THE β-CATENIN INTERACTOME IN MYOGENIC CELLS

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Abstract

β-catenin is a versatile protein implicated in a wide range of cellular processes including cell fate determination, proliferation, differentiation, adhesion, and cell survival. β-catenin performs its cellular functions through protein-protein interactions since it lacks the ability to interact directly with DNA. A β-catenin interactome study was performed in myogenic cells to investigate the protein network of β-catenin during myogenesis. The study utilized a novel GFP Nano-trap based affinity purification approach followed by LC-MS/MS analysis. The mass spectrometry dataset included a list of well-known and potential β-catenin protein interactors. Established β-catenin interactors present in the dataset included components of the β-catenin destruction complex such as GSK3-β and APC, in addition to components of the adherens junction such as α-catenin and Cadherins. After informatic processing of the mass spectrometry dataset, the interaction between β-catenin and FHL3 was further characterized. These studies indicate that FHL3 interacts with β-catenin in the nuclei of cells. The β-catenin responsive TOPFLASH reporter gene system was used to demonstrate that FHL3 inhibits β-catenin activity. FHL3 also inhibited the activation induced by β-catenin on a *myog* reporter gene assay in myogenic cells. It was further documented that FHL3 translocates to the nucleus during the early stages of myogenic differentiation, possibly to delay the onset of myogenesis. Experiments in which FHL3 expression was silenced using siRNAs targeting FHL3 transcripts lead to a small but significant increase in β-catenin activity in myogenic cells. Collectively, these data indicate a repressive role for FHL3 on β-catenin activity and the differentiation program in myogenic cells.

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List of Abbreviations

APC – Adenomatois spolyposis coli

β-TrCP – Beta-transducin repeat containing E3 ubiquitin protein ligase

BMD – Becker muscular dystrophy

BMP – Bone morphogenetic protein

CAMKII – Calcium/calmodulin-dependent protein kinase II

CBP – CREB binding protein

CHD4 – Chromodomain-helicase-DNA-binding protein 4

CtBP2 – C-Terminal Binding Protein 2

CK1 – Casein kinase 1

Dvl – Dishevelled

DMD – Duchenne muscular dystrophy

FHL1/2/3/5 – Four and a half LIM domain 1/2/3/5

Fzd7 – Frizzled 7

FOXO – Forkhead box O

GSK3 – Glycogen synthase kinase-3

IDR – Intrinsically disordered regions

IP3 – Inositol trisphosphate

JAK/STAT – Janus kinase/signal transducers and activators of transcription

JNK – c-Jun N -terminal kinase

BKLF/KLF3 – Krüppel-like Factor 3

LLPS – Liquid-liquid phase separation

LRP5/6 – Low density lipoprotein receptor-related protein 5/6

MCK – Muscle creatine kinase

MED12 – Mediator complex subunit 12

MEF2 –Myogenic regulatory factor

MRF – Myogenic regulatory factor

MyHc – Myosin heavy chain

Myf5/6 – Myogenic factor 5/6

MyoD – Myoblast determination protein 1

MyoG – Myogenin

MZF-1 – Myeloid Zinc Finger 1

Pax3/7 – Paired pox gene 3/7

PCP – Planar Cell Polarity

PKC – Protein kinase C

ROCK – Rho Kinase

ROR – Retinoic acid receptor-related orphan receptor

SC – Satellite cell

Shh – Sonic hedgehog

Smad – Suppressor of mothers against decapentaplegic

TCF/LEF – T-cell factor/lymphoid enhancer factor

TGF-β – Transforming growth factor-β

TBP – TATA binding protein

Wnt – Wingless integration site signaling pathway

Chapter I: Literature review

1. Skeletal muscles

Skeletal muscles are striated muscle cells that have important bodily functions such as locomotion (through muscle contraction) and maintaining an upright posture (1-3). In addition, skeletal muscles regulate various metabolic processes such as thermoregulation, nutrient storage, and energy metabolism (4, 5). Since skeletal muscles are involved in a variety of physiological processes, maintaining healthy skeletal muscles is essential for good life quality. Skeletal muscle dysfunction can lead to many diseases such as Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), cachexia, and rhabdosarcoma all of which can severely impact daily life activities or be lethal in some cases (6-8). Hence, it is crucial to understand the molecular mechanisms associated with muscle development or myogenesis. During vertebrate embryonic myogenesis, skeletal muscles develop from the paraxial mesoderm (9). The paraxial mesoderm, which flanks the neural tube in the developing embryo, contributes to the formation of most types of skeletal muscles through somitogenesis (Figure 1). The paraxial mesoderm also gives rise to the anterior paraxial mesoderm which does not form somites and develop into the head and neck muscles (10). During somite morphogenesis, the somites develop into the dermomyotome which contributes to the formation of skeletal muscles and muscle stem cells (satellite cells) in response to signals received from surrounding structures such as the neural tube, notochord, and dorsal ectoderm. The secreted signals, such as Wnt and Shh signals, induce the dermomyotome to express Pax3/7 and MRFs such as MyoD, Myf5 and MyoG which induce the skeletal muscle differentiation program in the embryo (10). Many aspects of skeletal myogenesis appear to be conserved between vertebrates and invertebrates. In both groups, the mesoderm responds to Wnt signals to produce skeletal muscles. In the nematode *Caenorhabditis*

elegans, Wnt signaling is involved in the formation of body wall muscle cells from the MS lineage (11). In addition, the mesodermal cells in the invertebrate *Drosophila melanogaster* also respond to external signals such as Wingless (Wg) and Hedgehog (Hh) to produce myogenic progenitor cells (12). Wg and Hh are Wnt and Shh homologs (respectively) in *D. melanogaster.* Additionally, in *C. elegans*, CeMyoD which is the MyoD homolog in *C. elegans* encoded by the *hlh-1* gene is involved in embryonic myogenesis (13). Ectopically activating the expression of the *hlh-1* gene induces the conversion of most *C. elegans* embryonic cells into myogenic cells (14). *D. melanogaster* also expresses a MyoD homolog called Nautilus (Nau). Nau is expressed in muscle cell precursors and is capable of inducing cells to commit to the muscle differentiation program (15, 16).

Vertebrate skeletal muscle cells possess a high regenerative capacity in response to certain stimuli such as stress or injury. The ability to regenerate in adult skeletal muscles is dependent on a population of resident stem cells called satellite cells (SC). These cells localize between the basal lamina and the sarcolemma of mature multinucleated muscle cells (17, 18). SCs are mononucleated and mitotically quiescent under normal conditions but are activated when stimulated (19, 20). Non-proliferating SCs predominantly express Pax7 and Myf5, however a subset of the SC population does not express Myf5 and contributes to the SC reservoir (21, 22). Upon activation, SC re-enter the cell cycle, start expressing MyoD and commit to the differentiation program. These $MyoD^{+}$ cells are termed myoblasts. Then, myoblasts start expressing MyoG and other MRFs which induce the fusion of myoblasts to form the terminally differentiated multinucleated myofibers (23, 24). A schematic demonstrating the stages of adult skeletal myogenesis is depicted in figure 2.

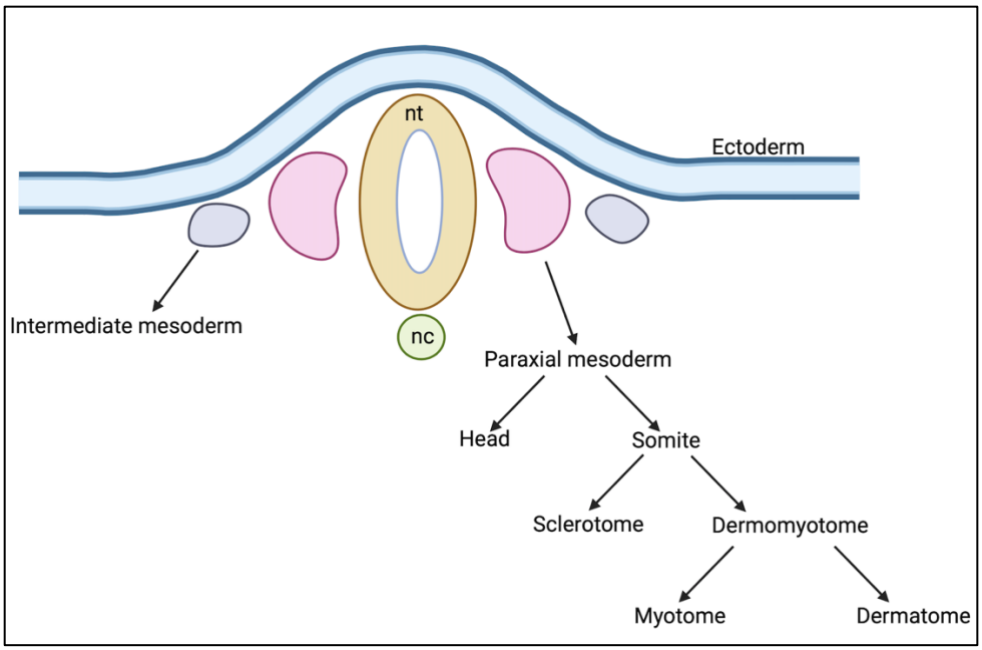

Figure 1: Embryonic development of skeletal muscle. Skeletal muscle cells originate from the paraxial mesoderm which gives rise to somites. Somites then develop to the sclerotome and dermomyotome. Skeletal muscles develop from the dermomyotome. nt: neural tube, nc: notochord. Created with BioRender.com.

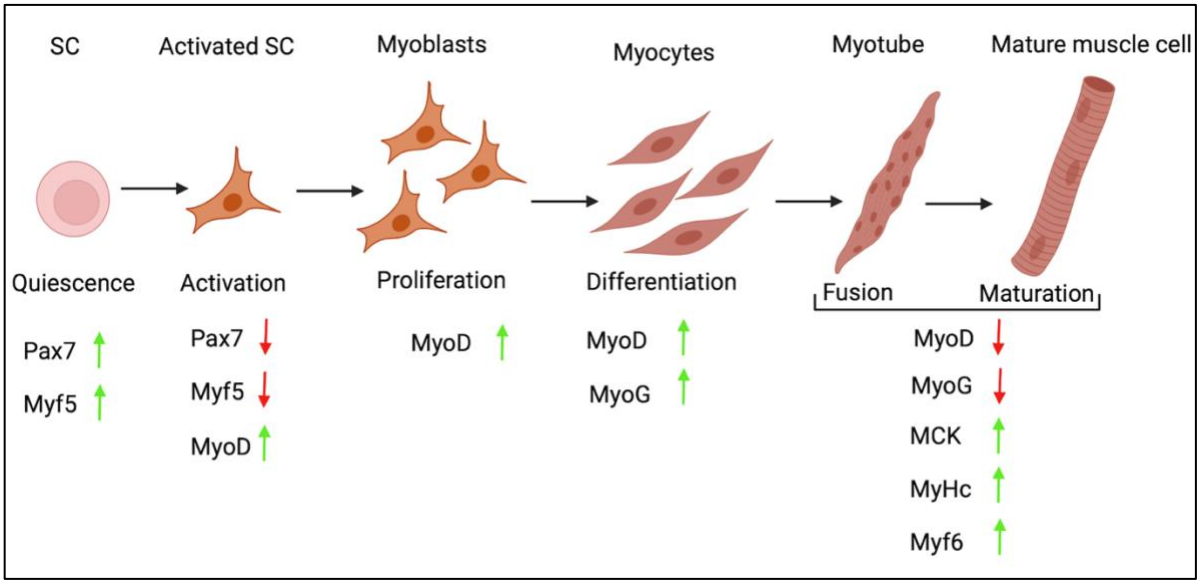

Figure 2: Regeneration in skeletal muscles. In response to an external stimulus, quiescent SCs expressing Pax7 and Myf5 are activated. Activated SCs upregulate MyoD, proliferate and differentiate into mature multinucleated muscle cells. Created with BioRender.com

2. β-catenin

β-catenin is the vertebrate homolog of the armadillo protein originally discovered in drosophila as a segment polarity gene (25). β-catenin is primarily known for its role as the co-activator of the canonical Wnt signaling pathway where it interacts with TCF/LEF transcription factors to activate Wnt target genes. However, the relationship between β-catenin and the TCF/LEF family of transcription factors is not monogamous as β-catenin has been shown to interact with a variety of other transcription factors. Data demonstrates that β-catenin cooperates with the MEF2 family of transcription factors to activate the expression of myogenic genes (26). β-catenin can also associate with FOXO transcription factors and enhance FOXO transcriptional activity in mammalian cells (27). In addition, the Wnt/ β-catenin signaling pathway cross talks with other pathways such as the BMP signaling pathway where β-catenin cooperates with Smads to regulate genes implicated in cell fate determination (28).

In addition to its role in signal transduction, β-catenin also has a structural function as one of the components of the adherens junction (29). The adherens junctions are cell-cell adhesion complexes that consist of Catenin-Cadherin complexes that interact with the Actin cytoskeleton. β-catenin interacts directly with Cadherins and interacts with the Actin cytoskeleton through α-catenin (30). A schematic portraying the functions of β-catenin in the cell is shown in figure 3.

β-catenin is made up of 13 armadillo repeats consisting of ~42 amino acids each (31). The armadillo repeats are flanked by an N-terminal domain consisting of ~130 amino acids and a C-terminal domain of ~100 amino acids (32). The armadillo repeats form a relatively rigid structure that is the binding site for many proteins such as the cell adhesion molecule Cadherin, the TCF/LEF transcription factors, and some components of the β-catenin destruction complex such as Axin and APC (33-35). Compared to the armadillo repeats, the N-terminal and C-terminal

domains of β-catenin are less structured and are more flexible. The N-terminal domain of β-catenin links it to the Actin cytoskeleton through α-catenin. In addition, degradation of β-catenin is mediated by its N-terminal domain where CK1 phosphorylates Ser45 and GSK-β phosphorylates Ser33, Ser37 and Thr41 at the N-terminus (36, 37). The C-terminal domain of β-catenin is essential for its signalling activity as it is the binding site for many transcriptional co-factors such as CBP, TBP and MED 12 (38-40). A diagram depicting the structure of β-catenin and the binding domains of some of its interactors is shown in figure 4.

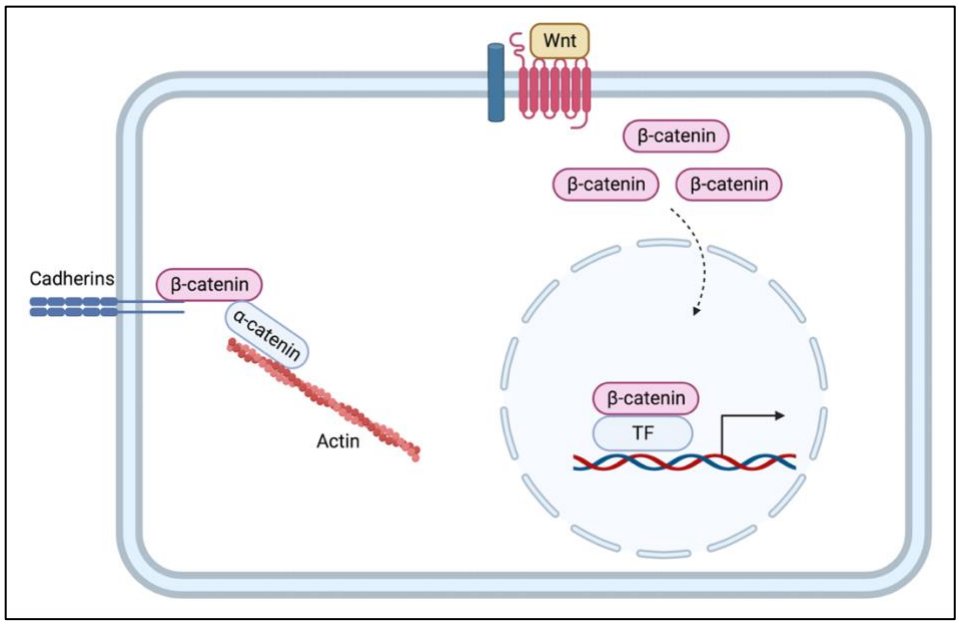

Figure 3: The role of β-catenin in the cell. β-catenin is involved in cell signaling and cell-cell adhesion. When β-catenin accumulates in the cytoplasm, it translocates to the nucleus and activates gene expression. As a component of the adherens junction, β-catenin interacts with Cadherins and interacts with the Actin cytoskeleton through α-catenin. Created with BioRender.com

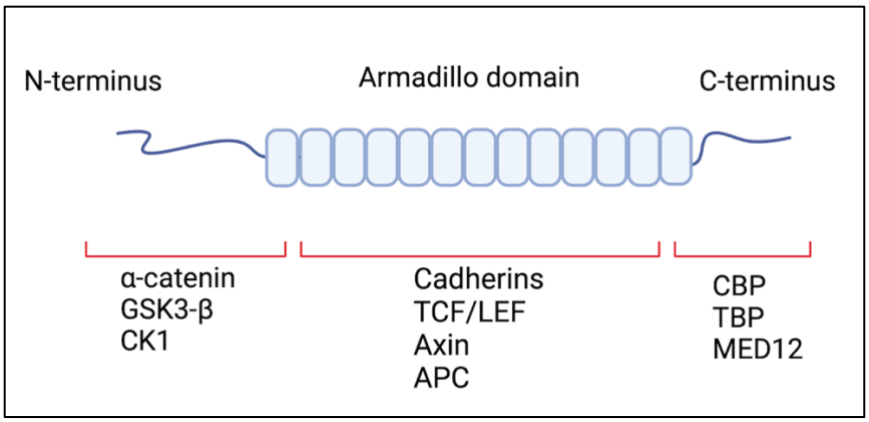

Figure 4: Structure of β-catenin. β-catenin consists of three main domains: the N-terminal domain, a central domain termed the Armadillo domain and a C-terminal domain. The N-terminal domain is the site of phosphorylation that marks β-catenin for degradation. The armadillo domain is the site of interaction with the TCF/LEF family of transcription factors. Both the N-terminal and armadillo domains link β-catenin to the cytoskeleton. The C-terminal domain is the transactivation domain of β-catenin. Created with BioRender.com

3. The Wnt Signaling pathway

a. The canonical Wnt signaling pathway

The Wnt signaling pathway is an evolutionary conserved pathway involved in a variety of cellular processes such as cell proliferation, migration, differentiation, and response to infection (41-44). The canonical Wnt pathway depends on β-catenin for signal transduction while the non-canonical Wnt pathway is β-catenin independent. Both pathways are activated when Wnt ligands, which are secreted glycoproteins rich in cysteine residues, interact with surface receptors (45).

The canonical Wnt pathway is activated when a canonical Wnt ligand (such as Wnt1, Wnt2, Wnt3, Wnt3a and Wnt8a) interacts with the transmembrane receptor Frizzled and the low density LRP5/6 co-receptors activating the scaffolding protein Dvl and recruiting it to the Frizzled/LRP receptor complex (46). This leads to the multimerization of Dvl and the recruitment of the other components of the β-catenin destruction complex to the receptor complex inducing the formation of the Wnt

signalosome (47). The formation of the Wnt signalosome releases β-catenin from the destruction complex leading to its accumulation in the cytoplasm and subsequently its translocation to the nucleus. In the nucleus, β-catenin interacts with the TCF/LEF transcription factors activating the expression of Wnt target genes (48).

The levels of β-catenin are tightly regulated within the cell. In the absence of a Wnt signal, β-catenin is marked for degradation by the destruction complex which consists of the tumor suppressors Axin and APC, and the serine/threonine kinases GSK3- β and CK1. In this process, the phosphorylation of β-catenin by CK1 primes it for phosphorylation by GSK3- β. These phosphorylation events then prime β-catenin for ubiquitylation by the β-TrCP E3 ligase complex which leads to the clearance of β-catenin through proteasomal degradation (49). A schematic summarizing the Wnt signaling pathway is shown in figure 5.

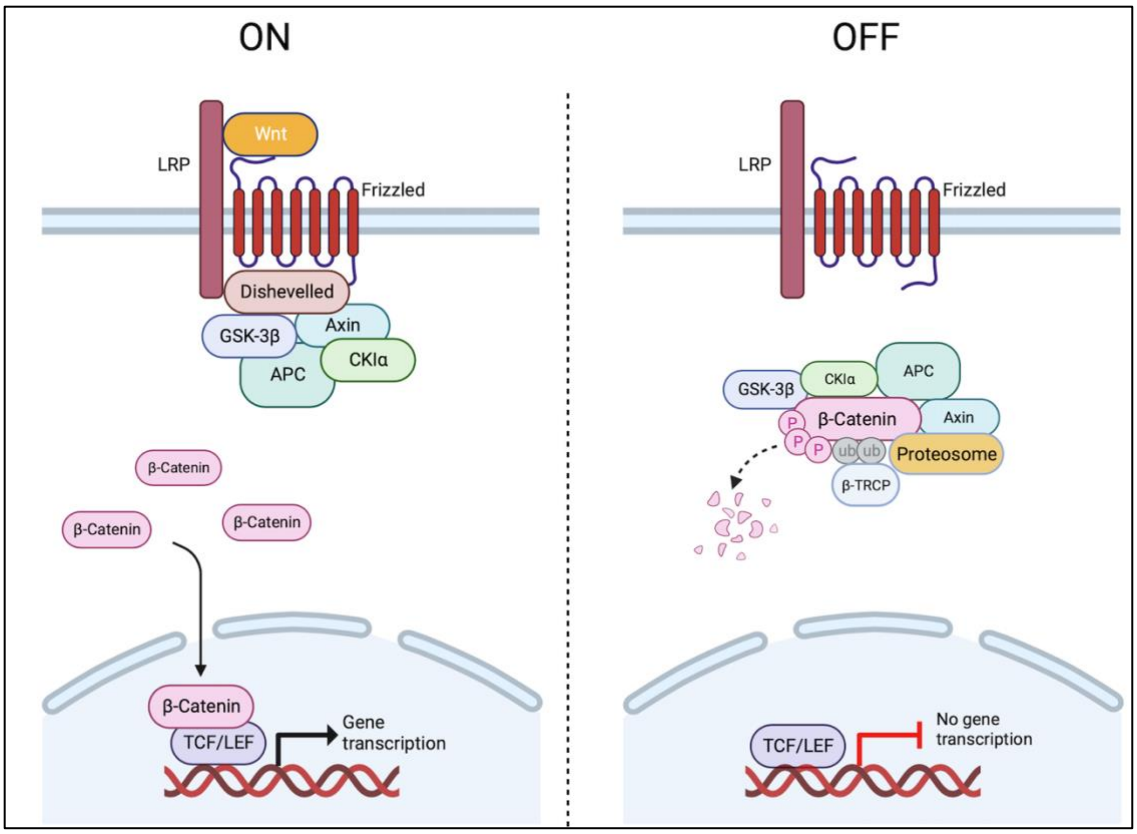

Figure 5: The Wnt/β-catenin signaling pathway. In the absence of a Wnt ligand (Wnt pathway OFF), β-catenin is degraded in the cytoplasm by the β-catenin destruction complex. CK1 and GSK3-β phosphorylate β-catenin which marks it for ubiquitination by β-TRCP ligases. β-catenin is then degraded by proteosomes. In the presence of a Wnt ligand (Wnt pathway ON), the destruction complex is recruited to the Frizzled/LRP co-receptor complex which leads to the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus where it activates gene expression. Created with BioRender.com

b. The non-canonical Wnt signaling pathway

The non-canonical Wnt signaling pathway does not involve the transducing function of β-catenin and is activated when a non-canonical Wnt ligand (such as Wnt4, Wnt5 and Wnt11) interacts with its corresponding surface receptors (46). The non-canonical Wnt pathway is divided into the Wnt/Ca^{+} and the PCP pathways (50). The Wnt/Ca^{2+} pathway is activated when a non-canonical Wnt ligand binds to its corresponding Frizzled receptor inducing the activation of phospholipase C. This enzyme activates molecules like IP3 which triggers the release of Ca^{2+} leading to the activation of PKC, CAMKII and Calcineurin. The activation of these molecules regulates the expression of Ca^{2+} target genes (51). The PCP pathway is also an example of a non-canonical Wnt pathway. This pathway is activated when a non-canonical Wnt ligand interacts with the ROR-Frizzled receptor complex to activate cascades such as JNK signaling and ROCK signaling (52-54).

4. Wnt signaling in adult skeletal myogenesis

Wnt signaling is involved in all stages of adult skeletal myogenesis including satellite cell proliferation, renewal, and differentiation (55-58). When satellite cells are activated in response to stress or injury, they undergo an extensive phase of proliferation before entering the differentiation program (59). Not all proliferating cells commit to the differentiation program, some cells divide to maintain the pool of satellite cells which can be achieved through symmetric or asymmetric cell expansion. Symmetric expansion occurs when a satellite cell produces two daughter satellite cells, while asymmetric expansion occurs when the dividing cell produces a satellite cell and a myogenic progenitor cell (60). Wnt signalling, through its non-canonical division, contributes to satellite cell renewal by symmetrical expansion. When the Wnt ligand Wnt7a activates the PCP pathway through interacting with its receptor Fzd7, two identical satellite cells are produced (55). The

Wnt/β-catenin signaling pathway has also been implicated in inducing myoblasts proliferation through activating cell cycle regulators such as *ccna2* and *cdc25c* (61).

Wnt signaling is implicated in the differentiation of myogenic precursor cells through the canonical division of this pathway. Activating the canonical Wnt signaling pathway through supplementing C2C12 cell cultures (a myoblast cell line) with Wnt-3a, a canonical Wnt ligand, has been shown to enhance myoblast fusion during differentiation (62). In addition, β-catenin has been shown to enhance the interaction of MyoD with certain myogenic loci such as the *myog, mymk, mymx* and *ckm* promoters (63-65). However, the role of β-catenin in priming myogenic loci for MyoD binding is suggested to be TCF-LEF independent. β-catenin null primary mouse myoblasts transfected with a mutated β-catenin construct that does not interact with TCF/LEF showed a differentiated morphology that was indistinguishable from that of β-catenin null primary mouse myoblasts transfected with the wild-type β-catenin suggesting that the binding of β-catenin at myogenic loci is TCF/LEF independent (64).

5. β-catenin in phase separation

Biomolecular condensates, also called liquid-liquid phase separation (LLPS) condensates, are membrane-less compartments that exhibit liquid-like properties. These condensates can be localized in both the nucleus and the cytosol (66, 67). LLPS condensates are capable of concentrating proteins and nucleic acids at specific cellular locations which is thought to regulate some cellular physiological activities as well as some pathological conditions (68). The formation of LLPS condensates is in part dependent on the intrinsic properties of the molecules. For example, the presence of certain domains like intrinsically disordered regions (IDRs), modular domains or other domains that can induce multivalent interactions can contribute to the formation of biomolecular condensates (69). β-catenin has an intrinsically disordered region at its N-terminal

and C-terminal domains which helps the protein in interacting with many other molecules through weak intermolecular interactions such as hydrophobic interactions, hydrogen bonds, van der Waals interactions and electrostatic interactions. These interactions induce the formation of a large complex with a high concentration of molecules (such as co-factors and transcription factors) which leads the complex to phase separate (70). Data demonstrates that β-catenin, SMAD3 and STAT3, which are the terminal signaling factors for the Wnt, TGF-β and JAK/STAT pathways respectively, use their IDRs to concentrate into LLPS condensates to activate gene expression. In addition, LLPS is suggested to contribute to the formation of super-enhancers (SEs) which are clusters of enhancers with high occupancy of transcription machinery components. Activating SE is suggested to enhance gene expression (71). Therefore, an understanding of the role of β-catenin in biomolecular condensates will be an important step in the future.

In summary, β-catenin is a multifaceted protein implicated in an array of cellular processes. It is a component of the adherens junction that maintains cell-cell interactions and a signaling molecule that modulates gene expression. Due to its involvement in a myriad of cellular processes, deregulation of β-catenin can have deleterious effects on cellular function. Owing to its association with multiple physiological and pathological processes, understanding the molecular mechanisms underlying the function of β-catenin through the characterization of novel β-catenin protein interactors is of great significance.

Statement of Purpose

β-catenin is a versatile transcription co-activator implicated in a wide range of cellular processes. When β-catenin is not targeted for degradation by the destruction complex in the cytoplasm, it translocates to the nucleus where it interacts with transcription factors to modulate the expression of various genes. Upregulation of β-catenin is associated with many pathological conditions such as many types of cancers, tissue fibrosis, metabolic diseases, and skeletal myopathy (72-75). β-catenin has been demonstrated to have a role in both proliferation and differentiation of muscle cells which suggests that β-catenin has temporally specific roles (61). This suggested to us that these temporally specific roles are dependent on β-catenin's protein interactors during the different stages of myogenesis. β-catenin is a co-activator of transcription that does not interact with DNA, so its role is highly dependent on its protein-protein network. Our group has characterized the β-catenin protein interactome in myogenic cells using a GFP-Nanotrap based affinity purification approach followed LC-MS/MS analysis which produced a comprehensive list of established and potential β-catenin interactors. **The objective of this study was to investigate the β-catenin interactome dataset to better understand the molecular mechanisms underlying the function of β-catenin in myogenic cells**. To address this objective, we have used bioinformatic and biochemical approaches directed at identifying one potential β-catenin binding partner and investigating its effect on β-catenin's function.

Chapter II: The β-catenin interactome in myogenic cells

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Conducting Experiments

Fatima Farhat: Figure 6B-C

Figure 7A-C

Figure 8A-G

Figure 9A-C

Figure 10B, E

Figure 11A-C

Figure 12A-D

Abstract

β-catenin is a versatile protein implicated in a wide range of cellular processes including cell fate determination, proliferation, differentiation, adhesion, and cell survival. β-catenin performs its cellular functions through protein-protein interactions since it lacks the ability to interact with DNA. We have previously performed a β-catenin interactome study in myogenic cells to investigate the protein network of β-catenin during myogenesis. The study was performed using a novel GFP-Nanotrap based affinity purification approach followed by LC-MS/MS analysis. The mass spectrometry dataset included a list of well-known and potential β-catenin protein interactors. Established β-catenin interactors present in the dataset included components of the β-catenin destruction complex such as GSK3-β and APC, in addition to components of the adherens junction such as α-catenin and Cadherins. After processing the mass spectrometry dataset, we focused on the interaction between β-catenin and FHL3. We showed that FHL3 interacts with β-catenin in the nuclei of cells. The TOPFLASH reporter system demonstrated that FHL3 inhibits β-catenin activity. FHL3 also inhibited the activation induced by β-catenin on the MyoG reporter assay in myogenic cells. We demonstrated that FHL3 translocates to the nucleus during the early stages of myogenic differentiation and performs an inhibitory function on β-catenin activity and myogenesis. We also showed that silencing FHL3 using siRNAs targeting FHL3 transcripts leads to a small but significant increase in β-catenin activity in myogenic cells. This data demonstrates a repressive role for FHL3 on β-catenin which we suggest contributes to the regulation of muscle differentiation.

Introduction

β-catenin has been implicated in a plethora of cellular processes including structural and signaling functions. It is involved in maintaining the structure of the cell through its interaction with cytoskeletal proteins such as Actin and α-catenin (30). The signaling function of β-catenin is mediated through its protein-protein network as it is a transcriptional co-activator that lacks a DNA-binding domain. When β-catenin is not targeted for degradation by the β-catenin destruction complex, it accumulates in the cytoplasm and then translocates to the nucleus to perform its co-activator role through interactions with various transcription factors and chromatin remodelers (27, 76, 77). β-catenin's major nuclear binding partners are the TCF/LEF family of transcription factors that induce the expression of Wnt target genes (78). In addition to its role in Wnt signaling, β-catenin is involved in activating other genes including myogenic genes such as the *myog* gene through its interaction with the myogenic transcription factor MyoD (64).

β-catenin has been demonstrated to be a positive regulator of myogenesis (61). Both proliferation and differentiation of myogenic cells are enhanced by the stabilization of β-catenin in the cytoplasm (57, 61, 64). Since β-catenin is active throughout the different stages of myogenesis, it is important to understand the molecular mechanisms underlying its regulation in muscle cells as deregulation of β-catenin target genes is usually associated with several pathological conditions. Excessive stimulation of β-catenin signaling alters muscle stem cell fate and increases fibrosis (79). On the other hand, reduced β-catenin activity leads to abnormal myofiber disruption and detachment from the sarcolemmal membrane (80).

Since β-catenin relies on protein-protein interactions to perform its signal transduction roles, we reasoned that examining β-catenin's network of interactors might give us insight into the molecular

mechanism underlying its regulation during myogenesis. To investigate this objective, we used a GFP-Nanotrap based affinity purification approach followed by LC MS/MS analysis to obtain a list of putative β-catenin binding partners in myogenic cells. Analysis of the mass spectrometry dataset included a list of well-known β-catenin binding partners such as Cadherins, α-catenin, TCFs and components of the destruction complex. In addition to the comprehensive list of established β-catenin interactors, many novel potential interactors were also documented. The purpose here was to study the interaction between β-catenin and the scaffolding protein FHL3. We document an interaction between β-catenin and FHL3 that fulfills a repressive function on β-catenin's transcriptional activation properties in muscle cells.

Materials and methods

Cell cultures

HEK 293T and C2C12 cells were obtained from the American Type Culture Collection (ATCC). Cells were maintained in growth media (GM) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% fetal bovine serum (FBS) supplemented with 1% penicillin–streptomycin (Invitrogen, ThermoFisher). Differentiation of C2C12 myoblasts was induced by switching GM to differentiation media (DM) made up of DMEM, 2% FBS and 1% penicillin–streptomycin. Cells were maintained in an incubator at 95% humidity, 5% CO_2, and 37 °C.

Rat tissue isolation

Neonatal rat pups were sacrificed by decapitation. Tissue was dissociated and digested with RIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140mM NaCl).

Transfections

Ectopically expressing proteins in C2C12 cells and HEK 293T cells was performed using polyethylenimine (PEI) at a DNA:PEI ratio of 1:3. Cells were re-fed 16 hrs post-transfection and harvested after 6 hrs.

Transfections for the purpose of gene silencing were performed using Lipofectamine RNAiMAX (Thermo-Fisher) and mission small interfering RNA (siRNA) purchased from Sigma-Aldrich. C2C12 cells were transfected with siFHL3 #1 (SASI_Mm02_00318055), siFHL3 #2 (SASI_Mm02_00318056), siFHL3 #3 (SASI_Mm01_00141082) and universal siControl (SIC001) at a final concentration of 50 nM.

Cell harvesting

Cells were washed three times with cold PBS then collected using a scraper. Cells were pelleted by centrifuging at 4 °C, 1.2k RPM for 10 min then resuspended with NP-40 lysis buffer (0.5 % NP-40, 50 mM Tris-HCL pH 7.6, 150 mM NaCl, 100 mM NaF,10 mM Sodium pyrophosphate,2 mM EDTA, 1 mM of Na3VO4, 1 mM PMSO). Cells were incubated for 30 min on ice then centrifuged at maximum speed for 10 min at 4°C. Supernatant constituting the soluble protein was collected.

For reporter gene assay experiments, cells were washed three times then 1x reporter lysis buffer (Promega, #E4030) was added to each well. Cells were incubated on a shaker for 30 min then collected in Eppendorf tubes. Lysates were vortexed for 15 min at 4 °C then centrifuged at maximum speed for 10 min at 4°C.

Western blot analysis

Protein concentrations were determined by standard Bradford assay (Bio-Rad laboratories). Protein extracts were denatured in 3x SDS loading buffer (0.6 ml of 1 M Tris-HCl pH = 8, 2.4 ml of glycerol, 2.4 ml of 10% SDS, 0.6 ml of Beta-Mercaptoethanol, Bromo blue dye) at 100 °C for 10 min then run on 10% SDS-PAGE gel for 1 h at 100V. The gel was transferred to a PVDF membrane (Millipore) and run in 1X transfer buffer (10% 10X tranfer buffer: 930.3g Tris-HCL, 144.2 g Glycine, 10 g SDS, equilibrated to 1L with ddH_20, 80% ddH_20, 10% of methanol). Then, the membrane was blocked in 5% blocking buffer (5% nonfat dry milk in TBS-T; 0.1% Tween® 20 in 1X TBS) on a shaker for 1 hr at room temperature. Membranes were incubated with primary antibodies solution (primary antibody diluted 1:1000 in 1% nonfat dry milk in TBS-T) on a shaker overnight at 4 °C. Membranes were washed with TBS-T then incubated in HRP-conjugated secondary antibody solutions (1:2000

secondary antibodies in 1% nonfat dry milk in TBS-T) on a shaker for 1 hr at room temperature. Membranes were washed three times with TBS-T protein/antibody immune-complexes were visualized iBright CL1500 Imaging System (ThermoFisher Scientific).

Co-immunoprecipitation (co-IP)

Anti-FLAG M2 magnetic beads (Sigma, #M8823) were washed three times with PBS then incubated with 1 mg of HEK 293T lysates overnight on a rotator at 4 °C. Magnetic beads were washed three times with PBS then protein complexes were eluted by incubating the beads in 500 μg/ml FLAG 3x peptide solution (Sigma, #4799) on a rotator at room temperature for 45 min.

Cell fractionation

The subcellular fractionation of C2C12 cells was performed using the NE-PER nuclear and cytoplasmic extraction reagents (Thermofisher scientific) according to the manufacturer's protocol.

Immunofluorescence analysis

C2C12 cells were seeded on collagen coated glass-bottom dishes (MatTek) and fixed with 4% paraformaldehyde (PFA) after washing three times with PBS. Cells were washed three times with PBS and permeabilized with 90% ice cold methanol for 10 min. Cells were washed again three times with PBS then incubated with IF blocking buffer (5% FBS in PBS) for 1 hr at room temperature. After removing blocking buffer, cells were incubated with appropriate primary antibody in IF blocking buffer overnight at 4 °C. Then, cells were washed three times with PBS and incubated with appropriate Alexa fluor conjugated secondary antibody (Life Technologies) in IF blocking buffer for 1 hr at room temperature. After washing two times with PBS, cells were stained with DAPI (Hoechst 33342) and subjected to Confocal fluorescent imaging with a Zeiss Observer Z1 microscope equipped with a Yokogawa CSU-X1 spinning disk. Images were

recorded by AxioCam MRm camera (Zeiss) and processed using zen 2.5 (blue edition) software and ImageJ.

Gene reporter assays

Gene reporter assays were performed with luciferase reporter plasmids containing the TCF/LEF consensus sequence or the MyoG promoter. Renilla plasmid (Promega, pRL-Renilla) was used as an internal control. Cells were harvested as described above. Transcriptional activity was measured on a luminometer (Berthold, Lumat LB) using Luciferase assay substrate (Promega, #E1501) and Renilla assay substrate (Promega, #E2820). Luciferase values were normalized to Renilla values to control for transfection efficiency.

Antibodies

Antibodies for β-catenin (rabbit, polyclonal, add #9562S), Myc (mouse, monoclonal, #9B11), α-tubulin (rabbit, polyclonal, #2144S) and Histone H3 (rabbit, polyclonal, #9715S) were purchased from Cell Signaling. Antibodies for MyoG (mouse, monoclonal, #F5D) and MyoD (mouse, monoclonal, #2A5) were purchased from Developmental Studies Hybridoma Bank (DSHB). Mck (mouse, monoclonal, #sc-365046) and β-actin (mouse, monoclonal, #sc-47778) antibodies were purchased from Santa Cruz Biotechnology. GFP (Rat, monoclonal, #3H9) was from ChromoTek. FHL3 antibody (rabbit, polyclonal #11028-2-AP) was purchased from proteintech.

Plasmids

The myc-FHL3 plasmid was a gift from Gregg Semenza (81). pcDNA3 was a gift from William Sellers (Addgene plasmid #10792). TOP-Flash and HOP-Flash were gifts from Randall Moon (Addgene plasmid #12456 & # 12457) (82). Myogenin core promoter was a gift from Michael Chin (Addgene plasmid #134722) (83). Renilla (pRL-Renilla) plasmid was purchased from Promega. For the myc-β-catenin construct, β-catenin ORF was amplified by PCR using cDNA

derived from C2C12 cells. The ORF was inserted at EcoRI and Xho1 sites into pcDNA3 (Invitrogen). A myc tag was inserted into pcDNA3 at HindIII and EcoRI sites of β-catenin ORF. For EYFP-FHL3 and mCherry-FHL3, FHL3 ORF was amplified by PCR using cDNA derived from C2C12 cells. The ORF was inserted at EcoRI and Xba1 sites into pcDNA3 (Invitrogen). A EYFP and mCheryy tags were inserted into pcDNA3 at HindIII and EcoRI sites of FHL3 ORF. For NLS-FHL3, a nuclear localizing signal (NLS, PKKKRKV) was inserted at the BamHI/EcoRI sites of pcDNA3-FHL3.

Statistical analysis

All statistical analysis were conducted using GraphPad PrismV8. Each experiment was performed at least in triplicate. Student's t-test was used to compare the means of two groups. The error bars represent SEM. P values less than 0.05 were considered statistically significant.

Results

Affinity purification/mass spectrometry analysis of the β-catenin interactome in myogenic cells

β-catenin maintains cellular structure and regulates gene expression through its protein network as β-catenin lacks a DNA binding domain. For this reason, we aimed to characterize the β-catenin interactome in myogenic cells. We reasoned that exploring β-catenin's protein interactors would further our understanding of the molecular mechanisms by which β-catenin performs its functional activity in muscle cells. To achieve this objective, we used a novel GFP-Nanotrap affinity purification approach followed by LC MS/MS analysis. EYFP-β-catenin was used as bait for the samples destined for mass spectrometry analysis and EYFP alone was used as bait for the negative control. The bait proteins were ectopically expressed in HEK 293T cells, and the lysates were complexed with GFP-Nanotrap magnetic beads coupled with GFP binding protein (GBP) nanobodies. C2C12 whole cell lysates were incubated with the GFP-Nanotrap-bait complex then the elution was analyzed by LC MS/MS (Figure 6A). To filter out proteins that interacted non-specifically with the EYFP-β-catenin bait proteins, a threshold of EYFP-alone to EYFP-β-catenin was set to a ratio of 1:3 or greater spectral counts. Proteins that met or exceeded this threshold were labelled "enriched proteins". This ratio was selected based on well-characterized β-catenin interactors being identified in the dataset around this value. Proteins that interacted with the EYFP-β-catenin bait and did not interact with the EYFP negative control were labelled "unique proteins" and were included in the list of candidates. After processing the dataset, a list of 179 total protein candidates was identified (Figure 6B). The list of candidates included many well-known β-catenin interactors which demonstrated the efficacy of the GFP-Nanotrap approach used. The list of established β-catenin binding partners included APC and GSK3-β which are core components of

the β-catenin destruction complex (35, 84). It also included Cadherins (Cdh2 and Cdh15), α-catenin and Actin which interact with β-catenin at the adherens junction (85-87). In addition to the mentioned cytoplasmic interactors, β-catenin nuclear interactors such as TCF transcription factors (TCF7l1 and TCF7l2) were also identified in the list (88, 89) (Figure 6C).

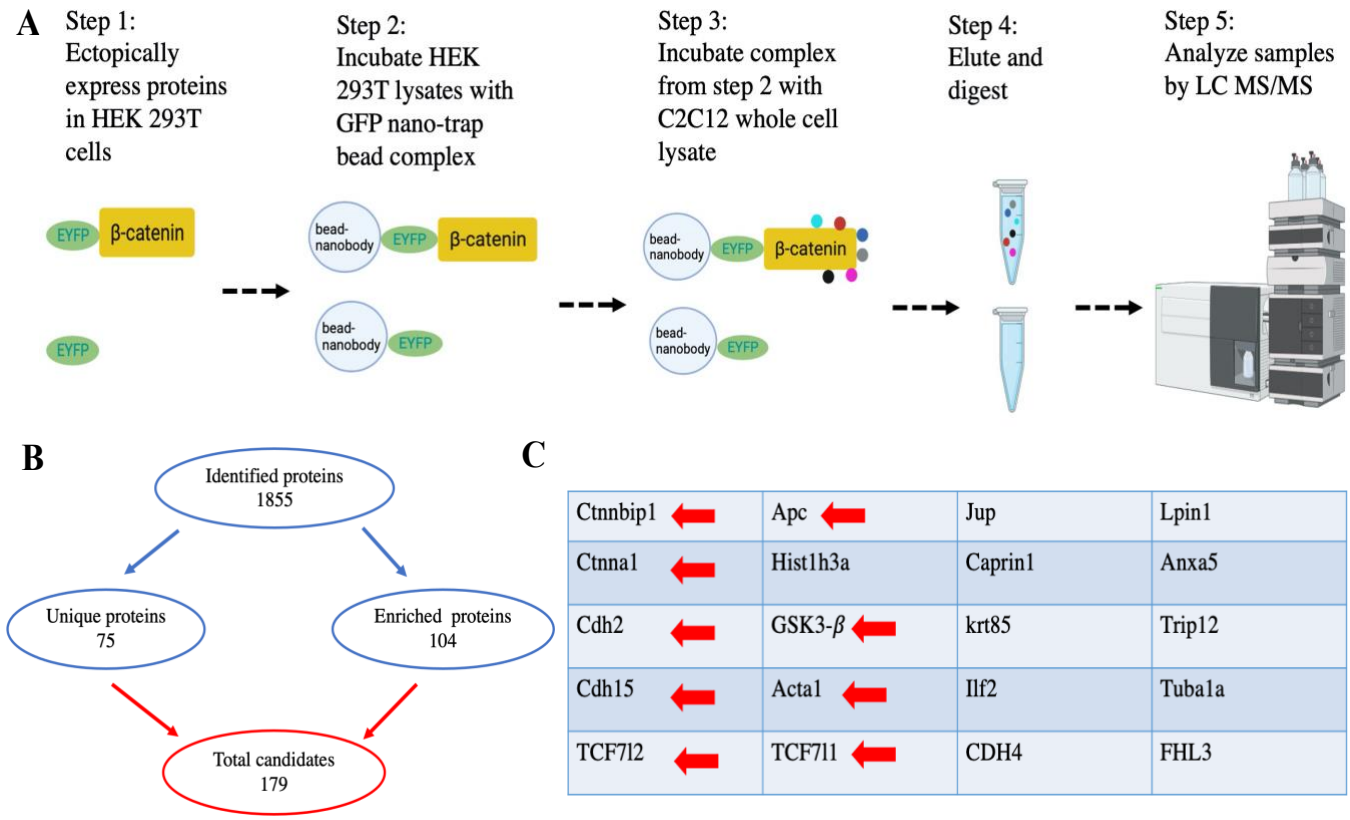


Figure 6: The β-catenin interactome in myogenic cells. (A) Schematic representation summarizing the steps performed to characterize the β-catenin interactome in C2C12 cells using the GFP-Nanotrap approach followed by LC MS/MS. Created with BioRender.com **(B)** Flow chart illustrating the number of proteins identified from the mass spectrometry dataset after data processing and analysis. **(C)** Summary of some well-characterized and novel β-catenin interactors identified in the dataset. Well-characterized β-catenin interactors are marked by a red arrow.

Bioinformatic analysis of the β-catenin interactome in myogenic cells

To investigate the signaling pathways and cellular processes associated with the list of 179 protein candidates obtained from processing the original dataset, KEGG and Reactome pathway analysis were performed. All pathways identified by KEGG and Reactome pathway analysis were associated with β-catenin function which further validated the approach used to obtain the list of β-catenin interactors. The pathway analyses revealed interactions related to the adherens junction: #1 in KEGG analysis pathway (Figure 7A) and #18 in Reactome analysis pathway (Figure 7B). Wnt signaling (#8 in KEGG and #10 in Reactome) was also present in both analysis pathways. The KEGG analysis pathway demonstrated pathways associated with β-catenin deregulation such as cancers (#3, #5, #11, #17 and #19, figure 2A) and pathologies related to the nervous system (#14 and #19, figure 2A). Most importantly, the Reactome pathway analysis revealed that the list of protein candidates is associated with pathways related to myogenesis (#2, Figure 7B).

To investigate which of these proteins were related to myogenesis, the ingenuity pathway analysis (IPA) was used. IPA helps interpreting proteomic data by placing the data within the context of different biological systems. Since the objective of my project was to investigate β-catenin interactors within the context of myogenesis, an IPA network associating proteins from the mass spectrometry dataset with muscle formation was explored. The network included well-characterized β-catenin interactors and novel potential interactors. The well-known interactors present in the network were APC, GSK3-β and the Cadherin Cdh2 while the novel interactors were FHL3 and CHD4. In this master's project, the interaction between β-catenin and FHL3 was pursued. FHL3 (Four and a half LIM domain 3) has been demonstrated to impact myogenesis by many groups. When ectopically expressed in a muscle cell line (C2C12 cells), FHL3 forms a complex with MyoD and inhibits the expression of MyoD target genes, such as *myog*, which leads

to the retardation of muscle cell differentiation (90). The interaction of FHL3 with MyoD has also been implicated in regulating the expression of MyHC isoforms. FHL3 has been shown to inhibit the expression of MyHC 1/slow through inhibiting the transcriptional activity of MyoD (91). Since β-catenin and FHL3 perform opposing functions in regulating myogenesis, we were interested in exploring the effect of FHL3 on β-catenin activity in a myogenic context.

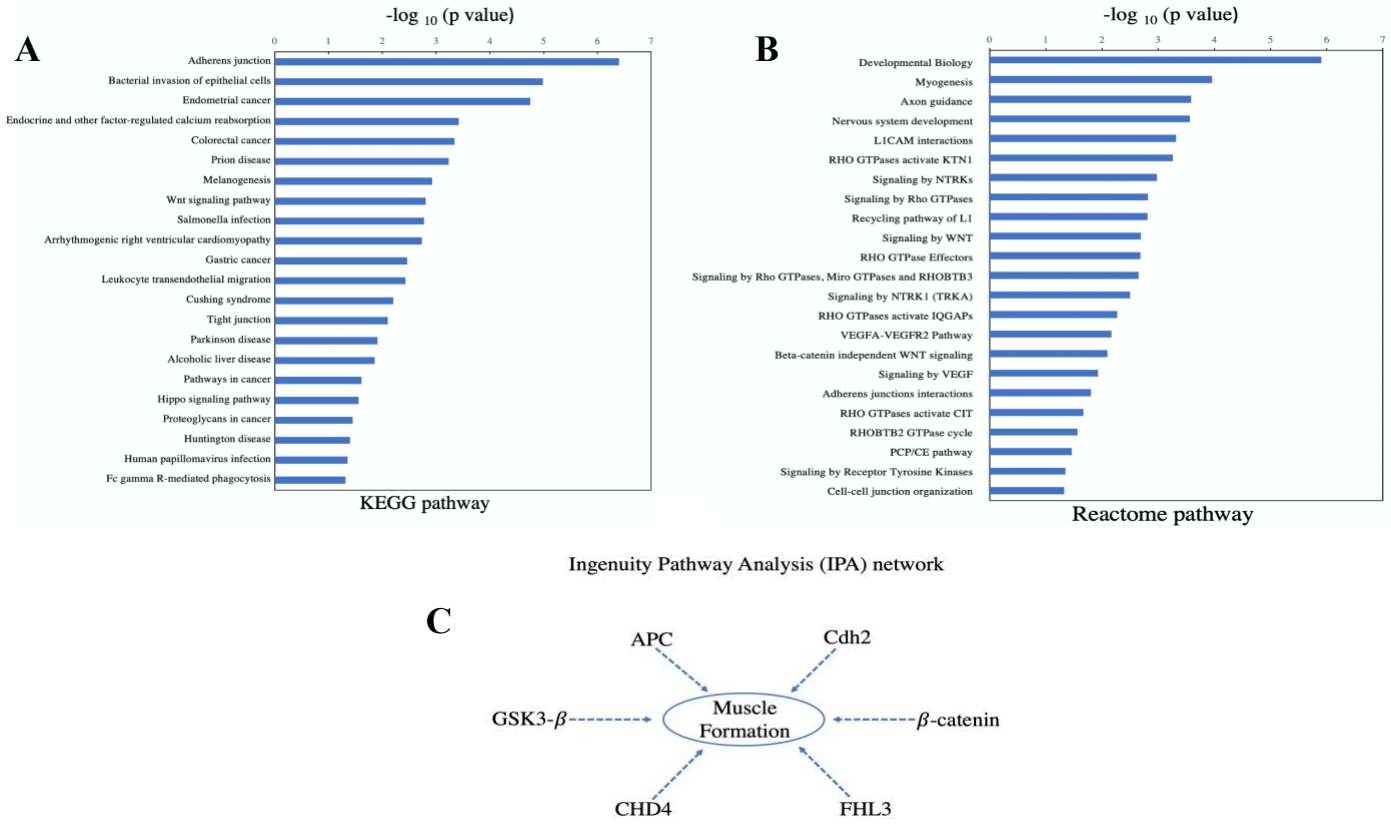


Figure 7: Bioinformatic analysis of the β-catenin interactome in myogenic cells. (A) KEGG pathway analysis associated with the list of 179 β-catenin interactors in C2C12 cells. **(B)** Reactome pathway analysis associated with the list of 179 β-catenin interactors in C2C12 cells. **(C)** Ingenuity pathway analysis (IPA) network showing β-catenin's interactors in the context of muscle formation. The network is associated with the original mass spectrometry dataset in C21C2 cells.

FHL3 inhibits β-catenin activity

To explore the protein expression levels of FHL3 in different tissue types, we performed SDS-PAGE analysis on different neonatal rat tissue types. Data demonstrated that FHL3 is highly expressed in skeletal muscle tissue (Figure 8A). Then, to assess whether the interaction between β-catenin and FHL3 predicted by the mass spectrometry analysis had a functional significance on β-catenin activity, we used luciferase gene reporter assays responsive to β-catenin induction. We initially tested the effect of FHL3 on the activity of β-catenin by using the TOPFLASH reporter system, which is a plasmid-based luciferase assay controlled by the TCF/LEF enhancer element (CCTTTGATC). The FOPFLASH system, which contains a mutated TCF/LEF enhancer element, was used as the corresponding negative control for the TOPFLASH system. β-catenin activity in response to the ectopic expression of FHL3 was assessed in C2C12 cells (an adult myogenic cell line) and HEK 293T cells (an embryonic kidney cell line). We observed that FHL3 significantly inhibited the β-catenin driven activation of the TOPFLASH system in both C2C12 cells and HEK 293T cells (Figure 8C and 8D). Also, a significant repression induced by the ectopic expression of FHL3 was observed when FHL3 and β-catenin were co-expressed (Figure 8C and 8D). Since β-catenin is a well-known activator of MyoG transcriptional activity (64), the *myog* luciferase reporter assay was used to explore the effect of ectopic FHL3 expression in a myogenesis context. Our data demonstrated that ectopic expression of FHL3 induced a significant repression on the *myog* reporter when expressed alone or in combination with β-catenin in C2C12 cells (Figure 8E). The inhibition induced by FHL3 at the level of *myog* gene expression was also reflected at the protein expression level. MyoG protein levels were significantly reduced in response to the ectopic expression of FHL3 in C2C12 cells differentiated for 72 hr (Figure 8F). In addition, immunofluorescence analysis (IF) aimed at visualizing the effect of ectopically expressing FHL3

at the individual cell level demonstrated that C2C12 cells expressing mCherry-FHL3 did not express MyoG (Figure 8G).

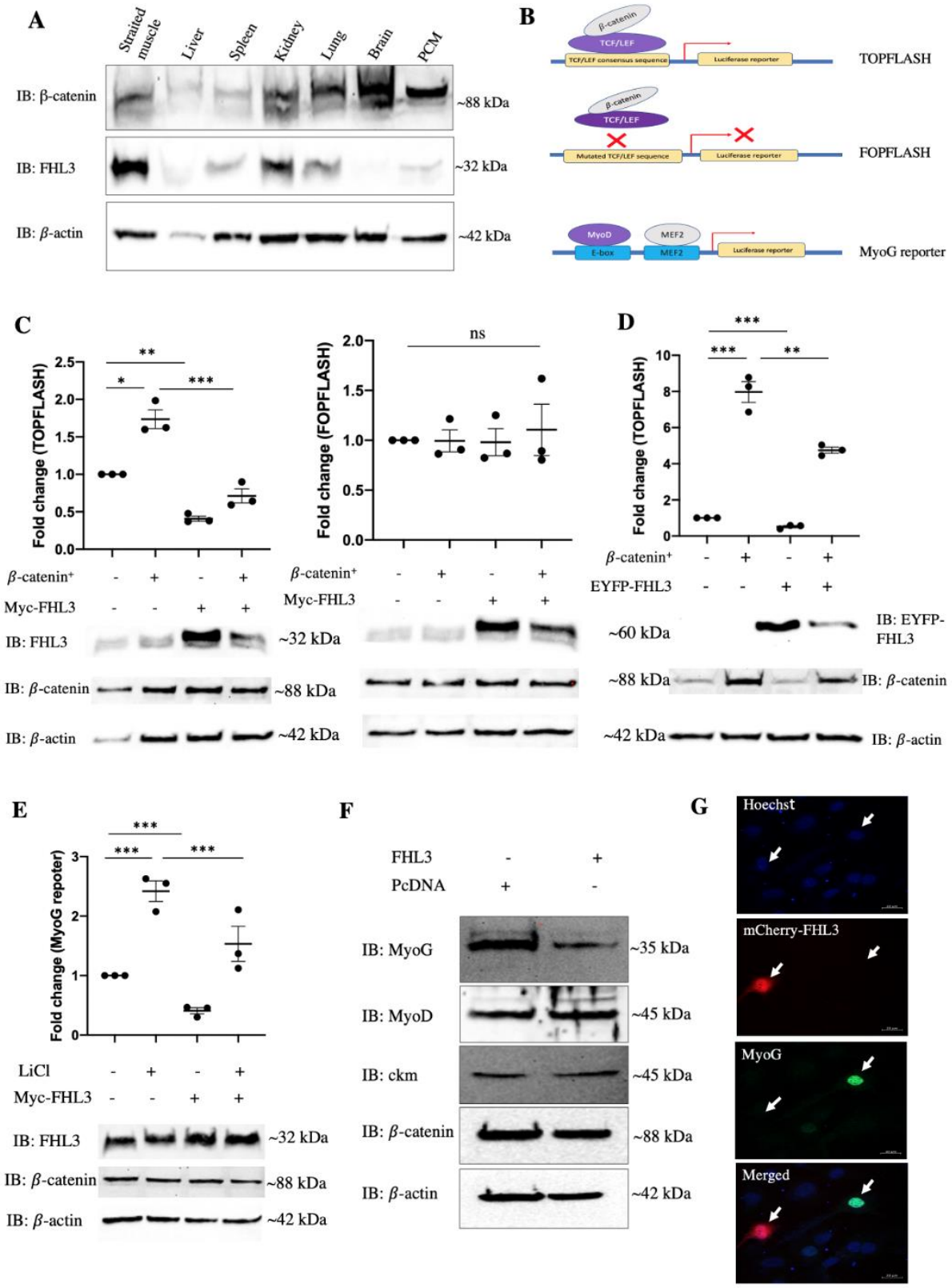
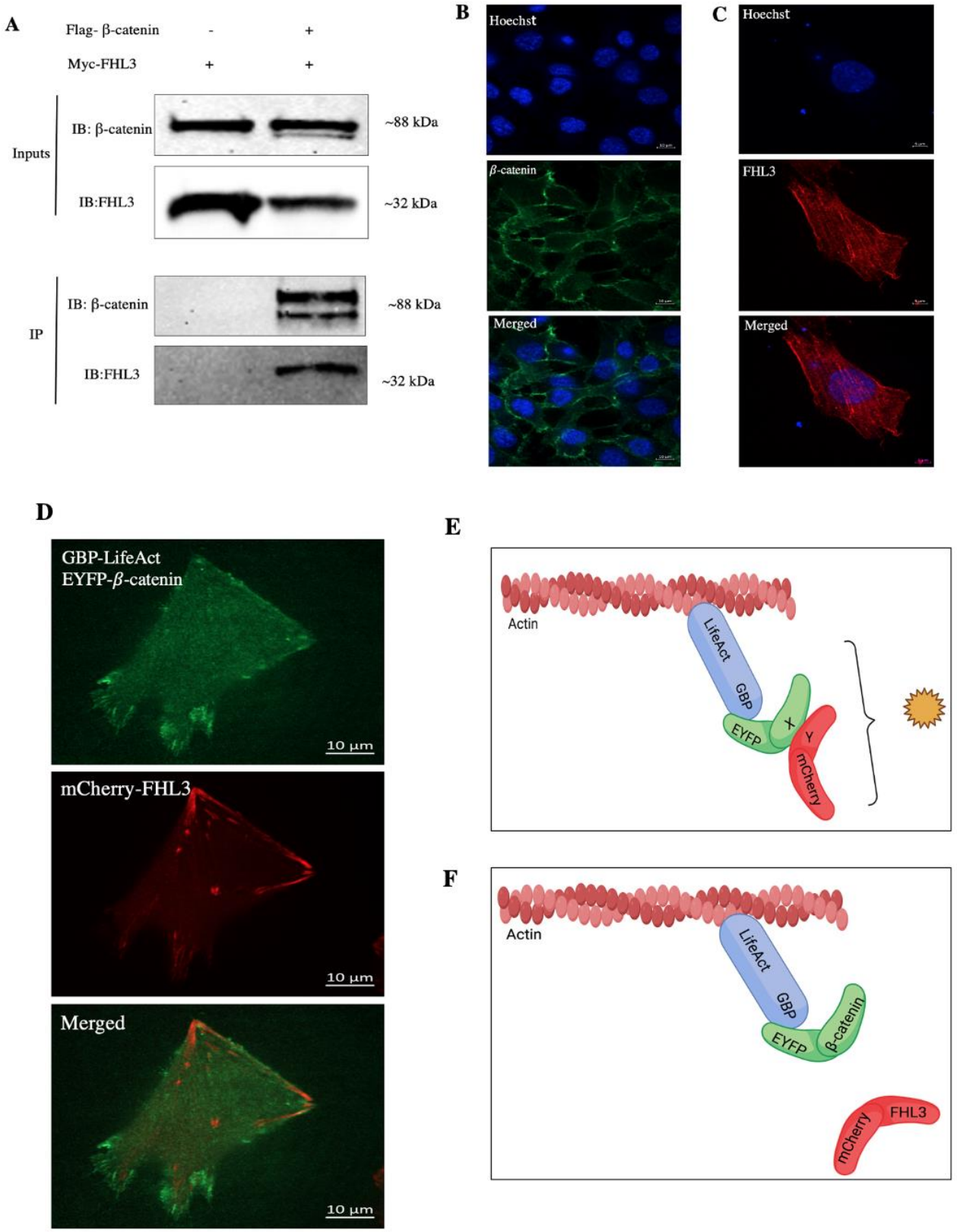

A
Straited muscle
Liver
Spleen
Kidney
Lung
Brain
PCM
IB: β-catenin
~88 kDa
IB: FHL3
~32 kDa
IB: β-actin
~42 kDa
B
β-catenin
TCF/LEF
TCF/LEF consensus sequence
Luciferase reporter
TOPFLASH
Mutated TCF/LEF sequence
FOPFLASH
MyoD
MEF2
E-box
MyoG reporter
C
Fold change (TOPFLASH)
Fold change (FOPFLASH)
ns
β-catenin+
Myc-FHL3
IB: FHL3
IB: β-catenin
IB: β-actin
D
Fold change (TOPFLASH)
EYFP-FHL3
~60 kDa
IB: EYFP-FHL3
E
Fold change (MyoG repoter)
LiCl
F
FHL3
PcDNA
IB: MyoG
~35 kDa
IB: MyoD
~45 kDa
IB: ckm
G
Hoechst
mCherry-FHL3
MyoG
Merged

Figure 8: FHL3 inhibits β-catenin activity in C2C12 cells and HEK 293T cells. (A) Different tissue types were isolated from neonatal rats and digested with RIPA buffer. Lysates were subjected to western blot analysis. **(B)** schematic representation of the reporter gene assays used in investigating the effect of FHL3 on β-activity. **(C)** activated β-catenin (mutated at the N-terminal domain to prevent degradation with GSK3-β) and myc-FHL3 were expressed in C2C12 cells, alone or in combination, with a TOPFLASH/FOPFLASH reporter gene. **(D)** EYFP-FHL3 and activated β-catenin were expressed in HEK293T cells, alone or in combination, with a TOPFLASH reporter gene. **(E)** myc-FHL3 was expressed in C2C12 cells with the *myog* reporter assay. LiCl was used to increase β-catenin activity (LiCl stabilizes β-catenin by inhibiting GSK3-β). **(C, D and E)** *Renilla* was used to control for transfection efficiency. Empty vector (pcDNA) was used as a control for ectopic expression. Lysates were collected 6 hrs after switching media and luciferase measurements were taken. The firefly luciferase activity was normalized to *Renilla* values. Fold change was obtained by averaging the luciferase: *Renilla* values for each treatment and comparing it to the control. N=3 biological replicates. Three replicates per condition. The error bars represent standard error of the mean (SEM). Statistical analysis was determined using T test. *P≤0.05, **P≤0.01, ***P≤0.001, ns indicated no statistical significance. **(F)** myc-FHL3 and pcDNA3 were ectopically expressed in C2C12 and grown for 24 h before switching to differentiation media for 72 hr. Lysates were collected and assessed for expression of the indicated proteins by western blot analysis. **(G)** mCherry-FHL3 was ectopically expressed in C2C12 cells and cells were differentiated for 72 hr after growing in growth media for 24 hr. Immunofluorescence analysis was performed by fixing C212C cells and staining for MyoG.

Nuclear FHL3 inhibits β-catenin activity in myogenic cells

To confirm the interaction between β-catenin and FHL3 identified by the mass spectrometry analysis biochemically, a co-immunoprecipitation (co-IP) in HEK 293T cells was performed. Flag-β-catenin and myc-FHL3 were ectopically expressed in HEK 293T cells, and the lysates were subjected to co-IP using anti-flag M2 magnetic beads. Co-IP results demonstrated that β-catenin was enriched in the Co-IP sample compared to the input and that FHL3 was co-eluted with β-catenin indicating an interaction between the two proteins (Figure 9A). To investigate the cellular localization of this interaction, C21C2 cells were immunostained with antibodies against endogenous β-catenin or FHL3 and IF analysis was performed under growth conditions. The IF analysis revealed that both β-catenin and FHL3 localize at the plasma membrane (Figure 9B and 9C) prompting us to propose that the interaction might be cytoplasmic. To further explore the

localization of the β-catenin/FHL3 interaction, we ectopically expressed GBP-Lifeact (an F-actin interacting protein), EYFP-β-catenin and mCherry-FHL3 in C2C12 cells and examined the localization of the ectopically expressed proteins. IF analysis showed that mCherry-FHL3 was not recruited to the GBP-Lifeact/EYFP-β-catenin complex (Figure 9D and 9F) suggesting that the β-catenin/FHL3 interaction might be occurring in the nucleus. To investigate the possibility of a nuclear interaction, we ectopically expressed EYFP-β-catenin and mCherry-FHL3 in C2C12 cells and performed live cell imaging. IF images demonstrated that ectopically expressed β-catenin and FHL3 localize to the same biomolecular condensates (Figure 10A) indicating that this interaction might be part of the same protein complex in the nucleus as β-catenin has been shown to form biomolecule condensates when involved in gene expression (Figure 10B) (70). To further investigate the nuclear localization of the β-catenin/FHL3 interaction, we used an EYFP-FHL3 construct containing a nuclear localizing signal (EYFP-NLS-FHL3) and performed IF analysis and TOPFLASH reporter assays. IF imaging indicated a co-localization between FHL3 and β-catenin within biomolecular condensates in the nucleus (Figure 10C). The TOPFLASH system demonstrated that both EYFP-FHL3 and EYFP-NLS-FHL3 significantly inhibit β-catenin activity (Figure 10E). The level of β-catenin activity inhibition was not significantly different between EYFP-FHL3 and EYFP-NLS-FHL3 which can be explained by the tendency of FHL3 to translocate to the nucleus when ectopically expressed (Figure 9E). Since restricting the localizing of FHL3 to the nucleus did not remove the inhibitory effect of FHL3 on β-catenin, we suggested that it is nuclear FHL3 that inhibits the activity of β-catenin.


A
Flag- β-catenin
-
+
Myc-FHL3
+
+
Inputs
IB: β-catenin
~88 kDa
IB:FHL3
~32 kDa
IP
IB: β-catenin
~88 kDa
IB:FHL3
~32 kDa
B
Hoechst
β-catenin
Merged
C
Hoechst
FHL3
Merged
D
GBP-LifeAct
EYFP-β-catenin
10 µm
mCherry-FHL3
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10 µm
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Actin
LifeAct
GBP
EYFP
X
Y
mCherry
F
Actin
LifeAct
GBP
EYFP
β-catenin
mCherry
FHL3

Figure 9: FHL3 interacts with β-catenin. (A) Immunoprecipitation was performed by ectopically expressing flag-β-catenin and myc-FHL3 in HEK 293T cells. Anti-flag M2 magnetic beads were used to trap flag-β-catenin to examine the interaction between β-catenin and the ectopically expressed myc-FHL3. For the negative control, cells were transfected with myc-FHL3 only and lysates were incubated with the anti-flag M2 magnetic beads. A β-catenin antibody was used to blot for flag-β-catenin (bait protein) and FHL3 antibody was used to blot for myc-FHL3 (bait protein). **(B)** and **(C)** Immunofluorescence analysis of fixed C2C12 cells immunostained for β-catenin (B) or FHL3 (C). Nuclei were stained with Hoechst 33342 (blue). **(D)** C2C12 cells were co-transfected with GBP-LifeAct, EYFP-β-catenin and mcherry-FHL3. Cells were visualized by live cell confocal imaging. **(E)** Schematic representation of the GBP LifeAct technique. **(F)** Schematic explaining the results obtained in (D). Created with BioRender.com

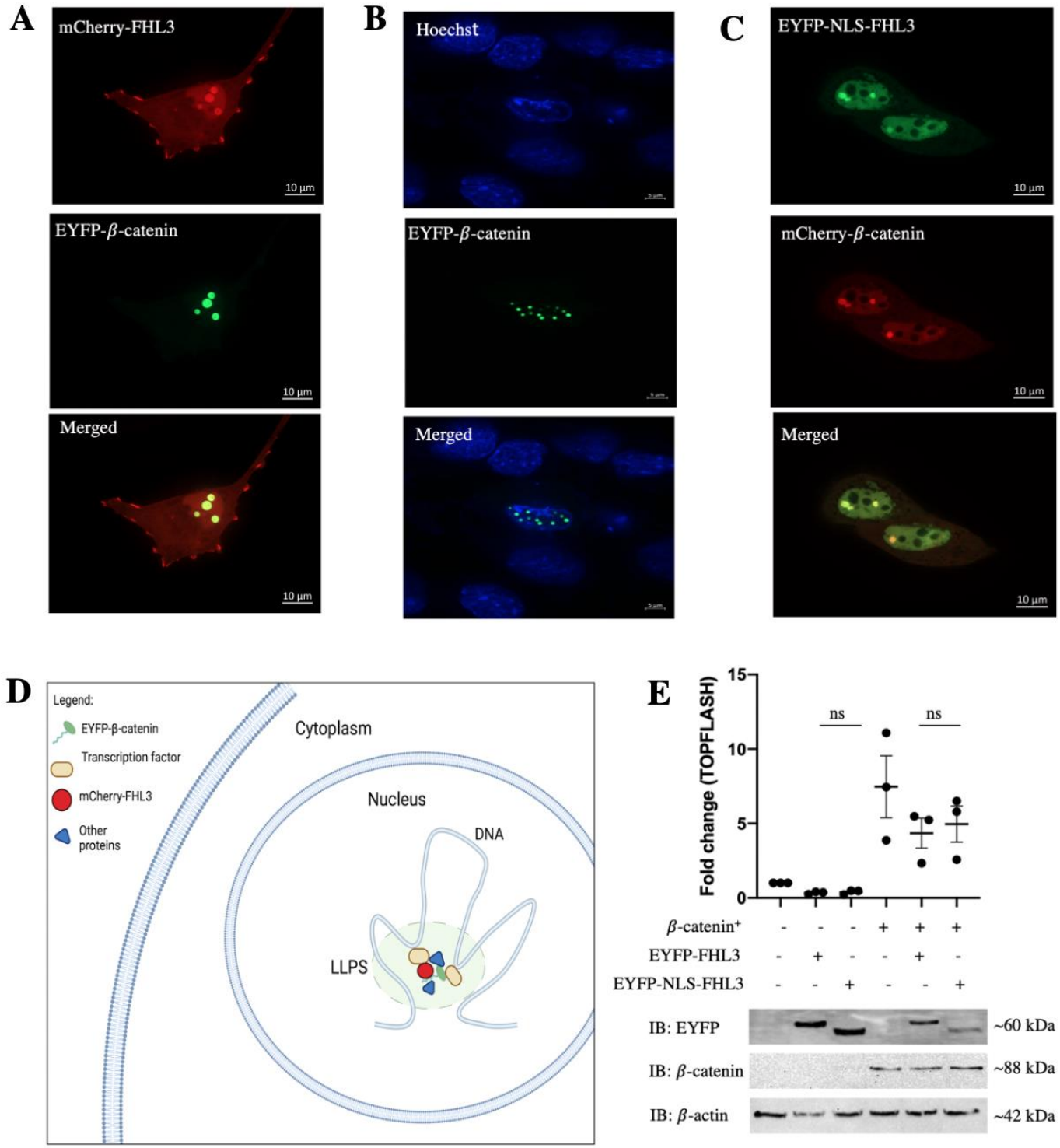

Figure 10: FHL3 interacts with β-catenin in nuclei of myogenic cells. (A), **(B)** and **(C)** C2C12 cells were transfected with the indicated constructs and visualized by live cell confocal imaging. **(D)** schematic representation of the suggested interaction between β-catenin and FHL3 in a biomolecular condensate. Created with BioRender.com **(E)** EYFP-FHL3 (WT-FHL3) and EYFP-

NLS-FHL3 were expressed in HEK 293T cells, with a TOPFLASH reporter gene. *Renilla* was used to control for transfection efficiency. Empty vector (pcDNA) was used as a control for ectopic expression. Lysates were collected 6 hrs after switching media and luciferase measurements were taken. The firefly luciferase activity was normalized to *Renilla* values. N=3 biological replicates. The error bars represent SEM. Statistical analysis was determined using T test, ns indicated no statistical significance.

FHL3 interacts with β-catenin during the differentiation stages of myogenesis

To investigate the temporal features of the interaction between β-catenin and FHL3 during the myogenic process, C2C12 cells were cultured for 24 hr in GM after which cells were induced to differentiate through switching media to DM for 24 hr, 48 hr and 72 hr. Western blot analysis demonstrated that FHL3 protein levels peaked at 48 hr during the differentiation program then decreased after the 48 hr timepoint. β-catenin protein levels also increased at 48 hr and remained high at 72 hr in DM (Figure 11A). Based on our previous data which suggested that the interaction between β-catenin and FHL3 was nuclear (Figure 10), we reasoned that performing a subcellular fractionation on C2C12 cells to separate the nuclear and cytoplasmic fractions under growth and differentiation conditions would give us insight into the timing at which the interaction between β-catenin and FHL3 occurs (Figure 11B and 11C). Western blot analysis showed that FHL3 translocates to the nucleus during the early stages of differentiation (24 hr in DM) and peaks at 48 hr in DM (Figure 11C). β-catenin protein expression levels in the nucleus also increased when cells were cultured in DM for 24 hr after which β-catenin levels remained constant (Figure 11C). Collectively, these data suggested that FHL3 interacts with β-catenin in the nuclei of myogenic cells during the differentiation stage of the myogenic program to negatively modulate the transcriptional activity of β-catenin.

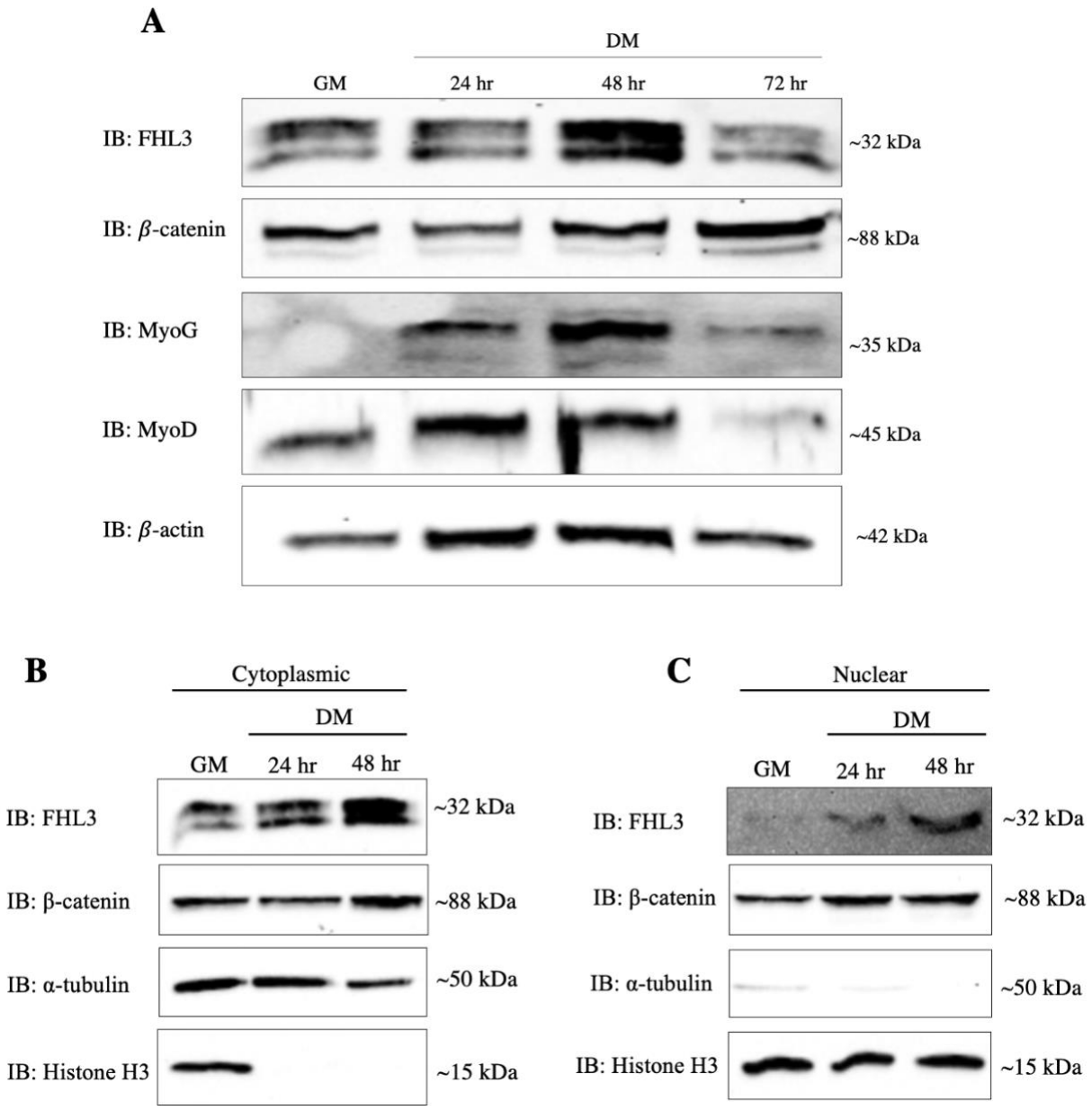

Figure 11: FHL3 interacts with β-catenin during the differentiation stages of myogenesis. (A) C2C12 cells were cultured in growth media for 24 hr before switching to differentiation media. Cells were collected at each time point and lysates were subjected to western blot analysis. MyoG and MyoD expression levels were used as myogenic markers for the differentiation process. The subcellular fractionation of the cytoplasmic **(B)** and nuclear **(C)** fractions of C2C12 cells was performed under growth conditions and differentiating conditions for 24 hr and 48 hr. α-tubulin and Histone H3 were used as markers for the cytoplasmic and nuclear fractionations respectively.

Silencing FHL3 increases β-catenin activity

To assess the effect of FHL3 depletion on the activity of β-catenin, we used siRNA targeting FHL3 transcripts (siFHL3) to deplete the endogenous pool of FHL3. Our data demonstrated that in HEK 293T cells, silencing FHL3 induced a two-fold increase in β-catenin activity on the TOPFLASH reporter system when siFHL3 was expressed alone and an eight-fold increase when siFHL3 and β-catenin were co-expressed (Figure 12A). In C2C12 cells, FHL3 depletion induced a significant but small increase (~20% increase) in β-catenin activity when cells were cultured in GM (Figure 12B) and 48 hr in DM (Figure 12C). In addition, depleting FHL3 induced a small increase in MyoG protein levels at 48 hr in DM (Figure 12D). Collectively, this data suggests that the role of FHL3 in regulating myogenesis might be redundant where in the absence of FHL3 other mechanisms are employed to regulate β-catenin activity.

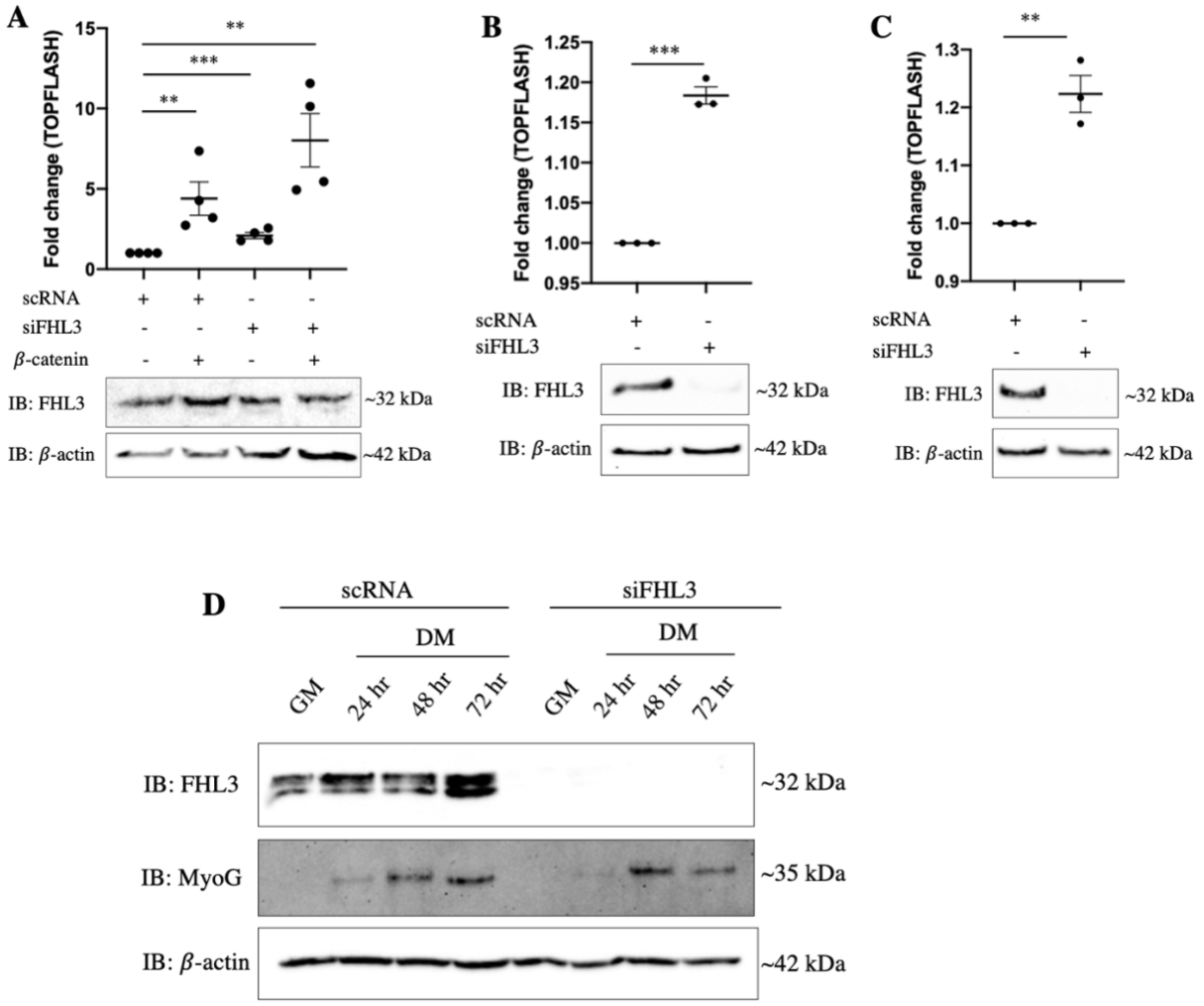

Figure 12: Silencing FHL3 increases β-catenin activity. (A) HEK 293T cells were transfected with siFHL3 or scRNA (negative control) alone or in combination with β-catenin to assess β-catenin activity on the TOPFLASH reporter system. Media was switched after 24 hr and cells were allowed to recover for 24 hr in GM before harvesting. Empty vector (pcDNA) was used as a control for ectopic expression. N= 4 biological replicates. **(B)** C2C12 cells were transfected with siFHL3 or scRNA with a TOPFLASH reporter gene. Media was switched after 24 hr and cells were allowed to recover for 24 hr in GM before harvesting. N= 3 biological replicates **(C)** C2C12 cells were transfected with siFHL3 or scRNA with a TOPFLASH reporter gene. Media was switched after 24 hr and cells were allowed to recover for 24 hr in GM then cells were differentiated in DM for 48 hr. N= 3 biological replicates **(A, B, C)** *Renilla* was used to control for transfection efficiency. The error bars represent SEM. Statistical analysis was determined using T test. *P≤0.05, **P≤0.01, ***P≤0.001. **(D)** C2C12 cells were transfected with siFHL3 or scRNA. Media was switched after 24 hr and cells were allowed to recover for 24 hr in GM before switching to DM. Cells were harvested at each timepoint and lysates were subjected to SDS-PAGE analysis.

Discussion

β-catenin has been the subject of extensive research due to its role in the highly evolutionary conserved Wnt signaling pathway. In this pathway, β-catenin performs its signaling transducing role as a co-activator through interacting with the TCF/LEF family of transcription factors. Although largely known for its role in the Wnt/β-catenin pathway, β-catenin also interacts with a variety of transcription factors and co-factors in the nucleus to induce gene expression. Besides its transducing function, β-catenin maintains cell-cell adhesion through its association with Cadherins and the Actin cytoskeleton.

Since β-catenin is incapable of directly interacting with DNA, it relies extensively on its protein-protein interaction network to accomplish its transcriptional regulatory function. Hence, exploring the β-catenin protein interactome offers valuable insights into the mechanisms by which β-catenin modulates gene expression. To investigate the β-catenin protein network in myogenic cells, our group performed a β-catenin protein interactome study in the C2C12 myogenic cell line using a novel GFP-Nanotrap based affinity purification followed by LC-MS/MS. Among the identified proteins in the interactome dataset were well-known β-catenin protein binding partners such as APC, GSK3-β, Cadherins, TCFs and Actin which validated the approach used to generate the interactome list. In addition to the established interactors identified, the interactome dataset characterized many novel potential β-catenin binding partners. Our bioinformatic analysis of the dataset predicted a relationship between β-catenin and the scaffolding protein FHL3 that influences muscle formation, so we focused on characterizing this interaction. We document a novel interaction between β-catenin and FHL3 in the nuclei of myogenic cells that has negative regulatory effects on β-catenin activity during the differentiation stages of myogenesis.

β-catenin interaction with the FHL family of proteins

FHL3 belongs to the FHL family of proteins which, in humans, is made up of four members: FHL1, FHL2, FHL3 and FHL5 (92-94). FHL1 and FHL3 are highly expressed in skeletal muscles while FHL2 is highly expressed in cardiac muscles (95). FHL5, also called ACT (activator of CREM in testes), is mainly expressed in testes (93). FHL3 shares 40% similarity in terms of amino-acid sequence with FHL1 and FHL2, but less than 20% sequence similarity with FHL5 (96). This suggests that FHL3 has unique functions in the cell.

FHL1 and FHL2 (but not FHL5) have been shown to modulate the function of β-catenin depending on the context. Studies have depicted a relationship between β-catenin and FHL1 in multiple cell lines. In C2C12 cells, ectopic expression of β-catenin significantly increases gene expression of the *fhl*1 promoter which enhances muscle differentiation (97). In ATDC5 cells, exogenous expression of β-catenin or treatment with LiCl increases *fhl1* gene expression leading to the inhibition of chondrogenesis (98). In colorectal cancer (CRC) cells, FHL1 has a negative effect on the transcriptional activity of β-catenin which leads to the inhibition of colorectal cancer progression (99). FHL2 has been shown to directly interact with β-catenin leading to the activation or inhibition of the transcriptional activity of β-catenin. Studies have demonstrated that FHL2 activates the transcription of β-catenin target genes in HEK 293 and SW480 cell while an inhibitory effect has been observed in the C2C12 cell line (100-102).

Although some aspects of the interaction between β-catenin and the two members of the FHL family of proteins (FHL1/2) have been explored, there is a surprising lack of research investigating the influence of FHL3 on β-catenin activity. The only study that explored the effect of FHL3 on Wnt signaling demonstrated that FHL3 positively regulates the canonical Wnt signaling pathway through regulating the BMP signaling pathway which triggers neural crest specification during

embryonic development (103). In our study, we have revealed an inhibitory effect for FHL3 on β-catenin activity in myogenic cells. Our study also demonstrated that silencing FHL3 leads to a small increase in β-catenin activity but does not have significant effects on MyoG expression levels suggesting that the loss of FHL3 is compensated for by other regulatory mechanisms.

Many studies have also depicted FHL3 as a co-repressor of gene expression. FHL3 has been shown to interact with the potent transcriptional repressors BKLF/KLF3 and CtBP2 to inhibit gene expression (104). FHL3 is also involved in the negative regulation of the high-affinity IgE receptor FcεRI expression through interacting with the transcription factor MZF-1 (105). In addition, FHL3 functions as a tumor suppressor by inhibiting cell cycle regulators such as Cyclin D1 and Cyclin B1 in certain breast cancer cell lines (106).

Chapter III: Summary, limitations, and future directions

Summary

β-catenin is a multifaceted protein involved in cell-cell adhesion and in activating gene expression. β-catenin is implicated in the positive regulation of myogenesis through enhancing myoblast proliferation in addition to inducing myoblast fusion (61, 63-65). β-catenin's function is highly dependent on its protein-protein network due to the absence of a DNA binding domain so, exploring the protein interactors of β-catenin would enhance our understanding of the molecular mechanisms underlying β-catenin's function. Our group has previously characterized the β-catenin interactome in myogenic cells using a novel GFP-Nanotrap affinity purification approach followed by mass spectrometry analysis (LC-MS/MS). The mass spectrometry analysis produced a comprehensive list of established and potential β-catenin protein interactors. In our study, we pursued the interaction between β-catenin and the scaffolding protein FHL3 after performing bioinformatic analysis on the mass spectrometry dataset. Previous studies depicted a negative regulatory role for FHL3 on the myogenic program achieved through repressing the transcriptional activity of MyoD (90, 91). In our study, we portray an inhibitory role for FHL3 on β-catenin activity. We also depict an interaction between β-catenin and FHL3 that we suggest takes place in the nuclei of myogenic cells during the early differentiation stages of the myogenic differentiation program. In addition, we show that FHL3 depletion induces a small increase in β-catenin activity but does not significantly modulate MyoG levels. We suggest that the inhibitory effect of FHL3 on β-catenin keeps the activity of β-catenin in check during skeletal myogenesis possibly to inhibit premature differentiation.

Limitations and future directions

This study has presented data demonstrating a novel inhibitory effect for FHL3 on the transcriptional activation properties of β-catenin. The inhibitory effect of FHL3 on the activity of β-catenin was solely based on reporter gene assay data (TOPFLASH and *myog* repoter assays). To further examine the effect of FHL3 on β-catenin activity, the expression of β-catenin target genes can be assessed by manipulating FHL3 expression, through knocking down or ectopically expressing FHL3, then performing RNA sequencing or qPCR for β-catenin target genes.

In addition, our data suggested a nuclear interaction between β-catenin and FHL3 during the early stages of the myogenic differentiation program. First, the co-immunoprecipitation performed to confirm the β-catenin/FHL3 interaction predicted by the mass spectrometry analysis does not indicate whether the interaction is direct or bridged by other proteins. To investigate a direct interaction between β-catenin and FHL3, an affinity purification approach can be used. Additionally, the interaction interphase on both proteins can be mapped by performing deletion mutations on β-catenin then performing co-immunoprecipitation or affinity purification experiments. Also, the requirement for the presence of posttranslational modifications (PTMs) on β-catenin for the interaction with FHL3 to occur can be explored through introducing specific mutations on β-catenin residues known to undergo PTMs then assessing the β-catenin/FHL3 interaction. Secondly, the temporal characteristics of this interaction were inferred based on data suggesting a nuclear localization for the interaction and not on a direct experimental approach. To confirm the nuclear localization of the interaction during the early stages of the myogenic differentiation program, performing a co-immunoprecipitation of nuclear β-catenin and FHL3 cultured for 24 hr and 48 hr in DM is required. In addition, to examine whether β-catenin and FHL3 interact at myogenic promoters in the nucleus, a chromatin immunoprecipitation (Ch-IP)

followed by qPCR analysis for myogenic loci can be performed in C2C12 cells using β-catenin and FHL3 antibodies.

Moreover, all the experiments in our study were performed *in vitro* in a controlled environment using the C2C12 cell line that mimics a healthy state of a myogenic cell. Performing the experiments in a deregulated cell line, such as a rhabdosarcoma cell line, may be pertinent for understanding the effect of FHL3 on β-catenin activity in a dysfunctional system. Rhabdosarcoma is a type of soft tissue cancer that originates from cells committed to the skeletal muscle lineage (107). These cells are incapable of terminally differentiating despite the expression of muscle regulatory factors (108). Studies have demonstrated that the Wnt/β-catenin signaling pathway is downregulated in certain rhabdosarcoma types, such as embryonal rhabdosarcoma, which leads to the inhibition of muscle differentiation (109). Therefore, investigating the relationship between FHL3 and β-catenin in rhabdosarcoma cells might have clinical significance.

Furthermore, performing *in vivo* studies is important to examine whether the results obtained in our study are reproducible in an organ system. For example, performing FHL3 knock-out or knock-in experiments in mice using CRISPR-Cas9 technology would give us more insights into the physiological function of FHL3 and its effect on β-catenin activity. In addition, to understand the specific effect of FHL3 on skeletal muscle cells, skeletal muscle specific knock-out experiments of FHL3 can be performed using Cre-Lox recombination where FHL3 is floxed and muscle cell specific deletions can be achieved through Pax7 promoter driven Cre expression. Also, investigating the expression of FHL3 during the different stages of embryonic development using *in situ* hybridization of mouse embryo sections can further our understanding on the role of FHL3 during the embryonic developmental process.

In addition to the interaction between β-catenin and FHL3, the bioinformatic analysis performed using IPA predicted an interaction between β-catenin and CHD4. CHD4 is a component of the chromatin remodeling complex Nurd that has been shown to have silencing effects on gene expression (110, 111). In a myogenic context, CHD4 has been shown to maintain the stem cell identity of satellite cells during skeletal muscle cell regeneration (112). In addition, CHD4 has been implicated in repressing the skeletal and smooth muscle programs in cardiomyocytes (113). It would be worthy to investigate the potential interaction between CHD4 and β-catenin and the implications of such an interaction on β-catenin activity and subsequently its consequences on the myogenic program.

References

1. Charvet, B., Ruggiero, F., & Le Guellec, D. (2012). The development of the myotendinous junction. A review. *Muscles, ligaments and tendons journal*, *2*(2), 53–63.
2. Lieber, R. L., Roberts, T. J., Blemker, S. S., Lee, S. S. M., & Herzog, W. (2017). Skeletal muscle mechanics, energetics and plasticity. *Journal of neuroengineering and rehabilitation*, *14*(1), 108. https://doi.org/10.1186/s12984-017-0318-y
3. Huxley, H.E., & Kress, M. (1985). Crossbridge behaviour during muscle contraction. *J Muscle Res Cell Motil,* 6, 153–161. https://doiorg.ezproxy.library.yorku.ca/10.1007/BF00713057
4. Hargreaves, M., & Spriet, L. L. (2020). Skeletal muscle energy metabolism during exercise. *Nature metabolism*, *2*(9), 817-828. https://doi.org/10.1038/s42255-020-0251-4
5. Frontera, W.R., & Ochala, J. (2015). Skeletal Muscle: A Brief Review of Structure and Function.*Calcif Tissue Int* **96**, 183–195. https://doi.org/10.1007/s00223-014-9915-y
6. Wilson, K., Faelan, C., Patterson-Kane, J. C., Rudmann, D. G., Moore, S. A., Frank, D., Charleston, J., Tinsley, J., Young, G. D., & Milici, A. J. (2017). Duchenne and Becker Muscular Dystrophies: A Review of Animal Models, Clinical End Points, and Biomarker Quantification. Toxicol Pathol, 45(7), 961-76.https://doi.org/10.1177/0192623317734823
7. Baracos, V. E., Martin, L., Korc, M., Guttridge, D. C., & Fearon, K. C. H. (2018). Cancerassociated cachexia. Nat Rev Dis Primers, 4, 17105. https://doi.org/10.1038/nrdp.2017.105
8. Skapek, S. X., Ferrari, A., Gupta, A. A., Lupo, P. J., Butler, E., Shipley, J., Barr, F. G., & Hawkins, D. S. (2019). Rhabdomyosarcoma. *Nature Reviews. Disease Primers*, *5*(1), 1. https://doi.org/10.1038/s41572-018-0051-2
9. Chal, J., & Pourquié, O. (2017). Making muscle: skeletal myogenesis *in vivo* and *in vitro*. *Development (Cambridge, England)*, *144*(12), 2104–2122. https://doi.org/10.1242/dev.151035
10. Kopantseva, E. E., & Belyavsky, A. V. (2016). Key regulators of skeletal myogenesis. *Molecular Biology*, *50*(2), 169–192. https://doi.org/10.1134/S0026893316010076
11. Kaletta, T., Schnabel, H., & Schnabel, R. (1997). Binary specification of the embryonic lineage in Caenorhabditis elegans. *Nature*, *390*(6657), 294–298. https://doi.org/10.1038/36869
12. Baylies, M. K., Arias, A. M., & Bate, M. (1995). wingless is required for the formation of a subset of muscle founder cells during Drosophila embryogenesis. *Development*, *121*(11), 3829-3837.
13. Krause M. (1995). MyoD and myogenesis in C. elegans. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *17*(3), 219–228. https://doi.org/10.1002/bies.950170308
14. Fukushige, T., & Krause, M. (2005). The myogenic potency of HLH-1 reveals wide-spread developmental plasticity in early C. elegans embryos. *Development (Cambridge, England)*, *132*(8), 1795–1805. https://doi.org/10.1242/dev.01774
15. Michelson, A. M., Abmayr, S. M., Bate, M., Arias, A. M., & Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in Drosophila

embryos. *Genes & development*, *4*(12A), 2086–2097. https://doi.org/10.1101/gad.4.12a.2086
16. Abmayr, S. M., & Keller, C. A. (1998). Drosophila myogenesis and insights into the role of nautilus. *Current topics in developmental biology*, *38*, 35–80. https://doi.org/10.1016/s0070-2153(08)60244-6
17. Mauro A. (1961). Satellite cell of skeletal muscle fibers. *The Journal of biophysical and biochemical cytology*, *9*(2), 493–495. https://doi.org/10.1083/jcb.9.2.493
18. Zammit, P. S. (2008). All muscle satellite cells are equal, but are some more equal than others? *Journal of Cell Science*, *121*(18), 2975–2982. https://doi.org/10.1242/jcs.019661
19. Schultz, E., Gibson, M. C., & Champion, T. (1978). Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. *The Journal of Experimental Zoology*, *206*(3), 451–456. https://doi.org/10.1002/jez.1402060314
20. Chargé, S. B. P., & Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiological Reviews*, *84*(1), 209–238. https://doi.org/10.1152/physrev.00019.2003
21. Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., & Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell*, *102*(6), 777–786. https://doi.org/10.1016/s0092-8674(00)00066-0
22. Beauchamp, J. R., Heslop, L., Yu, D. S., Tajbakhsh, S., Kelly, R. G., Wernig, A., Buckingham, M. E., Partridge, T. A., & Zammit, P. S. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *The Journal of cell biology*, *151*(6), 1221–1234. https://doi.org/10.1083/jcb.151.6.1221
23. Wright, W. E., Sassoon, D. A., & Lin, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell*, *56*(4), 607–617. https://doi.org/10.1016/0092-8674(89)90583-7
24. Yablonka-Reuveni, Z., & Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Developmental biology*, *164*(2), 588–603. https://doi.org/10.1006/dbio.1994.1226
25. Perrimon, N., & Mahowald, A. P. (1987). Multiple functions of segment polarity genes in drosophila. Dev. Biol. *119*(2): 587-600. https://doi.org/10.1016/0012-1606(87)90061-3
26. Ehyai, S., Dionyssiou, M. G., Gordon, J. W., Williams, D., Siu, K. W., & McDermott, J. C. (2015). A p38 Mitogen-Activated Protein Kinase-Regulated Myocyte Enhancer Factor 2-β-Catenin Interaction Enhances Canonical Wnt Signaling. *Molecular and cellular biology*, *36*(2), 330–346. https://doi.org/10.1128/MCB.00832-15
27. Essers, M. A., de Vries-Smits, L. M., Barker, N., Polderman, P. E., Burgering, B. M., & Korswagen, H. C. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. *Science (New York, N.Y.)*, *308*(5725), 1181–1184. https://doi.org/10.1126/science.1109083
28. Trompouki, E., Bowman, T. V., Lawton, L. N., Fan, Z. P., Wu, D. C., DiBiase, A., Martin, C. S., Cech, J. N., Sessa, A. K., Leblanc, J. L., Li, P., Durand, E. M., Mosimann, C., Heffner, G. C., Daley, G. Q., Paulson, R. F., Young, R. A., & Zon, L. I. (2011). Lineage regulators direct BMP and Wnt pathways to cell-specific programs during differentiation and regeneration. *Cell*, *147*(3), 577–589. https://doi.org/10.1016/j.cell.2011.09.044

29. Ozawa, M., Baribault, H., & Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *The EMBO journal*, *8*(6), 1711–1717. https://doi.org/10.1002/j.1460-2075.1989.tb03563.x
30. Hartsock, A., & Nelson, W. J. (2008). Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochimica et Biophysica Acta*, *1778*(3), 660–669. https://doi.org/10.1016/j.bbamem.2007.07.012
31. Huber, A. H., Nelson, W. J., & Weis, W. I. (1997). Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell*, *90*(5), 871–882. https://doi.org/10.1016/s0092-8674(00)80352-9
32. Xing, Y., Takemaru, K., Liu, J., Berndt, J. D., Zheng, J. J., Moon, R. T., & Xu, W. (2008). Crystal structure of a full-length beta-catenin. *Structure (London, England : 1993)*, *16*(3), 478–487. https://doi.org/10.1016/j.str.2007.12.021
33. Huber, A. H., & Weis, W. I. (2001). The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. *Cell*, *105*(3), 391–402. https://doi.org/10.1016/s0092-8674(01)00330-0
34. Graham, T. A., Weaver, C., Mao, F., Kimelman, D., & Xu, W. (2000). Crystal structure of a beta-catenin/Tcf complex. *Cell*, *103*(6), 885–896. https://doi.org/10.1016/s0092-8674(00)00192-6
35. Eklof Spink, K., Fridman, S. G., & Weis, W. I. (2001). Molecular mechanisms of beta-catenin recognition by adenomatous polyposis coli revealed by the structure of an APC-beta-catenin complex. *The EMBO journal*, *20*(22), 6203–6212. https://doi.org/10.1093/emboj/20.22.6203
36. Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X., & He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell*, *108*(6), 837–847. https://doi.org/10.1016/s0092-8674(02)00685-2
37. Wu, G., & He, X. (2006). Threonine 41 in beta-catenin serves as a key phosphorylation relay residue in beta-catenin degradation. *Biochemistry*, *45*(16), 5319–5323. https://doi.org/10.1021/bi0601149
38. Taurin, S., Sandbo, N., Qin, Y., Browning, D., & Dulin, N. O. (2006). Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase. *The Journal of biological chemistry*, *281*(15), 9971–9976. https://doi.org/10.1074/jbc.M508778200
39. van Veelen, W., Le, N. H., Helvensteijn, W., Blonden, L., Theeuwes, M., Bakker, E. R., Franken, P. F., van Gurp, L., Meijlink, F., van der Valk, M. A., Kuipers, E. J., Fodde, R., & Smits, R. (2011). β-catenin tyrosine 654 phosphorylation increases Wnt signalling and intestinal tumorigenesis. *Gut*, *60*(9), 1204–1212. https://doi.org/10.1136/gut.2010.233460
40. Kim, S., Xu, X., Hecht, A., & Boyer, T. G. (2006). Mediator is a transducer of Wnt/beta-catenin signaling. *The Journal of biological chemistry*, *281*(20), 14066–14075. https://doi.org/10.1074/jbc.M602696200
41. Wend, P., Runke, S., Wend, K., Anchondo, B., Yesayan, M., Jardon, M., Hardie, N., Loddenkemper, C., Ulasov, I., Lesniak, M. S., Wolsky, R., Bentolila, L. A., Grant, S. G., Elashoff, D., Lehr, S., Latimer, J. J., Bose, S., Sattar, H., Krum, S. A., & Miranda-Carboni, G. A. (2013). WNT10B/β-catenin signalling induces HMGA2 and proliferation in metastatic triple-negative breast cancer. *EMBO molecular medicine*, *5*(2), 264–279. https://doi.org/10.1002/emmm.201201320

42. Wellenstein, M. D., Coffelt, S. B., Duits, D. E. M., van Miltenburg, M. H., Slagter, M., de Rink, I., Henneman, L., Kas, S. M., Prekovic, S., Hau, C. S., Vrijland, K., Drenth, A. P., de Korte-Grimmerink, R., Schut, E., van der Heijden, I., Zwart, W., Wessels, L. F. A., Schumacher, T. N., Jonkers, J., & de Visser, K. E. (2019). Loss of p53 triggers WNT-dependent systemic inflammation to drive breast cancer metastasis. *Nature*, *572*(7770), 538–542. https://doi.org/10.1038/s41586-019-1450-6
43. Suzuki, A., Minamide, R., & Iwata, J. (2018). WNT/β-catenin signaling plays a crucial role in myoblast fusion through regulation of nephrin expression during development. *Development (Cambridge, England)*, *145*(23), dev168351. https://doi.org/10.1242/dev.168351
44. Blumenthal, A., Ehlers, S., Lauber, J., Buer, J., Lange, C., Goldmann, T., Heine, H., Brandt, E., & Reiling, N. (2006). The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation. *Blood*, *108*(3), 965–973. https://doi.org/10.1182/blood-2005-12-5046
45. Bodine, P. (2008). Wnt signaling control of bone cell apoptosis. *Cell Res 18*, 248–253 https://doi.org/10.1038/cr.2008.13
46. He, S., Lu, Y., Liu, X., Huang, X., Keller, E. T., Qian, C.-N., & Zhang, J. (2015). Wnt3a: functions and implications in cancer. *Chinese Journal of Cancer*, *34*(3), 50. https://doi.org/10.1186/s40880-015-0052-4
47. Bilic, J., Huang, Y. L., Davidson, G., Zimmermann, T., Cruciat, C. M., Bienz, M., & Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science (New York, N.Y.)*, *316*(5831), 1619–1622. https://doi.org/10.1126/science.1137065
48. Clevers, H., & Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell*, *149*(6), 1192–1205. https://doi.org/10.1016/j.cell.2012.05.012
49. Stamos, J. L., & Weis, W. I. (2013). The β-catenin destruction complex. *Cold Spring Harbor Perspectives in Biology*, *5*(1), a007898. https://doi.org/10.1101/cshperspect.a007898
50. Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, *4*(2), 68–75. https://doi.org/10.4161/org.4.2.5851
51. Patel, S., Alam, A., Pant, R., & Chattopadhyay, S. (2019). Wnt Signaling and Its Significance Within the Tumor Microenvironment: Novel Therapeutic Insights. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02872
52. Katoh, M. (2005). WNT/PCP signaling pathway and human cancer (Review). *Oncol Rep.* 14:1583–1588. https://doi.org/10.1158/1535-7163.MCT-09-0282
53. Tao, W., Pennica, D., Xu, L., Kalejta, R. F., & Levine, A. J. (2001). Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. *Genes & development*, *15*(14), 1796–1807. https://doi.org/10.1101/gad.894301
54. Boutros, M., Paricio, N., Strutt, D. I., & Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell*, *94*(1), 109–118. https://doi.org/10.1016/s0092-8674(00)81226-x
55. Le Grand, F., Jones, A. E., Seale, V., Scimè, A., & Rudnicki, M. A. (2009). Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. *Cell stem cell*, *4*(6), 535–547. https://doi.org/10.1016/j.stem.2009.03.013

56. Eliazer, S., Muncie, J. M., Christensen, J., Sun, X., D'Urso, R. S., Weaver, V. M., & Brack, A. S. (2019). Wnt4 from the Niche Controls the Mechano-Properties and Quiescent State of Muscle Stem Cells. *Cell stem cell*, *25*(5), 654–665.e4. https://doi.org/10.1016/j.stem.2019.08.007
57. Pansters, N. A., van der Velden, J. L., Kelders, M. C., Laeremans, H., Schols, A. M., & Langen, R. C. (2011). Segregation of myoblast fusion and muscle-specific gene expression by distinct ligand-dependent inactivation of GSK-3β. *Cellular and molecular life sciences: CMLS*, *68*(3), 523–535. https://doi.org/10.1007/s00018-010-0467-7
58. Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., & Rando, T. A. (2008). A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell stem cell*, *2*(1), 50–59. https://doi.org/10.1016/j.stem.2007.10.006
59. Hawke, T. J., & Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. *Journal of applied physiology (Bethesda, Md. : 1985)*, *91*(2), 534–551. https://doi.org/10.1152/jappl.2001.91.2.534
60. Motohashi, N., & Asakura, A. (2014). Muscle satellite cell heterogeneity and self-renewal. *Frontiers in cell and developmental biology*, *2*, 1. https://doi.org/10.3389/fcell.2014.00001
61. Suzuki, A., Pelikan, R. C., & Iwata, J. (2015). WNT/β-Catenin Signaling Regulates Multiple Steps of Myogenesis by Regulating Step-Specific Targets. *Molecular and cellular biology*, *35*(10), 1763–1776. https://doi.org/10.1128/MCB.01180-14
62. Tanaka, S., Terada, K., & Nohno, T. (2011). Canonical Wnt signaling is involved in switching from cell proliferation to myogenic differentiation of mouse myoblast cells. *Journal of molecular signaling*, *6*, 12. https://doi.org/10.1186/1750-2187-6-12
63. Kim, C. H., Neiswender, H., Baik, E. J., Xiong, W. C., & Mei, L. (2008). Beta-catenin interacts with MyoD and regulates its transcription activity. *Molecular and cellular biology*, *28*(9), 2941–2951. https://doi.org/10.1128/MCB.01682-07
64. Cui, S., Li, L., Yu, R. T., Downes, M., Evans, R. M., Hulin, J.-A., Makarenkova, H. P., & Meech, R. (2019). β-Catenin is essential for differentiation of primary myoblasts via cooperation with MyoD and α-catenin. *Development (Cambridge, England)*, *146*(6). https://doi.org/10.1242/dev.167080
65. Tripathi, S., Miyake, T., & McDermott, J. C. (2019). Smad7:β-catenin complex regulates myogenic gene transcription. *Cell death & disease*, *10*(6), 387. https://doi.org/10.1038/s41419-019-1615-0
66. Mao, Y. S., Zhang, B., & Spector, D. L. (2011). Biogenesis and function of nuclear bodies. *Trends in genetics : TIG*, *27*(8), 295–306. https://doi.org/10.1016/j.tig.2011.05.006
67. Wolozin, B., & Ivanov, P. (2019). Stress granules and neurodegeneration. *Nature reviews. Neuroscience*, *20*(11), 649–666. https://doi.org/10.1038/s41583-019-0222-5
68. Wu H. (2013). Higher-order assemblies in a new paradigm of signal transduction. *Cell*, *153*(2), 287–292. https://doi.org/10.1016/j.cell.2013.03.013
69. Banani, S. F., Lee, H. O., Hyman, A. A., & Rosen, M. K. (2017). Biomolecular condensates: organizers of cellular biochemistry. *Nature reviews. Molecular cell biology*, *18*(5), 285–298. https://doi.org/10.1038/nrm.2017.7
70. Zamudio, A. V., Dall'Agnese, A., Henninger, J. E., Manteiga, J. C., Afeyan, L. K., Hannett, N. M., Coffey, E. L., Li, C. H., Oksuz, O., Sabari, B. R., Boija, A., Klein, I. A.,

Hawken, S. W., Spille, J. H., Decker, T. M., Cisse, I. I., Abraham, B. J., Lee, T. I., Taatjes, D. J., Schuijers, J., … Young, R. A. (2019). Mediator Condensates Localize Signaling Factors to Key Cell Identity Genes. *Molecular cell*, *76*(5), 753–766.e6. https://doi.org/10.1016/j.molcel.2019.08.016

71. Sabari, B. R., Dall'Agnese, A., Boija, A., Klein, I. A., Coffey, E. L., Shrinivas, K., Abraham, B. J., Hannett, N. M., Zamudio, A. V., Manteiga, J. C., Li, C. H., Guo, Y. E., Day, D. S., Schuijers, J., Vasile, E., Malik, S., Hnisz, D., Lee, T. I., Cisse, I. I., Roeder, R. G., … Young, R. A. (2018). Coactivator condensation at super-enhancers links phase separation and gene control. *Science (New York, N.Y.)*, *361*(6400), eaar3958. https://doi.org/10.1126/science.aar3958
72. Braeuning, A., Sanna, R., Huelsken, J., & Schwarz, M. (2009). Inducibility of drug-metabolizing enzymes by xenobiotics in mice with liver-specific knockout of Ctnnb1. *Drug metabolism and disposition: the biological fate of chemicals*, *37*(5), 1138–1145. https://doi.org/10.1124/dmd.108.026179
73. Doo, D. W., Meza-Perez, S., Londoño, A. I., Goldsberry, W. N., Katre, A. A., Boone, J. D., Moore, D. J., Hudson, C. T., Betella, I., McCaw, T. R., Gangrade, A., Bao, R., Luke, J. J., Yang, E. S., Birrer, M. J., Starenki, D., Cooper, S. J., Buchsbaum, D. J., Norian, L. A., Randall, T. D., … Arend, R. C. (2020). Inhibition of the Wnt/β-catenin pathway enhances antitumor immunity in ovarian cancer. *Therapeutic advances in medical oncology*, *12*, 1758835920913798. https://doi.org/10.1177/1758835920913798
74. Huang, C., & Ogawa, R. (2012). Fibroproliferative disorders and their mechanobiology. *Connective tissue research*, *53*(3), 187–196. https://doi.org/10.3109/03008207.2011.642035
75. Okada, K., Naito, A. T., Higo, T., Nakagawa, A., Shibamoto, M., Sakai, T., Hashimoto, A., Kuramoto, Y., Sumida, T., Nomura, S., Ito, M., Yamaguchi, T., Oka, T., Akazawa, H., Lee, J.-K., Morimoto, S., Sakata, Y., Shiojima, I., & Komuro, I. (2015). Wnt/β-Catenin Signaling Contributes to Skeletal Myopathy in Heart Failure via Direct Interaction With Forkhead Box O. *Circulation: Heart Failure*, *8*(4), 799–808. https://doi.org/10.1161/CIRCHEARTFAILURE.114.001958
76. Kaidi, A., Williams, A. C., & Paraskeva, C. (2007). Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nature cell biology*, *9*(2), 210–217. https://doi.org/10.1038/ncb1534
77. Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., & Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *The EMBO Journal*, *20*(17), 4935–4943. https://doi.org/10.1093/emboj/20.17.4935
78. Schuijers, J., Mokry, M., Hatzis, P., Cuppen, E., & Clevers, H. (2014). Wnt-induced transcriptional activation is exclusively mediated by TCF/LEF. *The EMBO journal*, *33*(2), 146–156. https://doi.org/10.1002/embj.201385358
79. Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., & Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science (New York, N.Y.)*, *317*(5839), 807–810. https://doi.org/10.1126/science.1144090
80. Alexander, M. S., Kawahara, G., Motohashi, N., Casar, J. C., Eisenberg, I., Myers, J. A., Gasperini, M. J., Estrella, E. A., Kho, A. T., Mitsuhashi, S., Shapiro, F., Kang, P. B., & Kunkel, L. M. (2013). MicroRNA-199a is induced in dystrophic muscle and affects

WNT signaling, cell proliferation, and myogenic differentiation. *Cell Death and Differentiation*, *20*(9), 1194–1208. https://doi.org/10.1038/cdd.2013.62

81. Hubbi, M. E., Gilkes, D. M., Baek, J. H., & Semenza, G. L. (2012). Four-and-a-half LIM domain proteins inhibit transactivation by hypoxia-inducible factor 1. *The Journal of biological chemistry*, *287*(9), 6139–6149. https://doi.org/10.1074/jbc.M111.278630
82. Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H., & Moon, R. T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Current biology: CB*, *13*(8), 680–685. https://doi.org/10.1016/s0960-9822(03)00240-9
83. Sun, J., Kamei, C. N., Layne, M. D., Jain, M. K., Liao, J. K., Lee, M. E., & Chin, M. T. (2001). Regulation of myogenic terminal differentiation by the hairy-related transcription factor CHF2. *The Journal of biological chemistry*, *276*(21), 18591–18596. https://doi.org/10.1074/jbc.M101163200
84. Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., & Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes & development*, *10*(12), 1443–1454. https://doi.org/10.1101/gad.10.12.1443
85. Piedra, J., Miravet, S., Castaño, J., Pálmer, H. G., Heisterkamp, N., García de Herreros, A., & Duñach, M. (2003). p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. *Molecular and cellular biology*, *23*(7), 2287–2297. https://doi.org/10.1128/MCB.23.7.2287-2297.2003
86. Chen, Y. T., Stewart, D. B., & Nelson, W. J. (1999). Coupling assembly of the E-cadherin/beta-catenin complex to efficient endoplasmic reticulum exit and basal-lateral membrane targeting of E-cadherin in polarized MDCK cells. *The Journal of cell biology*, *144*(4), 687–699. https://doi.org/10.1083/jcb.144.4.687
87. Dickinson, D. J., Nelson, W. J., & Weis, W. I. (2011). A polarized epithelium organized by beta- and alpha-catenin predates cadherin and metazoan origins. *Science (New York, N.Y.)*, *331*(6022), 1336–1339. https://doi.org/10.1126/science.1199633
88. Hatzis, P., van der Flier, L. G., van Driel, M. A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I. J., Koster, J., Santo, E. E., Welboren, W., Versteeg, R., Cuppen, E., van de Wetering, M., Clevers, H., & Stunnenberg, H. G. (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. *Molecular and cellular biology*, *28*(8), 2732–2744. https://doi.org/10.1128/MCB.02175-07
89. Norton, L., Fourcaudot, M., Abdul-Ghani, M. A., Winnier, D., Mehta, F. F., Jenkinson, C. P., & Defronzo, R. A. (2011). Chromatin occupancy of transcription factor 7-like 2 (TCF7L2) and its role in hepatic glucose metabolism. *Diabetologia*, *54*(12), 3132–3142. https://doi.org/10.1007/s00125-011-2289-z
90. Cottle, D. L., McGrath, M. J., Cowling, B. S., Coghill, I. D., Brown, S., & Mitchell, C. A. (2007). FHL3 binds MyoD and negatively regulates myotube formation. *Journal of cell science*, *120*(Pt 8), 1423–1435. https://doi.org/10.1242/jcs.004739
91. Bai, W., Zhang, Y., Ma, J., Du, M., Xu, H., Wang, J., Zhang, L., Li, W., Hou, Y., Liu, X., Zhang, X., Peng, Y., Li, J., Zhan, X., Jiang, W., Liu, S., Liu, X., Li, Q., Miao, Y., … Zuo, B. (2023). FHL3 promotes the formation of fast glycolytic muscle fibers by interacting with YY1 and muscle glycolytic metabolism. *Cellular and Molecular Life Sciences*, *80*(1), 27. https://doi.org/10.1007/s00018-022-04680-w

92. Chan, K. K., Tsui, S. K., Lee, S. M., Luk, S. C., Liew, C. C., Fung, K. P., Waye, M. M., & Lee, C. Y. (1998). Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart. *Gene*, *210*(2), 345–350. https://doi.org/10.1016/s0378-1119(97)00644-6
93. Morgan, M. J., & Madgwick, A. J. (1999). The LIM proteins FHL1 and FHL3 are expressed differently in skeletal muscle. *Biochemical and biophysical research communications*, *255*(2), 245–250. https://doi.org/10.1006/bbrc.1999.0179
94. Morgan, M. J., & Whawell, S. A. (2000). The structure of the human LIM protein ACT gene and its expression in tumor cell lines. *Biochemical and biophysical research communications*, *273*(2), 776–783. https://doi.org/10.1006/bbrc.2000.3006
95. Samson, T., Smyth, N., Janetzky, S., Wendler, O., Müller, J. M., Schüle, R., von der Mark, H., von der Mark, K., & Wixler, V. (2004). The LIM-only proteins FHL2 and FHL3 interact with alpha- and beta-subunits of the muscle alpha7beta1 integrin receptor. *The Journal of biological chemistry*, *279*(27), 28641–28652. https://doi.org/10.1074/jbc.M312894200
96. Huang, Z., Yu, C., Yu, L., Shu, H., & Zhu, X. (2022). The Roles of FHL3 in Cancer. *Frontiers in Oncology*, *12*, 887828. https://doi.org/10.3389/fonc.2022.887828
97. Lee, J. Y., Chien, I. C., Lin, W. Y., Wu, S. M., Wei, B. H., Lee, Y. E., & Lee, H. H. (2012). Fhl1 as a downstream target of Wnt signaling to promote myogenesis of C2C12 cells. *Molecular and cellular biochemistry*, *365*(1-2), 251–262. https://doi.org/10.1007/s11010-012-1266-2
98. Lee, H., Lee, J., & Shih, L. (2013) Proper *Fhl1* expression as Wnt signaling is required for chondrogenesis of ATDC5 cells, *Animal Cells and Systems*, *17*(6), 413-420. https://doi.org/10.1080/19768354.2013.856341
99. Liu, Y., Wang, C., Cheng, P., Zhang, S., Zhou, W., Xu, Y., Xu, H., & Ji, G. (2021). FHL1 Inhibits the Progression of Colorectal Cancer by Regulating the Wnt/β-Catenin Signaling Pathway. *Journal of Cancer*, *12*(17), 5345–5354. https://doi.org/10.7150/jca.60543
100. Labalette, C., Renard, C. A., Neuveut, C., Buendia, M. A., & Wei, Y. (2004). Interaction and functional cooperation between the LIM protein FHL2, CBP/p300, and beta-catenin. *Molecular and cellular biology*, *24*(24), 10689–10702. https://doi.org/10.1128/MCB.24.24.10689-10702.2004
101. Wei, Y., Renard, C. A., Labalette, C., Wu, Y., Lévy, L., Neuveut, C., Prieur, X., Flajolet, M., Prigent, S., & Buendia, M. A. (2003). Identification of the LIM protein FHL2 as a coactivator of beta-catenin. *The Journal of biological chemistry*, *278*(7), 5188–5194. https://doi.org/10.1074/jbc.M207216200
102. Martin, B., Schneider, R., Janetzky, S., Waibler, Z., Pandur, P., Kühl, M., Behrens, J., von der Mark, K., Starzinski-Powitz, A., & Wixler, V. (2002). The LIM-only protein FHL2 interacts with beta-catenin and promotes differentiation of mouse myoblasts. *The Journal of cell biology*, *159*(1), 113–122. https://doi.org/10.1083/jcb.200202075
103. Alkobtawi, M., Pla, P., & Monsoro-Burq, A. H. (2021). BMP signaling is enhanced intracellularly by FHL3 controlling WNT-dependent spatiotemporal emergence of the neural crest. *Cell reports*, *35*(12), 109289. https://doi.org/10.1016/j.celrep.2021.109289
104. Turner, J., Nicholas, H., Bishop, D., Matthews, J. M., & Crossley, M. (2003). The LIM Protein FHL3 Binds Basic Krüppel-like Factor/Krüppel-like Factor 3 and Its Co-

repressor C-terminal-binding Protein 2. *Journal of Biological Chemistry*, *278*(15), 12786–12795. https://doi.org/10.1074/jbc.M300587200

105. Takahashi, K., Matsumoto, C., & Ra, C. (2005). FHL3 negatively regulates human high-affinity IgE receptor beta-chain gene expression by acting as a transcriptional co-repressor of MZF-1. *The Biochemical Journal*, *386*(Pt 1), 191–200. https://doi.org/10.1042/BJ20040775
106. Niu, C., Yan, Z., Cheng, L., Zhu, J., Zhang, H., Xu, X., Lin, J., & Ye, Q. (2011). Downregulation and antiproliferative role of fhl3 in breast cancer. *IUBMB Life*, *63*(9), 764–771. https://doi.org/10.1002/iub.502
107. Dagher, R., & Helman, L. (1999). Rhabdomyosarcoma: an overview. *The oncologist*, *4*(1), 34–44.
108. Dionyssiou, M. G., Ehyai, S., Avrutin, E., Connor, M. K., & McDermott, J. C. (2014). Glycogen synthase kinase 3β represses MYOGENIN function in alveolar rhabdomyosarcoma. *Cell death & disease*, *5*(2), e1094. https://doi.org/10.1038/cddis.2014.58
109. Pal, A., Leung, J. Y., Ang, G. C. K., Rao, V. K., Pignata, L., Lim, H. J., Hebrard, M., Chang, K. T., Lee, V. K., Guccione, E., & Taneja, R. (2020). EHMT2 epigenetically suppresses Wnt signaling and is a potential target in embryonal rhabdomyosarcoma. *eLife*, *9*, e57683. https://doi.org/10.7554/eLife.57683
110. Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Cote, J., & Wang, W. (1998). NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell, 2(6), 851-861. https://doi.org/10.1016/s1097-2765(00)80299-3
111. Ho, L., & Crabtree, G. R. (2010). Chromatin remodelling during development. *Nature*, *463*(7280), 474–484. https://doi.org/10.1038/nature08911
112. Sreenivasan, K., Rodríguez-delaRosa, A., Kim, J., Mesquita, D., Segalés, J., Arco, P. G., Espejo, I., Ianni, A., Di Croce, L., Relaix, F., Redondo, J. M., Braun, T., Serrano, A. L., Perdiguero, E., & Muñoz-Cánoves, P. (2021). CHD4 ensures stem cell lineage fidelity during skeletal muscle regeneration. *Stem cell reports*, *16*(9), 2089–2098. https://doi.org/10.1016/j.stemcr.2021.07.022
113. Wilczewski, C. M., Hepperla, A. J., Shimbo, T., Wasson, L., Robbe, Z. L., Davis, I. J., Wade, P. A., & Conlon, F. L. (2018). CHD4 and the NuRD complex directly control cardiac sarcomere formation. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(26), 6727–6732. https://doi.org/10.1073/pnas.1722219115

Supplementary data

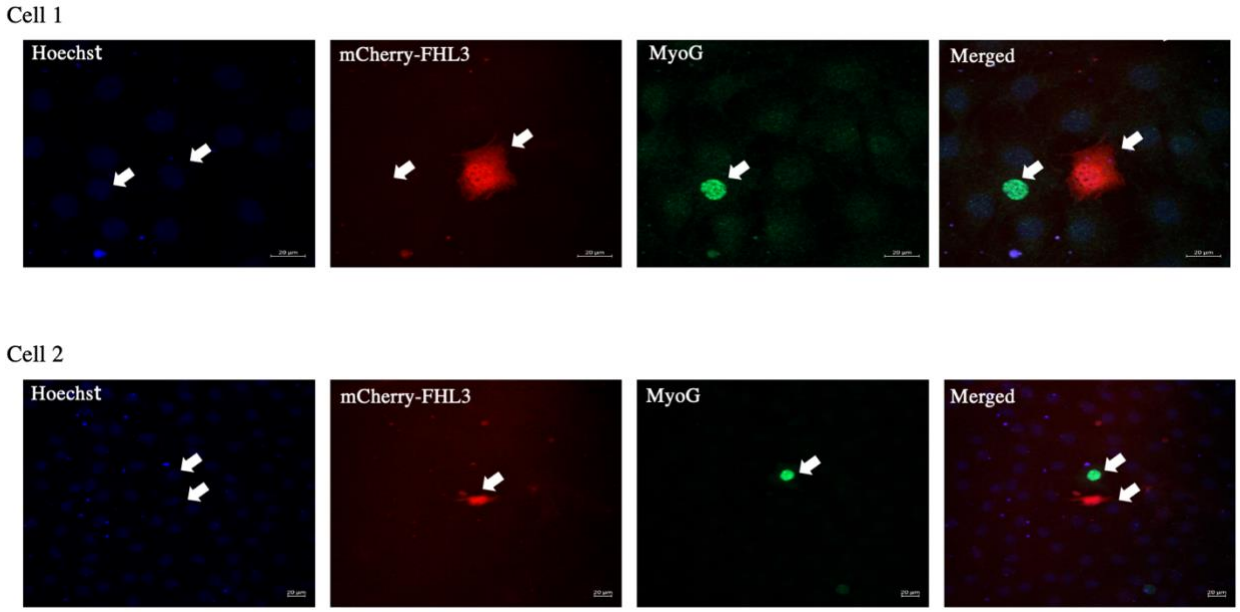

Figure 13: FHL3 inhibits MyoG expression. mCherry-FHL3 was ectopically expressed in C2C12 cells and cells were differentiated for 72 hr after growing in growth media for 24 hr. Immunofluorescence analysis was performed by fixing C212C cells and staining for MyoG.