

THE ROLE OF A β -CATENIN - FMRP COMPLEX IN SKELETAL MUSCLE

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Abstract

Skeletal muscle consists of multinucleated myofibers which generate contractile force and regulate glucose levels in the human body. Over time, skeletal muscle can become damaged and therefore requires a robust capacity to self-renew. One of the emerging areas of skeletal muscle research involves studying the post-transcriptional mechanisms of gene regulation which mediate this self-renewal pathway. Diseases processes such as sarcopenia and cachexia can impair the ability of skeletal muscle to regenerate and therefore pharmaceutical targets which can reverse this pathological process must be identified. One of the promising avenues of treatment for these disorders involves increasing the level of protein synthesis in skeletal muscle which has previously been shown to mediate both hypertrophy and regeneration of muscle fibers.

Our group has previously identified a novel post-transcriptional role of β -catenin in smooth muscle. It was found that β -catenin could interact with the RNA binding protein Fragile X Mental Retardation Protein (FMRP) in smooth muscle and mediate repression of protein synthesis. Due to the role of this complex on protein synthesis, we sought to study this interaction in skeletal muscle in order to determine whether it may serve as a druggable target in muscle wasting disorders. While β -catenin is a key transcriptional regulator and activator of myogenesis in skeletal muscle, it has never been identified as a post-transcriptional regulator.

In this study, we characterized the interaction between β -catenin and FMRP in skeletal muscle and explored the role of this complex on translational activity. We confirmed that β -catenin and FMRP interacted through biochemical assays and overexpression of β -catenin and FMRP fluorescent fusion proteins. Next, we used the SUnSET methodology in order to quantify translational activity of skeletal muscle at different stages of differentiation. We found that

knockdown of β -catenin in C2C12 myoblasts undergoing the growth phase led to a significant increase in global translational activity. This finding suggested that β -catenin serves as a translational repressor in skeletal muscle precursors. We then sought to determine whether the β -catenin-FMRP complex could be identified at the translational machinery which would further suggest a post-transcriptional role of this complex. Using polysome profiling, we identified the presence of β -catenin at the preinitiation complex along with FMRP. Furthermore, we validated the presence of the complex at the preinitiation complex by conducting an M7GTP immunoprecipitation assay which captures the translational machinery. Finally, we conducted an RNA immunoprecipitation assay (RIP) to capture RNAs that interact with FMRP in order to identify them by microarray analysis. We identified various classes of RNAs enriched in FMRP RIP samples including microRNAs which target regulators of myogenesis, Cajal Body RNAs, nucleolar RNAs, and ribosomal associated RNAs.

In conclusion, we have identified a novel post-transcriptional role of β -catenin in skeletal muscle and identified the presence of the FMRP- β -catenin in skeletal muscle. We postulate that pharmacological targets which interfere with the interaction of this complex may lead to an increase in global translational activity and reverse the pathology of muscle wasting disorders. Furthermore, we have identified RNA targets of FMRP in skeletal muscle which have deepened our understanding of the possible targets of the FMRP- β -catenin complex. These findings contribute to an emerging field of research regarding post-transcriptional control in skeletal muscle and represent a possible pharmacological target to reverse the pathology of muscle wasting disorders.

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List of Abbreviations

APC- adenomatosis polyposis coli

CA - Constitutively Active

c-Met - Tyrosine Kinase Protein Met

CYFIP- cytoplasmic FMRP binding protein

DM - Differentiation Media

eIF4E – Eukaryotic Initiation Factor 4E

FGF- Fibroblast Growth Factor

FMR1 - Fragile X Mental Retardation Gene 1

FMRP - Fragile X Mental Retardation Protein

FXR1/2 - Fragile X Mental Retardation Isoforms 1/2

FOXO- Forkhead Box Transcription Factors

GM- Growth Media

GSK3 β - glycogen synthase 3 β

JNK - c-Jun N-terminal kinase

KO – conditional knockout

LRP5/6 - Low Density Lipoprotein Receptor-Related Protein 5/6

m⁷GTP - Methyl Guanosine Triphosphate

MAP1B - Microtubule Associated Protein 1

MEF2 - Myocyte Enhancer Factor 2

MRF- Myogenic Regulatory Factor

MRF4 - Myogenic Regulatory Factor 4

Myf5 - Myogenic Factor 5

MyoD - Myoblast Determination Factor 1

Nlk- Nemo-Like Kinase

PABP – Poly-A Binding Protein

Pax3 - Paired Box Transcription Factor 3

Pax7 - Paired Box Transcription Factor 7

PCP – Planar Cell Polarity Pathway

PKC - Protein Kinase C

PLC - Phospholipase C

RPL9 - Large Ribosomal Subunit 9

SUnSET - Surface Sensing of Translation

TA - Tibialis Anterior

TCF/LEF - T-cell Factor/Lymphoid Enhancer Transcription Factor

TGF- β - Transforming Growth Factor Beta

TNIK - Traf2/Nck-interacting kinase

Wnt - Wingless Integration Site Signaling Pathway

Literature Review:

Overview of Skeletal Muscle

Human skeletal muscle consists of bundles of multinucleated cells known as myofibers which are responsible for a wide range of processes including movement, breathing and metabolism [1]. These myofibers can contract through the hydrolysis of ATP which allows for the interaction of actin and myosin proteins within the fiber and the subsequent generation of mechanical force [2]. Skeletal muscle is also responsible for the uptake of free glucose in the bloodstream and its conversion into glycogen [3]. Due to its ability to generate mechanical force and regulate blood glucose levels, skeletal muscle plays an essential role within the human body [1]. As individuals get older, they suffer from sarcopenia or muscle wasting which can lead to pathological symptoms such as insulin resistance, negative alterations in fat distribution and other metabolic changes [4]. Clearly, skeletal muscle significantly influences quality of life by allowing people to carry out day-to-day activities normally taken for granted and regulating important metabolic processes [4]. The functionality of skeletal muscle can be impaired in people afflicted with genetic disorders, sarcopenia or skeletomuscular injuries [1]. The dramatic reduction in quality of life due to skeletal muscle impairment significantly increases in Canadians between the ages of 60-79 as well as those with genetic disorders such as Duchenne's muscular dystrophy which impacts between 10.7 and 27.8 males per 100,000 individuals [5,6]. For these reasons, it is important to understand the molecular pathways that can mediate the repair of this indispensable organ system.

Physiology of Skeletal Muscle

Skeletal muscle consists of multinucleated cells derived from progenitor cells known as myoblasts. The main components of the myofiber include the cell membrane or sarcolemma, the myofibril containing the contractile filaments actin light chain and myosin heavy chain, the sarcoplasmic reticulum which sequesters calcium, and the troponin-tropomyosin regulatory complex which modulates the interaction between actin and myosin ([7]; Figure 1A/B).

Upon the release of acetylcholine by motor neurons at the neuromuscular junction, the sarcoplasm of a myofiber is depolarized leading to the propagation of an action potential [8]. This action potential activates dihydropyridine receptors on the sarcolemma which interact with ryanodine receptors on the membrane of the sarcoplasmic reticulum [7]. This interaction causes the sarcoplasmic reticulum to open and release calcium into the sarcoplasm which regulates the conformation of the troponin-tropomyosin complex ([9]; Figure 1C). This regulatory complex prevents actin from interacting with myosin under low calcium concentrations. In this state, tropomyosin wraps around myosin binding sites on actin while troponin regulates the conformational state of tropomyosin [9]. When calcium binds to troponin, the conformation of tropomyosin is altered and the interaction between actin and myosin can freely occur leading to the generation of mechanical force [9]. Unfortunately, this generation of mechanical force mediated by myofibrils can be impaired due to the expression of dysfunctional structural proteins required for the stability of the myofiber as well as a reduction in the quantity of force generating myofibers [10].

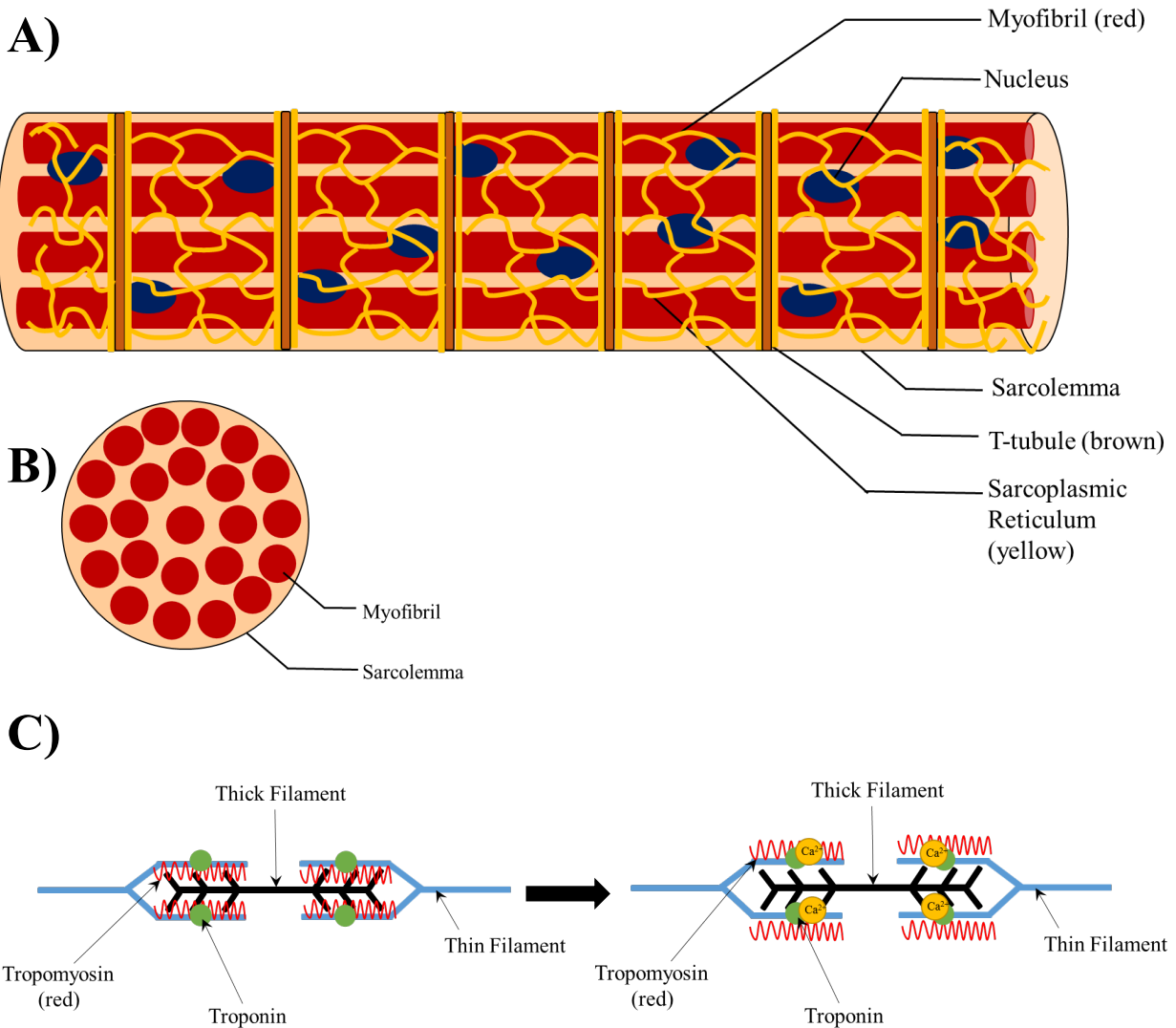


Figure 1: Schematic of a myofiber and the generation of mechanical force. **A)** Lateral view of a multinucleated myofiber containing the force-generating myofibrils. T-tubules (straight yellow lines) are invaginations in the sarcolemma of the myofiber that allow for the propagation of action potentials across the fiber. The sarcoplasmic reticulum spans across the inside of the myofiber just underneath the sarcolemma and releases calcium upon stimulation by an action potential. **B)** Cross sectional view of a myofiber showing the force generating myofibrils and the sarcolemma. **C)** Generation of mechanical force in skeletal muscle. Tropomyosin (red) wraps around the thin actin filament of the sarcomere and prevents its association with the thick myosin filament (left-side). A regulatory protein known as troponin (green) binds to tropomyosin and alters its conformational state upon binding to calcium. When an action potential causes the release of calcium from the sarcoplasmic reticulum, troponin binds to calcium and alters the conformation of tropomyosin. This allows for the interaction of thick and thin filaments and the subsequent generation of mechanical force.

Prenatal Development of Skeletal Muscle

Fortunately, skeletal muscle has a remarkable capacity to regenerate in healthy individuals. In order to understand the mechanism of regeneration in adults, the developmental processes occurring during embryogenesis must first be explored. Skeletal muscle is derived from myogenic precursors known as somites [11]. Somites are derived from the paraxial mesoderm of the embryo and are bilaterally positioned along the anterior-posterior axis [12]. The concentration gradients of key morphogens such as those involved in the Sonic Hedgehog, Wntless and Decapentaplegic pathways, previously identified in *Drosophila*, play a key role in the development of organs and appendages [13]. These morphogens are typically signaling molecules secreted from various types of tissue which induce a significant cellular response by regulating downstream signaling pathways [13]. It has been shown that an anterior to posterior gradient of retinoic acid is responsible for promoting the development of the next stage of the somite, the dermomyotome ([11]; Figure 2). The ventral part of the dermomyotome becomes the sclerotome which develops into cartilage and bone [14]. Muscle cells delaminate from the hypaxial dermomyotome and travel to the limb field where they will eventually form the dorsal and ventral muscle masses ([11]; Figure 2).

This developmental process is highly regulated by a key group of transcription factors and signaling molecules. An important family of regulators involved in myogenesis include the Paired box genes, transcription factors characterized by a paired DNA binding domain which play a role in lineage determination [15]. Delamination and migration of the hypaxial dermomyotome is regulated by the transcription factor Paired box domain protein 3 (Pax3) which activates the expression of c-Met, a tyrosine kinase receptor ([16]; Figure 2). Migration of skeletal muscle was absent in embryos of mice treated with antisense RNA against Pax3,

demonstrating its role in muscle progenitor migration [17]. Pax3 expression has also shown to be activated by ligands of the Wingless Integration Site (Wnt) signaling pathway [18]. Another Paired box gene that plays an important role in myogenesis is Paired box domain protein 7 (Pax7) which will be discussed later in the context of postnatal muscle regeneration.

Once the somite develops into the dermomyotome, muscle progenitors begin to express a key group of transcription factors known as Myogenic Regulatory Factors (MRFs) to form the myotome (Figure 2). These transcription factors are characterized by a helix-loop-helix domain and serve as important regulators of myogenesis by activating the expression of muscle specific genes [19]. The MRFs bind to E-Box sequences (CANNTG) on DNA in order to activate the myogenic transcriptional program [20]. One of the first MRFs to be expressed in the dermomyotome is Myogenic Factor 5 (Myf5) followed by myogenin, Myogenic Regulatory Factor 4 (MRF4) and Myoblast Determination Factor 1 (MyoD), respectively [20]. The progenitors within the hypaxial dermomyotome initially express low levels of Myf5 before delamination occurs [14]. Once MyoD is expressed, the central part of the hypaxial dermomyotome disintegrates leading to the development of the myotome and the terminal specification of muscle lineage [14]. Together, the MRFs turn on the myogenic program during development and activate the expression of muscle specific genes [14]. Some of these genes include those involved in contraction such as myosin heavy chain and muscle creatine kinase or structural proteins such as desmin and titin [21]. The regenerative stem cells in skeletal muscle known as satellite cells are derived from the central dermomyotome and will be discussed in the next section. These regenerative muscle progenitors are derived from muscle precursors which originated from the dermomyotome and migrated into the myotome ([22]; Figure 2). These satellite cells are marked by the expression of two Paired box domain proteins, Pax3 and Pax7

which maintain them in a stem-cell like state capable of repairing damaged muscle fibers ([20]; Figure 2).

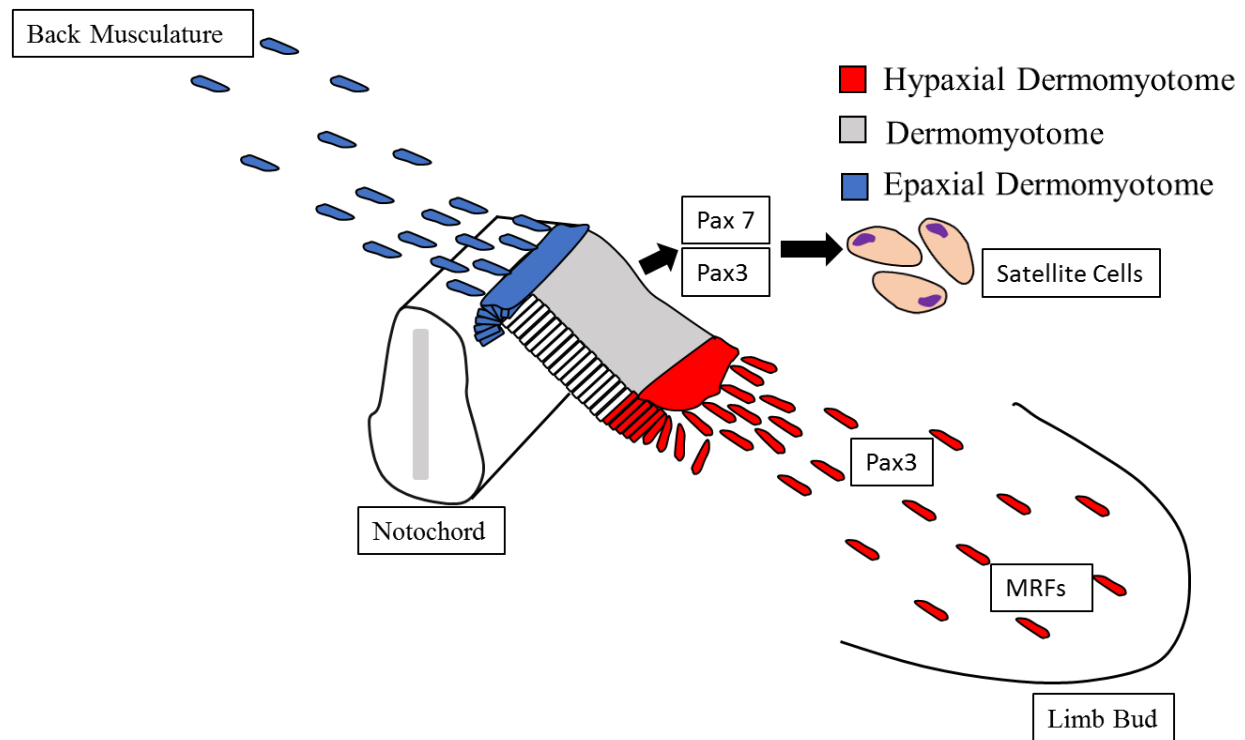


Figure 2: Schematic of prenatal muscle development. Skeletal muscle is derived from the paraxial mesoderm which eventually develops into the somite and then the dermomyotome (shown above). Muscle precursors located at the dermomyotome can delaminate once they express the myogenic marker Pax3. Delaminated muscle precursors from the hypaxial dermomyotome go on to form the muscle of the limbs while the precursors from the epaxial dermomyotome form the back musculature and other regions. Once myogenic precursors reach their final location they express myogenic regulatory factors and differentiate into muscle fibers. Some precursors continue to express the paired box transcription factors Pax 3/7 and go on to form the regenerative stem cells in skeletal muscle known as satellite cells. Adapted from Buckingham M, Bajard L, Chang T, et al. The formation of skeletal muscle: from somite to limb. *J Anat.* 2003;202(1):59-68.

Post-Natal Regeneration of Skeletal Muscle

Skeletal muscle has an ability to respond to injury in healthy individuals and regenerate through a pool of muscle progenitors known as satellite cells [23]. Satellite cells reside in a niche between the sarcolemma of muscle fibers and the basal lamina which encapsulates the myofiber [24]. Satellite cells remain in a state of quiescence where they reside in their niche until activated

by injury [25]. Another Paired box gene that is essential for the maintenance and proper functionality of satellite cell populations is Pax7 [26]. Activated satellite cells treated with siRNA against Pax7 prematurely differentiate into muscle fibers and show impaired proliferative capacity [26]. Induction of muscle damage using cardiotoxin in adult mice with a floxed Pax7 gene, was found to impair the regenerative capacity of skeletal muscle [26]. Activated satellite cells can undergo a proliferative phase to regenerate the pool of progenitor cells [27]. Furthermore, satellite cells can become activated in order to differentiate and fuse with damaged myofibers through a complex process of transcriptional regulation by MRFs as well as various signaling pathways.

Process of Skeletal Muscle Regeneration

Satellite cells can be activated through a variety of manners including the disruption of the sarcolemma due to muscle injury, nitric oxide produced by damaged muscle fibers, secretion of chemokines or cytokines from immune cells, as well as the release of growth factors from the bloodstream such as Wnt ligands and fibroblast growth factor (FGF) ([24]; Figure 3). Activated satellite cells undergo asymmetric cell division which allows the original pool of satellite cells to be maintained while also providing myogenic progenitors for muscle repair [27]. Satellite cells can also undergo symmetric division in which one activated satellite cell divides into two quiescent satellite cells in order to increase the population of satellite cells [27]. The ultimate result of satellite cell activation is the expression of myogenic regulatory factors starting with Myf5, followed by MyoD and myogenin ([24]; Figure 3). Satellite cells that underwent miR-33 mediated knockdown of Myf 5 could not initiate the myogenic program and had decreased expression of skeletal muscle specific markers such as myogenin [28]. Similarly, MyoD

expression was found to be required for the formation of myotubes in the C2C12 cell line, a model system for skeletal muscle [29]. Finally, myogenin, a MRF expressed after Myf5, was indispensable for the proper development of hind leg muscles *in vivo* as shown by decreased fiber size in mice lacking myogenin expression [30]. The coordinated expression of these MRFs allows for the initiation of the myogenic program in satellite cells and the eventual fusion of these cells with damaged myofibers.

Once satellite cells have been activated and the myogenic program has been initiated, they differentiate into a muscle precursor known as a myoblast. The final step in the repair of skeletal muscle is fusion of myoblasts with the damaged muscle fiber ([32]; Figure 3). This process involves the adhesion of an activated myoblast to a damaged myofiber, the invasion of the myoblast into the myofiber and eventual fusion pore formation which allows for the exchange of cytoplasmic contents [32]. A key protein recently discovered in the fusion process was coined the term Myomaker. Primary myoblasts from mice expressing a dysfunctional copy of this gene could express myogenic specific markers but could not fuse and form multinucleated myofibers [33]. Furthermore, the Myomaker gene appears to be regulated by MRFs due to the presence of many E-box sites located at its promoter [34]. It is clear that the process of muscle regeneration is a highly complex and coordinated process. Understanding the molecular mechanisms that mediating skeletal muscle regeneration will allow for the development of treatments that restore its remarkable ability of self-repair and restore quality of life to patients impacted by various disorders.

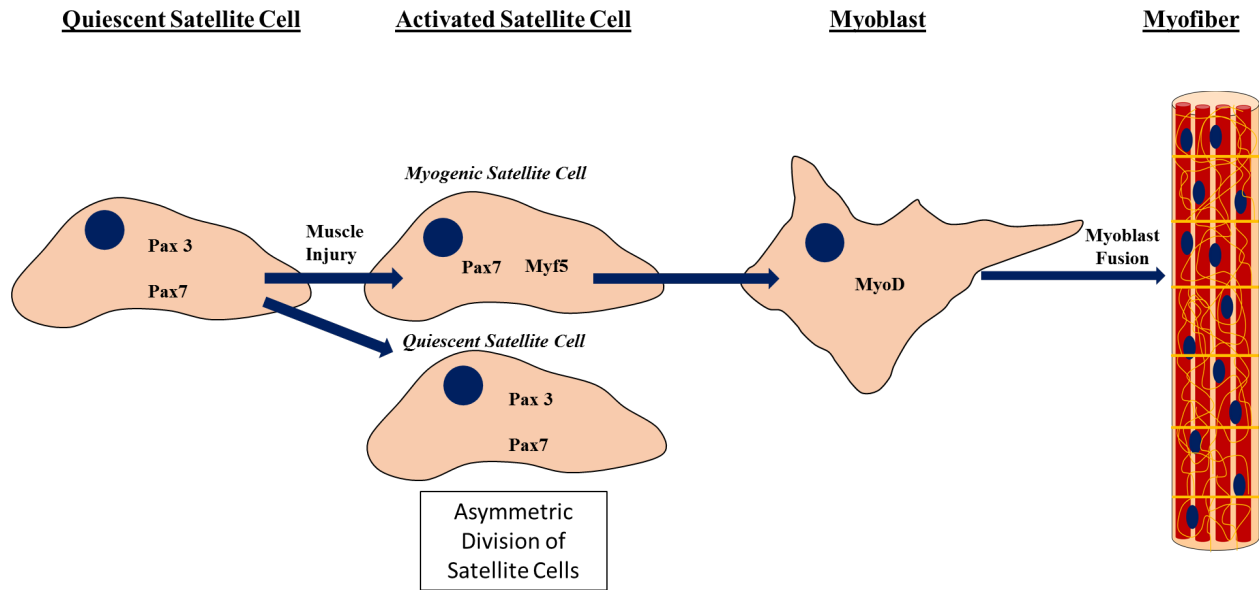


Figure 3: Process of satellite cell differentiation into a functional myofiber. Satellite cells can be activated through a variety of factors including muscle injury which leads to the expression of myogenic regulatory factors starting with Myf5 followed by MyoD and myogenin. These transcription factors activate the expression of muscle specific genes leading to the differentiation of a satellite cell into a myoblast which can fuse with damaged myofibers. Activation of a satellite cell can lead to asymmetric division which generates an activated satellite cell and a quiescent satellite cell. Satellite cells can also renew their population through symmetric division which generates 2 quiescent daughter cells.

The Role of Signaling Pathways in Skeletal Muscle

Various signaling pathways such as Notch, Transforming Growth Factor Beta (TGF- β) and Wnt converge upon skeletal muscle to regulate regenerative capacity by controlling the proliferation and differentiation of myogenic precursors [35,36]. Previous research established that Notch signaling is responsible for the proliferative expansion of myogenic precursors that mediate the regeneration of skeletal muscle [27]. Myostatin, a signaling molecule found to activate TGF- β signaling, acts as a negative regulator of muscle growth [36,37]. Finally, activation of Wnt signaling has been shown to play a key role in the differentiation of myoblast

precursors into myotubes [29]. It appears that in order to understand the mechanisms mediating the regeneration of skeletal muscle, we must understand how these signaling pathways facilitate their wide range of effects.

Wnt Signaling in Skeletal Muscle

Wnt signaling plays a crucial role in the regulation of gene expression in myogenic precursors of skeletal muscle. The major signaling receptors involved in this pathway include the Low Density Lipoprotein Receptor Related Protein 5/6 and Frizzled receptors which can bind to cysteine rich glycoproteins known as Wnt ligands [38]. These signaling receptors can activate a downstream cascade which regulates the activity of effector proteins in the Wnt pathway [38]. The main Wnt effector protein is β -catenin which has the ability to shuttle between the cytosol and the nucleus and ultimately regulate gene expression [38]. Whether β -catenin has the capacity to activate Wnt target genes depends on its subcellular localization. In the cytosol, β -catenin is degraded through interactions with a group of proteins that make up the “destruction complex” [39]. In the nucleus, β -catenin can interact with transcription factors such as those of the T-cell factor/lymphoid enhancer factor (TCF/LEF) and myogenic regulatory factors in order to activate the expression of Wnt target genes [29,36]. The regulation of Wnt signaling activity plays a key role in developmental processes, pathophysiological disorders such as cancer as well as the regeneration of skeletal muscle [38]. Therefore, it is essential to understand the process by which effector proteins involved in Wnt signaling such as β -catenin mediate the repair of skeletal muscle.

Mechanism of Canonical Wnt Signaling

The canonical Wnt pathway remains in an inactivated state without a Wnt ligand available to bind to the membrane-bound Frizzled receptor [39]. In order to understand the downstream processes occurring during Wnt inactivation, the role of the destruction complex must first be examined. The destruction complex contains the proteins glycogen synthase 3 β (GSK3 β), adenomatosis polyposis coli (APC) and Axin1 which interact with β -catenin in the cytosol [40]. During a state of Wnt inactivation, β -catenin is phosphorylated at serine and threonine residues on its N-terminus by casein kinase 1 and GSK3 β , leading to its ubiquitination and proteasomal-mediated degradation ([40,41]; Figure 4). The destruction complex prevents the activation of Wnt target genes by β -Catenin through this proteasomal mediated process [40].

The Wnt pathway can be activated once a Wnt ligand binds to the Frizzled receptor [40]. This binding event recruits LRP5/6 and activates a downstream cascade which inhibits the phosphorylation of Beta-catenin by the destruction complex [40]. The main event leading to the inactivation of the destruction complex is the recruitment of Dishevelled, a 650 amino acid protein that can shuttle between the nucleus and cytoplasm [42]. When Dishevelled interacts with the Axin in the destruction complex, it removes GSK3 β from the destruction complex, thereby suppressing the proteasomal mediated degradation of β -Catenin ([42]; Figure 4). The inhibition of the destruction complex allows β -Catenin accumulate in the cytosol and translocate into the nucleus where it interacts with TCF/LEF transcription factors as well as myogenic regulatory factors such as MyoD and Myocyte Enhancer Factor 2 (MEF2). ([29,43]; Figure 4). The interaction of β -catenin with transcription factors or myogenic regulatory factors activates the transcription of Wnt target genes and muscle specific genes [29]. Interestingly, different subtypes of Wnt ligands and Wnt receptors play a diverse role within different tissue types.

While the Wnt1/3a ligands are known to activate β -catenin in skeletal muscle, other Wnt ligands such as Wnt11 can play a role in the development of the dorsal axis [40,44]. For this reason, Wnt signalling has a multitude of effects on organ systems including regulation of development, pathological disorders of the brain and T-cell differentiation [45].

Regulation of Canonical Wnt Signaling

There is also an intricate interplay between regulatory proteins that can modulate the activity of the Wnt signaling pathway. Post-translational modifications on the components of the destruction complex, effector proteins such as β -catenin and the Frizzled or LRP5/6 receptors can have a dramatic impact on Wnt activity [42]. As previously discussed, post-translational phosphorylation of β -catenin leads to its proteasomal targeted degradation by the destruction complex [42]. Furthermore, upon binding of a Wnt ligand to the Frizzled receptor, LRP5/6 is phosphorylated via an unknown mechanism which leads to the downstream signaling cascade that frees β -catenin from the destruction complex [42]. Phosphorylation of TCF/LEF transcription factors by kinases such as Traf2/Nck-interacting kinase (TNIK) has been found to increase their affinity to β -catenin and transform them from transcriptional repressors to transcriptional activators [46]. Opposite of this stimulating effect on TCF mediated transcription, other protein kinases such as Nemo (Nlk) have been found to phosphorylate TCFs such as TCF4 which decreased the expression of its target genes [47]. The mechanism in which Nlk was found to reduce the expression of Wnt target genes was by decreasing the ability of the β -catenin/TCF complex to effectively bind to DNA targets [47].

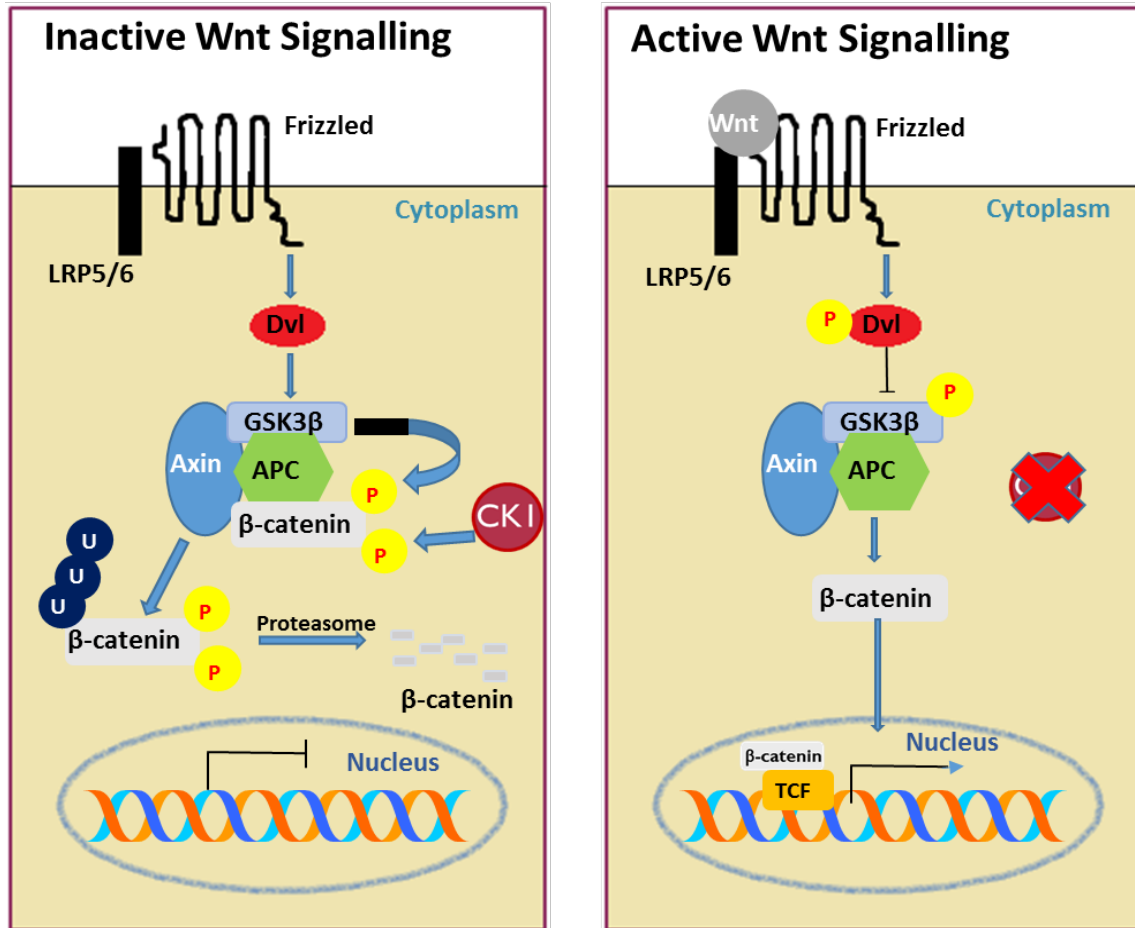


Figure 4: Process of Wnt signaling. When the Wnt pathway is inactive, β -catenin is sequestered in the destruction complex, phosphorylated by CK1 and GSK3 β and targeted for proteasomal mediated degradation. When a Wnt ligand binds to the Frizzled receptor, Wnt signaling is activated which leads to the recruitment of LRP5/6 at the cell membrane and the phosphorylation of the Dishevelled protein. Dishevelled then deactivates the destruction complex allowing β -catenin to be freed and translocate into the nucleus. *Adapted from Macdonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009;17(1):9-26.*

Multifunctional Role of β -Catenin

β -catenin is a multifunctional protein that can bind to various classes of proteins such as transcription factors, muscle regulatory factors, chromatin remodeling proteins and structural proteins [29,48]. Interestingly, β -catenin plays a key structural role in cell-cell adhesion as well

as the activation of Wnt target genes and muscle specific proteins. β -catenin was originally found to function as a structural protein when it was discovered that it could interact with the adhesion protein E-cadherin and link adhesion junctions to the cytoskeleton through an allosteric interaction with α -catenin ([49,50]; Figure 5).

It is also believed that this cell-cell adhesion role of β -catenin regulates its activity by sequestering the protein in the cytosol [51]. Sequestration of β -catenin at the adherens complex was shown to act as a site of residence until stimulation by Wnt ligands could allow for the subsequent release of β -catenin towards the nucleus [51]. As previously discussed, β -catenin also acts as a transcriptional regulator by interacting with TCF/LEF transcription factors and myogenic regulatory factors ([29]; Figure 5). Clearly, β -catenin plays a role in a multitude of cellular processes including both structural stability and transcriptional regulation by interacting with a diverse group of proteins which mediate its function.

β -catenin contains 3 major domains including a 150 amino acid N-terminal region, 12 Armadillo domain repeats (40 amino acids each) and a 100 amino acid C-terminal domain [52]. The N-terminus of β -catenin contains a key regulatory regions rich in serine residues which are targeted by members of the destruction complex in order to regulate the stability and localization of β -catenin [52]. The N-terminus was also found to harbor the region where α -catenin, a key adherens junction protein, binds with β -catenin to allosterically regulate the connection between cytoskeletal components of the cell with adherens junction proteins such as E-cadherin ([50]; Figure 5). Deletion mutagenesis studies have shown that the Armadillo repeat region of β -catenin plays a key structural role by interacting with cadherin proteins at cell-cell junctions ([49]; Figure 5). This region is also responsible for binding to members of the destruction complex such as APC in order to regulate the stability of β -catenin ([49]; Figure 5). Transcription factors

belonging to the TCF/LEF and Forkhead transcription factor (FOXO) family have also been shown to bind to the Armadillo region ([48,53]; Figure 5). Finally, transcriptional co-activators such as the Mediator complex and chromatin remodeling proteins have been found to interact with the C-terminal domain of β -catenin ([54]; Figure 5). It is not currently known whether β -catenin plays other roles in skeletal muscle but its high degree of multifunctionality suggests that this may be a possibility. Interestingly, β -catenin contains no enzymatic domains, therefore exploring its binding partners may provide insight on how β -catenin plays such a multifunctional role and whether there are other possible avenues of regulation mediated by β -catenin in skeletal muscle.

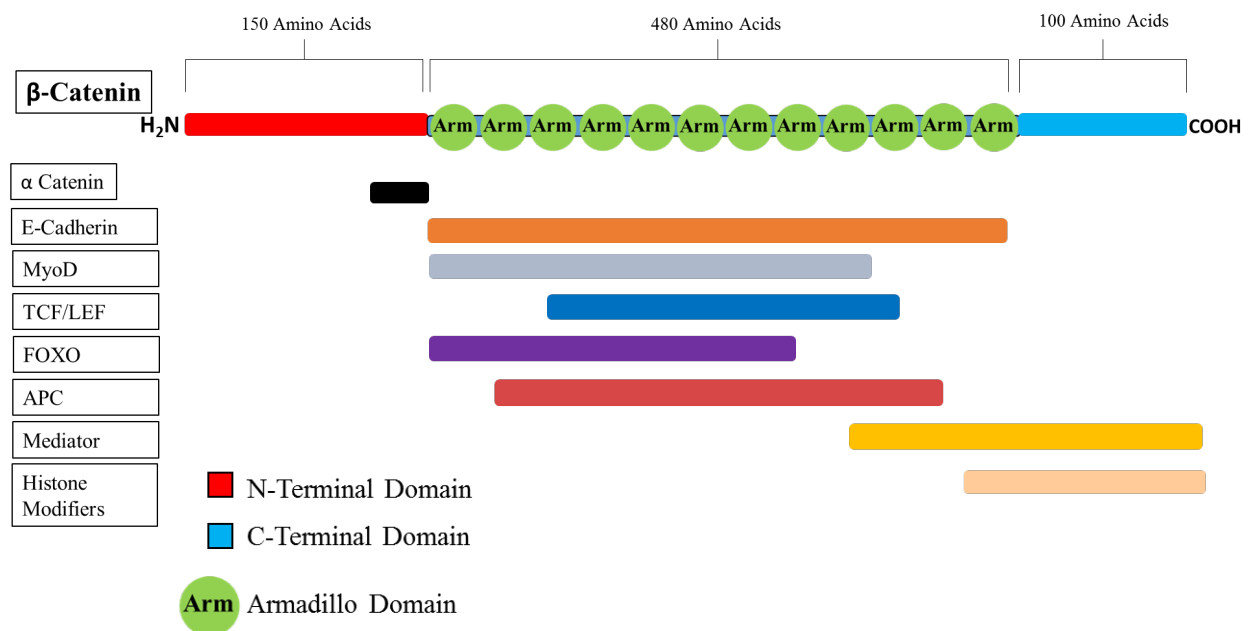


Figure 5: Schematic of β -catenin protein domains and major binding partners. The N-terminal domain is responsible for interacting with α -catenin in order to interact with structural proteins at the adherens junction. The Armadillo domains interact with other transcription factors and myogenic regulatory factors as well as APC, a member of the destruction complex. Finally the N-terminal domain interacts with components of the Mediator complex as well as histone modifying proteins. *Adapted from Valenta T, Hausmann G, Basler K. The many faces and functions of β -catenin. EMBO J. 2012;31(12):2714-36.*

Recent research has established that β -catenin can interact with key regulatory proteins including post-transcriptional regulators in smooth muscle [55]. It was found that β -catenin could interact with the Fragile X Mental Retardation Protein (FMRP), an important post-transcriptional regulator originally studied in neurons [55]. This β -catenin-FMRP complex adopted a localization at the translational machinery and repressed translational activity [55]. Interestingly, the β -catenin-FMRP mediated translational repression was abrogated by Wnt signalling activation due to the translocation of β -catenin into the nucleus and away from FMRP at the translational machinery [55]. This recent study has contributed to the theory of β -catenin as a multifunctional protein by establishing its novel role as a post-transcriptional regulator.

Pathophysiological Role of Wnt Signaling and β -Catenin

Aberrant activation of Wnt signaling is implicated in diseases such as cancer since this signaling pathway regulates the expression of cell growth genes such as C-Myc and Cyclin-D1 and has such a significant effect on the composition of the transcriptome [56]. A commonly implicated mutation in cancers caused by the Wnt signaling pathway involves the serines residues located on the N-terminal domain of β -catenin [52]. Typically, these residues are phosphorylated by the destruction complex leading to the degradation of β -catenin [52]. Mutations at these residues prevent the phosphorylation of β -catenin at these sites thereby altering its stability and subcellular localization leading to excessive cell growth [52].

β -catenin has also been identified as an interacting partner of FOXO transcription factors which have previously been found to cause skeletal muscle atrophy and alterations in muscle type when overexpressed in mouse models ([57,58]; Figure 5). Wnt activation was found to

promote the interaction between β -catenin and FOXO1 and shift the expression of fatigue-resistant muscle fiber markers such as Myosin Heavy Chain 4 (MyH4) to that of fatigueable muscle fibers including MyH2/7 [58]. In addition to cancer and muscle atrophy, aberrant activation of Wnt signaling and β -catenin has been implicated in a wide range of other diseases including metabolic, developmental, cardiac and bone related disorders [59]. In conclusion, targeting of the Wnt signaling pathway and the activity of its downstream effector protein, β -catenin, provides a promising avenue for the targeted treatment of a wide range of disorders including those related to skeletal muscle.

The Role of Canonical Wnt Signaling and β -Catenin in Myogenesis

Wnt signaling and β -catenin have been studied in a wide variety of contexts including developmental biology, cancer development and skeletal muscle. Wnt signaling is essential for the development of skeletal muscle through its role in dermomyotome/myotome formation as well as through regulation of Pax3/7 expression [11]. Interestingly, activation of the Wnt signaling pathway has been shown to maintain the somite in an undifferentiated, mesenchymal state [14]. Furthermore, β -catenin appears to play a key role in development as mice embryos lacking β -catenin expression do not survive past 8 days of development [60].

Looking at the process of myogenesis in skeletal muscle, β -catenin has also been found to interact with key myogenic regulatory factors such as MyoD to drive the transformation of myoblasts into myotubes in C2C12 cells [29]. Similarly, Wnt signaling activation was found to be required to increase the myogenic potential of myoblasts in order to fuse into myofibers [61]. An adenovirus induced Cre-recombinase model of skeletal muscle in mice with a floxed β -

catenin locus found that muscle fibers lacking β -catenin expression had significantly smaller cross-sectional area than wild type muscle fibers [62]. Wnt 4a stimulation of C2C12 cells was also found to inhibit myostatin, a negative regulator of skeletal muscle differentiation [63]. Finally, Wnt stimulation using Wnt1/3a/5a ligands induced a higher rate of proliferation in activated satellite cells [64].

It has been suggested that there is an optimal level of β -catenin activity required to activate skeletal muscle differentiation *in vivo* [36]. Tibialis anterioris (TA) muscles of mice with a constitutively active (CA) form of β -catenin or a conditional knockout (KO) of β -catenin were injected with cardiotoxin in order to measure the regenerative capacity of muscle after 30 days. It was found that in both of these models, TA muscles were found to have a higher degree of fibrotic and inflammatory markers in both CA and KO mice compared to control wild type mice [36]. This pro-fibrotic effect was believed to be mediated through increased transcription of TGF- β signaling components, such as TGF- β 2/3, previously known to positively regulate fibrosis, in both constitutively active and knockout mice [36]. Based on this study, it appears that there is an optimal level of β -catenin activity that maintains the regenerative capacity of skeletal muscle. It is clear that Wnt signaling, mediating its effect through β -catenin, plays an indispensable role in multiple facets of myogenesis including development, repopulation of myogenic progenitors and differentiation of myogenic cells.

Non-Canonical Wnt Signalling

Non-canonical Wnt signaling refers to an alternative mechanism of signaling which can be activated by certain Wnt ligands but does not use β -catenin as a downstream effector. The two

forms of non-canonical Wnt signaling include the planar cell polarity (PCP) pathway as well as the Wnt-Ca²⁺ pathway [65]. The PCP pathway activates downstream effectors such as GTPases from the Rac1/Ras family and c-Jun N-terminal kinase (JNK) which play a key role in developmental processes such as neurulation and cell migration [65,66]. Alternatively, the downstream effectors of the Wnt-Ca²⁺ pathway includes G-proteins which activate protein kinase C (PKC) and phospholipase C (PLC) [67]. The activation of PKC has a wide range effects on gene expression while the activation of PLC leads to increased intracellular calcium levels and activation of calmodulin dependent protein kinases [65,67]. Different Wnt ligands have the ability to activate either the canonical or non-canonical Wnt signaling pathway. For example, Wnt 1, 2, 3, 8a, 8b, 10a, and 10b ligands activate the canonical pathway while Wnt 4, 5a, 5b, 6, 7a, 7b, and 11 activate the non-canonical pathway [68]. It is important to understand that the process of Wnt signaling requires a complex interplay between downstream effector proteins and their interacting partners which can have a host of downstream effects including transcriptional regulation, developmental processes, cell migration and changes in intracellular ion concentrations.

Non-canonical Wnt signaling has also been proposed to play a role in myogenesis. Non-canonical Wnt7a ligand levels were found to be upregulated during regeneration after induction of muscle injury using cardiotoxin on the tibialis anterior muscle [69]. As previously mentioned, satellite cells can undergo asymmetric division to restore the pool of quiescent progenitor cells while also providing myogenic precursors that can regenerate damaged muscle fibers [27]. In contrast, symmetric division is the process by which one quiescent satellite cell divides into two quiescent satellite cells [69]. Stimulation with non-canonical Wnt7a was found to lead to an increase in the proportion of symmetric divisions occurring in satellite cells [69]. In conclusion,

non-canonical Wnt signalling plays a role in myogenesis by promoting the restoration of the quiescent satellite cell population in skeletal muscle [69].

Post-Transcriptional Regulation in Skeletal Muscle

Although signaling pathways such as Wnt signaling appear to play a major role in mediating the transcriptional regulation of muscle specific genes, another avenue of control in gene expression involves post-transcriptional regulation. Satellite cells activated by muscle injury were isolated and subjected to microarray analysis which uncovered an enrichment of genes involved in post-transcriptional control relative to inactivated satellite cells [70]. These genes included RNA processing proteins as well as splicing proteins which were found to have roles in the developmental formation of the limb bud, regulation of stem cell activity as well as regulation of muscle differentiation [70]. Post-transcriptional regulation in skeletal muscle has also been studied by exploring the role of Stauffen, an RNA binding protein which was previously found to regulate RNA localization [71]. It was found that Stauffen could bind to MyoD RNA and repress the translation of this MRF in order to preserve the stemness of skeletal muscle progenitors [72]. There is a large amount of post-transcriptional regulators expressed in skeletal muscle which have not yet been explored or have only been studied in other organ systems. One of these key post-transcriptional regulators was found to be the Fragile X Mental Retardation Protein (FMRP) which was initially studied in the context of neuronal gene expression [73].

The Fragile X Mental Retardation Protein

FMRP is an RNA binding protein, encoded by the FMR1 gene, which functions to regulate translation through interactions with the 5' cap of mRNA [73]. There are multiple isoforms of FMRP that each possess their own unique expression patterns in the human body. FMRP has typically been studied in the brain but recent studies have shown that it is also expressed in skeletal muscle [73,74]. These isoforms are encoded by the FXR1 and FXR2 genes. While FXR1 was originally discovered in muscle, both FXR1 and FXR2 localize in the brain [75,76]. It is believed that these isoforms have similar functions since they all possess an identical RGG RNA binding domain but may be differentially regulated due to truncations in phosphorylation sites on exon 15 [77]. FMRP was originally studied in neurons located within the hippocampus of mice where it was found to repress the translation of Microtubule Associated Protein 1 or Map1b [78]. The role of FMRP as a translational repressor is essential for proper synapse development since individuals who possess a mutated version of this gene on the X chromosome suffer from Fragile X Mental Retardation Syndrome [78]. FMRP mutations are characterized by an increased number of CGG trinucleotide repeats on the FMRP promoter which leads to its hypermethylation and a loss of FMRP expression [79]. Individuals with Fragile X Mental Retardation Disorder suffer from mental disabilities, emotional difficulties and facial abnormalities [79]. Interestingly, it appears that isoforms of FMRP may have different regulatory roles since only FMRP and not the other related proteins were shown to rescue synapse development in a *Drosophila* model system originally lacking expression of this protein [80].

Mechanism of FMRP Mediated Post-Transcriptional Regulation

The manner in which FMRP binds mRNA is through a KH1/KH2 and RGG domain ([81]; Figure 6). The consensus RNA binding sequences recognized by the KH2 region have previously been discovered and it was determined that these sequences can bind KH2 by associating with a “kissing complex” on RNA target sequences ([82]; Figure 6). The kissing complex consists of a duplex of stem loops in an RNA molecule that form intramolecular bonds with each other and stabilize tertiary structure [82]. Mutations in the KH2 domain have been found to prevent FMRP from associating with the ribosomal machinery [82]. The RGG domain of FMRP also binds to RNA through the formation of a G-quartet or quadruplex structure (Figure 6). The G-quartet is formed by the binding of arginine and glycine rich regions of the RGG domain in FMRP to guanine rich RNA sequences [83].

The mechanism by which FMRP regulates translational activity occurs during the initiation stage of translation. The eukaryotic ribosome consists of a large 60S subunit which carries out translation and a smaller 40S subunit that binds to RNA to recruit the 60S subunit [84]. Typically, the 40S subunit of the ribosome associates with various eukaryotic initiation factors that function to activate translation and becomes the 43S subunit [85]. This complex then binds to an mRNA and scans it until the start codon is found [85]. In order to mediate binding of the 43S subunit to the mRNA, eIF4E associates with the 5^l cap and forms the eIF4F complex which recruits the 43S subunit [85]. FMRP has been found to repress translation by forming a complex with CYFIP and binding to eIF4E during the initiation of translation ([73]; Figure 7). It was proposed that FMRP helped mediate the binding of CYFIP to eIF4E and that this disrupted the formation of the eIF4F complex at the 5^l cap, thereby stalling translation of FMRP bound transcripts ([73]; Figure 7).

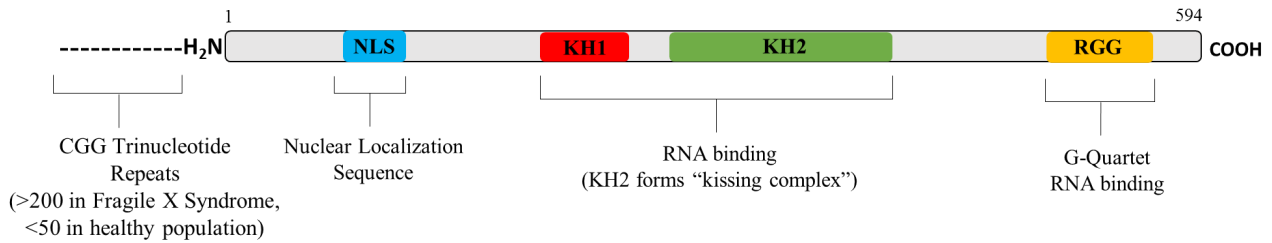


Figure 6: Schematic of FMRP protein domains. The major mutation involved in Fragile X Mental Retardation disorder involves an increase in CGG trinucleotide repeats on the FMR1 promoter which prevent the protein from being translated. FMRP can be shuttled through the nucleus by its nuclear localization signal and binds to RNA through its KH1/KH2 and RGG domain. Adapted from Valverde R, Pozdnyakova I, Kajander T, Venkatraman J, Regan L. Fragile X mental retardation syndrome: structure of the KH1-KH2 domains of fragile X mental retardation protein. *Structure*. 2007;15(9):1090-8.

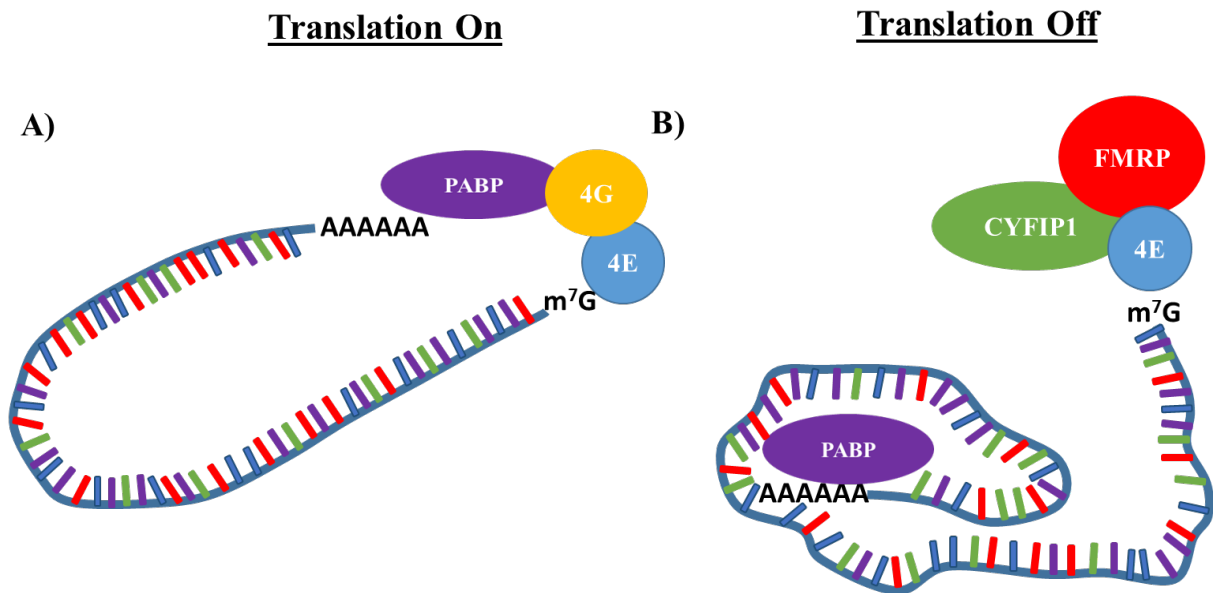


Figure 7: Mechanism of FMRP mediated translational repression. **A)** Under normal conditions, an mRNA transcript is circularized through interactions between the Poly-A binding protein and members of the eukaryotic initiation factors. This allows the translational machinery to process the transcript and translate the proper protein. **B)** When FMRP associates with CYFIP1, this protein complex binds to the eukaryotic initiation factor protein 4E at the 5 prime cap of the mRNA transcript. This prevents the full assembly of the eukaryotic initiation factors and subsequent recruitment of the translational machinery. Adapted from Napoli I, Mercaldo V, Boyl PP, et al. *The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP*. *Cell*. 2008;134(6):1042-54.

Regulation of Skeletal Muscle by FMRP

A recent area of research has focused on the role of FMRP in muscle. One of the isoforms of the FMR1 gene, FXR1, was found to play a key role in the development of skeletal muscle. FXR1 double knockout mice were found to die soon after birth while mice with a conditional knockout of FXR1 showed abnormalities in development of limb musculature and the number of nuclei present within each myofiber [86]. Further studies looked at the role of FXR1 in somite formation during development of *Xenopus* embryos. Morpholino treatment against FXR1 in *Xenopus* embryos lead to a reduction of MyoD expression in the developing somites at stage 25 of development [87]. By the time stage 30 of development was reached, it was found that a lack of FXR1 expression lead to reduced levels of skeletal muscle related proteins such as myosin light chain, Na/K ATPase γ subunit, myogenin and myosin binding subunit 85 [87]. It was found that FMRP post-transcriptionally regulated the expression of muscle specific genes such as the myogenic regulatory factor Myf5 [74]. FMRP expression was required for the expression of Myf5 and MyoD in activated satellite cells as well as the Paired box gene Pax7 [74]. Satellite cells in FMR1 knockdown mice showed an impaired capability to self-renew, evident by decreased Pax7 expression [74]. Although FMRP has typically been studied in the context of neuronal development, it is becoming clear that the post-transcriptional control mediated by this protein has implications in other tissue types including skeletal muscle.

Statement of Purpose:

Clearly, β -catenin plays a vital role in shaping the transcriptome of skeletal muscle by regulating the expression of muscle specific genes. Since the function of β -catenin is mediated by its interacting partners, our group conducted mass spectrometry to uncover the interacting partners of β -catenin in smooth muscle to determine whether β -catenin may possess other important regulatory functions. Interestingly, β -catenin was found to interact with proteins involved in both mRNA processing and translation.

One of the proteins involved in translational regulation identified in the study was FMRP. It was previously mentioned that FMRP could interact with CYFIP to regulate translational activity by preventing the binding of eukaryotic initiation factors, such as eIF4E, to the translational machinery [73]. Our group identified a role for β -catenin in modulating translational activity through its interaction with FMRP, a post-transcriptional regulator in smooth muscle cells [55]. These studies demonstrate the function of β -catenin is highly dependent on its interacting partners.

In view of the fundamental role played by β -catenin in skeletal muscle reviewed above, we conjectured that the β -catenin-FMRP interaction may also be operative in translational control in skeletal muscle. This tissue type requires a high degree of translational control for development, growth, adaptation and regeneration [88]. Our purpose therefore was to determine whether the interaction between β -catenin and FMRP is functional in control of gene expression in skeletal muscle. Exploring the β -catenin-FMRP interaction could allow us to identify a novel function of β -catenin in skeletal muscle and further develop our understanding of regulatory mechanisms acting on the myogenic program.

Introduction:

Human skeletal muscle consists of bundles of multinucleated cells known as myofibers which confer our ability to move and breathe as well as regulate metabolic processes [1]. Exploring the molecular mechanisms occurring at the cellular level deepens our understanding of this complex organ system and can help uncover potential therapeutic targets for those suffering from muscle wasting disorders. One of these potential targets involves the Wnt signaling pathway; implicated in mediating the regenerative capacity of skeletal muscle in adults by regulating the myogenic differentiation program of muscle precursors.

Wnt signaling plays an important role in activating the transition between muscle precursors known as myoblasts into fully functional myofibers in order to repair accumulated damage within skeletal muscle [61]. The key effector protein of the Wnt signaling pathway is β -catenin, a 730 amino acid protein with a wide class of interacting partners including TCF/LEF transcription factors, muscle regulatory factors and structural proteins found at adherens junctions [29,48]. Interestingly, β -catenin does not possess an enzymatic domain, therefore its function is mediated by its interacting partners. β -catenin has been identified as a key player in myogenesis where it was found to interact with the muscle regulatory factor MyoD and activate transcription of the myogenic program [29].

While β -catenin has been identified as a transcriptional regulator due to its interactions with transcription factors such as MyoD, an emerging field of research involves the role of post-transcriptional regulators in skeletal muscle. Recently, a protein originally studied in neurons, FMRP, has been found to play an important post-transcriptional role in skeletal muscle. This protein contains the RNA binding domains KH1/KH2 and RGG which allow it to bind to

mRNAs and regulate the translation of these transcripts by blocking the recruitment of initiation factors to the preinitiation complex of the ribosome [73]. Recently, FMRP was found to bind to the mRNA of another key myogenic regulatory factor, Myf5, in skeletal muscle and regulate its expression [74]. This suggested that FMRP plays an important role in the post-transcriptional regulation of muscle specific genes part of the myogenic program.

Previously, our group has sought to identify the interacting partners of β -catenin in smooth muscle in order to uncover novel regulatory functions of β -catenin. Interestingly, after conducting mass spectrometry, it was found that β -catenin interacted with FMRP and other components of the translational pre-initiation complex in smooth muscle [55]. Biochemical analysis confirmed the interaction and established β -catenin at the translational machinery in association with FMRP [55]. Furthermore, activation of Wnt signalling and subsequent activation of β -catenin was found to increase the overall translational activity of smooth muscle [55]. This suggested that the role of β -catenin may not only be restricted to transcriptional regulation but that it could also act as a post-transcriptional regulator by interacting with the RNA binding protein FMRP.

In this study we sought to further characterize the β -catenin-FMRP interaction in the context of skeletal muscle in order to further develop our understanding of its function in translational control. . Here we report that β -catenin interacts with FMRP in skeletal muscle and characterize the subcellular co-localization of these proteins. Furthermore, we identify the presence of β -catenin and FMRP at the translational machinery and the important role of this interaction in the overall translational activity of skeletal muscle. Finally, we provide preliminary findings regarding the mRNA transcripts predicted to interact with FMRP in skeletal muscle.

Materials and Methods:

C2C12 Myoblast Cell Culture - Growth Conditions (GM):

In order to maintain a proliferative/undifferentiated state, C2C12 myoblasts were grown in DMEM high glucose media (4.00mM L-Glutamine, 4500mg/L Glucose) supplemented with 10% FBS and 1% penstrap.

C2C12 Myoblast Cell Culture - Differentiation Conditions (DM):

In order to induce differentiation of myoblasts into myotubes, C2C12 myoblasts were grown in high glucose DMEM media (4.00mM L-Glutamine, 4500mg/L Glucose) supplemented with 2% horse serum for a period of 24-96h.

Primary Cell Culture:

Primary myoblasts were harvested from the limbs of neonatal mice between 1-3 days old. Muscle tissue was digested with a high glucose DMEM media solution containing 1.5U/ml collagenase, 2.4U/ml dispase and 2.5mM CaCl₂. Primary myoblasts were pre-plated 3 times to remove fibroblasts then grown in F10 media supplemented with 20% FBS, 2.5ng/mL fibroblast growth factor and 1% penstrap on 10% Matrigel coated plates. Differentiation was induced with high glucose DMEM media (4.00mM L-Glutamine, 4500mg/L Glucose) supplemented with 5% horse serum.

Fluorescence Confocal Microscopy of C2C12/ Primary Myoblasts

C2C12/primary myoblasts were fixed using a 4% formaldehyde solution for 15 minutes. These myoblasts were then permeabilized by adding 500ul of 90% methanol for 5 minutes. The fixed C2C12/primary myoblasts were blocked using 5% goat serum for 1hr followed by an overnight incubation with primary antibodies including mouse β -catenin (Abcam-19381), rabbit FMRP (Cell Signaling-4317) at a 1:300 ratio. After a 3x wash with PBS, C2C12/primary myoblasts were incubated with mouse TRITC (Sigma-T5393) and rabbit FITC (Sigma-F9887) secondary antibodies at a 1:1000 ratio for 2hr. C2C12/ and primary myoblasts were incubated with DAPI (Sigma-D9542) at a 1:1000 ratio for 30 minutes followed by a 3x PBS wash. C2C12 and primary myoblasts were visualized using a Zeiss Observer Z.1 confocal microscope with TRITC visualized using a 532 laser line and FITC visualized using a 488 nanometer laser line.

Single Fiber Isolation:

Single myofibers were harvested from the Extensor Digitorum Longus (EDL) muscle in 6-8 week old C57 mice (The Jackson Laboratory). EDL muscles were digested in 400U/mL collagenase solution for 2hr followed by 3 washes with PBS. Disassociated EDL muscle fibers were immediately fixed using a 4% formaldehyde solution for a period of 5 minutes followed by 3 washes with PBS.

Fluorescence Confocal Microscopy of Single Myofibers

Fixed single fibers were blocked using 5% goat serum for 1hr followed by an overnight incubation with primary antibodies including mouse Pax7 (Sigma), rabbit FMRP (Cell Signaling-4317) at a 1:50 and 1:300 ratio, respectively. After washing 3 times with PBS, single myofibers were incubated with anti-mouse TRITC (Sigma-T5393) and anti-rabbit FITC (Sigma-F9887) secondary antibodies at a 1:500 ratio for 2hr. Single myofibers were incubated with DAPI (Sigma-D9542) at a 1:1000 ratio for 30 minutes followed by a 3x PBS wash. Single fibers were then placed into glass slides using Dako Fluorescent Mounting Medium. Single myofibers were visualized using a Zeiss Observer Z.1 confocal microscope with TRITC visualized using a 532 laser line and FITC visualized using a 488 nanometer laser line.

Co-Immunoprecipitation:

Cell lysate was harvested from C2C12 cells maintained under growth conditions. 50ul of Immunocruz IP/WB Optima C beads were incubated with a 1:50 ratio of FMRP antibody (#4317- Cell Signalling). Immunocruz IP/WB Optima C beads were washed 3 times with PBS and cell lysate containing 1000ug of protein was added to the beads followed by overnight incubation. Immunocruz beads were then washed 3 times with PBS followed by boiling at 95°C in 40ul of SDS loading buffer. 20ul of SDS loading buffer was added to a 10% SDS PAGE gel and probed for FMRP (#4317- Cell Signalling) and β -catenin (ab19381- Abcam).

Overexpression of β -catenin and FMRP

C2C12 myoblasts (GM) grown in 35mm glass bottom dishes were transfected using 10ug of polyethylenimine added to 500ul of serum free DMEM media containing 1ug of mCherry-FMRP and 2ug of eYFP- β -catenin DNA plasmids. C2C12 myoblasts were incubated with this transfection medium for a period of 4hr. Afterwards, 1.5mL of GM media was added for a period of 24hr. A 1:1000 ratio of DAPI was then added for a period of 30min to visualize the nuclei of the myoblasts. Imaging was conducted immediately after DAPI incubation using a Zeiss Observer Z.1 confocal microscope with mCherry visualized using the 532 laser line and eYFP visualized using the 488 nanometer laser line. ImageJ RGB profile plot tool was used to visualize fluorescent intensities of each fusion protein.

Puromycin Translational Activity Assay (SUnSET):

C2C12 cells in GM, 24h DM, 48h DM, 72h DM and 96h DM growth stages were exposed to 0.54ug/mL of puromycin for 5 minutes. After 5 minutes, C2C12 cells were washed 3 times with PBS and cell lysate was harvested with NP40 lysis buffer (see extended Material and Methods). 20ug of cell lysate was loaded into an SDS PAGE gel and probed for either puromycin (DSHB - PMY-2A4), β -catenin (ab19381- Abcam), Myosin Heavy Chain (MF-20 - Dako Cytomation) or the loading control Erk (sc-93 Santa Cruz Biotechnology). The absolute incorporation of puromycin under each condition was determined via Western Blotting against puromycin (DSHB - PMY-2A4). The intensity of each puromycin blot was quantified using the ImageJ Gels tool and then compared relative to the intensity of the loading control Erk.

siRNA Mediated Knockdown of β -catenin

In order to conduct the SUnSET assay under β -catenin knockdown conditions, C2C12 myoblasts (GM or 24h DM) maintained in 35mm dishes were transfected with 5uL of Lipofectamine 2000 and 1uL of 100uM siRNA targeting β -catenin (SASI_Rn01_00099923 [**si23**] or SASI_Rn01_00099924 [**si24**] – Sigma Aldrich). Furthermore, C2C12 myoblasts (GM or 24h DM) maintained in 35mm dishes were transfected with 5uL of Lipofectamine and 1uL of 100uM scrambled siRNA (SIC001 – Sigma Aldrich).

Analysis of SUnSET Assay Data

Relative puromycin incorporation was calculated in scrambled siRNA, siRNA 23 and siRNA 24 conditions in both GM/ DM 24h conditions in C2C12 myoblasts (N=3). Puromycin incorporation was compared relative to scrambled siRNA condition in both GM and DM 24h conditions. A 2 tailed T-test was conducted using Graphpad between the scrambled siRNA condition and both siRNA 23/ siRNA 24 conditions to determine whether there was a significant effect of β -catenin knockdown on puromycin incorporation.

Polysome Profiling Analysis:

C2C12 cells were treated with 100ug/mL of cycloheximide for 10 minutes and washed with PBS containing 100ug/mL cycloheximide in order to halt translation. Cells were harvested and lysed in hypotonic buffer containing 5mM Tris-HCL, 2.5mM MgCl₂, 1.5mM KCl, protease inhibitor,

10ug/mL cycloheximide, 0.5% Triton X-100 and 0.5% sodium deoxycholate. Cell lysate was loaded into a sucrose gradient containing a range of 20-50% sucrose and was spun in an ultracentrifuge at 21,000G for 2 hours at 4 °C. After separation of ribosomal machinery, 14 fractions were collected from the sucrose gradient using a fraction collector. Western blotting against FMRP (#4317- Cell Signalling), β -catenin (ab19381- Abcam) and eIF4E (#9742 – Cell Signalling) was conducted using 25uL of each collected fraction. 300uL of RPL9 (ab182556 – Abcam) and PABP (ab21060 - Abcam) fractions were concentrated using TCA precipitation and then probed for using Western Blotting (see extended material and methods).

M7-GTP Assay

C2C12 cells grown in GM media were harvested and lysed using NP-40 lysis buffer (see Extended Material and Methods). 3000ug of lysate was incubated overnight with 50uL of immobilized M7-GTP beads (AC-155S) or negative control immobilized γ -Amino-octyl-GTP (AC-106S). Beads were washed twice with TBS followed by 2 times with 0.1% TBST. Beads were boiled at 95°C for 5 minutes in 50uL of SDS loading buffer. 5uL of loading buffer was added to a 10% SDS PAGE gel to probe for eIF4E (#9742 – Cell Signalling) while 20uL was used to probe for each of β -catenin (ab19381 – Abcam) and FMRP (#4317 – Cell Signalling).

RNA Immunoprecipitation

C2C12 cells grown under GM conditions were fixed with 1% formaldehyde for 10 minutes. Following fixation, 0.25M of glycine was used to quench the reaction for a period of 5 minutes. C2C12 lysate was washed with PBS 3 times and then harvested in 100uL RIPA Buffer (50 mM

Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl). Cells were then sonicated 5 times for a period of 5 seconds and then centrifuged at 16,000g for 10 mins. C2C12 lysate was then collected and incubated with Dynabeads Protein G magnetic beads containing 4ul of FMRP (#4317 – Cell Signalling) antibody for 4hr. Protein G magnetic beads were then washed 6 times with 500ul High Stringency RIPA Buffer (50 mM Tris-Cl, pH 7.5, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 M NaCl, 1–4 M urea, and 0.2 mM phenylmethylsulfonyl fluoride). Protein G magnetic beads were then heated to 70°C for 45 minutes in 50ul of Collection Buffer (50 mM Tris-Cl, pH 7.0, 5 mM EDTA, 10 mM dithiothreitol (DTT) and 1% SDS). RNA from FMRP-IP elution was then isolated using 150ul of Trizol. Total RNA was also collected from C2C12 myoblasts maintained in GM conditions using NP40 lysis buffer followed by Trizol extraction.

Microarray Analysis

Microarray analysis was conducted in cooperation with the Sick Kids Microarray Facility (TCAG). Total RNA and FMRP-IP associated RNA was quality checked with an Agilent Bioanalyzer and then incubated in an Affymetrix Mouse Gene 2.0 Microarray (ThermoFisher-902119). This microarray contained 27,358 probesets against RNAs within the *mus musculus* transcriptome. Data was analyzed using the Transcriptome Analysis Console (ThermoFisher) by comparing the relative enrichment of FMRP-IP associated RNA to total RNA. A cutoff of 2 fold enrichment from FMRP-associated RNA compared to total RNA was applied to generate candidate RNAs that were proposed to interact with FMRP. The 2090 RNAs with a 2 fold enrichment in FMRP-IP samples were then subjected to a GO Ontology analysis using the Gene Ontology Consortium GO Analysis Tool.

Antibodies:

Poly-A Binding Protein (ab21060), Ribosomal Protein L9 (ab182556) and β -catenin (ab19381) antibodies were purchased from Abcam. Puromycin antibody (PMY-2A4) was purchased from Developmental Studies Hybridoma Bank. Myosin Heavy Chain antibody was purchased from Dako Cytomation. Eukaryotic Initiation Factor 4E (#9742) and Fragile X Mental Retardation Protein (#4317) antibodies were purchased from Cell Signaling. Myoblast Determination Protein 1 (sc-304), Myogenic Factor 5 (sc-302), and Mitogen Activated Protein Kinase 3 (Erk 1, sc-93) antibodies were purchased from Santa Cruz Biotechnology.

Results:

Characterization of FMRP and β -catenin Expression in Skeletal Muscle

Initially, we assessed the abundance of β -catenin and FMRP through a time course experiment. C2C12 myoblasts were maintained in a proliferative state and then differentiated into myotubes over a 96h period through withdrawal of growth factors. It was found that both β -catenin and FMRP were present in the proliferative stage as well as during each stage of differentiation into myotubes (Figure 8). β -catenin expression peaked at 72h while FMRP expression increased between GM to 48h DM and decreased between 48h DM and 96h DM (Figure 8). The levels of MRFs including Myf5 and MyoD and the muscle specific protein myosin heavy chain were analyzed to assess the stage of differentiation that was occurring (Figure 8). As expected, MyoD expression increased following the induction of differentiation at 24h DM and maintained this expression level through the duration of the time course experiment. An early stage myogenic regulatory factor Myf5, which was previously mentioned to be expressed only upon the onset of differentiation, was abundant at GM where C2C12 cells possess the most myogenic potential, and then progressively decreased upon the induction of the myogenic program (Figure 8; [24]). These experiments indicated that our model of skeletal muscle expressed myogenic regulatory factors in a similar manner to human skeletal muscle and that the expression of both β -catenin and FMRP could be detected in this model.

Next, we sought to characterize the localization pattern of β -catenin and FMRP using fluorescent confocal microscopy. We conducted the same time course experiment using C2C12 and primary myoblasts and captured images of β -catenin and FMRP at each stage of differentiation. In C2C12 myoblasts, β -catenin was localized at the cell membrane between GM

and 24h DM as well as 72h DM to 96h DM (Figure 9). This localization was indicative of β -catenin's important role at the adherens junctions where it interacts with structural proteins at the cell membrane [48]. Interestingly, the localization of β -catenin shifted from the cell membrane to the cytosol and within the nucleus at the 48h DM time point (Figure 9). The localization of β -catenin likely shifted to the nucleus due to its important role in activating the transcription of muscle specific proteins by interacting with myogenic transcription factors [29]. FMRP exhibited a characteristic cytoplasmic localization around the outside of the nucleus; indicative of its presence at the translational machinery which is highly concentrated in the rough endoplasmic reticulum (Figure 9; [73]). After immunostaining primary myoblasts grown in high serum conditions, β -catenin maintained a localization along the inside of the cell membrane (Figure 10). After 24h serum withdrawal in primary myoblasts, β -catenin was expressed only in differentiated, multinucleated myofibers along the cell membrane and in the cytosol (Figure 10). Finally, FMRP was localized around the outside of the nucleus in primary myoblasts while also present in the cytoplasm of differentiated myofibers, albeit to a lesser degree (Figure 10). Finally, we isolated single muscle fibers in order to determine whether we could observe FMRP in the regenerative satellite cells that associate with muscle fibers [24]. Here we document the presence of FMRP in Pax7 positive cells, a key marker of satellite cells (Figure 11). In conclusion, we established that β -catenin and FMRP are expressed in the C2C12 model of skeletal muscle and in primary myoblasts. Furthermore, we document the expression of FMRP in Pax7 positive satellite cells.

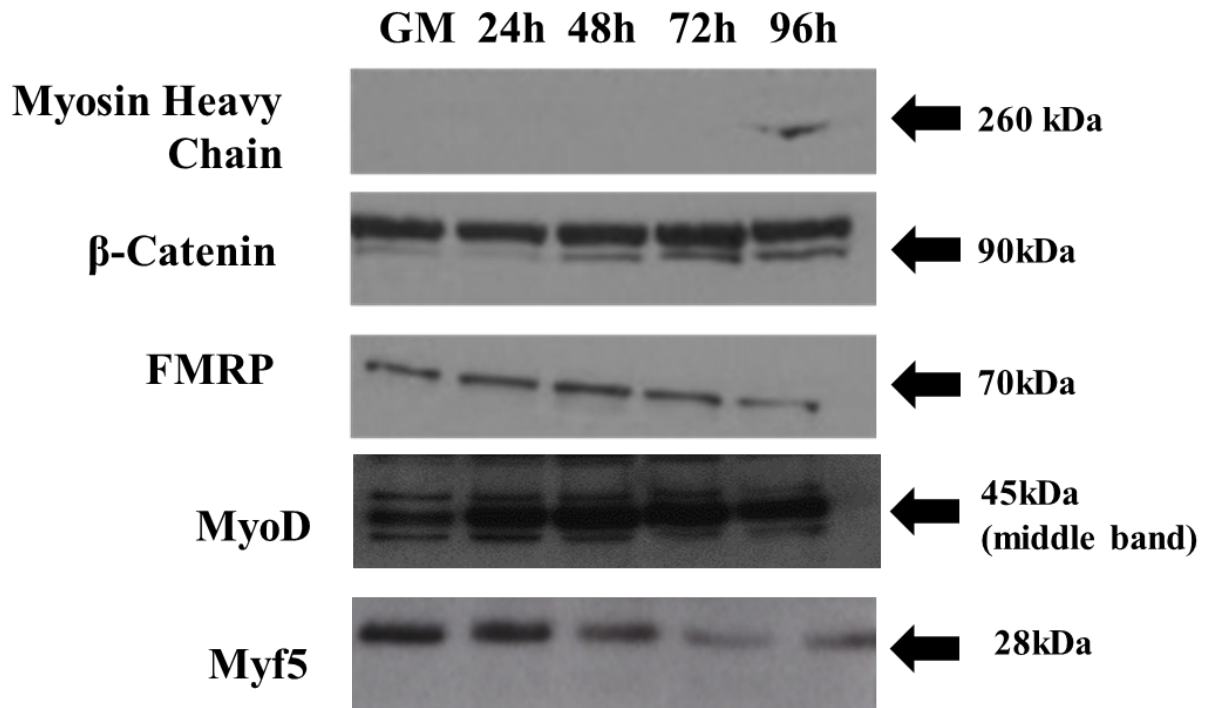


Figure 8: Expression of β -catenin and FMRP in C2C12 myoblasts at different stages of differentiation. C2C12 myogenic cells were grown under growth conditions and serum was withdrawn for periods of 24h, 48h, 72h and 96h to induce differentiation. Western blotting was conducted to probe for the presence of FMRP and β -catenin at each stage of differentiation. Western blotting was conducted against Myf5, MyoD and myosin heavy chain to measure stage of muscle differentiation.

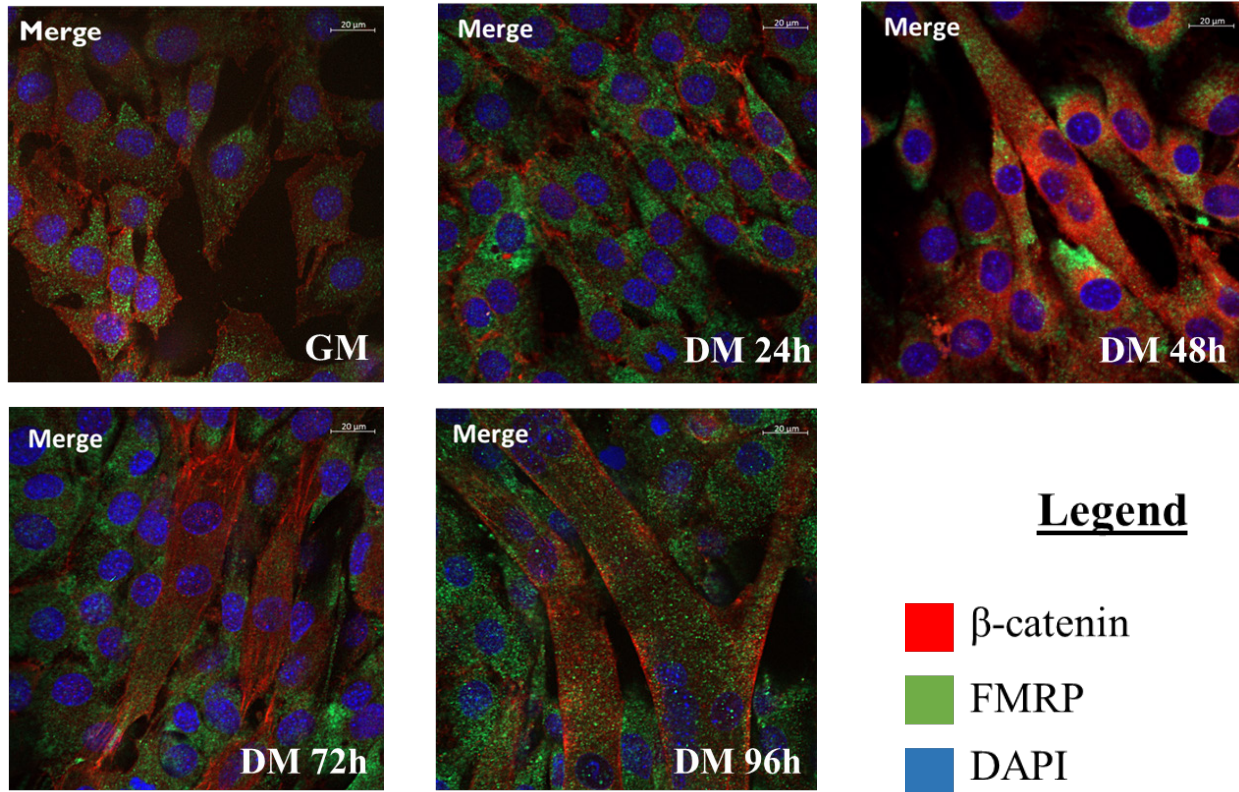
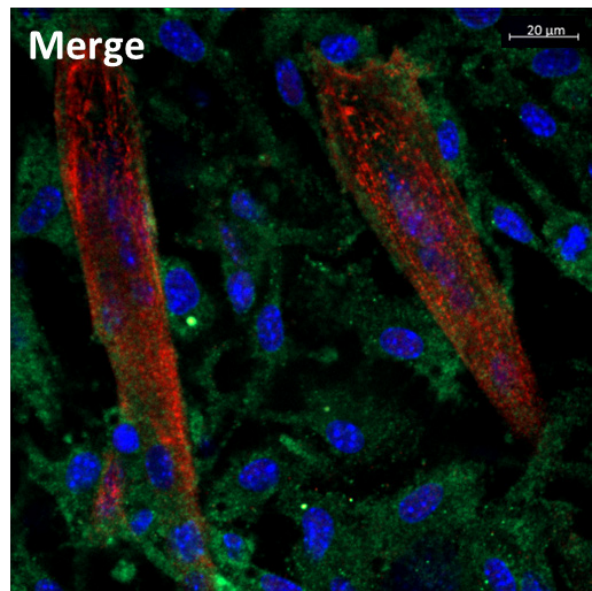
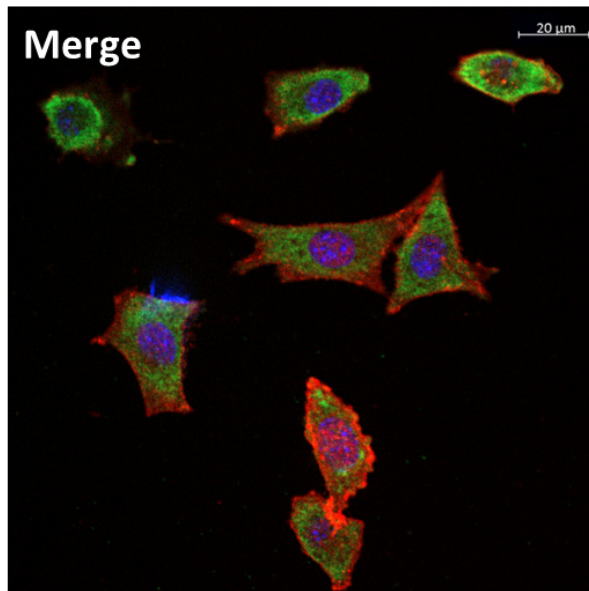


Figure 9: Subcellular localization pattern of β -catenin and FMRP in C2C12 myoblasts. C2C12 myogenic cells were grown under high serum growth conditions followed by serum withdrawal for periods of 24h, 48h, 72h and 96h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy. Individual fluorescent channels for each image are depicted in Figure S1-S5.



Legend

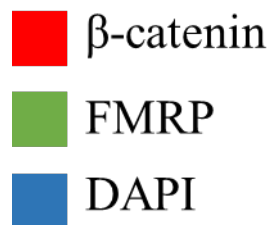


Figure 10: Subcellular localization pattern of β -catenin and FMRP in primary myoblasts. Primary myoblasts cells were grown under high serum conditions followed by serum withdrawal for a period of 24h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy. Individual fluorescent channels for each image are depicted in Figure S6 and S7.

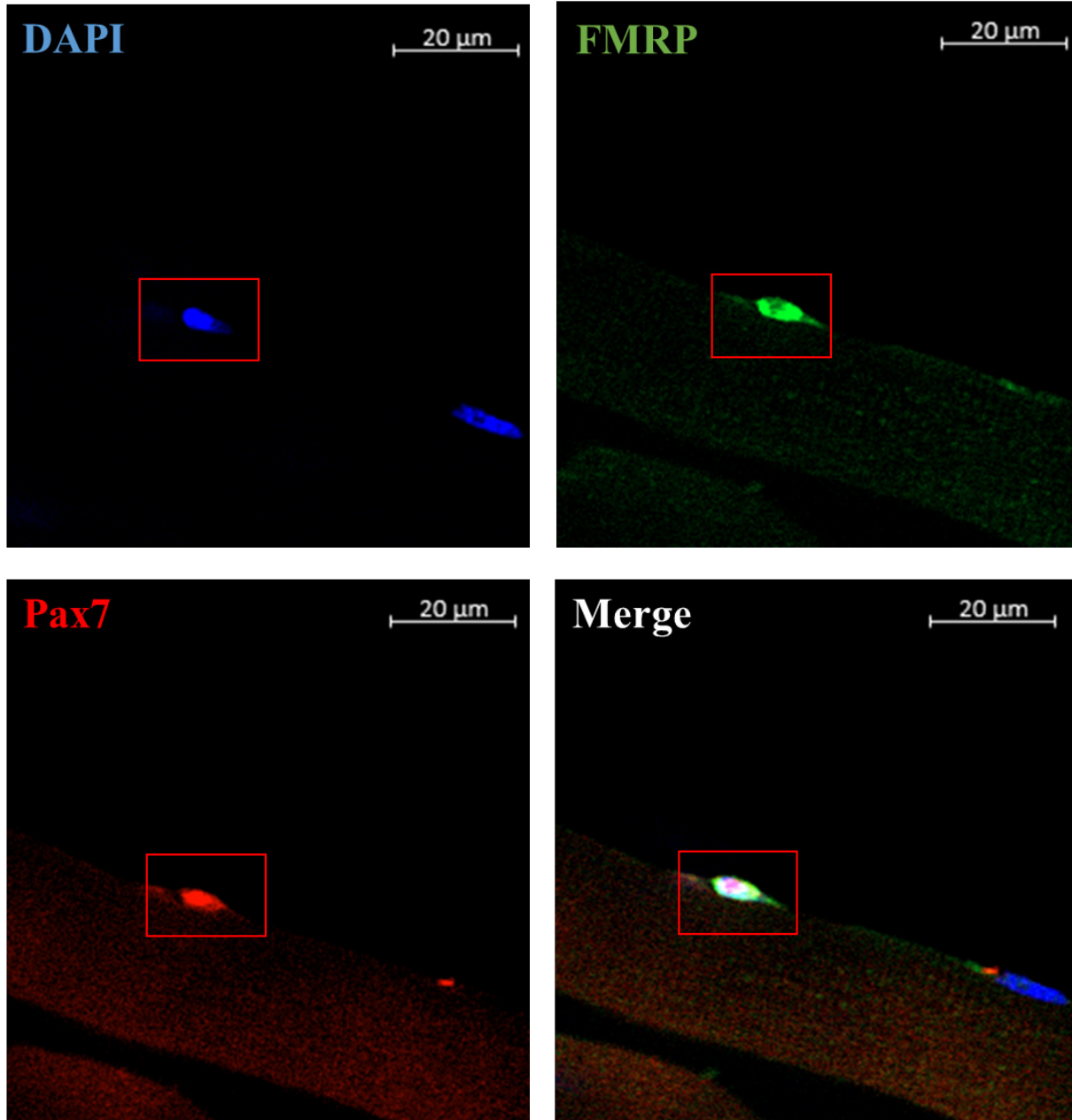


Figure 11: Expression of FMRP in satellite cells within single myofibers. Single myofibers were isolated from the Extensor Digitorum Longus muscle of 6-8 week year old mice and immunostained for the expression of Pax7 and FMRP. Pax7 was used as a marker to identify satellite cells.

Characterization of FMRP and β -catenin Interaction in Skeletal Muscle

After establishing that both FMRP and β -catenin were expressed in skeletal muscle, we sought to test the interaction biochemically using immunoprecipitation. To answer this question, we conducted an experiment in which we isolated FMRP using antibody-coupled agarose beads and probed for the presence of β -catenin in the immunoprecipitate. After isolating FMRP from GM C2C12 cellular lysate (Figure 12B), we also detected the presence of β -catenin (Figure 12A). In conclusion, this experiment indicated that FMRP and β -catenin interact biochemically. In order to confirm that this interaction was occurring in skeletal muscle, we utilized additional methodologies to assess this interaction.

Fluorescence microscopy has been used as a qualitative methodology that allows one to determine whether two proteins of interest colocalize with each other [89]. If colocalization between two proteins is observed then it can be inferred that they possibly interact and subsequently validated through other methodologies. We therefore used fluorescence confocal microscopy to analyze the interaction between β -catenin and FMRP.

In order to visualize the interaction between β -catenin and FMRP in cultured cells we generated plasmids containing fluorescently tagged fusion proteins of our proposed interactors. We transfected eYFP-FMRP and mCherry- β -catenin constructs into C2C12 cells grown in high serum conditions to determine whether colocalization of these protein occurs in a cellular context. After recording images of C2C12 cells expressing these fluorescently tagged proteins, we observed that both eYFP-FMRP and mCherry- β -catenin co-localized around the perinuclear region (Figure 13A). To further document the colocalization, ImageJ was used to generate RGB profile plots of transverse slices in the acquired image (red lines Figure 13A; Figure 13B/C/D). In theory, if colocalization was occurring at a proposed subcellular localization then one should

observe similar increases or decreases of intensity for the fluorescent channels that corresponded to the eYFP-FMRP and mCherry- β -catenin fusion proteins. After observing the RGB profile plots that were generated, we observed a characteristic colocalization pattern between eYFP-FMRP and mCherry- β -catenin (black arrows; Figure 13 B/C/D). Furthermore, we noticed that β -catenin appeared to shift from its more typical localization (Figure 9, GM) at the cell membrane and adopted a localization similar to FMRP when they were expressed together (Figure 13A). This observation, combined with the observation that β -catenin and FMRP interact biochemically, indicate a robust interaction between β -catenin and FMRP. The observations that β -catenin and FMRP interact biochemically and the imaging data depicting their cellular colocalization suggest a possible function in skeletal muscle.

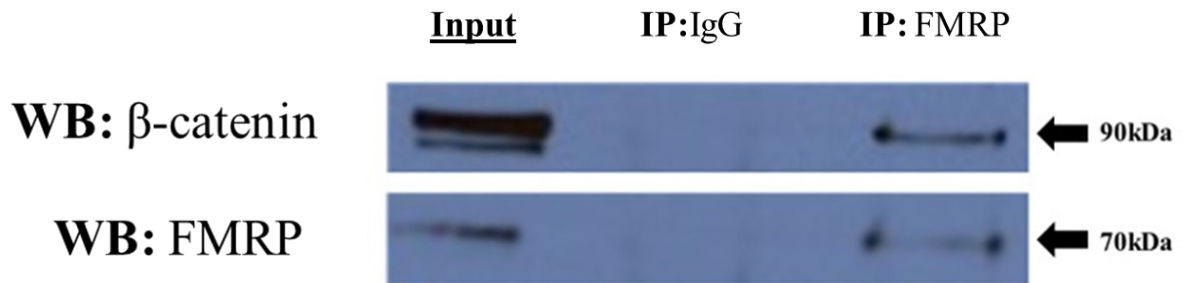


Figure 12: Coimmunoprecipitation of β -catenin and FMRP in C2C12 myoblasts. **A)** FMRP was isolated using an anti-FMRP antibody and Western blotting was conducted in eluted fraction to detect the presence of FMRP. An IgG antibody was used as a negative control. **B)** Western blotting was also conducted against β -catenin from the same eluted IgG and FMRP-IP fractions.

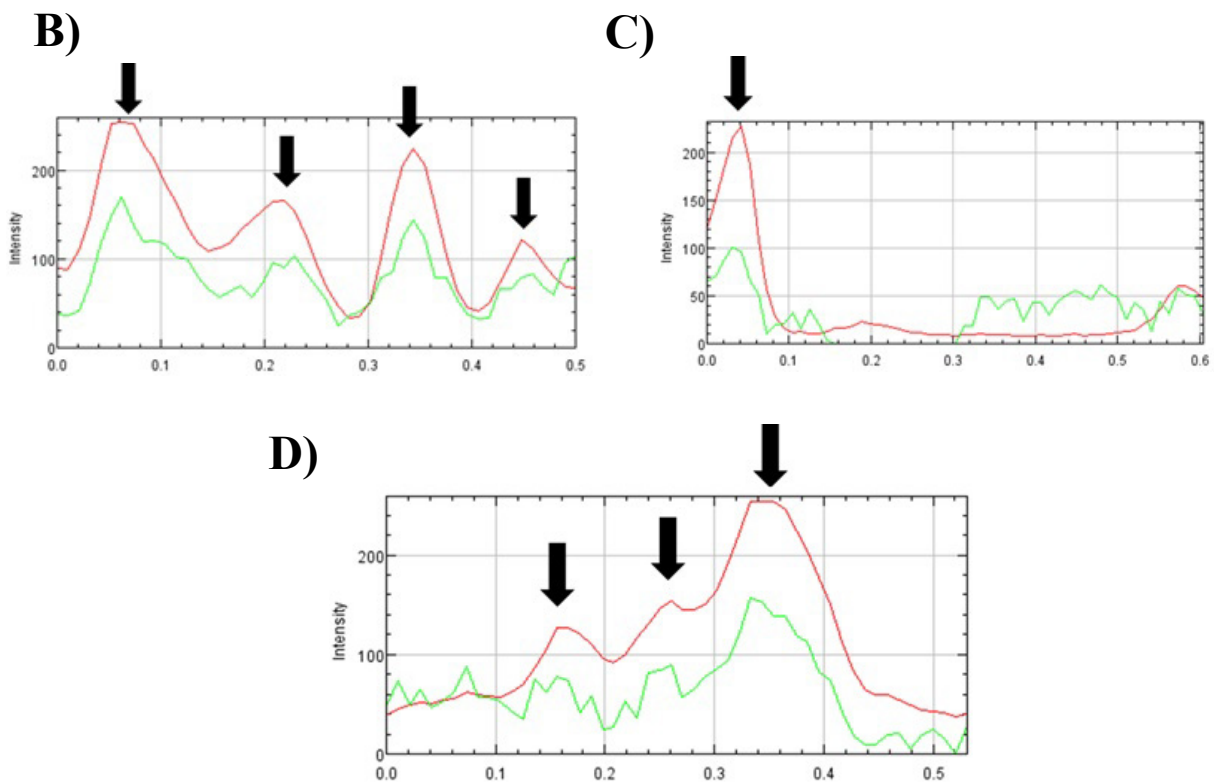
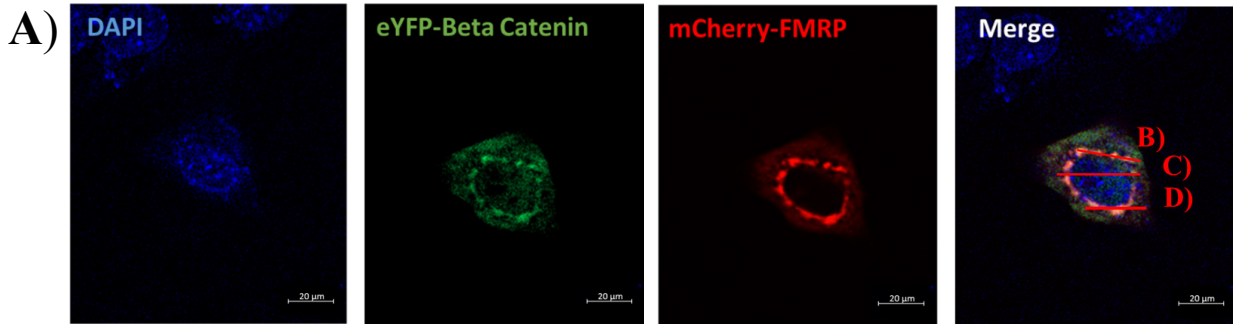


Figure 13: Colocalization of eYFP-FMRP and mCherry- β -catenin fusion proteins in C2C12 myoblasts grown under high serum conditions. Red-Green profile plots were acquired by using ImageJ software. **A)** Subcellular localization of eYFP-FMRP and mCherry- β -catenin in a C2C12 myoblasts. **B-D)** Red-Green profile plot of fluorescent channels from Figure XA (red lines in merged image). Black arrows represent areas in the image where similar changes in fluorescent intensity occurred indicating that colocalization occurred between each fluorescent fusion protein.

Role of FMRP and β -catenin Interaction in Skeletal Muscle on Translational Activity

The next step of our study was to determine whether the β -catenin-FMRP interaction played a role in the translational activity of skeletal muscle. In order to measure translational activity we employed the surface sensing of translation (SUnSET) technique (Figure 14; [90]). This methodology requires the use of puromycin, an analog of aminoacyl tRNAs, which incorporates into elongating polypeptide chains and stalls translation (Figure 14; [90]). The result of puromycin treatment is a buildup of stalled polypeptide chains containing puromycin (Figure 14; [90]). These polypeptide chains can then be visualized using a puromycin antibody in conjunction with western blotting (Figure 14). Therefore, when global translational activity is elevated there will be a higher rate of puromycin incorporation and subsequently stronger intensity of polypeptide chains when visualized through western blotting.

By exposing C2C12 myoblasts to puromycin we were able to acquire a readout of global translational activity at various stages of differentiation in a time course experiment (Figure 15). Interestingly, we found that the highest amount of puromycin incorporation occurred at 24h DM while the second highest rate of puromycin incorporation occurred at the GM phase (Figure 15). Previous studies characterizing the proteome of differentiating myoblasts have found that a wide range of muscle specific proteins such as transcription factors, signaling molecules and metabolic components must be translated in myoblasts as well as during their transition into a myotube [91]. β -catenin has also been proposed to play a key role in the onset of the myogenic program by activating the expression of muscle specific genes and regulating the fusion of myoblasts [29]. Furthermore, FMRP has recently been studied in skeletal muscle and found to post-transcriptionally regulate the expression of the MRF Myf5 which is required for the onset of myogenesis [74]. Clearly, there is a high degree of regulatory control occurring in the processes

that maintain a myoblast in a stem cell-like state as well as activate the process of differentiation from a myoblast to a myotube. For these reasons, we decided that studying the role of the β -catenin in both the myoblast (GM) and onset of differentiation (24h DM) stages would provide valuable insight regarding the possible post-transcriptional role of these proteins.

In order to study the role of the β -catenin in the post-transcriptional regulation of skeletal muscle we utilized the SUnSET assay in conjunction with siRNA treatment against β -catenin (Figure 16A). We were able to quantify global translational activity by measuring the intensity of puromycin incorporation and dividing it by the intensity of the loading control Erk using ImageJ software (Figure 16B/C). Interestingly, we found a 1.27 and 1.29 fold relative increase in translational activity in C2C12 myoblasts grown in GM conditions and treated with siRNAs against β -catenin (Figure 16B). After conducting a 2-tailed, equal variance T-test we found that our data was statistically significant with P values of 0.032 and 0.0039, respectively (Figure 16B). This suggested that β -catenin played a significant role in global translational inhibition since knocking down its expression increased translational activity in C2C12 myoblasts. Furthermore, C2C12 myoblasts that were induced to differentiate over a 24h period were found to have 1.20 and 1.75 fold relative decrease in translational activity when treated with siRNAs against β -catenin (Figure 16C). After conducting the same 2-tailed, equal variance T-test we determined that these data were not statistically significant with P values of 0.57 and 0.13, respectively (Figure 16C). While this data suggested that β -catenin could modulate translational activity in proliferating myoblasts, we wanted to understand whether it played a role directly at the translational machinery.

Based on a possible role in translational control we next determined whether β -catenin was present at the translational machinery using the polysome profiling technique. Polysome

profiling is a methodology that allows one to isolate various components of the translational machinery (Figure 17A). This includes the preinitiation complex which consists of the 40S ribosomal subunit, the ribosome which consists of the fully assembled 40S/60S subunits and the polysome which consists of multiple ribosomes translating an mRNA transcript [92]. By inhibiting protein translation using cycloheximide and loading cellular lysate containing the translational machinery into a sucrose gradient, one can collect fractions and isolate these translational components along with mRNAs and any regulatory proteins that associate with them (Figure 17A). As fractions are collected from the sucrose gradient, a spectrophotometer records the A_{254nm} absorbance of each sample which generates a characteristic polysome profile (Figure 17B). From here, one can probe for specific ribosomal proteins and analyze the pattern of the polysome profile to determine which component of the translational machinery is present in each fraction.

We collected cell lysate from C2C12 myoblasts growing under GM conditions in order to probe for the presence of β -catenin and FMRP in the translational machinery. In order to validate which components of the ribosome were present in each polysome fraction, we probed for other ribosomal proteins including eIF4E, large ribosomal subunit 9 (RPL9) and Poly-A Binding Protein (PABP). We determined that fraction 1-4 consisted of free RNA and the preinitiation complex due to an enrichment of eIF4E which was previously found to associate with the 40S subunit in the initiation of translation (Figure 17C; [85]). This was further validated by the presence of PABP which serves an important role in binding to the m⁷G cap during the process of translational initiation (Figure 17C; [93]). Finally, we probed for RPL9, a subunit of the 60S component of the ribosome that is recruited to the 40S component to create the complete

ribosomal complex [94]. From these data we found that fraction 6-14 contained the fully assembled ribosomes and polysomes due to an enrichment of RPL9 (Figure 17C).

After probing for β -catenin, we found that it was highly abundant in fractions 1- 4 which suggested that it was enriched in fractions containing the preinitiation complex (Figure 17C). We also found an abundance of FMRP in fractions 1 and 2 (Figure 17C). This corresponded with its previous role in the inhibition of translation at the preinitiation complex by preventing the binding of eukaryotic initiation factors [73].

In order to confirm the presence of β -catenin and FMRP at the translational machinery we conducted an m7GTP assay which uses modified agarose beads coupled with m7GTP to capture the pre-initiation complex. We detected an enrichment of β -catenin and FMRP bound to m7GTP beads relative to the GTP negative control (Figure 18). We also found a relative enrichment of the ribosomal protein eIF4E bound to the m7GTP beads, confirming that we successfully pulled down the translational machinery in this assay (Figure 18). Collectively, these observations suggest that β -catenin and FMRP are present at the pre-initiation complex of the translational machinery. In conclusion, these data indicate that β -catenin can modulate translational activity and that it can be found along with FMRP at the preinitiation complex of the translational machinery.

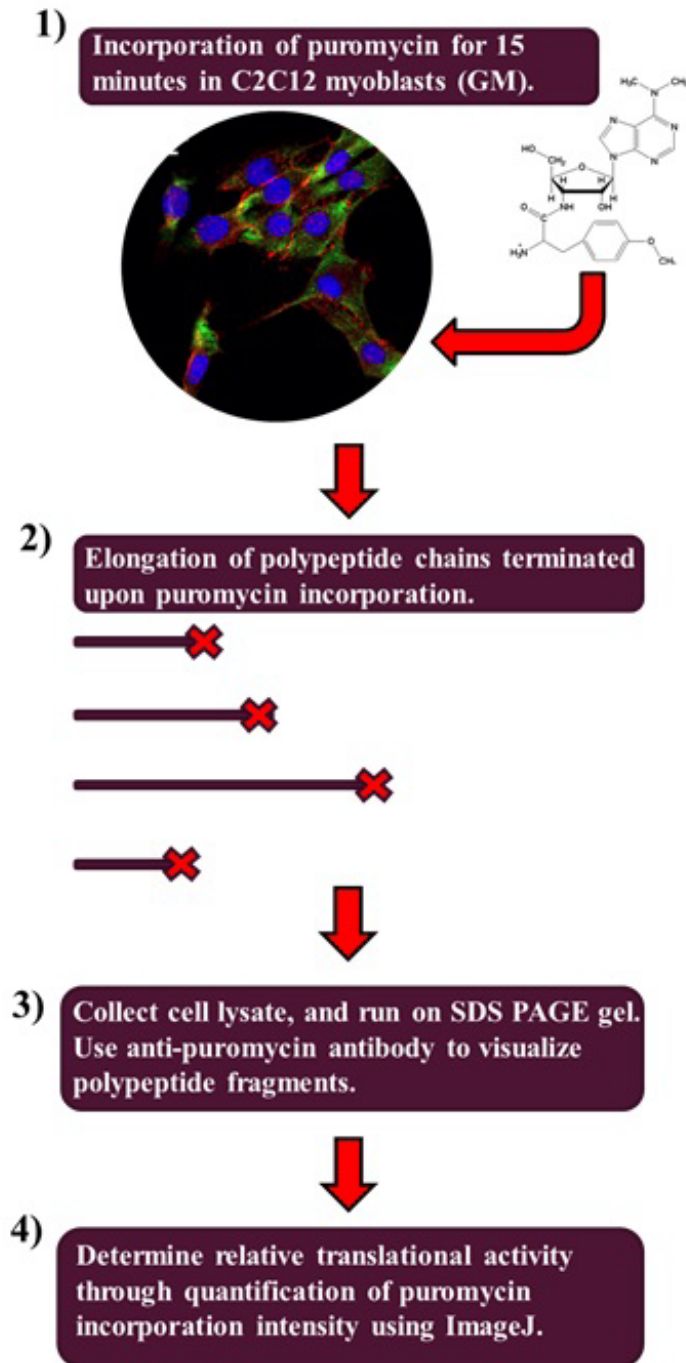


Figure 14: Workflow of the SUnSET technique. C2C12 myoblasts were exposed to a puromycin concentration of 0.544 μ g/ml for a period of 15 minutes. Elongating polypeptide chains were terminated upon the incorporation of puromycin. Cell lysate was collected and polypeptide chains were visualized upon Western Blotting using a puromycin antibody. Translational activity was quantified by measuring the intensity of puromycin incorporation; with more translational activity producing a more intense puromycin signal.

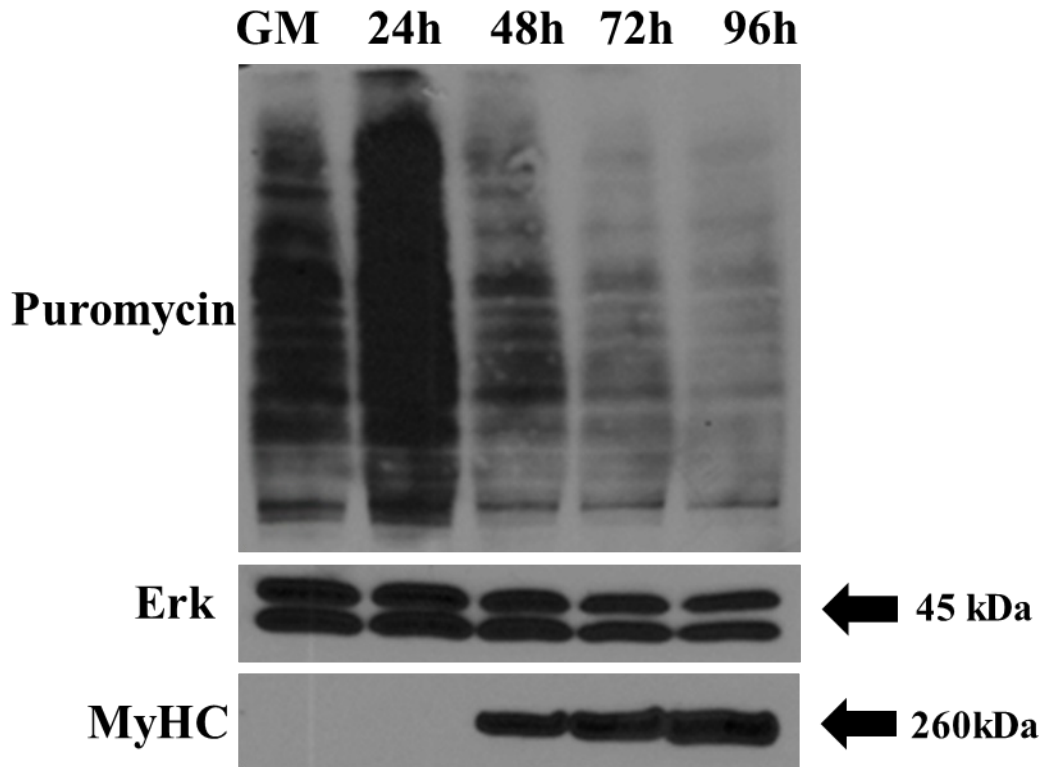


Figure 15: Puromycin incorporation of skeletal muscle in growth and differentiation conditions. C2C12 myogenic cells were maintained under growth conditions and serum was withdrawn for periods of 24h, 48h, 72h and 96h to induce differentiation. Immediately after these time periods elapsed, C2C12 cells were exposed to 0.544ug/ml of puromycin for 5min. Western blotting was conducted against puromycin to measure translational activity. Western blotting was conducted against Erk as a loading control and myosin heavy chain as a marker of differentiation.

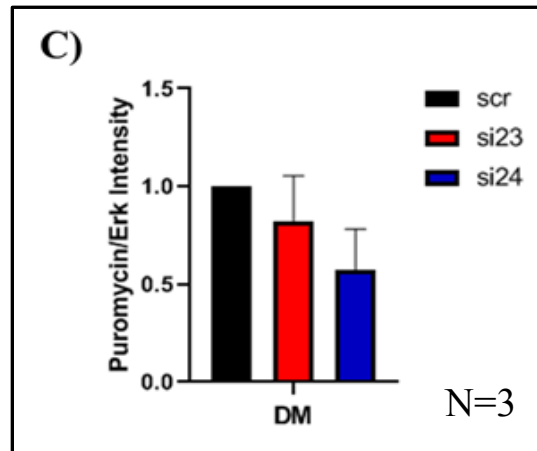
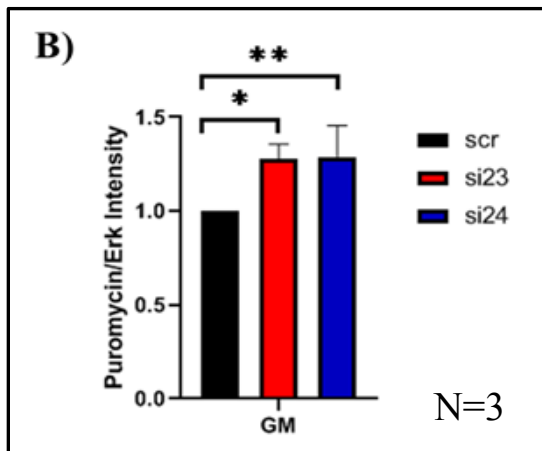
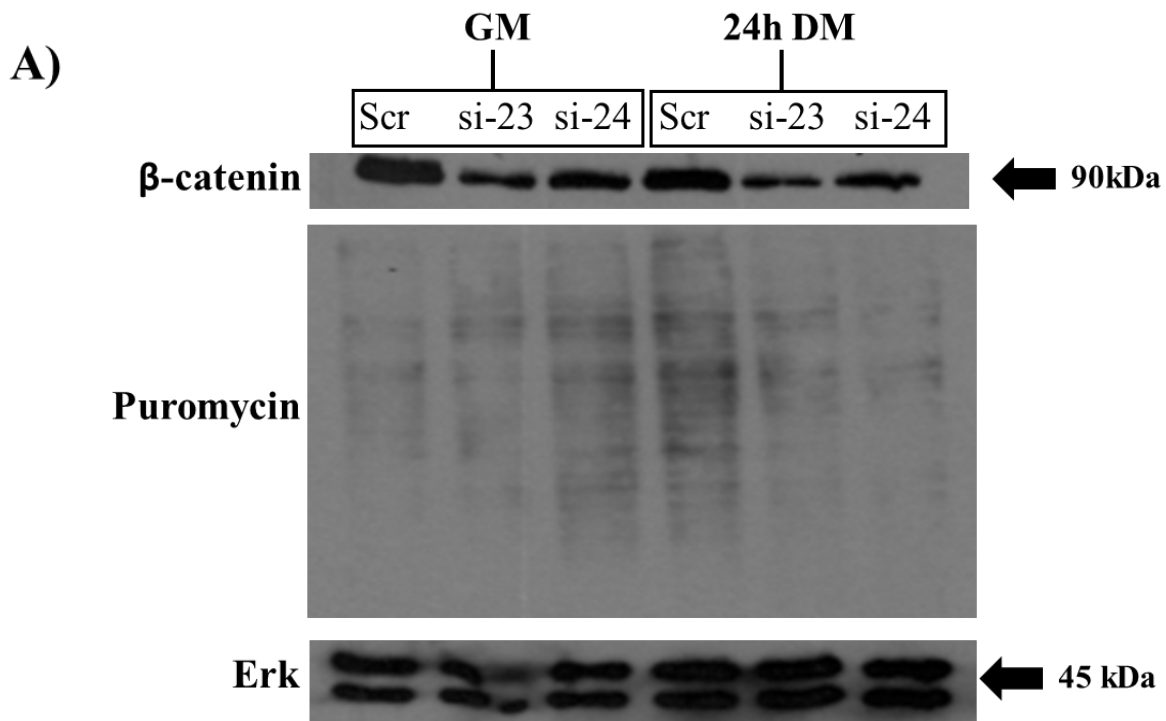


Figure 16: Puromycin incorporation (0.544ug/ml) of skeletal muscle in GM and DM 24h conditions. **A)** C2C12 myogenic cells were maintained under growth conditions and serum was withdrawn for a periods of 24h to initiate the differentiation program. C2C12s were treated with si-23 or si-24 to knock down expression of β -catenin (n=3). Scrambled (scr) siRNA was used as a control (n=3). Relative translational activity in both GM and DM 24h was quantified by dividing the intensity of puromycin incorporation by the intensity of the Erk loading control. **B)** Relative puromycin incorporation of C2C12s grown under GM and treated with either si-23, si-24 or scr siRNA (n=3). Intensity of puromycin incorporation was compared relative to scr DM 24h siRNA treatment. **C)** Relative puromycin incorporation of C2C12s grown in DM 24h and treated with either si-23, si-24 or scr siRNA. Intensity of puromycin incorporation was compared relative to scr siRNA treatment.

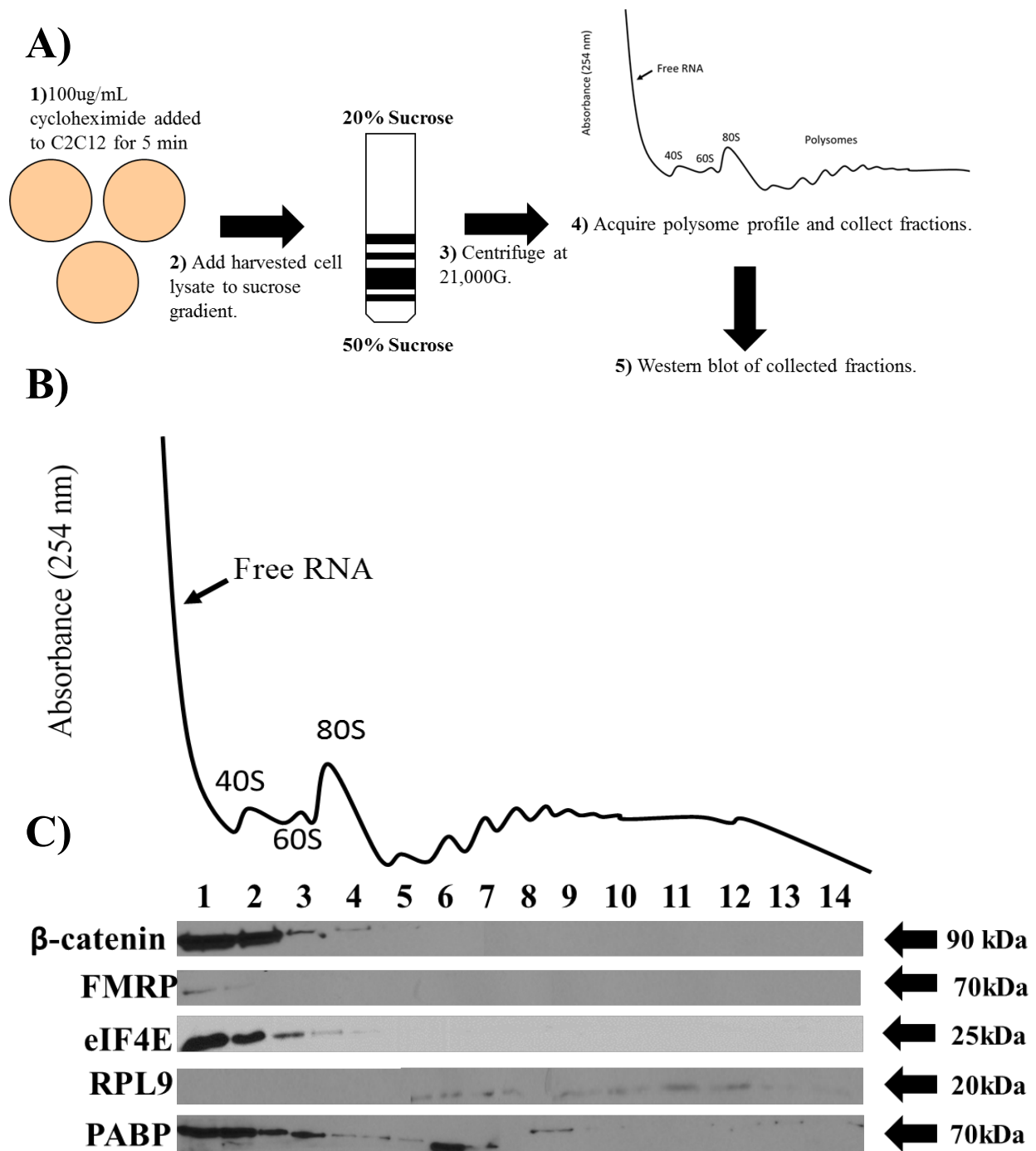


Figure 17: Polysome profile of β -catenin and FMRP in C2C12 myoblasts maintained under GM conditions. **A)** Basic workflow of polysome profiling methodology. **B)** Expected 254nm absorbance peaks within collected fractions. **C)** Western blot of β -catenin and FMRP in collected fractions. Eukaryotic Initiation Factor 4E (eIF4E) was used to detect the presence of the preinitiation complex in the collected fractions. Ribosomal protein L9 was used to detect the presence of fully assembled ribosomes and polysomes in the collected fractions.

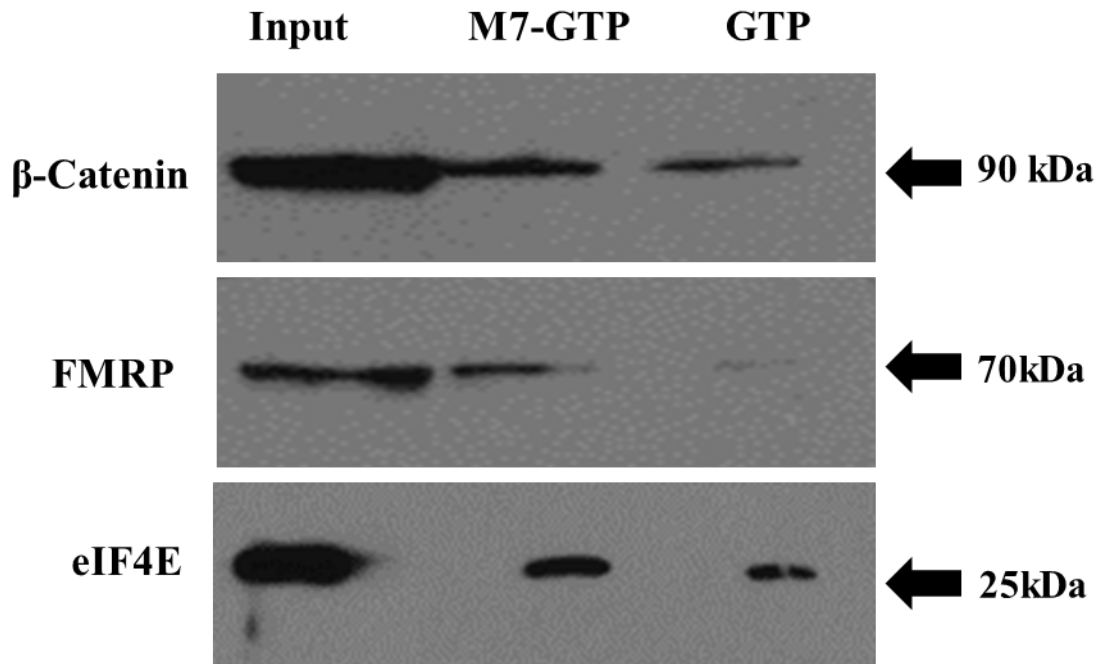


Figure 18: M7GTP assay of β -catenin and FMRP at in C2C12 myoblasts grown under GM conditions. M7-GTP coupled agarose beads were used to isolate the translational machinery and then probed for the presence of β -catenin and FMRP. eIF4E, a component of the preinitiation complex, was used to detect the presence of the translational machinery. GTP coupled agarose beads lacking an M7 tag were used as a negative control.

FMRP Associated RNA Transcripts in Skeletal Muscle

After establishing that the β -catenin-FMRP interaction exists at the pre-initiation complex in skeletal muscle and that β -catenin plays a potential role in modulating translational activity, we next wanted to test whether RNA transcripts that associate with FMRP are altered with manipulation of β -catenin. Since FMRP is an RNA binding protein, we postulated that identifying the FMRP associated RNAs in skeletal muscle could allow us to identify possible targets of the β -catenin-FMRP complex. Although the RNA binding partners of FMRP have previously been studied in neurons, no such study has thus far been carried out in skeletal muscle.

We isolated FMRP-associated transcripts by conducting an RNA immunoprecipitation experiment where we incubated cellular lysate from GM C2C12 cells with anti-FMRP agarose beads in order to pull down FMRP along with its associated RNAs (Figure 19A). We then conducted a microarray analysis of FMRP-associated RNAs using an Affymetrix Mouse Gene 2.0 Microarray. This microarray contained 27,358 probesets corresponding to RNAs within the *mus musculus* transcriptome. A cutoff of 2 fold enrichment from FMRP-associated RNA relative to a total RNA control was applied to generate candidate RNAs that were proposed to interact with FMRP. After applying a cutoff criteria of a 2 fold or greater enrichment relative to the total RNA control, we identified 2090 mRNA transcripts of interest that were found to associate with FMRP (Figure 19B). These enriched mRNA transcripts represented 6.06% of the entire transcriptome, demonstrating that FMRP binds to a significant amount of mRNA transcripts in skeletal muscle (Figure 19C). Interestingly, we were able to identify previously characterized FMRP mRNA targets such as MAP1B (2.13 fold enrichment; [78]) and Myf5 (1.23 fold enrichment; [74]) which provided validation of our methodology.

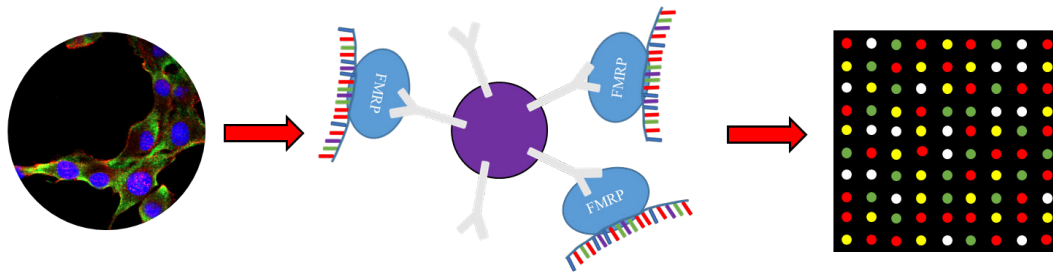
To begin to understand what types of mRNA transcripts might bind to FMRP in skeletal muscle, we conducted a GO biological function enrichment analysis. This analysis uncovered the FMRP-associated mRNAs with common biological function that were overrepresented in our samples relative to expected representation values (Figure 20A). In total, we found mRNAs associated with 13 different biological functions that were significantly overrepresented ($p < 0.05$) in our FMRP bound samples relative to the expected representation values generated by our GO enrichment analysis tool (Figure 20A/B). Interestingly, we identified an overrepresentation of mRNA transcripts involved in processes such as gene expression, transcriptional regulation by RNA polymerase II and RNA metabolism (Figure 20A). These findings suggest that not only does FMRP associate with β -catenin at the translational machinery, but that the mRNAs it associates with have a significant impact on the overall makeup of the transcriptome through the overrepresented biological processes we identified.

We then identified the top 20 mRNAs that were found to associate with FMRP in our microarray analysis of skeletal muscle (Figure 21). The enriched mRNAs that were isolated via FMRP-IP were found to have a 9.12-88.94 fold increase in abundance relative to the total RNA control (Figure 21). We found mRNAs with distinct functions enriched in our FMRP-IP samples including microRNAs, Cajal Body RNAs, nucleolar RNAs, and ribosomal associated RNAs (Figure 21). This microarray study identified mRNAs that could associate with FMRP in skeletal muscle and possible targets of the β -catenin-FMRP complex.

In conclusion, our study began by characterizing the expression and subcellular localization of FMRP and β -catenin in a model of skeletal muscle. We were also able to detect the presence of these proteins in primary myoblasts and the presence of FMRP in the satellite cells of single myofibers. We then established that the interaction between β -catenin and FMRP

occurred in skeletal muscle through biochemical and confocal microscopy techniques. Furthermore, we established the role of β -catenin as a regulator of global translational activity and found the presence of the β -catenin-FMRP complex at the preinitiation complex. Finally, we conducted a microarray analysis of FMRP-associated transcripts in skeletal muscle to determine the makeup of RNAs that may be regulated by the β -catenin-FMRP complex. These results provide novel insight into the role of β -catenin as a potential post-transcriptional regulator and suggests that β -catenin can interact with post-transcriptional proteins such as FMRP to regulate gene expression in skeletal muscle.

A)

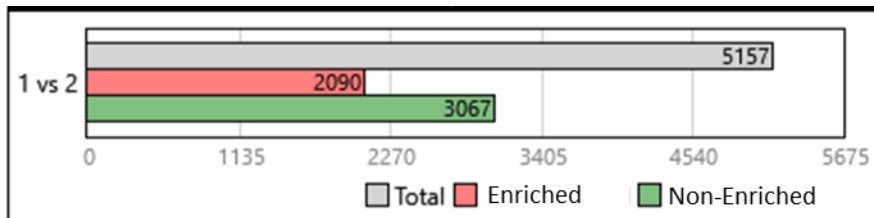


1) Harvest cell lysate from C2C12 myoblasts maintained in growth medium.

2) Crosslink RNA and add lysate to anti-FMRP magnetic beads.

3) Process samples in microarray and compare relative to total RNA.

B)



C)

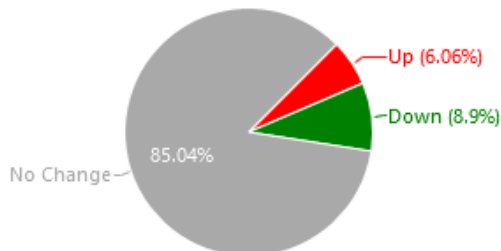


Figure 19: Microarray analysis of FMRP associated RNA transcripts. **A)** Workflow of the RNA immunoprecipitation methodology. C2C12 myoblasts maintained under GM conditions were crosslinked with formaldehyde and cell lysate was collected. Cell lysate was incubated with anti-FMRP magnetic beads to isolate FMRP and its associated RNAs. Next, RNA was collected and processed in an Affymetrix Mouse Gene 2.0 microarray. **B)** Relative enrichment of RNA from FMRP-IP compared to total RNA. We found 2090 FMRP-IP enriched RNAs that had at least a 2 fold increase relative to total RNA samples. In contrast, there were 3067 RNAs with a 2 fold or greater enrichment in total RNA relative to FMRP-IP RNA. **C)** Percentage of FMRP enriched RNAs within the entire transcriptome. 6.06% of 34,472 RNAs were enriched (2-fold increase) in FMRP-IP samples relative to total RNA while 8.9% of 34,472 RNAs were not enriched (2-fold decrease). 85.04% of 34,472 RNAs showed no significant change (less than 2-fold increase or decrease relative to total RNA).

A)

GO Ontology Number	GO Biological Function
1 (GO:0009889)	regulation of biosynthetic process
2 (GO:0031326)	regulation of cellular biosynthetic process
3 (GO:0010468)	regulation of gene expression
4 (GO:0010556)	regulation of macromolecule biosynthetic process
5 (GO:2000112)	regulation of cellular macromolecule biosynthetic process
6 (GO:0019219)	regulation of nucleobase-containing compound metabolic process
7 (GO:0051252)	regulation of RNA metabolic process
8 (GO:0006355)	regulation of transcription, DNA-templated
9 (GO:2001141)	regulation of RNA biosynthetic process
10 (GO:1903506)	regulation of nucleic acid-templated transcription
11 (GO:0006357)	regulation of transcription by RNA polymerase II
12 (GO:1902679)	negative regulation of RNA biosynthetic process
13 (GO:1903507)	negative regulation of nucleic acid-templated transcription

B)

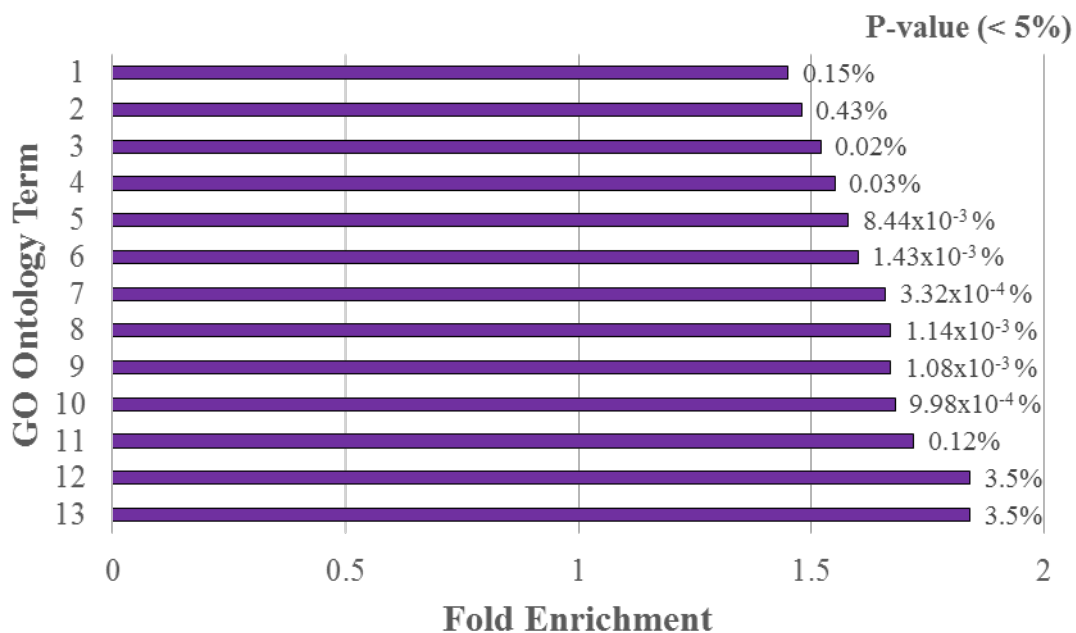


Figure 20: GO ontology analysis of biological function in FMRP enriched RNAs. A) The 2090 RNAs with a 2 fold enrichment in FMRP-IP samples were subjected to a GO Ontology analysis using the Gene Ontology Consortium GO Analysis Tool. We found 12 biological functions that were significantly overrepresented in FMRP-IP RNA samples ($P < 0.05$). B) P-values associated with each biological function. Each term in figure XA is assigned a number from 1 to 12 which corresponds to the P-value associated with this biological function in Figure XB.

Fold Change (FMRP to Total RNA)	Gene	Full Name
88.94	Snora62	small nucleolar RNA, H/ACA box 62
38.36	Scarna9	small Cajal body-specific RNA 9
36.14	Snord17	small nucleolar RNA, C/D box 17
20.63	Snora74a	small nucleolar RNA, H/ACA box 74A
19.83	Rpl6	ribosomal protein L6
18.98	Taf1d	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D
17.82	Snora81; Eif4a2	small nucleolar RNA, H/ACA box 81; eukaryotic translation initiation factor 4A2
17.73	Snora30	small nucleolar RNA, H/ACA box 30
17.3	Nop56	NOP56 ribonucleoprotein
16.64	Mir3473c	microRNA 3473c
14.02	Scarna10; Ncapd2	small Cajal body-specific RNA 10; non-SMC condensin I complex, subunit D2
12.97	n-R5s89	nuclear encoded rRNA 5S 89 [Source:MGI Symbol;Acc:MGI:4421937]
12.31	Scarna13; Mir3069	small Cajal body-specific RNA 1; microRNA 3069
12.24	Neat1	nuclear paraspeckle assembly transcript 1 (non-protein coding)
12.07	Mir148b	microRNA 148b
11.43	Mir140	microRNA 140
11.26	Mir365-2	microRNA 365-2
9.12	Rnu1b6	U1b6 small nuclear RNA
9.12	Rnu1b1; Rnu1b2	U1b1 small nuclear RNA; U1b2 small nuclear RNA
9.12	Rnu1b6	U1b6 small nuclear RNA

Figure 21: List of top 20 enriched RNAs in FMRP-IP sample relative to total RNA. The identity of RNAs with a 2 fold or greater increase in FMRP-IP samples relative to total RNA was determined using the Thermo Fisher Transcriptome Analysis Console. The RNAs with top 20 enrichment values (9.12-88.94 fold increase relative to total RNA) were recorded.

Discussion:

In this study we have explored the post-transcriptional role of β -catenin, a component of the Wnt signaling pathway which has been shown to activate the expression of muscle specific genes by interacting with MRFs such as MyoD [29]. Previous studies conducted by our group found that in smooth muscle, β -catenin interacts with the post-transcriptional regulator FMRP to modulate translational activity [55]. In this study, we have expanded the current scope of research by exploring the proposed interaction between β -catenin and FMRP in skeletal muscle, investigating the role of β -catenin in the translational activity of skeletal muscle progenitors and exploring the presence of β -catenin at the translational machinery in skeletal muscle. Finally, we present a novel preliminary study regarding the identity of RNAs which bind to FMRP in skeletal muscle.

As previously mentioned, β -catenin is a multifunctional protein with its role depending on both its localization and interacting partners [29,48]. While β -catenin does not have an enzymatic domain, it seems to confer functionality through its interacting partners, evident by its diverse functions such as regulating the expression of muscle specific proteins in myogenesis, maintaining the structural integrity of multiple cell types, regulating developmental processes, and controlling the process of cell division [40,95,96]. A mass spectrometry study was previously conducted in smooth muscle which identified potential interacting partners of β -catenin such as the post-transcriptional regulator FMRP [55]. In this study, we used multiple approaches to determine that the β -catenin-FMRP interaction occurs in skeletal muscle. By characterizing this interaction in skeletal muscle, we have identified a potential new role of β -catenin as a post-transcriptional regulator. This study expands on the current understanding of β -catenin's function in skeletal muscle as either a transcriptional regulator interacting with MyoD

or a structural protein which interacts with cadherin proteins at the adherens junction [29,97]. The discovery of the β -catenin-FMRP interaction in skeletal muscle uncovers a novel role of β -catenin and emphasizes the multifunctionality of β -catenin which is mediated by the binding partners it associates with.

Since we found that β -catenin acts as a translational repressor in undifferentiated skeletal myoblasts, we propose that it may serve multiple roles depending on the conditions of the extracellular environment. Previous work has found the activation of Wnt signalling by extracellular Wnt ligands in smooth muscle can cause the disassociation of the β -catenin-FMRP complex at the translational machinery due to the migration of β -catenin into the nucleus [55]. Based on our finding that β -catenin acts as a translational repressor in undifferentiated muscle progenitors, we believe this indispensable protein may serve dual functions in skeletal muscle. It has previously been discussed that the activation of Wnt signalling leads to the migration of β -catenin into the nucleus where it interacts with MyoD and is essential for differentiation of myogenic precursors into skeletal muscle [29,98]. Therefore, it is possible that β -catenin may act as a post-transcriptional regulator alongside FMRP in when Wnt signalling is inactive while acting as a transcriptional activator in differentiating myoblasts which have activated Wnt signalling.

In conclusion, while β -catenin may act as a transcriptional activator by interacting with MyoD and other transcription factors, it also possesses a post-transcriptional role by interacting with RNA binding proteins such as FMRP. During the initiation of translation, the recruitment of a multitude of proteins called eukaryotic initiation factors are required to form the preinitiation complex and begin the process of translation [99]. Complementary regulatory proteins such as FMRP can inhibit the formation of the preinitiation complex by preventing eukaryotic initiation

factors such as eIF4F from binding to their targets such as the 5' cap of mRNA or the translational machinery [73]. We propose a model in which β -catenin cooperates with FMRP at the translational machinery in order to mediate translational repression by preventing the assembly of eukaryotic initiation factors such as eIF4F in skeletal muscle (Figure 22).

Although the mechanism of β -catenin mediated post-transcriptional regulation has not yet been fully elucidated, previous research has found that GSK3 β inhibitors can reverse many of the symptoms of Fragile X Syndrome in FMR1 knockout mice [100]. As previously mentioned, GSK3 β is a protein part of the destruction complex which mediates the degradation of β -catenin by the proteasome [48]. This study demonstrates a possible link between β -catenin and FMRP outside of skeletal muscle since GSK3 β inhibition is known to lead the increased activation of β -catenin and seems to correct some of the pathologies associated with a lack of FMRP expression [100,101]. We postulate that β -catenin may act at the translational machinery by recruiting post-transcriptional regulators such as FMRP in order to control the rate of translation in skeletal muscle.

These findings have crucial implications within skeletal muscle research since a major cause of muscle atrophy involves pathways which activate protein turnover [102]. Due to the constant damage that skeletal muscle undergoes throughout day-to-day life, there is a strong requirement for activation of protein synthesis through signaling pathways such as IGF1 signaling [103]. In this study, we have identified an inhibitor of global translational activity within skeletal muscle. It may be possible to pharmacologically target the β -catenin-FMRP interaction and determine whether it could restore healthy levels of protein synthesis in people suffering from muscle atrophy. Furthermore, we identified the presence of FMRP in satellite cells which mediate the regeneration of skeletal muscle. It is possible that the β -catenin-FMRP

complex mediates post-transcriptional regulation in these progenitor cells and may play a role in skeletal muscle regeneration. Further understanding the role of post-transcriptional regulators such as β -catenin and FMRP in skeletal muscle represent a promising avenue of research that can identify pharmacological targets which that activate protein synthesis and improve both regenerative capacity and functionality of skeletal muscle.

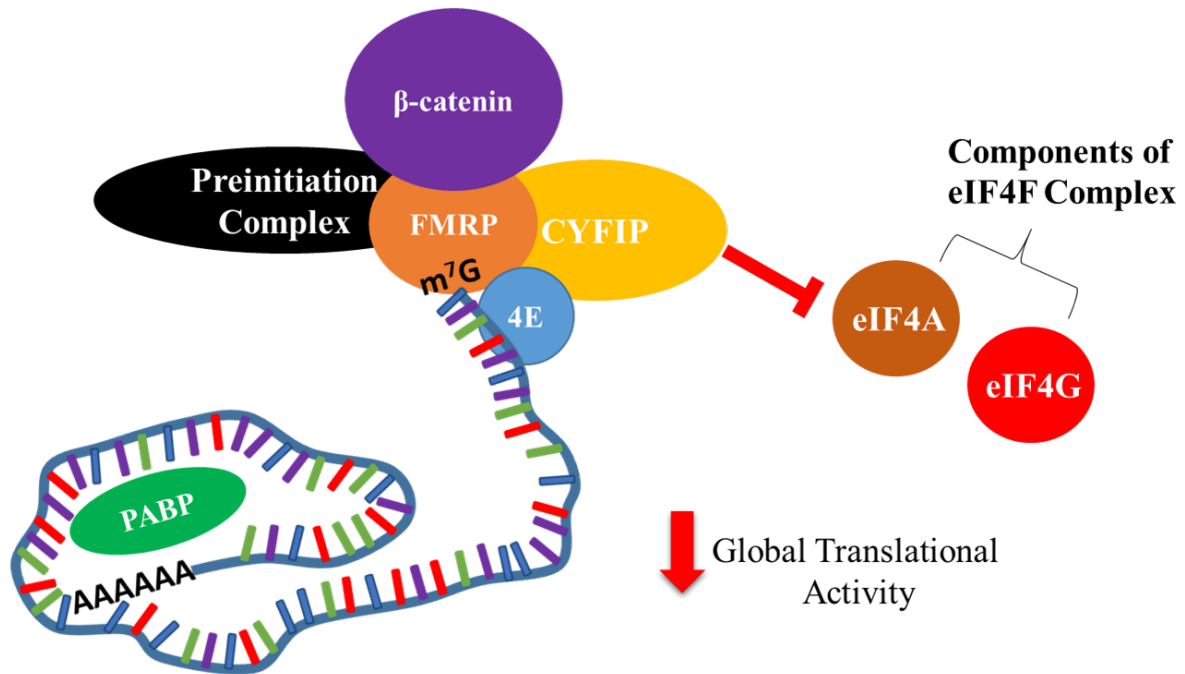


Figure 22: Proposed model of β -catenin mediated translational repression. Previous studies have found that FMRP associates with CYFIP at the translational machinery in order to inhibit the formation of the eIF4F complex and repress translation. In this study we show that β -catenin associates with the preinitiation complex along with FMRP. Although its mechanism of action is currently unknown, we propose that β -catenin associates with FMRP at the preinitiation complex in order to post-transcriptionally regulate gene expression and global translational activity.

Since we proposed that the FMRP- β -catenin complex functions post-transcriptionally to regulate gene expression, we sought to identify the population of RNAs which associate with FMRP. Understanding the targets of FMRP may provide evidence related to skeletal muscle related RNAs that are post-transcriptionally regulated by the β -catenin-FMRP complex. We identified a list of the top 20 RNAs enriched with FMRP which coded for important biological molecules such as microRNAs, nucleolar/nuclear RNA, Cajal body-specific RNA, transcriptional regulators and components of the translational machinery (Figure 21).

First, we identified 4 microRNAs enriched in FMRP RIP samples including Mir3473c, Mir148b, Mir140 and Mir365-2 (Figure 21). Of interest to our group was Mir148b (12.07 fold enrichment) which has been shown to regulate the expression of the Wnt1 ligand in neuronal cells, thereby modulating Wnt signaling activity (Figure 21;[104]). The expression of Mir148b has also been found to decrease the expression of β -catenin, a crucial mediator of myogenesis [104]. The fact that FMRP may post-transcriptionally regulate miRNAs associated with β -catenin expression suggests the existence of a possible feedback mechanism. It is possible that FMRP regulates the expression of β -catenin through miRNAs such as Mir148b while FMRP activity is subsequently regulated at the preinitiation complex by β -catenin.

Another candidate microRNA identified in our microarray screen was Mir140 (11.43 fold enrichment) which has been shown to regulate the expression of TGF- β 1/3, key members of the TGF- β signaling pathway (Figure 21). TGF- β signaling activation has previously been found to promote fibrosis and inhibit myogenesis in skeletal muscle upon injury [105]. Mir140 has currently been studied in osteoblasts and lung fibroblasts and was found to inhibit the expression of TGF- β 1 and 3, cytokines known to activate TGF- β signaling [106,107]. Due to the role of TGF- β in the inhibition of myogenesis, identifying regulators of miRNAs which inhibit the

expression of TGF- β signaling molecules provides potential pharmaceutical targets for those suffering from muscle wasting disorders.

Another group of RNAs found to associate with FMRP included Cajal body specific RNA 1, 9 and 10 (13.21 38.36 and 14.02 fold enrichment; Figure 21). Cajal bodies are complexes of RNA and proteins that are found near the nucleolus and play an important role in the production of spliceosomal small nuclear ribonucleoproteins or snRNPs [108]. Recent studies have found that certain isoforms of FMRP can associate with Cajal bodies and that snRNP formation may be regulated by RNA binding proteins such as FMRP [109]. Interestingly, we also found the association of FMRP with small nuclear RNA U1B1 and U1B2 (9.12 fold enrichment) which are precursors of snRNPs, further suggesting the importance of FMRP at Cajal bodies. Here we present evidence of three specific Cajal body RNAs and snRNP precursors that are proposed to associate with FMRP, suggesting that FMRP may play a role in regulating components of the spliceosomal machinery in skeletal muscle. These findings may have implications for understanding the alternative splicing patterns of muscle specific genes potentially regulated by the β -catenin-FMRP complex.

Finally, we identified various RNAs proposed to associate with FMRP that play a role in both transcription and translation. One of these RNAs included TATA box binding protein associated factor D (18.98 fold enrichment, Figure 21). This mRNA codes for a subclass of protein that regulates the activity of TATA binding protein in the initiation of transcription [110]. Another RNA with a proposed translational role which was enriched with FMRP was ribosomal protein L6 (RPL6). This rRNA codes for a component of the ribosomal machinery and plays a key role in translation. Furthermore, RPL6 has a role in the progression of the cell cycle by interacting with upstream regulators of P53, an important tumor suppressor which controls the

checkpoints of the cell cycle [111]. Mutations in RPL6 have been implicated in cancers such as gastric cancer due to upregulation in cell cycle proteins such as cyclin D1 and its previously mentioned role in regulating P53 activity [111,112].

In conclusion, we have identified β -catenin as a modulator of translational activity in skeletal muscle. We propose that β -catenin serves as a translational repressor in undifferentiated C2C12 myoblasts and that this form of regulation may be carried out through its interacting partner FMRP. The presence of β -catenin at the translational machinery along with its interacting partner FMRP further suggests that these proteins may cooperate in order to repress translational activity in undifferentiated myoblasts. Furthermore, we present preliminary work regarding the identity of RNAs that associate with FMRP in skeletal muscle. Our current model postulates that there is a sub fraction of β -catenin present at the translational machinery which serves to repress global translational activity in undifferentiated myoblasts and that this effect may be mediated through interactions with FMRP (Figure 22). We conclude that we have identified a novel post-transcriptional regulator in skeletal muscle and that by further understanding this mechanism of regulation we can identify pharmacological targets in those suffering from muscle wasting disorders.

Future Direction and Implications:

In this study we have identified FMRP as novel interacting partner of β -catenin, identified the presence of β -catenin in the translational machinery and found that β -catenin plays a role in modulating global translational activity in skeletal muscle. We have also identified potential mRNA targets of FMRP in skeletal muscle using microarray technology. Although these findings are significant, future work must focus on further understanding the role of this interaction in skeletal muscle.

First, while we have discovered that β -catenin and FMRP interact with each other, we must begin to explore the regions of each protein that mediate this interaction. β -catenin has previously been identified as an essential transcriptional regulator while FMRP has been previously studied as a post-transcriptional regulator [36,73]. In order to understand whether the interaction between β -catenin and FMRP truly plays a role in post-transcriptional regulation, the interaction between these proteins should be interfered with while maintaining the supplementary functions of these proteins. By understanding which regions of β -catenin and FMRP interact with each other, it may be possible to generate cell lines expressing mutant proteins by using methodologies such as CRISPR to gain a deeper insight on the specific role of this interaction. Conducting such experiments would allow us to determine whether this interaction has an impact on myogenesis as well as global translational activity.

Secondly, since we have found that β -catenin exerts a repressive effect on translational activity in myoblasts, we should seek to determine whether the β -catenin-FMRP complex is present at every stage of skeletal muscle differentiation. Previous work has found that Wnt stimulation in smooth muscle leads to the disruption of the β -catenin-FMRP complex due to the

translocation of β -catenin into the nucleus. Understanding whether this complex exists during each stage of muscle differentiation can be carried out through co-immunoprecipitation and fluorescent fusion colocalization experiments. These experiments will provide information regarding the temporal pattern of the β -catenin-FMRP complex and provide further insight on whether this complex plays a role only at specific stages of muscle differentiation.

Furthermore, our preliminary microarray study suggested that FMRP may associate with miRNAs that may play important roles within skeletal muscle. Future work should seek confirm the association of these candidate RNAs with FMRP using methodologies such as RT-PCR. Once it is established that FMRP binds to a candidate RNA, knockdown of FMRP in skeletal muscle should be conducted in order to determine the role of FMRP in the expression of that transcript. Specifically, we should seek to identify candidate mRNAs that are proposed to play a role in myogenesis such as *Myf5* which has previously been identified as being regulated by FMRP [74]. Doing so will further develop our understanding of the post-transcriptional mechanisms mediating myogenesis and identify possible pharmacological targets for skeletal muscle related disorders.

Additionally, the role of β -catenin in the β -catenin-FMRP interaction must be studied by understanding whether β -catenin can modulate the types of RNA transcripts that associate with FMRP. We plan to conduct an RNA immunoprecipitation assay in which transcripts bound to FMRP in skeletal muscle can be identified in both the presence of β -catenin and under knockdown conditions of β -catenin. By comparing the makeup of transcripts in the presence of β -catenin relative to knockdown conditions, we can understand whether β -catenin may regulate the makeup of RNAs associate with FMRP at the translational machinery. This experiment may

provide further evidence regarding the role of β -catenin as a post-transcriptional regulator and begin to provide a possible mechanistic explanation for its impact on translational activity.

Our findings have contributed to the large body of skeletal muscle research by establishing a novel role of β -catenin which may serve as a druggable target in muscle wasting disorders induced by aging or cachexia. Skeletal muscle requires a constant turnover of sarcomeric proteins in order to maintain optimal function [102]. Previous work has found that activation of signalling pathways which turn on protein synthesis such as the PI3K-Akt pathway lead to hypertrophy of skeletal muscle [113]. While the over-activation of Akt is implicated in diseases such as cancer, it may be possible to target the β -catenin-FMRP complex in order to reverse the process of muscle wasting by activating protein synthesis [114]. Understanding the targets of this complex through microarray technology will allow us to determine whether targeting this complex may serve as a viable treatment for muscle wasting disorders without having pathological effects. In conclusion, the identification of the β -catenin-FMRP complex in skeletal muscle has further developed our understanding of post-transcriptional regulation in skeletal muscle and identified a possible pharmacological target for muscle wasting disorders.

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Supplementary Figures:

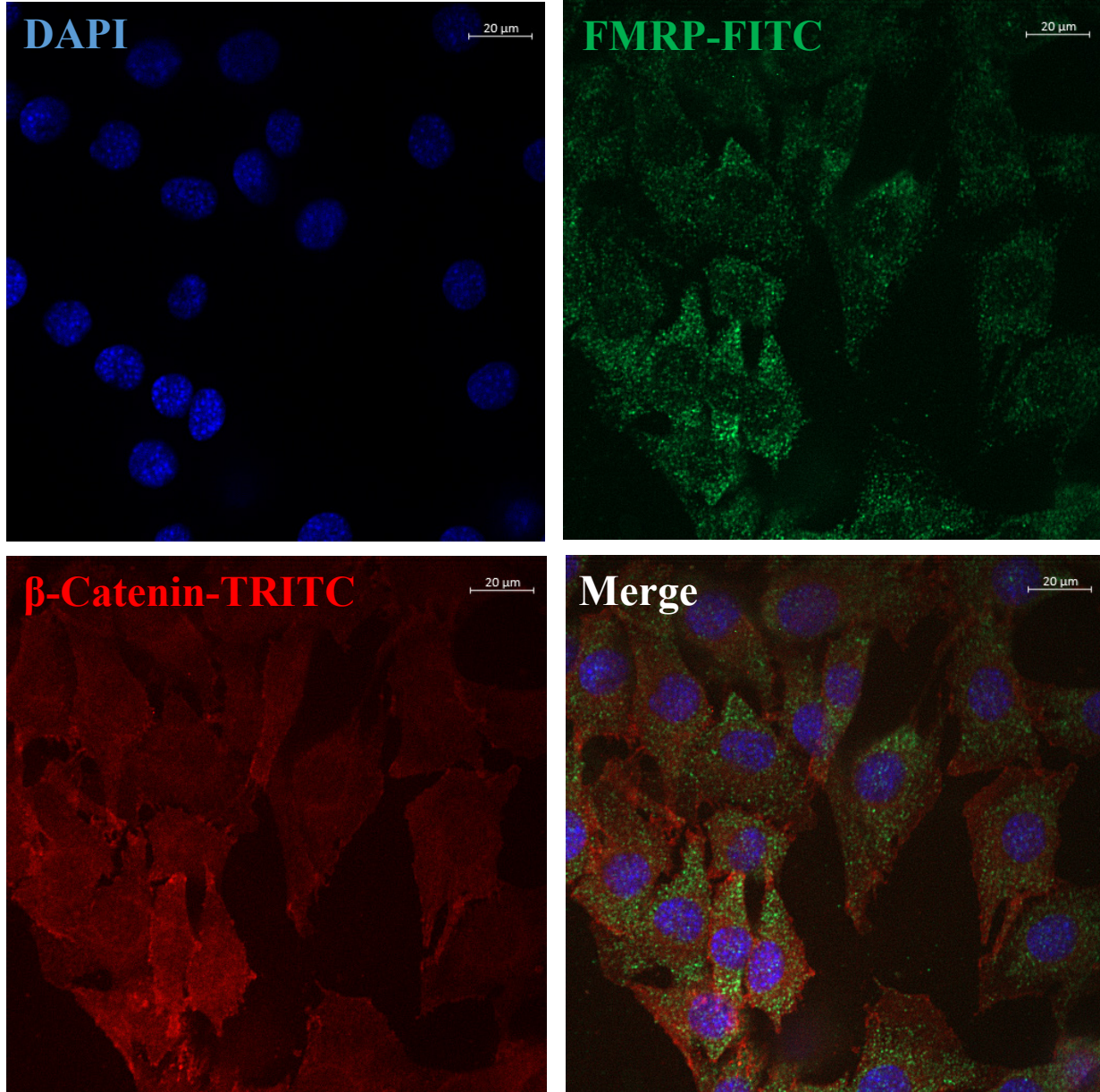


Figure S1: Subcellular localization pattern of β -catenin and FMRP in C2C12 myoblasts corresponding to Figure 9. C2C12 myogenic cells were grown under high serum growth conditions. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

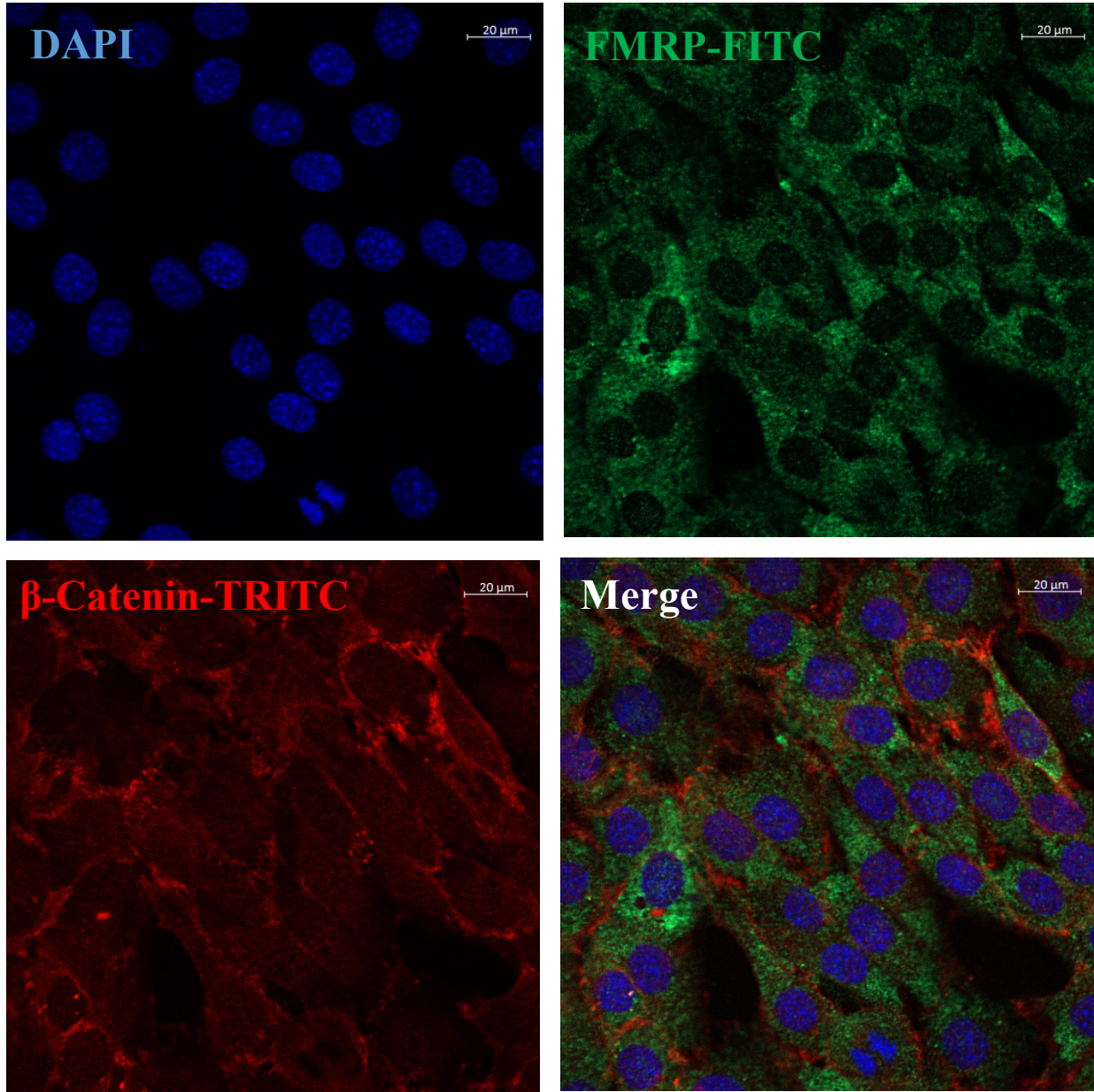


Figure S2: Subcellular localization pattern of β -catenin and FMRP in C2C12 myoblasts corresponding to Figure 9. C2C12 myogenic cells were grown under high serum growth conditions followed by serum withdrawal for a period of 24h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

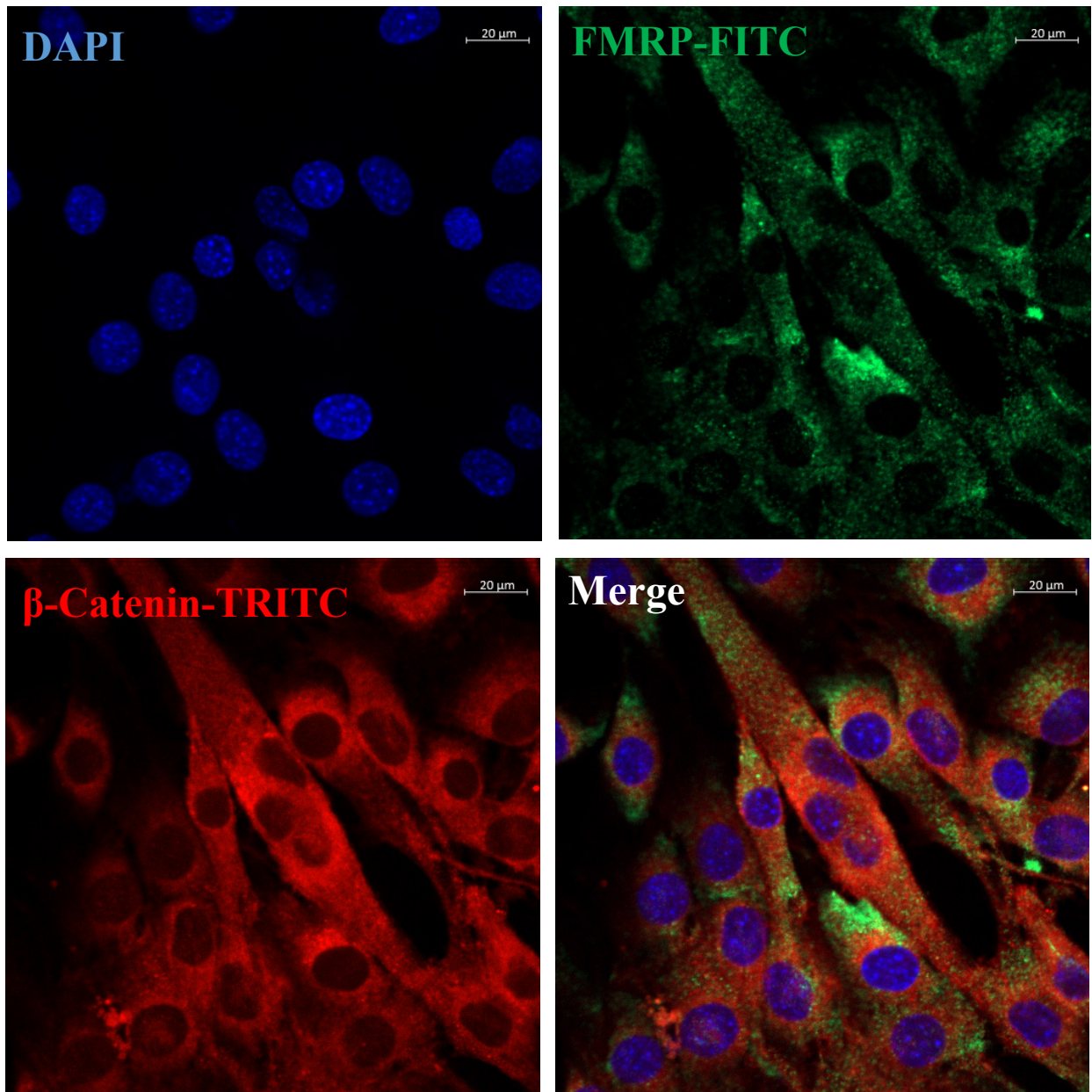


Figure S3: Subcellular localization pattern of β -catenin and FMRP in C2C12 myoblasts corresponding to Figure 9. C2C12 myogenic cells were grown under high serum growth conditions followed by serum withdrawal for a period of 48h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

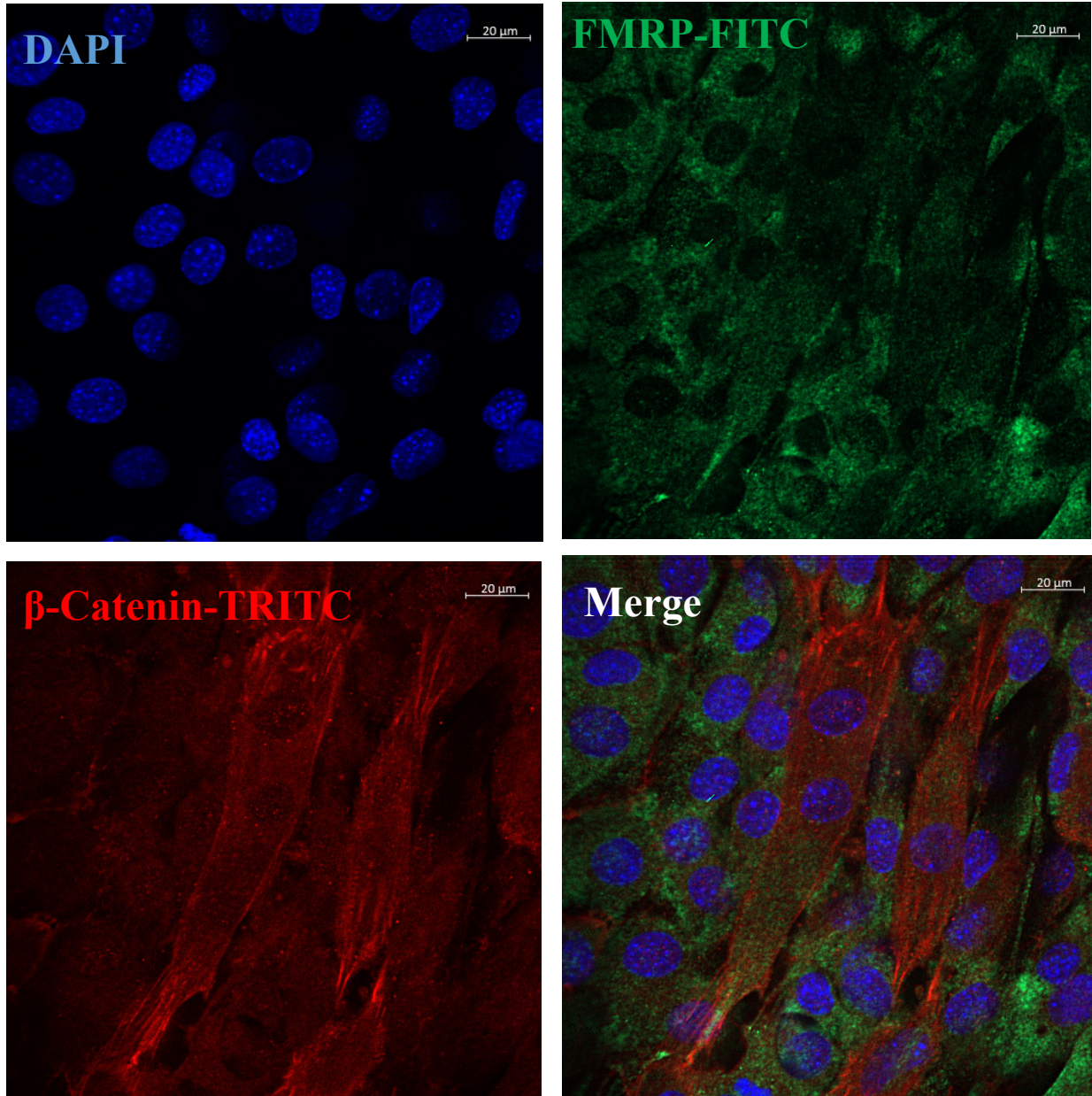


Figure S4: Subcellular localization pattern of β -catenin and FMRP in C2C12 myoblasts corresponding to Figure 9. C2C12 myogenic cells were grown under high serum growth conditions followed by serum withdrawal for a period of 72h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

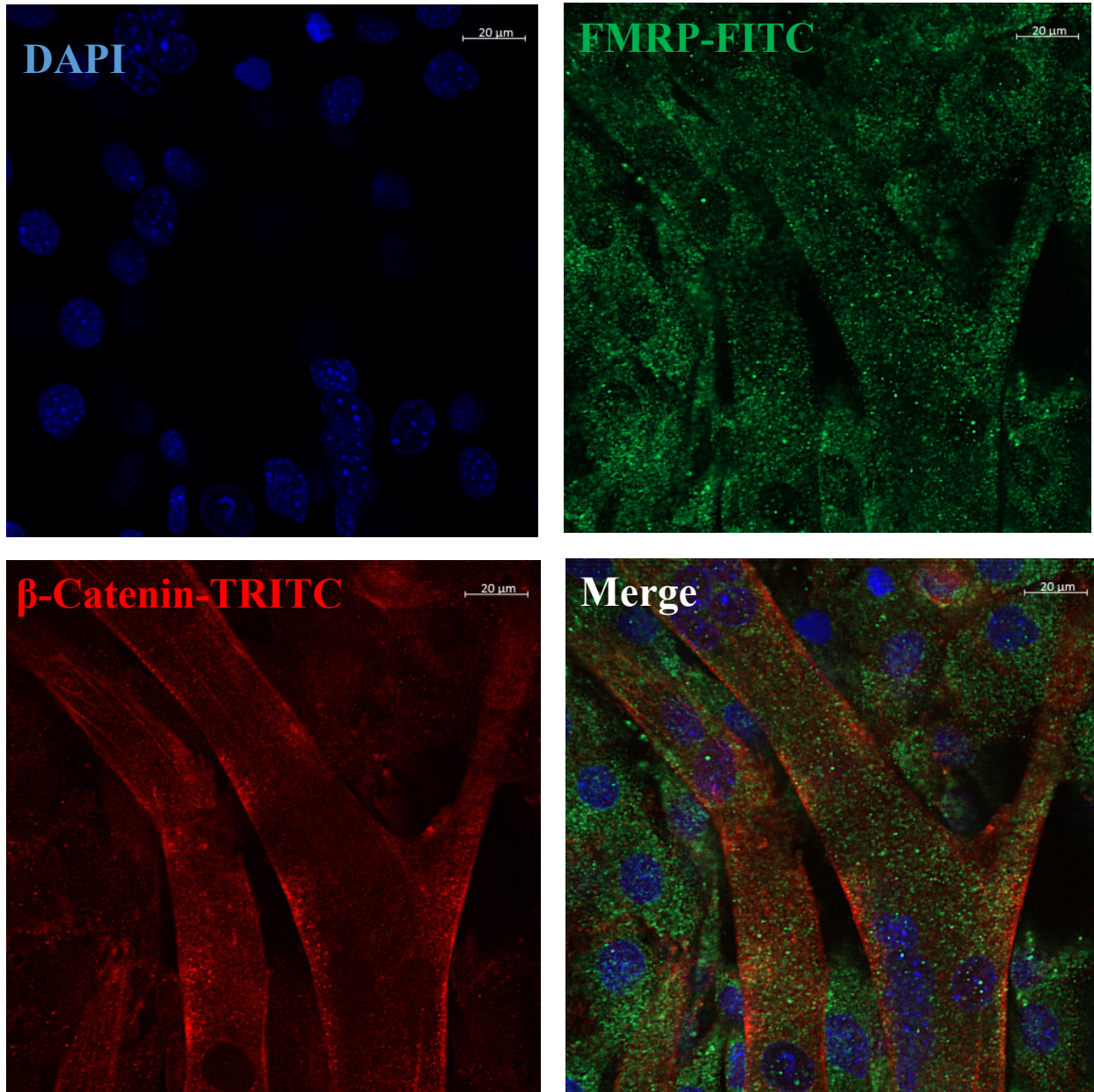


Figure S5: Subcellular localization pattern of β -catenin and FMRP in C2C12 myoblasts corresponding to Figure 9. C2C12 myogenic cells were grown under high serum growth conditions followed by serum withdrawal for a period of 96h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

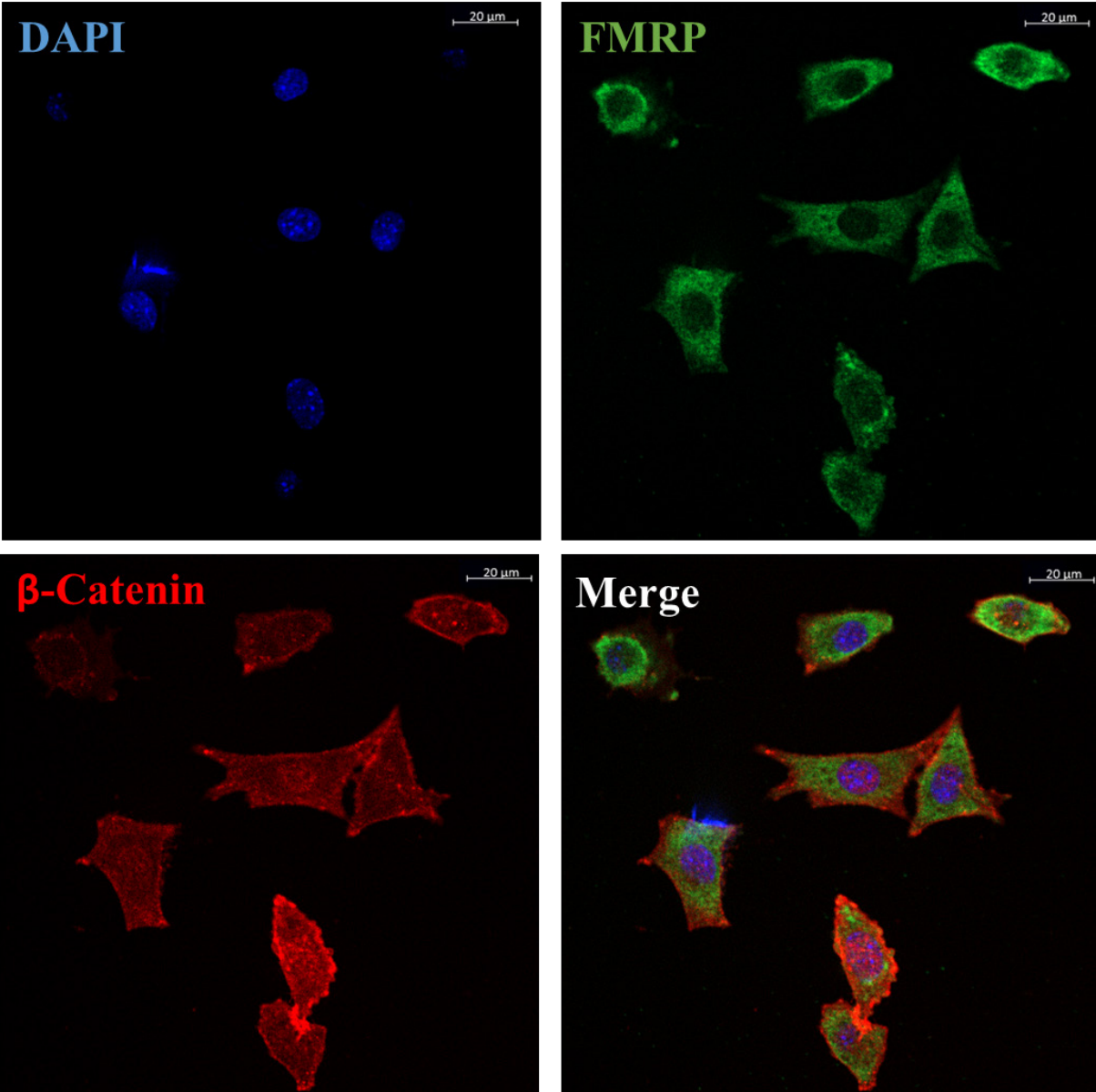


Figure S6: Subcellular localization pattern of β -catenin and FMRP in primary myoblasts corresponding to Figure 10. Primary myoblasts cells were grown under high serum conditions (GM). The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

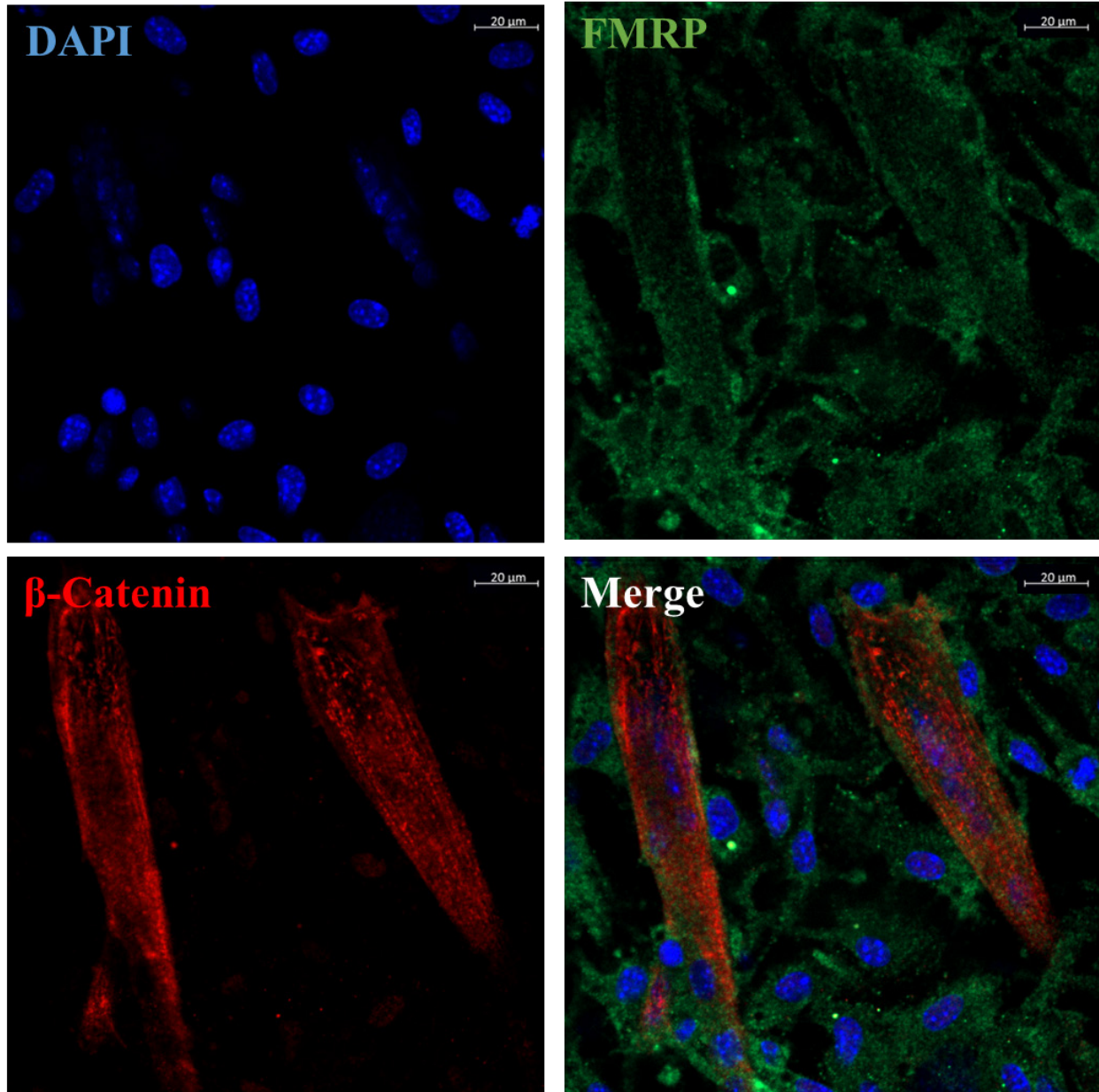


Figure S7: Subcellular localization pattern of β -catenin and FMRP in primary myoblasts corresponding to Figure 10. Primary myoblasts cells were grown under high serum conditions followed by serum withdrawal for a period of 24h to induce differentiation. The subcellular localization pattern of β -catenin and FMRP was observed by immunostaining against β -catenin and FMRP followed by confocal fluorescence microscopy.

Extended Material and Methods

NP-40 Lysis Buffer Recipe (25mL):

- 1) 1.25ml of 1M Tris (PH=8)
- 2) 0.75ml of 5M NaCl
- 3) 1.25ml of 10% NP-40
- 4) 100ul of 0.5M EDTA
- 5) 5ml of 0.5M NaF
- 6) 2.5ml of 0.1M Sodium Pyrophosphate

*Equilibrate volume to 25ml with ddH₂O.

*Per 1ml of NP-40 lysis buffer add 10ul 0.1M Na₃VO₄, 10ul 0.1M PMSF and protease inhibitor of choice.

Western Blotting Protocol:

1) Add cell lysate to 3X Laemmli buffer. Dilute as necessary with NP-40 lysis buffer to reach 1X dilution. Boil at 95°C for 5 minutes to fully denature proteins.

Per 8ml Laemmli Buffer: add 0.7ml 1M Tris-HCl [PH=8], 2.4ml glycerol, 2.4ml of 10% SDS, 0.6ml of Beta-Mercaptoethanol, Bromo blue dye)

2) Make an SDS PAGE gel according to the proper proportions of ddH₂O, polyacrylamide, SDS, APS, Tris and TEMED required. SDS PAGE gel should contain a separating section and a stacking section for loading samples.

3) Load gel into the apparatus containing the electrodes. Add 1X Laemmli buffer to the apparatus so that current can travel through the gel. Run between 120V-160V for approximately 1hr or until dye has almost migrated down the gel.

10X Laemmli Buffer: 30.3g Tris-HCl, 144.2g Glycine, 10g SDS in 1L of ddH₂O

4) Take the gel out of the electrode apparatus and add an activated PVDF membrane (use methanol) on top of the gel. Load the gel and PVDF membrane into the transfer sandwich and place into the electrode apparatus. Add the appropriate amount of Transfer buffer to the apparatus for current to travel. Run at 100V for 1hr. *Optional:* Place on ice for optimal transfer of proteins.

Transfer Buffer: 100ml of 10X Transfer Buffer (per 1L 10X Transfer Buffer, 30.3g Tris, 144g Glycine), 100ml of methanol and 800ml of ddH₂O.

5) Block with 5% milk solution in PBS for 30min and then wash 3 times using PBS. Add primary antibody of interest overnight followed by a 3 washes with PBS. Add HRP conjugated secondary antibody for 2h followed by 3 washes with PBS. Visualize the film in a dark room.

TCA Precipitation Protocol

- 1) Add a 100ul of a 100% TCA solution (dissolved in ddH₂O) per 1ml of protein sample. Vortex the sample.
- 2) Precipitate the protein for a period of 30 min on ice.
- 3) Centrifuge samples at 10,000G for 15 min at 4°C.
- 4) Aspirate the supernatant without disrupting the white pellet at the bottom of the tube.
- 5) Let the pellet dry and then dissolve it in 2X SDS PAGE loading buffer.