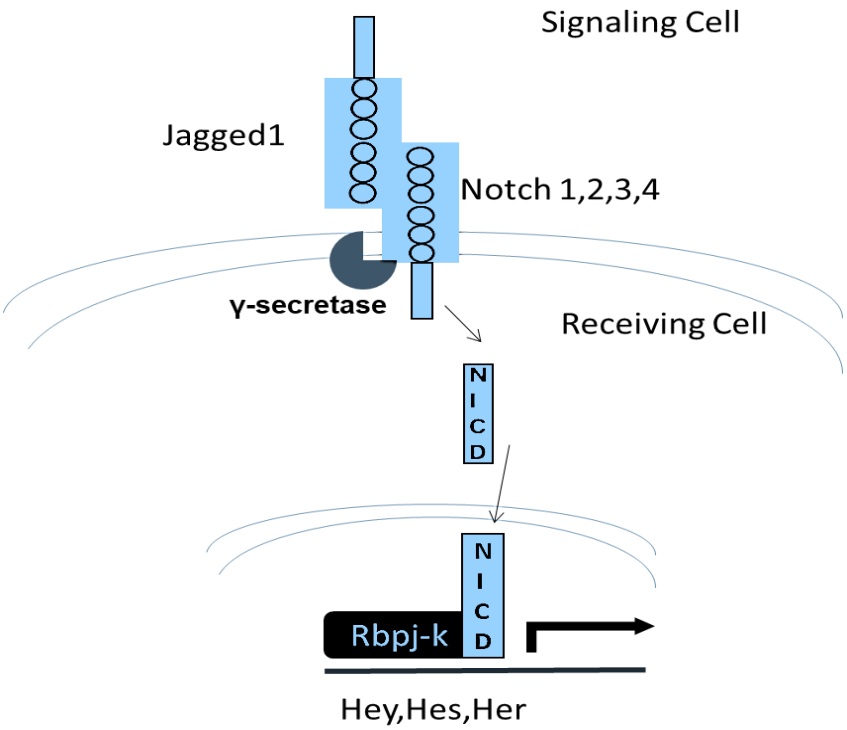

Figure 1.5: The Jag1-Notch signalling: The Jagged1 ligand can activate all four notch receptors (Notch1-4) in the neighbour signal-receiving cell. The sequential cleavage of the receptor releases the intracellular domain (NICD) from the trans-membrane domain by γ-secretase enzyme is a key step of the signalling event. In the nucleus, NICD can bind Rbpj-κ and activate transcription of Hes, Her family of transcription factors. (Adapted from Gridley, 2007)

Mutations in Jag1 are associated with Alagile Syndrome and Tetralogy of Fallot, affecting multiple organs. Jag1 is a ubiquitously expressed protein throughout developmental stages and adulthood. The deregulation of Jag1 expression is associated with many cancers of different origins like gliomas, carcinomas of the gastrointestinal tract, -breast, -ovarian and prostate cancers (Xiu et al., 2020). Though most studies show canonical notch receptors are required for Jag1 ligand function, it can activate JAK2 signalling in a notch-independent manner in osteoblasts (Kamalakar et al., 2019). These non-canonical receptor/s and signalling mechanisms are not well understood.

NOTCH RECEPTORS

The notch receptor pathway is highly conserved in multi-cellular animals. In mammals, five ligands, DLL1,3,4 and Jagged 1,2, can activate 4 receptors, Notch 1,2,3,4. It is a cell-cell contact-dependent pathway where cells expressing ligands can activate notch receptors on adjacent cells to induce binary cell fate and boundary formation during early embryonic development (Lewis et al., 2009). Once the pathway is activated, Notch Intracellular Domain (NICD or ICD) is released into the cell. In the 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 (Master Mind-Like) and CBP/p300 (Fryer et al., 2002). Well-known targets include the HEY (Iso et al., 2003) and HES (Kageyama et al., 2005) family of transcriptional repressors. These repressors have differing target specificities as homodimers and heterodimers, leading to a wide variety of responses.

PLEIOTROPISMS OF NOTCH SIGNALLING FOR CONTEXT-DEPENDENT OUTCOMES

The Notch pathway is very complex and highly context-dependent. There are differences in the ligand binding in *trans* to neighbor receiver cell and *cis* binding to self-receptors that result in receptor signalling in *trans* and inhibition in *cis* (Nandagopal et al., 2019). There are more than 20 proteins involved in more than 100 post-translational modifications of these ligands and receptors that fine-tune this signalling pathway (Antfolk et al., 2019). In addition to 5 ligands and 4 receptors, ADAM family metalloproteinases and γ-secretase sequentially cleave the extracellular domain (NECD) and intracellular domains from a transmembrane domain (TD) of Notch receptors and MEGFs that process the unbound or ligand bound cleaved extracellular domain (Vargas-Franco et al., 2022). The proteins NUMB, NUMB-like, Neutralized, etc., can regulate ligand and receptor endocytic cycling and half-life by ubiquitinoylation (Shao et al., 2017), contributing to the pleiotropisms of the pathway.

Notch signalling is involved in Inner Cell Mass (ICM) survival in the early embryo (Cormier et al., 2006), neurulation (de la Pompa et al.,1997), vascularization and smooth muscle development (Baeten & Lilly, 2017), as well as skeletal regeneration (Dong et al., 2014) in vertebrates. Notch signalling has been implicated in various processes in different tissues - maintenance and self-renewal of stem cells (Chiba, 2006), apoptotic (Yang et al., 2004) and

anti-apoptotic (Mungamuri et al., 2006), tumorigenic or tumor suppressor (Craig & Radtke., 2017) activities have been attributed to this pathway in a context-dependent manner. In addition to the vast array of activities in different tissues, within single tissues, the non-redundant, combinatorial nature of all the pathway components and multiple layers of regulation produce varied outcomes during homeostasis and disease.

NOTCH INVOLVEMENT IN MYOGENESIS

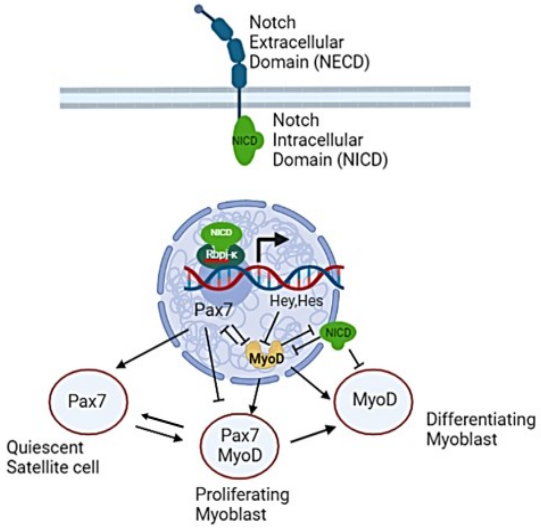

Figure 1.6: Notch signalling involvement in myogenesis. The notch signalling is important for maintenance of quiescence in the satellite cells during homeostasis. During injury repair, notch signalling improves self-renewal of committed myoblasts. Notch signalling delays the process directly by competing with MyoD or via its downstream effectors during differentiation. (Adapted from Wen et al., 2012)

In skeletal muscles, its function in myogenesis and regeneration has been studied extensively (Den Hartog & Asakura, 2022; Gioftsidi et al., 2022). Here its role is also highly context-dependent. In satellite cells, notch signalling helps maintain a quiescent state primarily by upregulating Inhibitors of Metalloproteases in the Extracellular Matrix (ECM) (Montarras et al., 2013; Pallafacchina et al., 2010). Metalloproteases digest ECM in case of injury and activate inflammatory responses and satellite cells; thus, notch receptor signalling blocks this process under homeostasis when satellite cells are required to maintain quiescence. When Notch activity is blocked experimentally, satellite cells lose the ability to stay quiescent (Bjornson et al., 2012; Mourikis et al., 2012), which in the long term lead to the depletion of the SC pool (Schuster-Gossler et al., 2007, Vasyutina et al., 2007). Both the high number of activated satellite cells (Kottlors & Kirschner, 2010) and the down-regulation of notch receptors and ligands have been observed in DMD patients (Schuster-Gossler et al., 2007).

Activating this pathway experimentally by co-culturing ligand and receptor-expressing populations (Lindsell et al., 1995), using antibodies against receptor, injecting NICD or overexpressing NICD (Kopan et al., 1994) by viral vector constructs gave ambiguous results with improved regeneration (Conboy & Rando, 2002) in some cases and poor regeneration in other studies (Church et al., 2014). On the other hand, blocking Notch activity stimulates pre-mature differentiation resulting in small and fragile muscles (Schuster-Gossler et al., 2007, Vasyutina et al., 2007).

In the activated myoblasts, it blocks differentiation and promotes self-renewal (Conboy & Rando, 2002). It was found that notch blocks MyoD and Myf5 expression and MyoD activity (Kopan et al., 1994) indirectly by occupying Myogenin and Mef2C regulatory regions and suppressing their expression (Buas, 2010), thus delaying muscle formation. Canonical Notch pathway targets/effectors Hes1 and MyoR act in blocking myogenic differentiation when overexpressed, but their knockdown does not affect the delay of myogenesis (Buas, 2009). The delay of myogenesis is independent of CSL, too (Shawber, 1996; Nofziger et al.,1999), suggesting that unknown multiple effector/s or non - transcriptional function of the Notch pathway may be involved.

The role of notch receptors in mature myofibers has been understudied. Though deregulation of the Notch pathway has been implicated in age-related (Carey et al., 2007), unloading induced and diabetic muscle atrophy (Fujimaki et al., 2022). The myofiber expression of ligands and Notch receptor/s is thought to create a niche to maintain satellite cell homeostasis (Gioftsidi et al., 2022). The satellite cells express Notch1,2, and 3 receptors and ligand Jag1 (Kitamoto & Hanaoka., 2010; Fujimaki et al., 2018), but its reciprocal effect on myofiber homeostasis remains unexplored.

NOTCH PATHWAY IN DMD

The Notch pathway components at transcript and protein concentration differ between mouse models and human patients (Church et al., 2014). The reduction of Notch1, Hes1 and increase in Jagged2, NUMB, and Notch3 at the transcript level from patient biopsy samples have been reported (Church et al., 2014). The protein levels of Hes1 were increased in the patient samples in the same study. The constitutive Notch1 activity in the myotube stage improved regeneration and exercise performance, which in muscle progenitors caused regeneration defects in the *mdx* background (Bi et al., 2016). Though it should be noted that Notch3 activity differs between

human and mouse dystrophic conditions (Church et al., 2014), which can influence the outcome of Notch1 activation as Notch 3 is considered an inhibitor of Notch1 (Kitamoto et al., 2010). Hence, it is difficult to extrapolate these signalling pathway-related findings from the *mdx* model to human dystrophic condition. In DMD patients derived from immortalized myoblast cultures, Jag1 was found to be upregulated, while its knockdown regained the mitogenic action of IL-1β on dystrophic myoblasts (Nagata et al., 2017). Studies focusing on the effect of Jag1 overexpressing myoblasts on surrounding dystrophic myofibers are still missing.

KEY PROBLEM AND THE HYPOTHESIS OF THE CURRENT STUDY

Vieira and coworkers (2015) found that some GRMD had escaped the disease and lived a normal life with mild muscle weakness. The study reported that a point mutation created a consensus Myogenin binding site in the promoter region of Jagged1. This resulted in increased expression of Jagged 1 in only muscles, as Myogenin is a muscle-specific transcription factor and somehow rescued the disease. It was further verified by injections of jagged1 mRNA in the single-cell embryo of *Sapje* zebrafish. It resulted in a ~70% decrease in the death of dystrophic larvae at one month. This study could not provide a mechanism of action as the Jagged1-Notch pathway has multiple functions in many tissues.

The same group's follow-up study showed that upregulation of PI3/pAKT due to the downregulation of α-PITPNA found in the "Escaper" transcriptomic data results in PTEN downregulation. The lentivector-shRNA mediated knockdown of PTEN increased per cent survival at 21-day post fertilization (dpf) and rescued muscle structure and function at 4 dpf *Sapje* Zebrafish (Vieira et al., 2017). Meanwhile, increasing PI3/pAKT stimulation via IGF treatment has failed to ameliorate motor function despite improving muscle pathology during clinical trials (Rutter et al., 2020). In *mdx*, PTEN inhibition reduced fibrosis and rescued other aspects of the disease (Yue et al., 2021). The PTEN is a classic tumour suppressor in all the tissues to control excess cell proliferation. Though skeletal muscle is post-mitotic tissue at a lesser risk of primary tumorigenesis than other tissues, PTEN inhibition or other such tumor suppressor inhibition can be dangerous for non-target tissues as a therapy approach. Additionally, myoblast-specific knockdown of PTEN leads to hypertrophy at a young age but faster exhaustion of myogenic potential in mice (Yue et al., 2016). The “escaper” GRMDs’ muscle biopsy showed higher than affected level fibrosis without any hypertrophy (Zucconi et al., 2010), and in this case, early exhaustion of myogenic potential should have manifested the

disease at least in old age “escapers”. All these together suggest that though involved indirectly, PTEN reduction might not be the primary rescue mechanism. The Notch receptors can reduce muscle fragility by increasing localization and translation of desmin and vinculin mRNA in zebrafish (Pascoal et al., 2013). Notch involvement in the regulation of RNA binding protein (Okabe et al., 2001), stress fiber formation (Srivastava et al., 2009), asymmetric cell division (Kuang et al., 2007; Jory et al., 2009) and metabolic regulation (Bi & Kuang, 2015) suggests its potential to ameliorate dystrophic pathology in multiple ways.

All these findings led us to hypothesize that the Jag1-mediated rescue involves reduced contraction-induced loss of muscle structure and function, which can prolong the overall time of cycles of degeneration-regeneration. This could contribute towards maintaining muscle progenitor self-renewal and thus the myogenic potential for a more extended period in dystrophic conditions. The difficulty in identifying the rescuing mechanism is that the Jag1 or Notch receptor(s) signalling components do not respond to exercise in muscles (Bubak et al., 2022), which is responsible for initiating pathology, as described in detail previously. Hence, identifying the disease aspects mitigated by Jag1 overexpression might help shed light on the mechanism. As the causal relations between pathological processes are clouded, this bonafide rescue could be used to clarify what should constitute a rescue in the preclinical models of DMD. Identifying mitigated pathology in the rescue could also help develop Jagged1 independent therapy approaches in the future.

Accordingly, the following objectives were set.

1) A comparative analysis of DMD pathology post Jagged1 upregulation on survival, muscle function, structure, cell proliferation, apoptosis and oxidative stress measurements.
2) Finding receptor(s) and known targets (HES and HEY family genes) involved in Jagged1 upregulated trunk muscles.
3) Substantiating newfound targets of this pathway by artificially blocking and upregulating these in cultured human muscles to validate the findings.