

MYOGENIC PROTEIN INTERACTIONS IN RHABDOMYOSARCOMA

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Abstract

Rhabdomyosarcoma (RMS), a pediatric soft tissue sarcoma, is characterized by impaired myogenic differentiation and uncontrolled proliferation, often driven by dysregulated transcriptional and signaling networks. In this thesis, we unveil the first comprehensive protein interactomes of MEF2A and β -catenin—two pivotal regulators of skeletal myogenesis and oncogenesis—within RMS subtypes using nanobody-mediated affinity purification coupled with high-resolution mass spectrometry. Strikingly, despite reduced MEF2A expression in RMS cells, RD cells paradoxically exhibited elevated transcriptional activity, implicating compensatory post-translational modifications or unique cofactor interactions. In contrast, β -catenin activity was nearly abolished in the aggressive RH30 subtype, suggesting Wnt pathway evasion and reliance on alternative oncogenic circuits.

Interactome mapping revealed subtype-specific regulatory landscapes: RD-derived MEF2A associated with splicing and transcriptional machinery, while RH30 networks were dominated by chromatin remodelers and epigenetic regulators. β -catenin's interaction profile likewise diverged, linking to tumor-suppressive pathways in RD cells but shifting toward mitotic and nuclear export regulators in RH30. Enrichment analyses identified novel protein hubs—such as TRIM32, HDAC1, XPO1, and RACK1—that modulate gene expression, RNA metabolism, and cell cycle control, and may represent uncharted therapeutic nodes.

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List of Abbreviations

α -MHC-Myocin heavy chain

AMOT – Angiomotin

AMOTL1/2 – Angiomotin-like 1/2

AMPK – 5' AMP-activated protein kinase

APC – adenomatois spolypsis coli

ARMS - Alveolar rhabdomyosarcoma (RH30)

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

BHLH-Basic helix-loop-helix

BMD – Becker muscular dystrophy

BMP – Bone morphogenic protein

BRD4 – Bromodomain-containing protein 4

CAMK – Ca²⁺/calmodulin-dependent protein kinase

CDK9 – Cyclin-dependent kinase 9

CK1 – Casein kinase 1

CTGF – Connective tissue growth factor

CYR61 – Cysteine-rich angiogenic inducer 61

DEGS- Differential expressed genes

DMD – Duchenne muscular dystrophy

DUX4 – Double homeobox 4

DVL – Disheveled

EGFR- Epidermal growth factor

EMT – Epithelial-to-mesenchymal transition

ERK5 – Extracellular-signal-regulated kinase 5

ERMS -Embryonal rhabdomyosarcoma (RD)

EYFP- Enhanced yellow fluorescent protein

F-actin – Filamentous actin

FGF2 – Fibroblast growth factor 2

FOX- Forkhead transcription factor

Fzd- Frizzled

FSHD – Facioscapulohumeral muscular dystrophy

GFP- Green fluorescent protein

GO-Gene ontology

GSK3 – Glycogen synthase kinase-3

HDACs- Histone deacetylases

HGH – Human growth hormone

HM – Hydrophobic motif

IDR – Intrinsically disordered region

IFG-1 – Insulin-like growth factor 1

JAK1 – Janus kinase 1

LATS1/2 – Large tumor suppressor kinase 1/2

LLPS – Liquid-liquid phase separation

LRP5/6-Lipoprotein receptor-related transmembrane proteins

MAP4K – Mitogen-activated protein kinase 4

MED1 – Mediator complex subunit 1

MEF2 –Myocyte enhancer factor 2

MYCBP-C-Myosin binding protein C

MOB1 – Mps1-binder-related 1 **MRFs** – Myogenic regulatory factor

mTOR – Mammalian target of rapamycin

MyoD – Myoblast determination protein 1

MyoG – Myogenin

NF2 – Neurofibromatosis type 2

NO – Nitrous oxide

NuRD – Nucleosome Remodeling Deacetylase

P73 – Protein 73

Pax3/7 – Paired pox gene 3/7

RMS- Rhabdomyosarcoma

Runx1/2 – Runt-related transcription factor 1

SARAH – Sav/Rassf/Hpo

SAV1 – Salvador 1

SC – Satellite cell

Shh – Sonic hedgehog

SMAD1/2/3/7 – Suppressor of mothers against decapentaplegic

STAT1 – Signal transducer and activator of transcription 1

STK25 – Serine-Threonine kinase 25

TAO – Thousand and one

TAZ – Transcriptional activator with PDZ-binding motif

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

TRAIL – TNF-related apoptosis-inducing ligand

TRIM 32-Tripartite motif 32

Wnt – Wingless integration site signaling pathway

ZFs-Zinc Fingers

ZNFX1- Zinc finger NFX-1 containing protein

1. Literature Review:

1.1 Overview of Myogenesis

Myogenesis is the biological process by which muscle tissue forms, primarily occurring during embryonic development and muscle regeneration in adults. This multi-step process involves the specification, proliferation, differentiation, and fusion of myoblasts—mononucleated precursor cells derived from mesodermal progenitors—into multinucleated myotubes that eventually mature into functional muscle fibers. This intricate process is governed by a network of molecular signals and transcription factors that regulate muscle cell proliferation, differentiation, and maturation. It is primarily regulated by a network of myogenic regulatory factors (MRFs), including MyoD, Myf5, myogenin, and 4. These factors orchestrate gene expression changes that drive the transition of myoblasts into myotubes and eventually into fully functional muscle fibers (Buckingham & Rigby, 2014). The process begins with the activation of Pax3 and Pax7, transcription factors that specify myogenic precursor cells (Relaix & Zammit, 2012) During embryogenesis. These precursor cells proliferate under the influence of growth factors such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF) (Kuang et al., 2007). As myoblasts exit the cell cycle, MyoD and Myf5 initiate differentiation, followed by myogenin, which further drives the formation of multinucleated myotubes (Tapscott, 2005). Myogenesis also involves interactions with signaling pathways such as Wnt, Notch, Hippo Pathway Hedgehog Signaling and IGF, which influence myoblast proliferation and differentiation. There are satellite cells, the resident stem cells of skeletal muscle, are crucial for postnatal muscle growth and repair, as they remain quiescent under normal conditions but become activated in response to injury. Additionally, epigenetic modifications and microRNAs contribute to the fine-tuning of myogenic gene expression. Any perturbation in this cascade, whether due to genetic mutations, epigenetic modifications, inflammatory responses, or environmental stressors, can have profound

consequences on muscle development, function, and repair lead to severe muscular disorders or impaired muscle regeneration. For instance, mutations in genes like DMD (Dystrophin) result in Duchenne muscular dystrophy (DMD), a progressive degenerative muscle disease characterized by muscle weakness and atrophy. (Blake et al., 2002). Similarly, dysregulated signaling pathways such as Wnt/ β -catenin, Notch, and TGF- β can hinder myoblast differentiation and fusion, leading to muscle wasting conditions like sarcopenia or cachexia (Brack et al., 2008). TGF- β signaling, particularly through Smad3 activation, has been implicated in fibrosis and myogenic inhibition, further exacerbating muscle degeneration (McCroskery et al., 2003). Additionally, oxidative stress and mitochondrial dysfunction disrupt cellular homeostasis and impair myoblast fusion, leading to defective muscle repair (Yin et al., 2013). Even such disruptions in myogenesis can lead to muscle cancer like Rhabdomyosarcoma, a pediatric cancer with poor survival rate.

The interplay between these molecular disruptions underscores the importance of targeted therapeutic strategies, such as gene therapy, CRISPR-based genome editing, and stem cell interventions, to restore proper myogenesis and mitigate muscle degeneration.

In recent years, significant progress has been made in understanding myogenesis at the molecular and genetic levels. CRISPR/Cas9 Genome Editing is used to investigate gene function in muscle development and to correct genetic mutations associated with muscular dystrophies (Long et al., 2016). Another approach, Single-Cell RNA Sequencing (scRNA-seq) provides insights into the heterogeneity of muscle stem cells and their dynamic gene expression patterns during regeneration (Giordani et al., 2019). Organoid and Tissue Engineering used the development of in vitro skeletal muscle models to study myogenesis and muscle-related diseases (Madden et al., 2015).

1.2 Skeletal myogenesis

Skeletal muscle is a highly intricate and diverse tissue that performs a wide range of functions within the body. Comprising over 600 individual muscles, skeletal muscle constitutes the body's largest tissue mass and plays a crucial role in movement and structural support (Jerome et al). Unlike cardiac and smooth muscles, skeletal muscles are unique in that they are under voluntary control by the organism. They consist of bundles of striated myofibers, which are elongated, multinucleated syncytia. These fibers are encased in a basal lamina and contain a well-organized cytoskeleton made up of myofibrils. The development of skeletal muscle is the complex process occurs in distinct stages, guided by tightly regulated genetic and molecular mechanisms.

The paraxial mesoderm consists of two symmetrical tissue strips located on either side of the neural tube and notochord. In the posterior region, these strips remain unsegmented, forming the pre- somatic mesoderm, whereas in the anterior region, they differentiate into somite, which establish the segments of the embryo (Buckingham et al). The specification of the paraxial mesoderm occurs as precursors leave the progenitor zone and enter the posterior pre-somatic mesoderm. As they mature, somite differentiate into three primary compartments: the sclerotome, myotome, and dermatome. This phase, known as the epithelial-to-mesenchymal transition (EMT), is marked by enhanced cellular mobility (Lim and Thierry, 2012). The dorsomedial region of the somite gives rise to the sclerotome, which differentiates into cartilage and bone precursor cells (Buckingham & Relaix, 2015). The dorsolateral portion, known as the dermomyotome, retains its epithelial structure and houses the earliest myogenic progenitor cells. These progenitors are distinguished by high expression of paired box transcription factors Pax3 and Pax7 and low levels of the basic helix-loop-helix transcription factor Myf5 (Jostes et al, 1990; Buckingham & Relaix, 2015). These processes are primarily regulated by the Wnt and fibroblast growth factor (FGF) signaling pathways (Takada et

al). Other influential signaling pathways plays an important role in the specification and fate of mesoderm are BMP (Bone Morphogenetic Proteins) and Sonic Hedgehog (Shh).

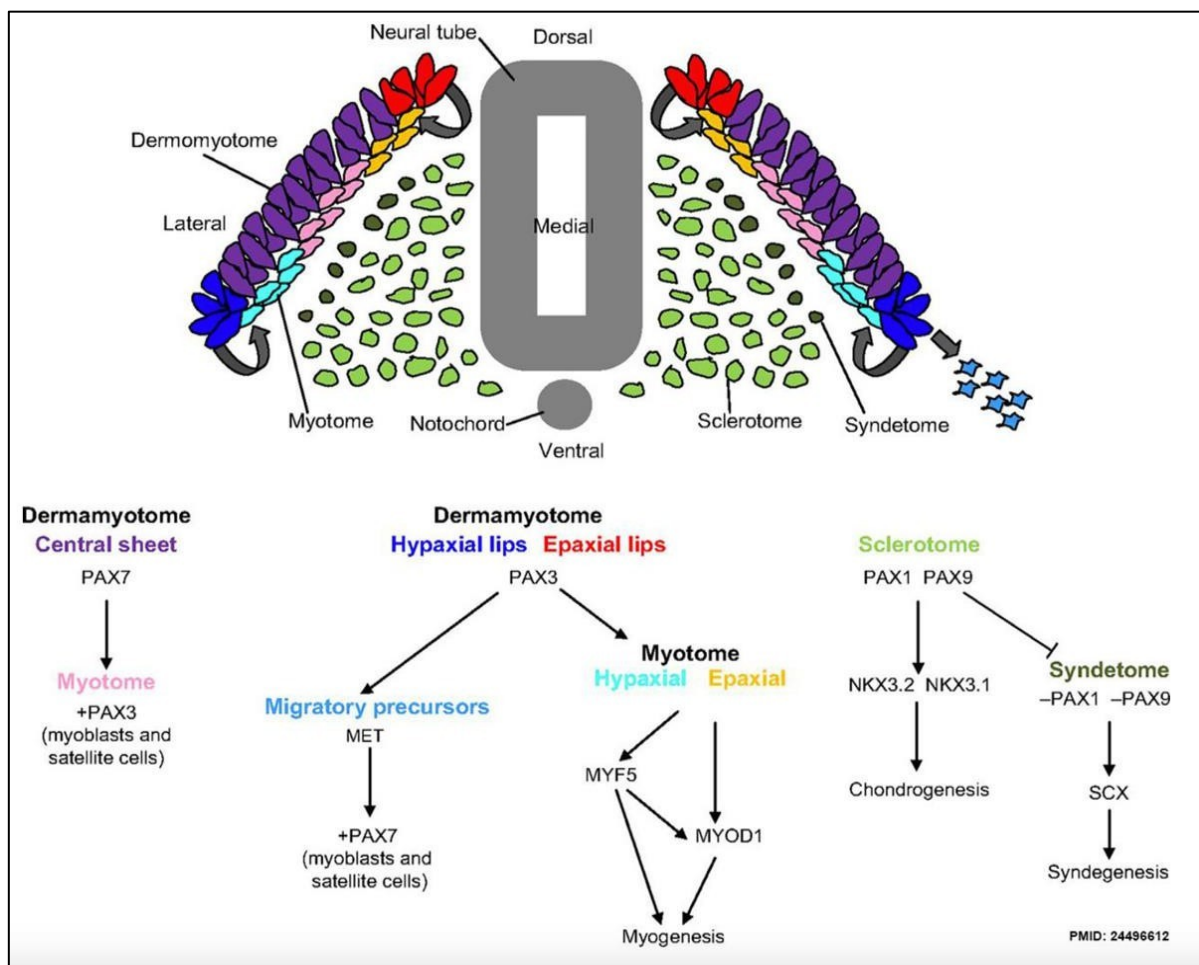
In mouse and chicken embryos, the earliest indication of myogenesis is the activation of the myogenic factor Myf5 in cells located in the dorsomedial region of the newly formed somite (Ott et al., 1991; Pownall & Emerson, 1992). The dorsal epithelial dermomyotome, which expresses Pax3, further differentiates into several distinct regions: a central domain, a dorsomedial lip, anterior and posterior lips, and a ventrolateral lip (Gros et al., 2004). Shortly after the formation of the dermomyotome, cells in the dorsomedial lip begin expressing Myf5 while simultaneously downregulating Pax3 (Bober et al., 1994; Ott et al., 1991; Williams & Ordahl, 1994). The primary myotome emerges as a cellular layer positioned between the dermomyotome dorsally and the sclerotome ventrally (Ordahl, 1993). This structure is later divided into two functional regions: the epaxial myotome, which develops into the muscles of the back and trunk, and the hypaxial myotome, which gives rise to the limb and body wall muscles (Buckingham & Thorsteinsdottir, 2016).

At the genetic level, Pax3 functions upstream of the four myogenic regulatory factors (MRFs)—a highly conserved group of class II basic helix-loop-helix (bHLH) transcription factors (TFs) that establish the genetic framework for myogenic progression (Rudnicki & Jaenisch, 1995). MRFs possess a conserved DNA-binding basic domain and a helix-loop-helix domain, both essential for binding to E-box motifs in the promoters of numerous muscle-specific genes (Massari & Murre, 2000). Under Pax3 regulation, the upregulation of Myf5 triggers a rapid induction of MyoD, a key marker of myogenic commitment. This is followed by the expression of MyoG (myogenin), which plays a critical role in myoblast differentiation and precedes the activation of genes encoding contractile proteins. Studies on MyoG knockout mice reveal that although myoblasts are present, they fail to differentiate and fuse, emphasizing MyoG's essential role in terminal myoblast differentiation (Hasty et al.,

1993).

Overall, the genetic hierarchy regulating myogenesis in limb muscles follows a sequential pattern: Six1/4 and Pax3 initiate the process, leading to Myf5 expression, followed by MyoD in MyoG expression, which is crucial for myogenic differentiation. Figure 1 illustrates the overview of skeletal myogenesis.

A.



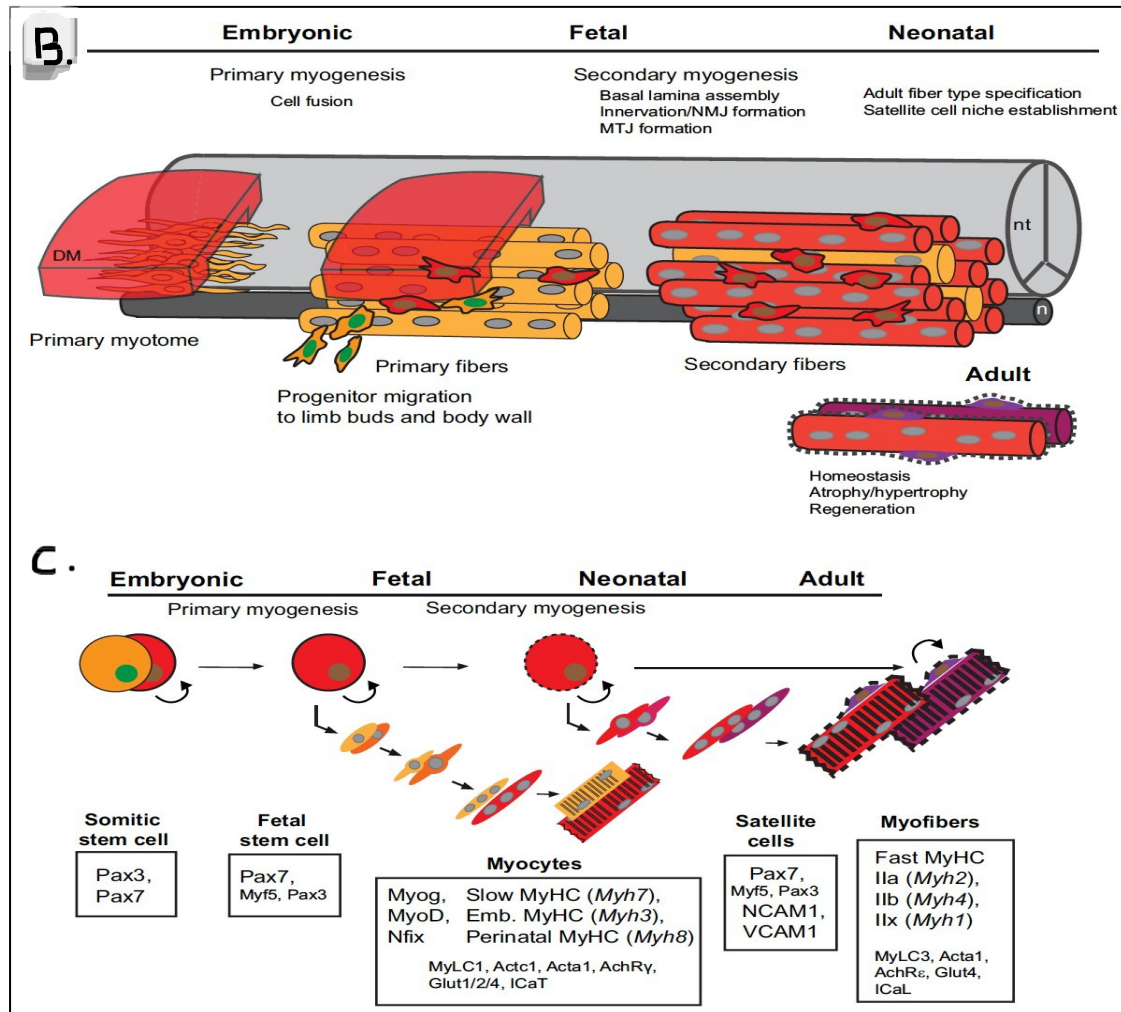


Figure 1. Somitogenesis and Myogenesis. (A) Schematic representation of early somite patterning and lineage specification. The developing somite is compartmentalized into the dermomyotome, myotome, sclerotome, and syndetome. The central sheet of the dermomyotome, marked by PAX7, gives rise to the myotome, contributing to myoblasts and satellite cells. The epaxial and hypaxial lips of the dermomyotome express PAX3 and give rise to migratory precursors that undergo epithelial-to-mesenchymal transition (EMT), ultimately contributing to muscle progenitors. These cells activate MYF5 and MYOD1 to initiate myogenic commitment. Meanwhile, the sclerotome, regulated by PAX1 and PAX9, differentiates into cartilage through NKX3.1/3.2, while the syndetome gives rise to tendon precursors under SCX regulation. This panel highlights the complex spatial and molecular signaling guiding muscle, cartilage, and tendon development. (B & C) Stages of myogenesis from embryonic to adult muscle development. (B) Illustrates the transition from somite-derived stem cells to differentiated myofibers across developmental stages. During embryonic myogenesis, PAX3 and PAX7-expressing somitic stem cells give rise to fetal progenitors expressing MYF5 and PAX3, which differentiate into myocytes expressing early and perinatal myosin heavy chains (MyHCs). (C) Shows progression into the neonatal and adult phases where secondary myogenesis occurs, accompanied by muscle fiber type specification, neuromuscular junction (NMJ) formation, and establishment of satellite cell

niches. Adult muscle maintenance involves satellite cells (PAX7, NCAM1, VCAM1) and mature fast/slow myofibers expressing distinct MyHC isoforms (e.g., Myh1, Myh2, Myh4, Myh7). This panel captures the dynamic and tightly regulated process of muscle development, maintenance, regeneration, and adaptation throughout life. Figures are adopted from Blake JA et al, 2014 and Chal J et al, 2017.

During the perinatal phase, muscle-resident myogenic progenitors undergo extensive proliferation. However, as the number of myonuclei stabilizes and myofibrillar protein synthesis reaches its peak, their proliferation declines (Schultz, 1996; Davis & Fiorotto, 2009). Once the muscle reaches full maturity, these progenitors transition into a quiescent state, becoming satellite cells, a specialized population of regenerative stem cells residing within skeletal muscle.

These satellite cells originate from the central dermomyotome, emerging from muscle precursors that migrate into the myotome during early development. They are distinguished by their expression of Pax3 and Pax7, transcription factors that maintain them in a stem-cell-like state, ensuring their capacity to repair and regenerate muscle fibers. Satellite cells are strategically positioned within a niche—situated between the sarcolemma of muscle fibers and the basal lamina—where they remain in a dormant state until activated by muscle injury (Schmalbruch & Lewis, 2000).

While most Pax3/7+ progenitors from the dermomyotome eventually express myogenic regulatory factors (MRFs) and commit to the myogenic lineage, a subset remains MRF-negative, continuing to proliferate and eventually adopting the satellite cell position. In the absence of muscle damage, quiescent satellite cells predominantly express Pax7 and Myf5, though a smaller subset remains Myf5-negative. Notably, quiescence is not a passive state; rather, it is an active regulatory process, as gene expression studies have identified over 500 genes upregulated in quiescent satellite cells compared to their activated counterparts (Fukada et al., 2007).

Like all self-renewing organs, adult skeletal muscle relies on a homeostatic mechanism that compensates for the natural turnover of terminally differentiated cells (Pellettieri & Sánchez Alvarado, 2007). This regenerative myogenesis is primarily driven by satellite cell activation, enabling these stem cells to differentiate and fuse with damaged myofibers, restoring muscle integrity (Charge & Rudnicki, 2004). The most extensively studied form of myogenesis occurs when mature muscle sustains injury, prompting large populations of satellite cells to proliferate, differentiate, and repair the damaged tissue, thereby restoring homeostasis (Rudnicki et al., 2008). This intricate process is tightly regulated by MRFs and multiple signaling pathways, ensuring precise control over muscle regeneration and functional recovery.

The role of transcription factors in myogenesis

Myogenesis, the process of skeletal muscle formation, is a meticulously coordinated biological event governed by a complex network of transcription factors. These factors regulate gene expression at various stages of muscle development and regeneration, ensuring the proper specification, proliferation, differentiation, and maturation of muscle cells. Recent research has provided deeper insights into the intricate interactions between these transcription factors and the regulatory mechanisms that fine-tune myogenic progression.

1.2.1 MRFs

Key players in this process include myogenic regulatory factors (MRFs), myocyte enhancer factors (MEFs), and serum response factors (SRFs), which work together to drive muscle cell fate. Among them, the highly conserved MRF family—comprising MyoD, Myf5, myogenin, and MRF4 (also known as Myf6 or herculin)—plays a pivotal role in skeletal muscle lineage commitment (Weintraub et al., 1991; Rudnicki & Jaenisch, 1995). These MRFs belong to the basic helix-loop-helix (bHLH) class of transcription factors and are essential for myoblast differentiation.

Myf5 is the first MRF to be expressed during early embryonic development, appearing transiently in the paraxial mesoderm before working in concert with other MRFs to form the myotome (Ott et al., 1991; Buckingham, 1992). Alongside MyoD, Myf5 plays a crucial role in the early commitment of progenitor cells to the muscle lineage, a process regulated by the Pax3 transcription factor. Functionally, Myf5 and MyoD act redundantly upstream of myogenin and MRF4 to drive myoblasts toward terminal differentiation (Florian Bentzinger et al., 2012). In contrast, myogenin and MRF4 are indispensable for the later stages of differentiation and the maturation of muscle fibers. Beyond their classical roles, MRFs have been shown to influence the expression of both myogenic and non-myogenic genes, suggesting a broader regulatory scope than previously recognized (Shirakawa et al., 2022).

1.2.2 PAX genes

The Pax (paired box) family of transcription factors plays a pivotal role in tissue formation, binding specifically to GTTCC and GTTAT sequences (Phelan & Loekan, 1998). These transcription factors are essential not only for embryonic development but also for adult muscle regeneration. By regulating the expression of Myf5 and MyoD, they control the entry of progenitor cells into the myogenic program. Notably, Pax3 has been shown to directly activate Myf5 transcription in muscle progenitor cells, emphasizing its role as an upstream regulator. The Pax gene family consists of nine members, categorized based on their tissue-specific expression and spatiotemporal distribution (Buckingham & Relaix, 2007). Among them, Pax3 and Pax7 are crucial for skeletal muscle development, as they govern myogenic cell fate. Mutations in these genes have been linked to alveolar rhabdomyosarcoma, an aggressive pediatric cancer of skeletal muscle origin (Barr et al., 1999).

Beyond their role in muscle formation, a distinct population of proliferating Pax3/7-positive cells has been identified within developing skeletal muscle masses (Ben-Yair & Kalcheim, 2005). These cells originate from the dermomyotome during somite maturation (Gros et al.,

2005; Kassar- Duchossy et al., 2005) and contribute to a reserve pool of muscle progenitor cells during embryonic and fetal development. The loss of Pax function in these reserve cells disrupts MyoD activation, preventing their entry into the myogenic program (Relaix et al., 2005). During embryogenesis, Pax3/7-positive progenitor cells emerge beneath the basal lamina surrounding developing muscle fibers, eventually giving rise to satellite cells in the trunk (Gros et al., 2005). In contrast, satellite cells of limb muscles originate from Pax3-expressing cells that migrate from the hypaxial dermomyotome (Schienda et al., 2006). Ultimately, Pax3 and Pax7 are indispensable for skeletal muscle formation, ensuring both the proper entry of progenitor cells into the myogenic program and their long-term survival.

1.2.3 MEF2

MEF2 (Myocyte Enhancer Factor 2) proteins are the family of transcription factors comprises four members: MEF2A, MEF2B, MEF2C, and MEF2D, each contributing to various stages of muscle development and differentiation with each gene located on different chromosomes (McDermott *et al.*, 1993, Hobson *et al.*, 1995). Studies have demonstrated that MEF2 proteins, in conjunction with myogenic regulatory factors (MRFs), synergistically enhance the expression of genes necessary for muscle differentiation and development (PS Naidu et al, 1995). Recent findings indicate that interactions between MEF2D and MRF4 can modulate transcriptional activity, thereby influencing muscle hypertrophy and the expression of MEF2 target genes.(J Manuel Hernández-Hernández et al, 2017). MEF2 protein belong to the MADS (MCM1, agamous, deficient, serum response factor) superfamily of transcription factors which contain a highly conserved 57a.a MADS-box that is located directly N-terminal to a 29 a.a MEF2 domain (Molkentin *et al.*, 1996). Together these two domains are involved in high affinity, DNA binding and protein interactions.

MEF2 factors and MRFs cooperate to activate skeletal muscle gene expression through direct protein-protein interactions between their DNA-binding domains (DBDs). While MRFs

alone can drive skeletal muscle differentiation, MEF2 proteins cannot. The bHLH domain of MRFs facilitates dimerization with E-proteins (E12, E47, HEB), forming heterodimers that bind to E-box sequences in myogenic gene regulatory regions. Forced MyoD or Myogenin expression in non-myogenic cells induces MEF2 DNA-binding activity, highlighting its role in the myogenic program. Additionally, a dominant-negative MEF2A can block myogenesis in multipotent cells. Interestingly, MEF2 enhances E-box promoter transactivation without binding to it, while MRFs boost MEF2 transcriptional activity despite the absence of an E-box, indicating they act as cofactors. Although MEF2 cannot independently activate MyoD, MRF4, or Myogenin expression, it is crucial for maintaining their transcription, especially Myogenin in myoblasts and embryos.

MEF2 and MRFs activate muscle differentiation via four mechanisms: (1) MEF2 recruitment to an E-box via an MRF/E-protein complex, (2) MRF/E-protein binding to a MEF2 box, (3) independent binding to adjacent sites with cooperative activation, or (4) binding to non-adjacent sites followed by DNA bending for interaction. The interaction mechanism varies by gene and context.

Other proteins, including HDACs, HATs, AP-1, and Smads, regulate myogenesis by binding MEF2/MRFs or muscle-specific genes. MEF2 and MRFs undergo complex regulation via alternative splicing and posttranslational modifications. The next section will explore MEF2/MRF phosphorylation and key signaling pathways regulating their transcriptional activation, focusing on Myogenin and Krüppel-like factor 6 (KLF6).

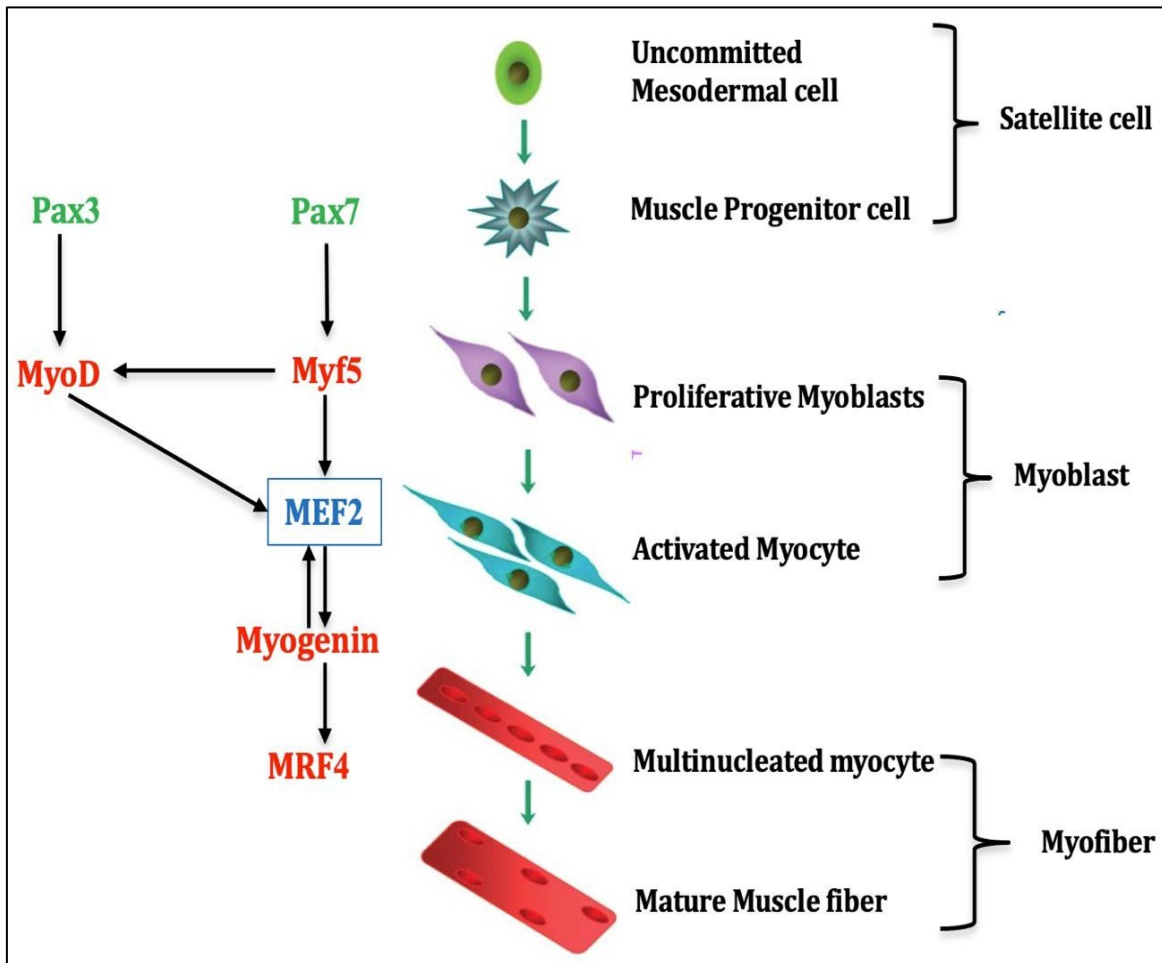


Figure 2: Genetic Factors Regulating Myogenesis. This schematic illustrates the sequential progression of myogenic differentiation from uncommitted mesodermal cells to mature myofibers, highlighting the key transcriptional regulators involved at each stage. The process begins with uncommitted mesodermal cells, which give rise to muscle progenitor cells marked by **Pax3** and **Pax7** expression. These progenitors differentiate into proliferative myoblasts, a stage regulated by early myogenic regulatory factors (MRFs) such as **Myf5** and **MyoD**. **Pax3** primarily induces **MyoD**, while **Pax7** activates **Myf5**, both of which are crucial for commitment to the myogenic lineage. These MRFs, in turn, activate the **MEF2** (myocyte enhancer factor 2) family of transcription factors, which cooperate with **MyoD** and **Myf5** to further drive the expression of downstream myogenic genes. As myogenesis progresses, **Myogenin** and **MRF4** promote terminal differentiation, leading to the formation of multinucleated myocytes and eventually mature muscle fibers. This figure underscores the tightly regulated transcriptional network controlling skeletal muscle development and highlights the interplay between Pax genes, MRFs, and MEF2 in orchestrating myogenic progression from stem-like progenitors to fully differentiated myofibers. Adopted from Mathew Dionyssiou et al., 2014.

MEF2 proteins are integral to the regulation of myogenesis, primarily through their interactions with MRFs. The cooperative dynamics between MEF2 and MRFs ensure the precise activation of muscle-specific genes, facilitating proper muscle development and regeneration.

1.2.4 Serum Response Factor (SRF)

Serum Response Factor (SRF) is a MADS-box transcription factor that plays a crucial role in muscle gene regulation, particularly genes involved in cytoskeletal organization and contractile function. It governs actin gene expression, essential for cytoskeletal remodeling during myogenesis. SRF's activity is modulated by extracellular signals such as mechanical stress and growth factors, influencing muscle hypertrophy and regeneration.

SRF functions alongside cofactors like Myocardin-Related Transcription Factors (MRTFs), which activate SRF-dependent muscle gene transcription, and Elk1, which is involved in early differentiation stages (Posern G, et al). As a highly conserved transcription factor encoded by a single gene (except in zebrafish), SRF integrates multiple signaling pathways to regulate immediate-early response and muscle-specific genes (Davis JL et al). It belongs to the MADS-box family and binds CArG-box sequences [CC(A/T)6GG] as a homodimer (Posern G, et al).

The role of SRF in myogenesis was initially suggested through in vitro studies, where it was shown to be essential for MyoD expression, myogenic differentiation, and muscle fiber maintenance. However, subsequent in vivo studies using Cre-loxP-mediated SRF deletion in myogenic cells revealed that MyoD expression, myoblast formation, and fusion were not entirely dependent on SRF (Gauthier-Rouviere C et al).

During postnatal muscle growth, skeletal muscle expands through increased synthesis of structural and contractile proteins and the fusion of myogenic stem cells with existing multinucleated fibers. Secreted factors like IGF-1 and Interleukin-4 (IL-4) contribute to this

process (Chavret C et al, 2006). In vivo studies using a skeletal actin promoter-driven Cre recombinase revealed that SRF deletion led to growth retardation, severe muscle mass reduction, and decreased expression of skeletal actin and other contractile genes. Additionally, SRF-deficient myofibers exhibited a lower myonuclear count and failed to regenerate, likely due to impaired satellite cell recruitment resulting from downregulated IL-4 and IGF-1 transcription.

Other than these genes, several additional genes play crucial roles in myogenesis. For instance, homeobox genes are essential for skeletal muscle generation, regulating the specification, proliferation, and differentiation of myogenic precursor cells. These genes are characterized by a conserved 180-base pair (bp) DNA sequence, which encodes a 60-amino acid DNA-binding domain known as the homeodomain. As transcription factors, homeobox genes recognize specific DNA sequences and regulate target gene expression (McGinnis & Krumlauf, 1992; Quiring et al., 1994). Figure 2 describes the role of genetic factors that regulates myogenesis.

The homeobox transcription factor family includes Hox, Pax, Msx, Six, and Pitx. Among these, Pax3 and Pax7 are critical for muscle progenitor cell survival and commitment, acting upstream of myogenic regulatory factors (MRFs) such as MyoD and Myf5, which drive muscle lineage specification (Relaix et al., 2005; Kumar, 2009). Additionally, Six1 and Six4, in cooperation with Eya proteins, promote the activation of MyoD and Myf5, facilitating the transition from muscle progenitors to differentiated myoblasts (Laclef et al., 2003). Conversely, Msx1, another homeobox gene, maintains myoblasts in an undifferentiated state by repressing MyoD, preventing premature differentiation and ensuring proper muscle formation (Woloshin et al., 1995).

During postnatal muscle growth and regeneration, Pax7 is vital for satellite cell maintenance and function, ensuring a reservoir of myogenic stem cells for muscle repair (Seale et al., 2000). Homeobox genes also play fundamental roles in cell identity and spatial positioning during embryonic development. Mutations in these genes can lead to severe developmental defects, including homeotic transformations, where body segments or structures adopt incorrect identities (Lewis et al., 1999a). Disruptions in homeobox gene function can result in severe muscle development abnormalities, highlighting their significance in muscle formation, regeneration, and repair. Understanding the regulatory mechanisms of homeobox genes in myogenesis has profound implications for regenerative medicine and muscle-related disorders.

1.3 Regulatory Mechanisms Modulating Myogenic Transcription Factors

The process of skeletal muscle formation (myogenesis) is tightly regulated by a network of myogenic transcription factors (MRFs). These factors control muscle cell fate, differentiation, and maturation, and their activity is modulated by various regulatory mechanisms at transcriptional, post-transcriptional, and epigenetic levels to ensure proper muscle development and regeneration (Bentzinger et al., 2012). The activity and stability of myogenic transcription factors are subject to multiple layers of regulation.

1.3.1 Post transcriptional and translational modifications

Post-transcriptional mechanisms play a crucial role in fine-tuning the activity of myogenic transcription factors, with microRNAs (miRNAs) serving as key regulators. RNA-binding proteins and non-coding RNAs, including microRNAs and long non-coding RNAs, meticulously orchestrate the stability, localization, and translation of mRNAs encoding myogenic transcription factors. This intricate control ensures the precise timing and spatial expression of these factors, which is essential for coordinated muscle development (Shen-Liang Chen, 2024). Notably, specific miRNAs such as miR-1 and miR-133 act as molecular

switches, directly regulating MyoD and myogenin expression to either promote or inhibit muscle differentiation, thereby influencing the balance between muscle growth and regeneration (Chen et al., 2006).

1.3.2 Epigenetic modifications

Epigenetic modifications fine-tune myogenic transcription factor activity through chromatin remodeling and histone modifications. MyoD recruits histone acetyltransferases (HATs) like p300/CBP and PCAF, which acetylate histones and enhance chromatin accessibility, promoting muscle-specific gene expression (Dilworth et al., 2004). Conversely, histone deacetylases (HDACs) suppress MyoD activity, keeping muscle genes in a repressed state (McKinsey et al., 2001).

1.3.3 Ubiquitination

Ubiquitination and proteasomal degradation serve as pivotal regulatory mechanisms in muscle biology, ensuring precise control over MyoD stability. The ubiquitin-proteasome system governs this process, with E3 ubiquitin ligases such as Murf1 and Atrogin-1 marking MyoD for degradation. This targeted regulation plays a crucial role in maintaining muscle homeostasis by balancing muscle atrophy and regeneration, preventing uncontrolled MyoD activity that could disrupt muscle function (Tintignac et al., 2005).

1.3.4 Phosphorylation

Phosphorylation is another critical regulator of myogenesis. MyoD activity is modulated by phosphorylation through MAPK and Akt signaling pathways, influencing its DNA-binding ability and interaction with co-factors (Lluis et al., 2006).

1.3.5 Protein-protein interactions

Protein-protein interactions also play a key role in modulating myogenesis. Transcription factors often interact with co-activators or repressors to fine-tune gene expression. For instance, the muscle LIM protein (MLP) enhances myogenesis by associating with MyoD,

myogenin, and MRF4, thereby promoting the transcription of muscle-specific genes (Arber et al., 1999). Figure 3 shows the genetic factors associated with different myogenic steps in a regulatory interactive manner.

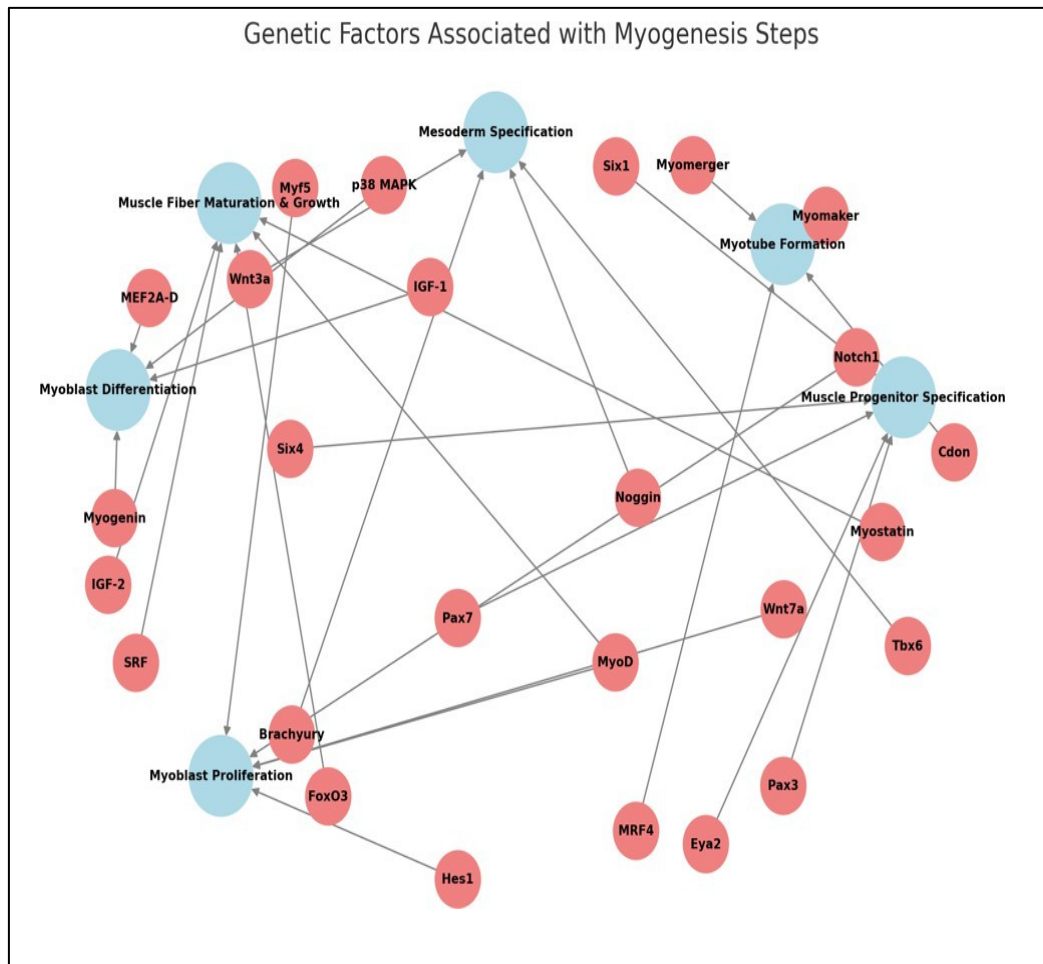


Figure 3: Genetic Factors Regulating Myogenesis in a regulatory network. This figure illustrates the genetic factors involved in each key stage of myogenesis, from mesoderm specification to muscle fiber maturation in a regulatory network. Blue nodes represent the sequential stages of myogenesis. Red nodes represent specific genes and transcription factors that regulate each stage. Edges (arrows) indicate the regulatory relationship between genes and the corresponding developmental stage. This diagram provides an overview of the complex genetic regulation of myogenesis, with interactions among transcription factors, signaling molecules, and epigenetic regulators shaping muscle formation and repair.

1.3.6 DNA methylation

DNA methylation is another epigenetic mechanism whereas methylation of MyoD regulatory elements can silence its expression in non-muscle cells, ensuring lineage specificity during development (Brunk et al., 1996). Epigenetic regulators can modulate the transcriptional activity of myogenic factors, thereby influencing muscle cell differentiation and growth (Marie-Claude Sincennes, 2016).

1.4 Signaling pathways controlling myogenesis

Myogenesis is a fundamental biological process responsible for the development and regeneration of skeletal muscle. It is a highly coordinated sequence of events, beginning with the specification of myogenic progenitor cells and followed by their proliferation, differentiation, and fusion into multinucleated myofibers. This process is governed by numerous signaling pathways that regulate the expression of myogenic regulatory factors (MRFs) such as MyoD, Myf5, myogenin, and MRF4. Understanding these signaling networks is essential for elucidating the molecular mechanisms of muscle development and repair.

Embryonic tissues surrounding the somite play a crucial role in skeletal myogenesis by providing essential inductive signals. This intricate process is finely regulated by a diverse array of signaling pathways, including Notch, Wnt, Shh, MAPK, and others. The notochord, neural tube, and dorsal ectoderm—key sources of Shh and Wnt signals—are particularly vital for myogenic initiation (Münsterberg et al., 1995; Münsterberg and Lassar, 1995; Rios et al., 2011). Additionally, neural crest cells migrating between the neural tube and somites contribute to myogenesis by activating Myf5 in dermomyotome cells. Wnt and FGF signaling maintain progenitor cells in an undifferentiated state within the posterior paraxial mesoderm, while the ventral region gives rise to the sclerotome. Except for certain cranial muscles, most skeletal muscle originates from the dorsally located dermomyotome, which

largely retains its epithelial nature. This region is characterized by high expression of paired box transcription factors (Pax3 and Pax7) and low levels of Myf5. Myogenic differentiation is further orchestrated by the induction of myogenic regulatory factors (MRFs), driven by key signaling molecules such as bone morphogenetic proteins (BMPs), Shh, and Wnt. Here, we explore the key signaling pathways governing myogenesis.

1.4.1 Wnt Signaling Pathway

Wnt signaling plays a pivotal role in regulating myogenesis at various stages, from the initial specification and differentiation of embryonic muscle cells to the maintenance of adult skeletal muscle homeostasis. This highly conserved pathway, found across species from humans to *Drosophila*, comprises 21 secreted glycoproteins related to the *Drosophila* Wingless protein. It operates through two primary mechanisms: the canonical Wnt/ β -catenin pathway and several non-canonical pathways.

In the canonical pathway, Wnt ligands bind to Frizzled receptors, triggering the stabilization and nuclear translocation of β -catenin. This, in turn, activates myogenic regulatory factors (MRFs) such as MyoD, Myf5, and Myogenin, which are essential for committing progenitor cells to the myogenic lineage. Research indicates that neural crest cells, which present Wnt1 on their surface and express delta-like 1 (Dll1), engage Notch signaling in the dorsomedial lip of the somite. Notch activation in these cells inhibits GSK3 β , stabilizing Snail and promoting the delamination of cells into the forming myotome (Rios et al., 2011; Serralbo and Marcelle, 2014; Sieiro et al., 2016).

Another study shows Wnt1, Wnt3a, Wnt4, and Wnt6 play a crucial role in sustaining the expression of Pax3 and Pax7 in myogenic precursor cells within the somite, mediated by signals originating from the surface ectoderm and neural tube (Otto et al, 2006). Disruptions in Wnt gene function have been linked to adverse effects on muscle development. Research on mice lacking Wnt1 and Wnt3a revealed a significant decline in the expression of key

skeletal muscle transcription factors, including Pax3 and Myf5, along with abnormalities in dermomyotome formation (Ikeya et al, 1998). Research conducted in 2009 revealed that Wnt7a plays a key role in promoting satellite cell expansion, thereby influencing the regenerative capacity of muscle tissue (Le Grand et al, 2009). Following muscle injury, satellite cells contribute to new myofiber formation either by fusing with one another or integrating into damaged myofibers. To ensure a continuous supply of muscle progenitors, a precise balance between their differentiation and self-renewal is essential. However, early activation of the canonical Wnt signaling pathway by Wnt3a during muscle regeneration can disrupt this balance, leading to premature differentiation of progenitor cells and a subsequent depletion of the satellite cell pool.

Non-canonical Wnt signaling operates independently of β -catenin's transcriptional regulation and encompasses pathways such as planar cell polarity (PCP), Wnt/ Ca^{2+} , and PI3K/AKT/mTOR. The Wnt7a/Fzd7-mediated PCP pathway plays a crucial role in balancing satellite cell self-renewal and differentiation (Bentzinger et al., 2014, 2013; Le Grand et al., 2009). These pathways are essential for cytoskeletal organization, facilitating myoblast migration and proper alignment during muscle formation. Although they also engage Frizzled receptors, their reliance on LRP co-receptors differs. Compared to the canonical pathway, non-canonical Wnt signaling remains less extensively studied (Kuhl et al., 2004). Overall, Wnt signaling is fundamental not only for embryonic muscle development but also for muscle regeneration and repair in adulthood.

1.4.2 Insulin-like Growth Factor (IGF) Signaling

Insulin-like growth factor-1 (IGF-1) is a circulating hormone that plays a vital role in skeletal myogenesis, influencing muscle mass, strength development, and degeneration while enhancing the proliferative capacity of muscle satellite cells (MSCs). Unlike many growth factors that act as negative regulators of myogenesis, IGFs contribute to both myoblast

proliferation and differentiation (Ewton and Florini, 1980, 1981). The IGF family consists of two primary subgroups, IGF-I and IGF-II (Husmann et al., 1996).

IGF-1 exerts its effects through IGF-1R, a transmembrane receptor with tyrosine kinase activity that activates the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway. This activation promotes the expression of myogenic regulatory factors (MRFs) while inhibiting myostatin, a well-known suppressor of muscle growth (Hamilton D.L et al., 2010). Studies indicate that the loss of IGF-I or IGF-II function in myoblasts leads to impaired muscle regeneration and inhibition of myogenic differentiation, respectively (Lefaucheur and Sebillé, 1995; Carter et al., 2009). Additionally, IGF-1 knockout mice exhibit muscle hypoplasia, further underscoring its essential role in muscle development.

Moreover, blocking myostatin activity stimulates the Akt/mTOR/S6K pathway, which is crucial for IGF-1-induced muscle hypertrophy. IGF-1 has significant therapeutic potential, particularly in managing Duchenne muscular dystrophy, preventing muscle atrophy, and promoting neurite growth (Syed Sayeed Ahmad et al., 2020). Through mTOR activation, IGF-1 enhances protein synthesis and facilitates muscle hypertrophy. In summary, IGF signaling is fundamental to muscle maintenance, regeneration, and overall muscular health.

1.4.3 p38 MAPK signaling pathways

The development of skeletal muscle is orchestrated by extracellular growth factors that initiate largely unknown intracellular signaling cascades, influencing the muscle transcriptional program. One key signaling pathway involved in myogenic differentiation is the p38 mitogen-activated protein kinase (MAPK) pathway. p38 MAPK regulates transcription factors from the MyoD and MEF2 families and plays a role in chromatin remodeling at specific muscle-regulatory regions (Aviad Keren et al., 2006). Evidence highlights the essential function of p38 signaling in activating MEF2 transcription factors during somite development in mice.

In vitro studies have demonstrated the significance of p38 MAPK in controlling myogenesis, showing that inhibiting p38 α/β (Mapk14/11) impairs myogenic differentiation and cell fusion (Cuenda and Cohen, 1999; Wu et al., 2000; Zetser et al., 1999). Initially, p38 was identified as a kinase that phosphorylates MEF2C at three specific residues (T293, T300, and S387), with alanine substitution at these sites diminishing MEF2C transcriptional activity (Han et al., 1997). Further research confirmed that p38 also phosphorylates and enhances the activity of MEF2A and MEF2D in both myoblasts and fibroblasts (Zhao et al., 1999; Penn et al., 2004; Rampalli et al., 2007).

Beyond its role in MEF2 phosphorylation, p38 also phosphorylates E47, facilitating the formation of MyoD/E-protein complexes, which are crucial for muscle-specific gene expression (Simone et al., 2004; Lluís et al., 2005). This interaction helps explain how p38 enhances MyoD transcriptional activity, although direct activation remains unconfirmed due to limited supporting evidence (Puri et al., 2000). Additionally, p38 signaling regulates myogenin transcriptional activity in differentiating myocytes, either indirectly via MEF2 and E-proteins or through direct phosphorylation. Dysfunction in this pathway has been strongly linked to rhabdomyosarcoma, emphasizing its importance in muscle development and disease (Puri et al., 2000).

1.4.4 Notch Signaling pathway

Among the various negative regulators of myogenesis, the Notch signaling pathway plays a crucial role. This pathway enables two neighboring cells within an organism to communicate through direct mechanical interactions mediated by the Notch protein. Notch signaling primarily regulates myoblast proliferation and prevents premature differentiation. Activation occurs when Notch ligands (Delta-like or Jagged) bind to Notch receptors, leading to the release of the Notch intracellular domain (NICD), which translocates into the nucleus and modulates gene expression (Matthew E Buas et al, 2010). *Notch* and *Myf5* genes are

expressed in early progenitor cells of skeletal muscles in mice (Kuang S et al, 2007). A study conducted at the Fred Hutchinson Cancer Research Center in Seattle discovered that the Notch protein inhibited myoblast determination, even when the pro-determination protein Myf5 was present. Researchers proposed that Notch suppresses myoblast differentiation while they migrate to their designated location in the embryo (Raphael Kopan et al., 1994). Moreover, Notch signaling maintains the myogenic progenitor pool by inhibiting MyoD and promoting Pax7, a key marker of muscle stem cells (Yefei Wen et al, 2012). Downregulation of Notch signaling allows myoblasts to exit the proliferative state and enter differentiation. Notch signaling is particularly important in muscle regeneration, where it governs satellite cell activation.

1.4.5. Hedgehog Signaling Pathway

Hedgehog (Hh) signaling, mediated by Sonic Hedgehog (Shh), plays a role in muscle precursor proliferation and differentiation influencing muscle development from embryonic stages through to adult muscle regeneration. Shh signaling enhances Myf5 and MyoD expression, promoting myogenic lineage commitment. It also supports muscle progenitor expansion by counteracting BMP-mediated inhibition (Giuseppe Straface et al, 2008). It also plays a role in the selection of muscle fiber types, such as fast and slow fibers, ensuring proper muscle patterning and function. Studies in zebrafish embryos have demonstrated that overexpression of Hh can convert the entire myotome into slow muscle fibers, highlighting its influence on muscle type specification (Huijuan Li et al, 2018). Hedgehog signaling is crucial for embryonic muscle development and has been implicated in muscle repair processes. Beyond embryonic development, Shh signaling remains active in adult tissues, particularly during muscle regeneration. In response to muscle injury, Shh is reactivated and contributes to the repair process by promoting the proliferation and differentiation of muscle progenitor cells. This reactivation underscores the pathway's versatility and its critical role in

maintaining muscle integrity throughout an organism's life (Giuseppe Straface et al, 2008).

1.4.6 Bone Morphogenetic Protein (BMP) Signaling

BMP signaling acts as a negative regulator of myogenesis by suppressing myogenic differentiation and promoting alternative cell fates. BMPs signal through Smad (particularly R- Smads 1, 5 and 8) transcription factors to inhibit MyoD activity (Richard N Wang et al, 2014). BMP antagonists, such as Noggin, facilitate myoblast differentiation by counteracting BMP- mediated suppression (Melanie Friedrichs, et al, 2011). Regulation of BMP signaling is vital for the balance between muscle and non-muscle cell fates.

1.4.7 Transforming Growth Factor- β (TGF- β) Signaling

TGF- β signaling is a critical inhibitor of myogenesis, acting through Smad proteins to suppress MyoD and Myogenin expression (Mia Jaffe et al, 2012). Myostatin, a member of the TGF- β family, negatively regulates muscle growth by inhibiting satellite cell proliferation and differentiation (Allen et al, 1987) while TGF- and myostatin signal through R-Smads 2 and 3 (Kollias et al, 2008). TGF- β signaling is involved in fibrosis and muscle wasting diseases, making it a potential therapeutic target for muscle regeneration strategies.

1.5 Wnt/ β -catenin signaling pathway in skeletal muscle

The Wnt/ β -catenin pathway is a highly conserved signaling pathway that plays crucial roles in embryonic development, tissue homeostasis, and cell fate determination. In the canonical Wnt signaling pathway, β -catenin serves as a central mediator. Under normal conditions, in the absence of Wnt ligands, β -catenin levels in the cytoplasm are kept low through continuous degradation. This degradation is facilitated by a destruction complex comprising adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3 β (GSK3 β), and casein kinase 1 α (CK1 α). Within this complex, β -catenin is phosphorylated by CK1 α and GSK3 β , marking it for ubiquitination and subsequent proteasomal degradation (Akiko Suzuki et al,

2018). When Wnt ligands bind to their receptors, the destruction complex is inhibited, leading to the stabilization and accumulation of β -catenin in the cytoplasm (fig4). The stabilized β -catenin then translocates into the nucleus, where it interacts with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors to activate the expression of Wnt target genes involved in cell proliferation, differentiation, and survival (Akiko Suzuki et al, 2015). Beyond its role in Wnt signaling, β -catenin is integral to cell-cell adhesion. It associates with the intracellular domain of cadherins, a class of transmembrane proteins essential for adherens junctions that maintain tissue architecture. Through its interaction with cadherins and α -catenin, β -catenin links the actin cytoskeleton to the cell membrane, facilitating strong intercellular adhesion. Disruption of β -catenin's adhesive functions can lead to increased cellular motility and invasiveness, characteristics that are hallmarks of cancer metastasis. For example, in certain cancers, the loss of E-cadherin expression or function leads to the release

of β -catenin from the cell membrane, increasing its availability in the cytoplasm and potentially enhancing Wnt signaling activity. This dual role of β -catenin in adhesion and signaling underscores its significance in maintaining cellular homeostasis and how its dysregulation can contribute to oncogenesis.

Research shows disruption in Wnt/ β -Catenin pathway affect myogenesis. The canonical Wnt pathway, involving β -catenin, is principal in regulating various stages of myogenesis, including myoblast proliferation, differentiation, and fusion. During the early stages of myogenesis, Wnt/ β -catenin signaling promotes the proliferation of myoblasts. This is achieved by regulating genes such as cyclin A2 (Ccna2) and cell division cycle 25C (Cdc25c), which are crucial for cell cycle progression. Inhibition of β -catenin disrupts this process, leading to reduced myoblast proliferation (Akiko Suzuki et al, 2015). β -Catenin is essential for the differentiation of myoblasts into mature muscle fibers. Studies utilizing β -

catenin-null primary myoblasts have demonstrated impaired morphological differentiation and delayed expression of myogenic regulatory factors, underscoring the necessity of β -catenin in muscle formation (Shuang Cui et al, 2019). Beyond proliferation and differentiation, β -catenin plays a critical role in the fusion of myoblasts to form multinucleated myotubes. The pathway regulates specific genes involved in membrane fusion and cytoskeletal remodeling; processes essential for effective myoblast fusion (Akiko Suzuki et al, 2015). Additionally, β -Catenin interacts with various signaling molecules to fine-tune myogenesis. For instance, it forms complexes with Smad7, influencing myogenic gene transcription and highlighting the crosstalk between Wnt and transforming growth factor-beta (TGF- β) signaling pathways (Soma Tripathi et al, 2019).

Aberrations in this pathway can lead to uncontrolled cell growth and cancer (Shuang Shang et al, 2017). For instance, mutations in the APC gene, which impair the formation of the destruction complex, result in the accumulation of β -catenin and the constitutive activation of Wnt target genes, contributing to tumorigenesis (D Kimelman et al, 2006). Such mutations are commonly observed in colorectal cancers and are associated with familial adenomatous polyposis, a condition characterized by the development of numerous polyps in the colon that have a high potential to progress to malignancy (Shuang Shang et al, 2017). Figure 4 illustrates the canonical Wnt/ β -catenin signaling pathways and how it regulates gene transcription.

The Wnt/ β -catenin signaling pathway is crucial for muscle development and maintenance, influencing gene expression related to cell cycle regulation and facilitating the assembly of cadherin/ β -catenin/actin complexes. This suggests that Wnt signaling interacts with distinct target molecules at different stages of development.

1.6 β -catenin dysregulation in Human diseases

β -Catenin, the key effector of canonical Wnt signaling, regulates both cell adhesion and gene

transcription. Its dysregulation contributes to various cancers due to multiple factors. Understanding the mechanisms by which β -catenin contributes to oncogenesis and how its activity is modulated by its protein network is crucial for developing targeted therapeutic strategies. The activity and stability of β -catenin are intricately regulated by a network of interacting proteins that modulate its localization, degradation, and transcriptional activity. Key components of this regulatory network include GSK3 β , Axin, APC, CK1 α , Dsh, T-cell factor (TCF)/lymphoid enhancement factor (LEF). Therefore, one major cause of disrupted Wnt/ β -catenin signaling pathway is mutations in the destruction complex (APC, Axin, GSK3 β , and CK1 α). For instance, AXIN1 mutations are linked to hepatocellular carcinoma, emphasizing its role in β -catenin homeostasis. Another regulatory mechanism is phosphorylation while GSK3 β -mediated phosphorylation promotes degradation, other kinases like CK1 δ modulate β -catenin stability differently. E-cadherin, a cell adhesion molecule, sequesters β -catenin at the membrane, but its loss, common in EMT during cancer metastasis, releases β -catenin, enhancing its signaling and promoting invasion. In the nucleus, β -catenin interacts with co-activators like BCL9 and Pygo to drive gene expression, and disrupting these interactions—such as targeting β -catenin-BCL9—has been proposed as a therapeutic approach in colorectal cancer. Additionally, sFRPs, extracellular Wnt antagonists, prevent Wnt ligand-receptor binding, but their downregulation or epigenetic silencing enhances β -catenin activation. Restoring sFRP1 expression inhibits tumor growth, highlighting its tumor-suppressive role.

Depending on the context both downregulation and upregulation of β -catenin can cause numerous diseases in human including cancers. When β -catenin is overactivated it translocates into the nucleus and binds to LEF-1/TCF4 to promote the transcription of target genes like Jun, c-Myc and CyclinD-1, cyclinB1 and C in tissue specific manner, most of which encode oncoproteins (Fang Hua et al, 2017). This can lead to different types of cancers

like colorectal cancer, hepatocellular carcinoma, lung cancer, pancreatic cancer, breast cancer, ovarian cancer and may be in more cancers. More than half of all cancer cases β -catenin accumulates within the nucleus or cytoplasm and promotes cancer cell proliferation and survival (Khramtsov et al, 2010; Tao J et al, 201; Kobayashi M et al, 2000; Damsky WE et al, 2011). Wnt/ β -catenin pathway regulates many key aspects of cancer development, including maintaining cancer stem cells (CSCs); promoting metastasis, cancer cell survival, and chemoresistance suppressing the immune response within the tumor microenvironment and enhancing tumor angiogenesis (Vu Hong Loan Nguyen et al, 2019). Indeed, nuclear accumulation of β -catenin can be observed in 80% of colorectal carcinoma (White BD et al, 2012). Besides, it promotes the progression of tumors via suppressing the T-cell responses (Hong Y et al, 2015). Study demonstrates that β -catenin upregulates the expression of Cyclin B1 and Cyclin C to promote carcinogenesis in Hepato-cellular carcinoma (Vilchez V et al, 2016). The hyperactivation of Wnt/ β -catenin pathway has been reported to promote cancer stem cell self-renewal, metastasis, and chemoresistance in all subtypes of EOC (endothelial ovarian cancer) (Vu Hong Loan Nguyen et al, 2019).

Other than cancer, hyperactive β -catenin is implicated in pulmonary fibrosis, kidney fibrosis, and cardiac fibrosis, where it drives excessive tissue remodeling (Jianling Song, et al, 2024) whereas Aberrant Wnt/ β -catenin signaling is associated with the development of pulmonary fibrosis, contributing to the replacement of normal lung tissue with fibrotic tissue and Chronic activation of Wnt/ β -catenin signaling has been shown to aggravate renal fibrosis by disrupting mitochondrial homeostasis, leading to excessive extracellular matrix deposition and impaired kidney function (Jianling Song, et al, 2024). Another study shows, Elevated levels of fibroblast growth factor 23 (FGF23) can activate β -catenin, which mediates the upregulation of pro-fibrotic factors such as TGF- β , collagen I, and collagen III, leading to myocardial fibrosis (Traian Chiuariu et al, 2024). Additionally, Elevated β -catenin activity

has been linked to Alzheimer's disease (AD), affecting neuronal function and survival. In the brain, Wnt/ β -catenin signaling is crucial for neuronal survival, neurogenesis, synaptic plasticity, and blood-brain barrier integrity. Activation of this pathway inhibits amyloid- β production and tau protein hyperphosphorylation, both key features of AD pathology. However, in AD, Wnt/ β -catenin signaling is often suppressed, leading to neuronal vulnerability (Lin Jia et al, 2019). Additionally, the Wnt antagonist DKK1 is upregulated in AD brains, leading to synapse loss and memory impairment. Reactivation of Wnt/ β -catenin signaling has been shown to restore synaptic function and cognitive abilities in experimental models (Lin Jia et al, 2019).

β -Catenin is frequently upregulated in various diseases, particularly in cancers. However, its downregulation has also been observed in specific contexts, with significant implications for cellular functions. Loss of β -catenin function can disrupt cell adhesion, enhance metastasis, impair differentiation and signal transduction, reduce proliferation, and even contribute to drug resistance in patients, ultimately influencing tumor progression through diverse mechanisms. Furthermore, the downregulation of this multifunctional protein has been implicated in several diseases, including developmental and neurodegenerative disorders. However, this section will focus specifically on the role of β -catenin downregulation in cancer development. One of the key mechanisms underlying β -catenin downregulation in cancer is the upregulation of the β -catenin destruction complex, which promotes its degradation and leads to diminished Wnt signaling. A notable example is alveolar rhabdomyosarcoma, a pediatric cancer in which muscle cells fail to undergo terminal differentiation, resulting in tumor formation. In this malignancy, enhanced activity of Glycogen Synthase Kinase 3 β (GSK-3 β) drives the proteasomal degradation of β -catenin, thereby impairing Wnt signaling (Mathew Dionyssiou et al., 2014).

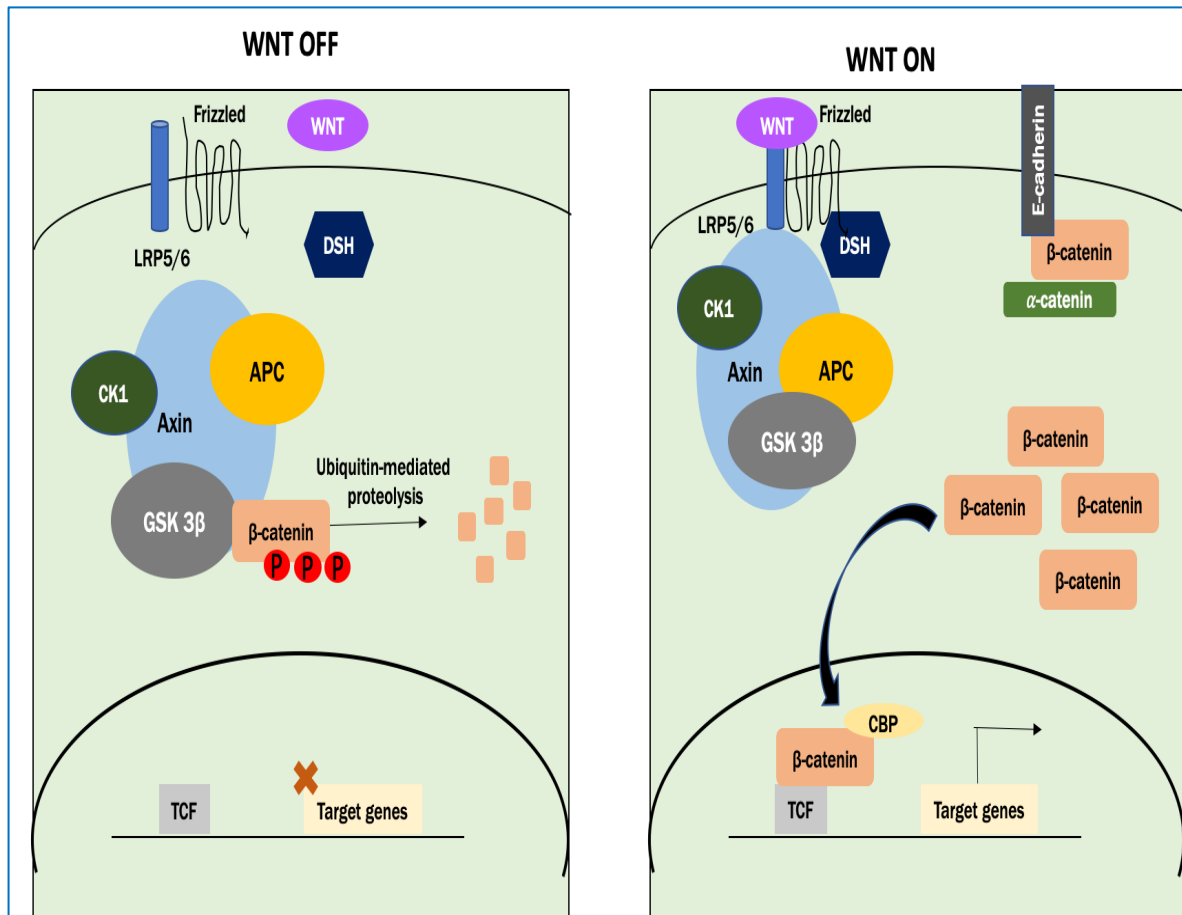


Figure 4. Wnt/β-Catenin Signaling Pathway. This diagram illustrates the canonical Wnt/β-catenin signaling pathway, comparing the WNT ON and WNT OFF states and their impact on β-catenin stability and downstream gene transcription. In the WNT OFF state (left panel), in the absence of Wnt ligand, the β-catenin destruction complex—composed of Axin, APC (adenomatous polyposis coli), CK1α, and active GSK-3β—phosphorylates β-catenin, marking it for ubiquitin-mediated proteasomal degradation. This prevents β-catenin from accumulating in the cytoplasm or entering the nucleus, thereby inhibiting Wnt target gene transcription and suppressing cell proliferation. In contrast, the WNT ON state (right panel) is initiated when Wnt ligands bind to the Frizzled receptor and co-receptor LRP5/6, leading to the recruitment of Dishevelled (not shown) and disruption of the destruction complex. GSK-3β becomes inactive, preventing β-catenin phosphorylation and degradation. As a result, stabilized β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it binds to Tcf/Lef transcription factors to activate Wnt target gene expression. This activation promotes various cellular processes, including cell proliferation, differentiation, and survival. This figure is adopted from (Tripathi et al, 2019).

A similar phenomenon is observed in certain hematological malignancies, where reduced β -catenin activity compromises the self-renewal capacity of cancer stem cells. The Wnt/ β -catenin signaling pathway plays a crucial role in the maintenance and proliferation of hematopoietic stem cells (HSCs). Studies have shown that disruptions in this pathway lead to impaired self-renewal and differentiation, hallmark features of various hematological cancers (Tiago C. Luis et al., 2009). Additionally, the interaction between β -catenin and transcription factors such as lymphoid enhancer-binding factor 1 (LEF1) is essential for the regulation of genes involved in HSC maintenance (Shuyang Yu et al., 2016). Dysregulation of this interaction can impair HSC function and has been linked to hematological malignancies. Thus, proper regulation of the Wnt/ β -catenin pathway is critical for HSC maintenance, and its disruption contributes to the pathogenesis of these cancers.

Moreover, β -catenin downregulation has been associated with increased metastatic potential in gastric cancer. A study published in the *Journal of Clinical Oncology* reported significantly reduced β -catenin expression in metastatic gastric cancer tissues. Notably, only one out of 18 metastatic lesions exhibited detectable β -catenin protein levels, highlighting the correlation between β -catenin loss and enhanced metastatic potential in gastric cancer (Matthias P. A. Ebert et al., 2003).

Collectively, these findings underscore the complex and context-dependent role of β -catenin in cancer biology, where both its upregulation and downregulation can contribute to disease progression through distinct mechanisms.

1.7 Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a malignant neoplasm arising from primitive mesenchymal cells committed to skeletal muscle differentiation. It represents the most prevalent soft tissue sarcoma in the pediatric population, accounting for approximately 4.5% of all childhood cancers. RMS can manifest throughout the body but predominantly occurs in the head and

neck regions, genitourinary tract, and extremities. Histologically, RMS is categorized into several subtypes, with embryonal and alveolar being the most common. Embryonal rhabdomyosarcoma typically affects younger children and is characterized by small, round blue cells with a myxoid stroma, often resembling developing skeletal muscle. Alveolar rhabdomyosarcoma, more common in adolescents and young adults, exhibits densely packed, uniform round cells arranged in an alveolar pattern, indicative of a more aggressive clinical course (Parham, D. et al, 2001). Advancements in multimodal therapeutic approaches, including surgery, chemotherapy, and radiation therapy, have significantly improved patient outcomes. However, the prognosis remains variable and is heavily influenced by factors such as tumor histology, anatomical location, and the presence of metastatic disease at diagnosis. Figure 5 depicts the regulators that drives normal muscle cells into cancerous cell in human, which is called Rhabdomyosarcoma, a lethal pediatric cancer.

Regardless of their cellular origin—whether derived from non-myogenic progenitors or through dedifferentiation of mature myocytes—rhabdomyosarcoma (RMS) cells consistently exhibit an inability to (a) exit the cell cycle, (b) undergo morphological differentiation, and (c) express muscle-specific functional and structural proteins, even when exposed to mitogen-depleted conditions in vitro. Numerous studies have demonstrated that despite the presence of MyoD and E-protein heterodimers in these cells, their myogenic potential remains significantly impaired, preventing efficient differentiation. This phenomenon highlights the fundamental disruption of myogenic regulatory pathways in RMS, as described in the seminal work *Myogenesis and Rhabdomyosarcoma: The Jekyll and Hyde of Skeletal Muscle*.

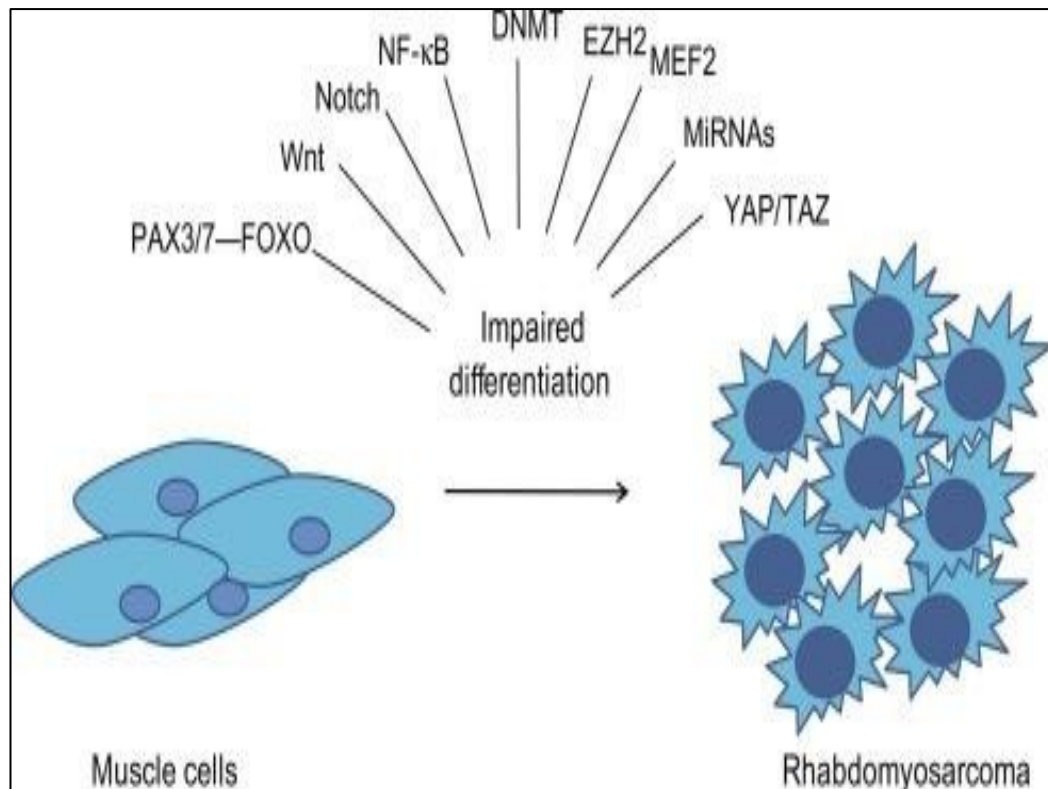


Figure 5: Molecular Regulators of Impaired Differentiation in Rhabdomyosarcoma.

This figure illustrates the transition from normal muscle cells to rhabdomyosarcoma (RMS) because of impaired myogenic differentiation. Multiple signaling pathways and transcriptional regulators are shown to converge and disrupt normal differentiation programs, contributing to tumorigenesis. Key pathways and factors involved in this dysregulation include Wnt, Notch, NF- κ B, and PAX3/7–FOXO signaling, all of which are known to play critical roles in muscle lineage commitment. Epigenetic modifiers such as DNMT (DNA methyltransferases) and EZH2 (a component of the Polycomb repressive complex 2), as well as post-transcriptional regulators like miRNAs, further contribute to silencing differentiation-promoting genes. Additionally, dysregulated transcription factors such as MEF2 and co-activators like YAP/TAZ—components of the Hippo signaling pathway—also impair terminal myogenic differentiation. Adapted from Yu PY et al., 2017.

1.8 Molecular mechanism of Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a malignant soft tissue tumor arising from mesenchymal progenitor cells with impaired skeletal muscle differentiation. The molecular mechanisms underlying RMS involve a complex interplay of genetic mutations, chromosomal translocations, and dysregulated signaling network that drive tumorigenesis, proliferation, and resistance to apoptosis. RMS is primarily categorized into embryonal rhabdomyosarcoma

or fusion negative RMS mutations (ERMS) and alveolar rhabdomyosarcoma (ARMS) referred to as fusion positive RMS, each with distinct molecular signatures (Silvia Pomella et al, 2021). ERMS is frequently associated with loss of heterozygosity at chromosome 11p15.5, leading to dysregulation of IGF2 (Insulin-like Growth Factor 2), a key player in cell proliferation and survival (John Anderson et al, 1999). Additionally, mutations in TP53, PTCH1, and NF1 contribute to defective cell cycle control and hyperactivation of the Hedgehog and RAS-MAPK pathways, promoting unchecked growth (John Anderson et al, 1999) (Shern JF et al, 2021). Conversely, ARMS is characterized by a somatic cell chromosomal translocation fusion occurred between t(2;13)(q35;q14) or t(1;13)(p36;q14) respectively which rearranges PAX3 and PAX7 which are members of paired box transcription factor and a crucial determining factor of somatic myogenesis (Dodou E et al, 2003) and this phenomenon is shown in figure 6. This chromosomal translocation fuses the PAX3 or PAX7 gene with FOXO1 gene (a member of fork-head box protein, plays an important role in regulating the expression in cell growth) resulting a chimeric fusion genes PAX3-FOXO1 or PAX7-FOXO1 encodes a dynamic oncogene which act as a transcription co-activator PAX3/7 FOXO1 and enhance oncogenic pathways, leading to impaired differentiation and increased tumor aggressiveness (Galili, N., et al, 1993). Figure 6 illustrates the molecular mechanism of alveolar rhabdomyosarcoma a subtype of pediatric cancer rhabdomyosarcoma.

The PI3K/AKT/mTOR and Hippo-YAP pathways are also highly dysregulated in RMS, fostering tumor cell survival and evasion of apoptosis. Recent studies highlight the role of microRNAs (miRNAs) and epigenetic modifications, such as DNA methylation and histone acetylation, in RMS pathogenesis, affecting gene expression and cellular plasticity (Elizabeth Stewart et al, 20218). Targeting these molecular pathways using small-molecule inhibitors, monoclonal antibodies, or gene-editing technologies like CRISPR-Cas9 holds promise for

novel therapeutic interventions. However, the heterogeneity of RMS and the presence of tumor-initiating cells pose significant challenges in treatment, necessitating further research into personalized medicine approaches.

1.9 MEF2A in Rhabdomyosarcoma

The MEF2 family of transcription factors plays a critical role in regulating various developmental programs, including myogenesis by enhancing their ability to activate muscle-specific gene expression and drive differentiation. MEF2A, in particular, governs the expression of muscle-specific genes by binding to their promoter regions, thereby influencing both early myogenic determination and terminal differentiation (Ya-Ning Wang et al., 2018). During myogenesis, MEF2A interacts synergistically with Myogenic Regulatory Factors (MRFs) such as MyoD and myogenin, enhancing the transcription of essential muscle development genes. This cooperative interaction underscores the pivotal role of MEF2A in facilitating effective myogenic differentiation (Ning Liu et al., 2014).

Beyond its function in differentiation, MEF2A has also been identified as a positive regulator of myoblast proliferation, highlighting its dual role in muscle development. As one of the late stages' myogenic transcription factors, alongside myogenin and myostatin, MEF2A is essential for orchestrating the transcriptional networks that drive myogenic differentiation and muscle maturation (Klaudia Skrzypek et al, 2015). Given its central role in muscle formation, any disruption in MEF2A or its regulatory network can significantly impair myogenic differentiation, potentially leading to muscle development disorders. Since, RMS cells have morphologic features like skeletal muscle cells and frequently express myogenic transcription factors such as MyoD and Myogenin, yet they remain unable to fully differentiate into mature muscle cells due to a disruption in the differentiation pathway.

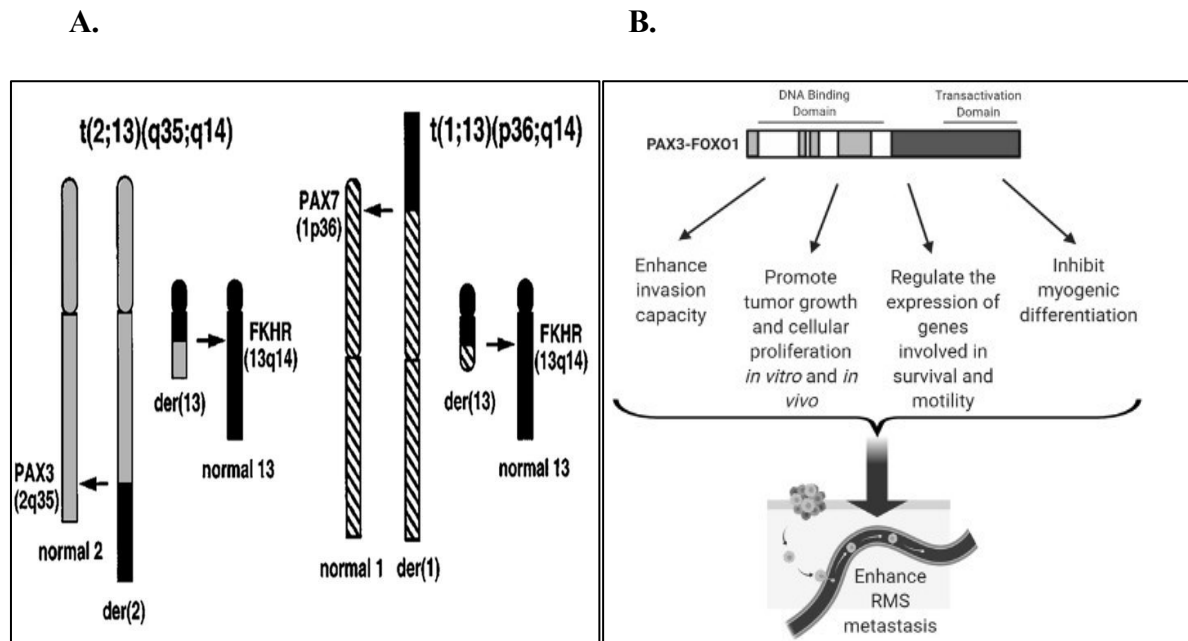


Figure 6. Chromosomal Translocations and Oncogenic Functions of PAX-FOXO1 Fusion Proteins in Rhabdomyosarcoma. (A) Schematic representation of the two primary chromosomal translocations associated with alveolar rhabdomyosarcoma (ARMS): $t(2;13)(q35;q14)$ and $t(1;13)(p36;q14)$. These translocations result in the fusion of PAX3 or PAX7 with FOXO1 (also known as FKHR), forming the oncogenic fusion genes PAX3-FOXO1 and PAX7-FOXO1, respectively. These fusions lead to the generation of derivative chromosomes (der) and rearranged transcription factors that retain the DNA-binding domain of PAX and the potent transactivation domain of FOXO1, driving tumorigenesis. (B) Functional consequences of the PAX3-FOXO1 fusion protein in RMS progression. The chimeric protein promotes several oncogenic processes, including (i) enhancement of cellular invasion capacity, (ii) increased tumor growth and proliferation both *in vitro* and *in vivo*, (iii) regulation of genes involved in survival and motility, and (iv) inhibition of myogenic differentiation. Collectively, these mechanisms contribute to the metastatic potential and aggressiveness of RMS tumors. (Barr et al., 2001 and Farah Ramadan et al., 2020).

The precise cause of this disruption remains unclear. However, given its critical role in myogenesis, MEF2A may be a key contributor to this dysregulation, as suggested by the previously discussed mechanisms.

A study investigating the effects of constitutive MET signaling activation in RMS cells found that sustained MET activity impairs myogenic differentiation. In this context, MEF2A expression was notably reduced, correlating with decreased levels of myogenin and myostatin, both critical for muscle differentiation. This suggests that aberrant MET signaling

may contribute to RMS pathogenesis by downregulating MEF2A and disrupting normal myogenic processes (Klaudia Skrzypek et al, 2015). Another Studies have demonstrated that RMS cells often exhibit altered expression of MEF2 family members, including MEF2A. This dysregulation contributes to the impaired differentiation observed in RMS. For instance, research indicates that the loss of MEF2D expression in RMS cells inhibits differentiation and contributes to tumorigenicity. While this study primarily focuses on MEF2D, it underscores the critical role of MEF2 family members in RMS pathology (Meiling Zhang et al, 2013).

Another research conducted on MEF2C suggests that myogenic co-factors such as MEF2C or E proteins play a crucial role in overcoming the differentiation block observed in rhabdomyosarcoma (RMS) cells. While MyoD is the master regulator of myogenesis, its expression alone is often insufficient to induce full differentiation in RMS cells. However, when co-expressed with these essential co-factors, MyoD can effectively activate the myogenic differentiation pathway, helping RMS cells transition from a partially differentiated state to a fully developed muscle lineage (Meiling Zhang et al, 2015). Additionally, previous research on MEF2C and MEF2D shows that exogenous expression of MEF2C or MEF2D has been shown to promote differentiation in rhabdomyosarcoma (RMS) cells, helping to overcome the differentiation block typically observed in these tumors. By enhancing the activity of myogenic regulatory factors (MRFs), MEF2C and MEF2D facilitate the activation of muscle-specific genes, driving RMS cells toward a more mature myogenic phenotype (Meiling Zhang et al, 2015). Although this research has focused on MEF2C and MEF2D, given the conserved nature of MEF2 family proteins, it is likely that MEF2A plays a similar role in regulating muscle gene expression in RMS cells.

Dysregulation of MEF2A expression and activity has been implicated in rhabdomyosarcoma (RMS), potentially contributing to the failure of RMS cells to undergo proper muscle differentiation. Introducing myogenic co-factors such as MEF2A alongside MyoD has been

shown to help overcome this differentiation block, facilitating the restoration of myogenic programs and promoting the formation of mature muscle-like structures. Given that RMS is a heterogeneous disease with distinct subtypes, the specific co-factors required to rescue differentiation may vary depending on the tumor type and its unique regulatory mechanisms. Despite MEF2A's established role in muscle differentiation, its function in RMS remains poorly understood due to the limited research available on this transcription factor in the context of RMS. Therefore, further studies are needed to explore the potential of MEF2A as a therapeutic target for restoring myogenic differentiation in RMS.

1.10 β -catenin in Rhabdomyosarcoma

β -Catenin is a key regulator of the Wnt signaling pathway, orchestrating crucial cellular functions such as proliferation, differentiation, and apoptosis. In rhabdomyosarcoma (RMS), a pediatric soft tissue sarcoma with skeletal muscle characteristics, dysregulation of this pathway has been linked to tumor invasion and metastasis. Understanding β -catenin's role in RMS not only sheds light on its contribution to tumorigenesis but also opens avenues for potential therapeutic strategies targeting this pathway.

Although the canonical Wnt/ β -catenin signaling pathway is crucial for skeletal muscle development, its role in the transformation of these cells into rhabdomyosarcoma (RMS) remains poorly understood due to limited available data. Research indicates that β -catenin may influence RMS development through either overactivation or downregulation. Increased Wnt/ β -catenin signaling can lead to nuclear accumulation of β -catenin, where it activates oncogenes that drive cell proliferation and uncontrolled cell cycle progression.

However, studies have shown that β -catenin is expressed in RMS tissues without mutations, and its localization varies between cytoplasmic/membranous regions and, less frequently, the nucleus. This suggests that β -catenin is not upregulated in RMS cells rather exists in limited quantities (Nada Ragab et al., 2018). Similarly, research indicates that β -catenin mutations do

not appear to contribute significantly to pediatric RMS tumorigenesis (Dorothee et al, 2009) suggesting dysregulation of Wnt signaling, rather than mutations in β -catenin itself, is essential for myogenesis, contributing to RMS tumorigenesis, and can have a tumor suppressor effect in this context (Sahar Choudhary et al., 2024). An analysis of 44 RMS cases found cytoplasmic/membranous β -catenin expression in both alveolar and embryonal subtypes, with nuclear expression detected in only a minority of embryonal RMS cases. These findings suggest that while β -catenin is present in RMS cells, its activation and nuclear translocation may be restricted (Srinivas R. Annavarapu et al., 2013).

Research has established that β -catenin is neither overexpressed nor mutated in rhabdomyosarcoma (RMS) cells; rather, it is downregulated compared to normal muscle cells. This disruption in Wnt signaling has been implicated in the tumorigenesis of embryonal RMS (ERMS), particularly in highly invasive tumors observed in p53/c-fos double mutant mice, as demonstrated by Shalini Singh and colleagues in 2010. Experimental activation of the Wnt/ β -catenin pathway using recombinant Wnt3a in RMS cell lines led to β -catenin stabilization and nuclear translocation. Notably, alveolar RMS (ARMS) cells exhibited reduced proliferation and upregulation of myogenic differentiation markers, including myogenin, MyoD1, and Myf5, in response to Wnt3a treatment (Srinivas R. Annavarapu et al., 2013). A similar study corroborated these findings, showing that Wnt/ β -catenin pathway activation in ARMS cells promoted differentiation while inhibiting proliferation (Shalini Singh et al., 2010).

In contrast, embryonal RMS cells displayed a less pronounced response to Wnt activation, suggesting subtype-specific variations in pathway sensitivity (Nada Ragab et al., 2018). Given the critical role of impaired Wnt/ β -catenin signaling in RMS, its downregulation has been attributed, at least in part, to elevated GSK3 β activity in ARMS cells (Mathew Dionyssiou et al., 2014). In vivo studies in ERMS-bearing zebrafish demonstrated that pharmacological

inhibition of GSK3 β led to Wnt/ β -catenin pathway activation, resulting in suppressed tumor growth, depletion of TPCs, and reduced self-renewal capacity. Additionally, GSK3 β inhibition was shown to prevent ERMS development and regeneration (Chen E.Y. et al., 2014).

Beyond GSK3 β , another key negative regulator of the Wnt/ β -catenin pathway is Dickkopf-1 (DKK-1), which antagonizes Wnt signaling by binding to the LRP6 co-receptor with high affinity, thereby preventing Wnt ligand interaction and subsequent pathway activation (Atsushi Niida et al., 2004). Notably, pharmacological inhibition of DKK-1 has been shown to restore Wnt/ β -catenin signaling and promote RMS cell differentiation. Specifically, the small-molecule inhibitor WAY-262611 blocks DKK-1-mediated suppression by preventing the formation of the DKK-1–LRP5–Kremen complex, thereby enabling Wnt/ β -catenin pathway activation (Irina Giralt et al., 2021).

The functional significance of Wnt/ β -catenin signaling in rhabdomyosarcoma (RMS) has been an area of active investigation. While some factors contributing to its downregulation in RMS cells have been identified, the underlying mechanisms remain incompletely understood. The multifaceted role of β -catenin in RMS underscores the complexity of targeting this pathway for therapeutic purposes. Although activation of Wnt/ β -catenin signaling has been shown to promote differentiation in certain RMS subtypes, its broader impact on tumor growth and progression is not yet fully elucidated. Consequently, therapeutic strategies aimed solely at β -catenin modulation may not be universally effective across all RMS cases.

A deeper understanding of the molecular context and subtype-specific signaling networks is essential for the development of targeted therapies that can precisely regulate β -catenin activity and its downstream effects in RMS. Investigating its regulatory network, including protein-protein interactions, is crucial for elucidating β -catenin's contribution to RMS pathogenesis.

In summary, β -catenin is expressed in RMS and can promote differentiation in specific contexts; however, its precise role in tumor proliferation and survival remains unclear. Future research should focus on defining the conditions under which β -catenin modulation may offer therapeutic benefits and identifying combination strategies that can enhance the efficacy of β -catenin-targeted interventions in RMS.

Statement of purpose

MEF2A is a key transcription factor involved in skeletal muscle differentiation and myogenesis. Given that rhabdomyosarcoma (RMS) arises from skeletal muscle progenitor cells that fail to properly differentiate, investigating MEF2A could provide insights into the differentiation defects observed in RMS. MEF2A interacts with various signaling pathways, including the MyoD transcriptional network and the PI3K/AKT pathway, which are frequently dysregulated in RMS. Moreover, MEF2A is known to be modulated by epigenetic modifications, and its altered expression could contribute to RMS pathogenesis by sustaining an undifferentiated, proliferative state.

Similarly, β -catenin (CTNNB1), a key component of the Wnt/ β -catenin signaling pathway, has been implicated in cancer progression, stemness, and resistance to apoptosis. Aberrant activation of β -catenin signaling is a hallmark of several sarcomas, including RMS, where it is frequently upregulated and contributes to tumor aggressiveness and metastasis. Wnt/ β -catenin signaling is essential for normal skeletal muscle development, and its dysregulation in RMS could lead to uncontrolled proliferation and impaired differentiation.

Given that both MEF2A and β -catenin regulate key aspects of muscle cell fate and tumor progression, their interplay in RMS could reveal novel mechanisms underlying tumor initiation, maintenance, and therapeutic resistance. Targeting these molecules could provide new avenues for differentiation therapy, aiming to push RMS cells toward terminal differentiation and reducing tumorigenic potential. Therefore, studying MEF2A and β -catenin in RMS could enhance our understanding of the disease and contribute to the development of targeted and differentiation-inducing treatments. Considering the described possibilities, it can be hypothesized that in human Rhabdomyosarcoma, MEF2 and β -catenin protein regulatory complex is dysregulated, therefore, the main goal of this thesis is to analyze the protein-protein interactions of MEF2A and β -catenin and compare their interactomes between

RMS and normal skeletal muscle cells using LC-MS/MS nanobody technology and proteomics analysis.

2. Materials and Method

2.1 Cell culture

- i. **Human RH-30:** The base medium for this cell line is ATCC-formulated RPMI-1640 medium. 10% of fetal bovine serum is added to get the complete growth media and the recommended culture condition is 37°C, 95% humidity, 5% carbon dioxide (CO₂), and in a controlled incubator until the culture plates were 75-80% confluent.

A.

B.

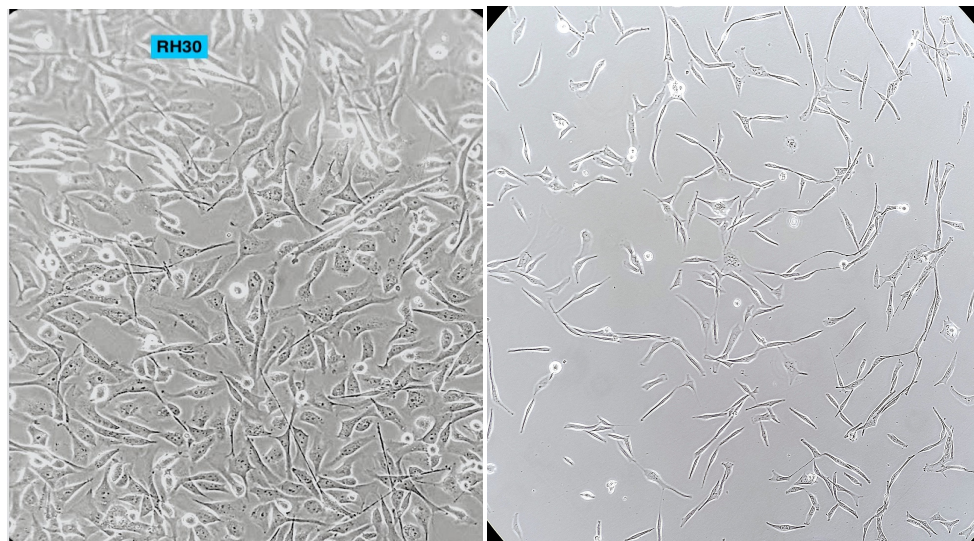


Fig 7: Morphological comparison of RH30 and RD cells under phase-contrast microscopy. A) Left panel: RH30 cells exhibit a densely packed, spindle-shaped morphology characteristic of rhabdomyosarcoma cells. The cells display prominent nuclei and extend cytoplasmic processes, with frequent overlapping and clustering, indicative of high proliferative activity. B) Right panel: RD cells display a more elongated, fibroblast-like morphology with a relatively sparse and organized distribution. The cells appear aligned and less confluent compared to RH30, reflecting their distinct lineage and lower proliferation under identical culture conditions.

- ii. **Human RD cell:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and 1% penstrap followed by incubation at 37°C in a controlled incubator until they were 75-80% confluent.

- iii. **Mouse C2C12 and HEK293T cells culture (growth condition):** Mouse C2C12 cells and HEK293T were obtained from the American Type Culture Collection (ATCC). They were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penstrap (penicillin-streptomycin Invitrogen, ThermoFisher). The cells were cultured under conditions of 95% humidity, 5% carbon dioxide (CO₂), and a temperature of 37 degrees Celsius in a controlled incubator.

N.B. No need to transfer the C2C12 to differentiation medium (DM) to induce myotube formation since we are comparing them to cancer cells, and they (RD&RH30) don't differentiate into myotubes. Therefore, we isolated the C2C12 cells when the growth plates were 70-80% confluent.

2.2 Cell harvesting:

To extract total protein, cells were harvested on ice using NP-40 solution supplemented with proteolytic inhibitors, including PMSF, sodium orthovanadate, and a general protease inhibitor. Prior to lysis buffer treatment, the cells were washed three times with chilled 1X PBS. After the final wash, the cells were scraped into 150 µL of 10% NP-40 lysis buffer. The resulting extracts were mixed thoroughly and incubated at 4 °C for 15 minutes in a cold room. Following this, the collected lysates were centrifuged at 4 °C at 10,000 RPM for 10 minutes. The supernatant, containing soluble proteins, was carefully collected.

2.3 Antibodies

Four antibodies were used initially, and they are:

- Anti-β-actin (mouse, monoclonal, #sc-47778 antibodies were purchased from Santa Cruz Biotechnology
- Anti MEF2A (Rabbit polyclonal antibody were produced in house with the assistance of the Animal Care Facility at York University, Toronto, ON, Canada)
- Anti-β-catenin

- MyoD (mouse, monoclonal, #2A5 antibodies were purchased from Developmental Studies Hybridoma Bank (DSHB)

2.4 Western blot analysis

Western blot was performed to identify and characterize the proteins from collected cell extracts. Both human embryonal rhabdomyosarcoma cell and alveolar rhabdomyosarcoma cell extraction were used to perform western blot. Mouse C2C12 cell extraction was used as a control. Protein samples (20-25 µg per well) along with 5 µL of pre-stained protein ladder (Bio-Rad, #PM008) were heated in 3x SDS loading buffer at 100°C for 10 minutes to denature. The samples were loaded onto 10% SDS-PAGE gels and electrophoresed at 100V for about an hour. Following separation, the gel was transferred onto a PVDF membrane (Millipore) using 1X transfer buffer and subsequently blocked with 5% blocking buffer for 1 hour on a room-temperature orbital shaker. Primary antibodies were diluted (1:1000) in 1% blocking buffer and applied to the membranes overnight at 4°C on a rocker. After quick washes with TBS containing 0.1% Tween® 20 (TBS-T), the blots were incubated with HRP- conjugated secondary antibodies diluted in 1% blocking buffer (1:2000) for 1 hour at room temperature. The blots were then washed three times with TBS-T, and the protein-antibody complexes were visualized by treating the blots with HRP substrate (Bio-Rad) and capturing the signals using the iBright CL1500 Imaging System (Thermo Fisher Scientific).

2.5 Plasmids

TOP-Flash and FOP-Flash were gifts from Randall Moon (Addgene plasmid #12456 & #12457) (Veeman et al., 2003). Renilla (pRL-Renilla) plasmid was purchased from Promega. The expression plasmid for full-length pMT2 MEF2A, 4xMEF2-Luc reporter gene constructs have been described by us previously (Du et al., 2008; Gordon et al., 2009; Perry et al., 2009). EYFP-MEF2A, EYFP-β-CATENIN. Details of the GFP constructs have been previously reported (Pagiatakis et al., 2017).

2.6 Gene reporter assays

To see the activity of endogenous level of MEF2A and β -catenin, reporter gene assay was performed in both RD, RH30 and C2 cell whereas C2 cell was used as a control. To see MEF2A activity level in both cancer cell line MEF2luc (has the MEF2A binding sites) reporter plasmid was co-transfected with PMT2 empty vector (as an internal control) and Renilla luciferase plasmid (as a positive control) and measure the activity in dual luciferase assay system.

Moreover, MEF2A plasmid was overexpressed in both RD and RH30 cells to see its effect on MEF2 transcription factor. Furthermore, to see the β -catenin activity, TOPflash (wt TCF binding sites) and FOPflash (mutant TCF binding sites) reporter plasmids were also transfected in both cancer cell lines along with PMT2 and Renilla reporter plasmid. The ratio of expression from TOPFlash to expression from FOPFlash (T/F) provides a precise measurement of canonical Wnt-specific transcriptional activity. After transfection, cells were rinsed three times with 1X PBS, followed by the addition of 100 μ L of 1X luciferase lysis buffer (20 mM Tris pH 7.4, 0.1% Triton X-100) to detach the cells by scraping, which were then collected into Eppendorf tubes. The tubes were placed on a shaker at 4°C for 10 minutes, and subsequently centrifuged at maximum speed in a 4°C microcentrifuge for 15 minutes. After lysis, 20 μ L of the cell lysate was transferred into test tubes for measuring enzymatic activity using Luciferase assay substrate (Promega, #E1501) or Renilla assay substrate (Promega, #E2820) on a luminometer (Lumat LB, Berthold). Luciferase activity was adjusted relative to Renilla activity within the same cell extracts and presented as a fold increase compared to the control.

2.7 GFP-Trap sample preparation for mass spectrometry (Affinity Purification)

We have used GFP-nanobody (GFP-nano trap) technology to pull-down GFP-fusion and their interacting proteins. GFP-nano trap consists of a GFP-binding protein called GBP based on a nanobody (a single antibody derived from *Lama alpaca*) coupled with a magnetic bead (9).

The requirements for the pull-down aspect are a mixture of protein extract from the Rhabdomyosarcoma cell lines and the availability of the bait protein which is a fusion of purified MEF2A or β -catenin tagged protein, in my case which were EYFP-MEF2A and EYFP- β -catenin and as a control I have used only GFP protein. MEF2A and β – catenin - EYFP fusion protein and GFP protein were overexpressed in HEK293 cell. After 24 hr of transfection the cell extracts were collected and then combined with GFP-nano trap magnetic beads.

The overexpression of bait proteins in HEK cells were confirmed by western blot. Then the bait protein complexes were incubated with the prey proteins (extract from Rhabdomyosarcoma cell lines) for a significant amount of time. Then the supernatant was removed, and the bait-prey complex proteins were sent to SPARC for liquid chromatography-mass spectrometry analysis.

2.8 Statistical Analysis

Unless otherwise noted, all experiments were conducted with N=4 biological replicates. Data is reported as MEAN $\pm$ SEM. Statistical analysis was conducted using one-way ANOVA, with subsequent pairwise comparisons performed using Tukey's Honestly Significant Difference (HSD) post hoc test for experiments involving more than two conditions. Statistical significance was defined as $p < 0.05$, with * indicating $p < 0.05$, ** indicating $p \leq 0.01$, and *** indicating $p \leq 0.001$. All analyses were conducted using GraphPad Prism 10, with specific experimental details provided under each figure.

3. Results

3.1 Identification of MEF2A and β -catenin proteins in Rhabdomyosarcoma cells

We performed western blot analysis to see the protein expressions in RMS cell lines and identified both MEF2A and β -catenin in both RD and RH30 cells. Figure 8 illustrates the expression of myogenic proteins in Rhabdomyosarcoma (RMS) cell lines, with C2C12 cells serving as a control. Western blot analysis was performed four times (N=4) to ensure the reproducibility and reliability of the results. The analysis shows that both MEF2A and β -catenin are significantly downregulated in the RMS cell lines compared to the normal C2C12 cells. These findings are crucial in understanding the differential protein expression patterns between RMS and normal cells, which will inform subsequent experimental investigations. The observed downregulation of MEF2A and β -catenin suggests a potential dysregulation of their associated protein networks, which are known to play key roles in myogenic differentiation. This dysregulation is further underscored by inactive MyoD activity in RMS cells. Since MyoD is a master regulator of muscle differentiation, its lack of expression strongly supports the hypothesis that RMS cells exhibit disrupted myogenic pathways. These results provide the basis for further exploration into the mechanisms driving this dysregulation and its implications for Rhabdomyosarcoma pathogenesis.

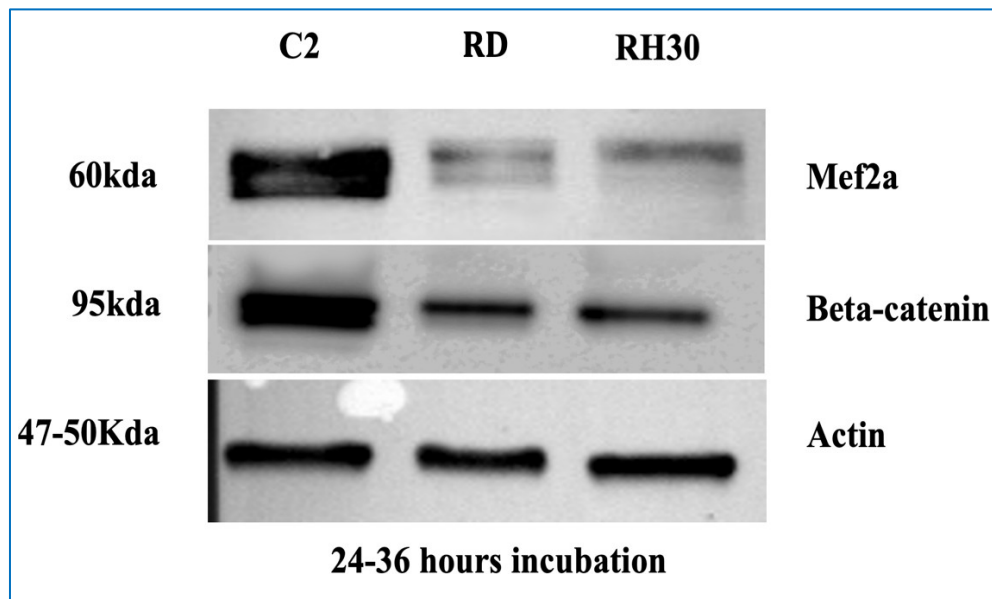


Figure 8: Western Blot Analysis of Myogenic Proteins. Western blot analysis was performed 15 times to assess the expression of Actin, MEF2A, β -catenin, and MyoD in C2C12, RD, and RH30 cell lines. Consistent results were observed across all experiments, confirming reliable expression patterns. Actin was used as a loading control, showing stable expression in all cell lines, which ensures even protein loading and transfer across samples

3.2 Divergent MEF2A Activity in Rhabdomyosarcoma Subtypes: Enhanced Transcriptional Response Despite Reduced Protein Expression in RD Cell

To evaluate MEF2A activity in cancer cell lines, we conducted a reporter gene assay in both RD and RH30 cells, using C2C12 myoblasts as a control. We employed a MEF2-luciferase (MEF2- luc) reporter plasmid, containing MEF2A-binding elements, along with a Renilla luciferase plasmid as an internal control for normalization within a dual-luciferase assay system. The experiment was performed under two conditions: the first employed only the MEF2-dependent reporter system with a pMT2 empty vector serving as a control, while the second involved MEF2A overexpression. As anticipated, RH30 cells exhibited reporter activity consistent with the Western blot analysis: under both conditions, MEF2-luc showed reduced or comparable activation levels relative to the C2C12 control, indicating diminished MEF2-dependent transcriptional activity in these cancer cells. Notably, in RH30 cells, MEF2A overexpression enhanced reporter activity significantly, confirming that MEF2A

positively regulates MEF2-luc activation (see fig 9).

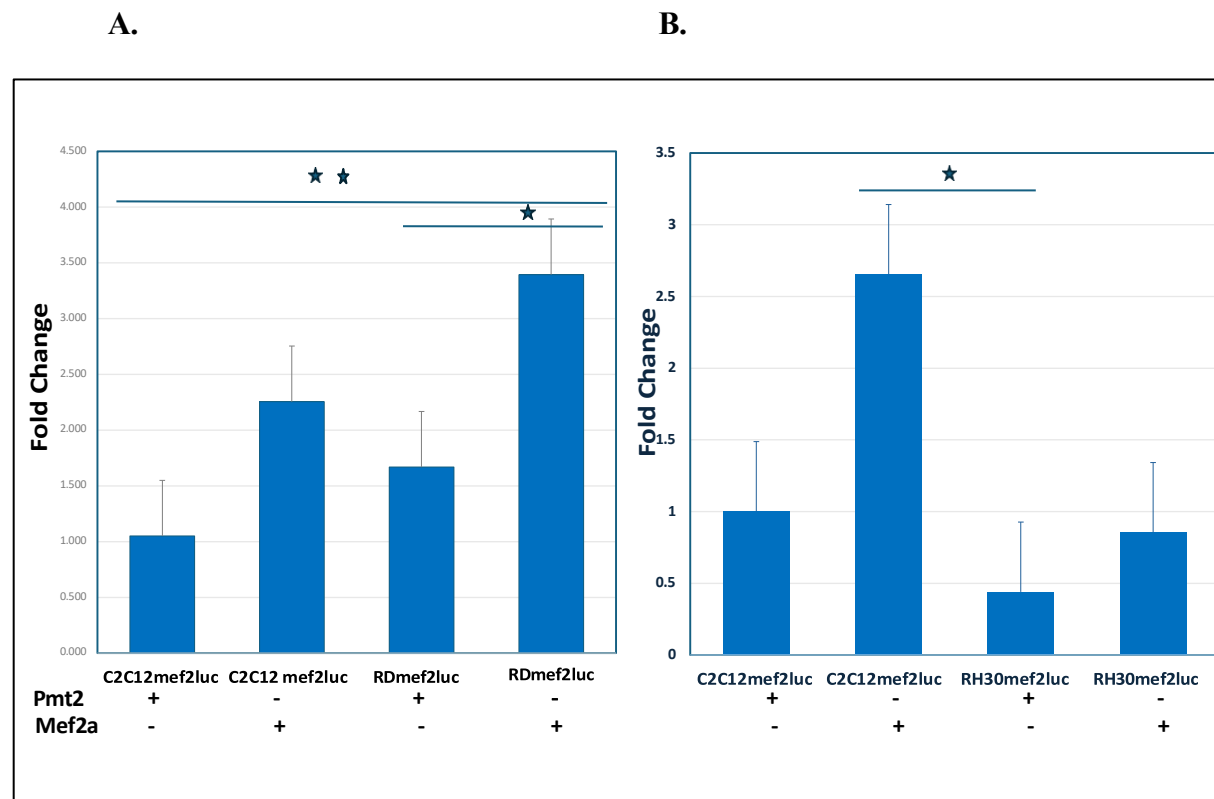


Figure 9: MEF2A activity level measurement by reporter assay in Rhabdomyosarcoma cell lines (RD and RH 30). A) MEF2 Luciferase Assay in C2, RH30:MEF2luc + Pmt2 Condition: C2 cells show higher baseline luciferase activity than RH30 and RH30*. MEF2luc + MEF2A Condition: MEF2A increases luciferase activity significantly in C2 cells, indicating strong MEF2 activation. RH30 and RH30* exhibit lower or comparable activity, suggesting reduced MEF2 activation. B) MEF2 Luciferase Assay in C2, RD: MEF2luc + Pmt2 Condition: C2 cells show similar luciferase activity as in Panel A, while RD cells exhibit even higher activity. MEF2luc + MEF2A Condition: Both C2 and RD cells show increased activity with MEF2A, with a stronger response in RD cells. Western Blot Analysis: Western blot shows higher MEF2A protein levels in C2 cells compared to RH30 and RD, supporting the luciferase results. Higher luciferase activity correlates with higher protein expression, especially under MEF2A overexpression. The data is derived from 4 independent experiments (N=4), performed in triplicates. Each bar represents the mean of four technical replicates. The error bars represent standard error of the mean +/- (SEM) and plus. Luciferase activity values obtained were normalized to Renilla activity in the same cell extracts and expressed as fold activation to the control. Statistical analysis was conducted with the Tukey's multiple comparisons test in one-way ANOVA using Microsoft excel was utilized to test for statistical significance; *** P < 0.001, ** P < 0.01, * P < 0.05.

Interestingly, the behavior of RD cells diverged sharply from expectations and was contradictory to the Western blot findings (see fig 8). Surprisingly, RD cells demonstrated

higher baseline MEF2-luciferase activity compared to C2C12 cells. Even more striking, the overexpression of MEF2A in RD cells led to a pronounced increase in luciferase activity, suggesting a heightened sensitivity of the MEF2 reporter to MEF2A in these cells. This enhanced transcriptional response of RD cells to MEF2A presents an intriguing paradox that warrants further exploration.

3.3 β -catenin activity is differentially regulated, revealing distinct mechanisms in

Rhabdomyosarcoma

Building upon our prior findings that highlighted the downregulation of β -catenin in Rhabdomyosarcoma (RMS) cells (fig8), we sought to explore its functional activity through a more precise assay system. To achieve this, we employed the TOPflash reporter gene, which features multimerized binding sites for TCF/LEF, serving as a robust indicator of β -catenin-mediated transcriptional activation. Conversely, the FOPflash reporter comprises mutated TCF/LEF binding sites, functioning as an appropriate negative control in this experimental setup. In this experiment RD cells exhibit some residual β -catenin activity when measured with the TOPflash reporter (fig 10A), indicating that this pathway is partially active. In contrast, RH30 cells displayed negligible β -catenin activity(fig10B), almost equivalent to the FOPflash control, suggesting that the pathway is largely suppressed in this cell line. The differing levels of β -catenin activity in RD and RH30 cells imply that these RMS subtypes engage the Wnt/ β -catenin pathway differently that influence their behavior, such as cell proliferation, differentiation, and tumor progression. The partial β -catenin activation in RD cells may contribute to features like enhanced differentiation potential, while the suppression in RH30 cells could correlate with aggressive tumor characteristics and a lack of differentiation. This insight not only underscores the complexity of RMS but also points toward potential avenues for targeted therapies.

3.4 Confirmation of bait protein overexpression in HEK 293 cell and Affinity purification- LC MS/MS analysis of the MEF2A and β -catenin interactomes in RMS cells

Since, we have distinct and interesting reporter assay results for both RD and RH30 cells in terms of MEF2A and β -catenin which support our idea of disrupted MEF2A and β -catenin regulatory protein networks in RMS cells. Their functional specificity, along with their selection of target genes, are shaped by their interactions with other proteins, including sequence-specific DNA-binding factors and components of transcriptional complexes. With this in mind, we hypothesized that uncovering the interacting partners of MEF2A and β -catenin would provide deeper insight into the transcriptional mechanisms driving their roles in cancer cells. Given our recent discovery of MEF2A and β -catenin as novel regulators of skeletal muscle gene expression, our primary objective was to explore their interactomes within RMS cells. To achieve this, we employed a cutting-edge proteomic approach, leveraging the power of NanoTrap technology to capture MEF2A and β -catenin-interacting proteins from cell lysates. First, we immobilized our ‘bait’ proteins—EYFP-MEF2A and EYFP- β -catenin—along with the EYFP control on beads. These ‘baits’ were produced in HEK293T cells and isolated using GFP- Nanotrap beads conjugated with GFP-binding protein (GBP) nanobodies. Afterward, the beads were incubated with RMS cell extracts from both RD and RH30 cells. Figure 11B, C illustrates the successful expression and immunoprecipitation of the GFP-tagged bait proteins employed in the GBP-Nanotrap. The overexpression of bait proteins in HEK293 cells were confirmed by western blot analysis (fig11A). A major benefit of this affinity-capture method is its capacity to retain the native configuration of the myogenic proteome by avoiding the overexpression of bait proteins within the target cells. This helps maintain the natural molecular balance of the cellular environment, preventing any artificial interference.

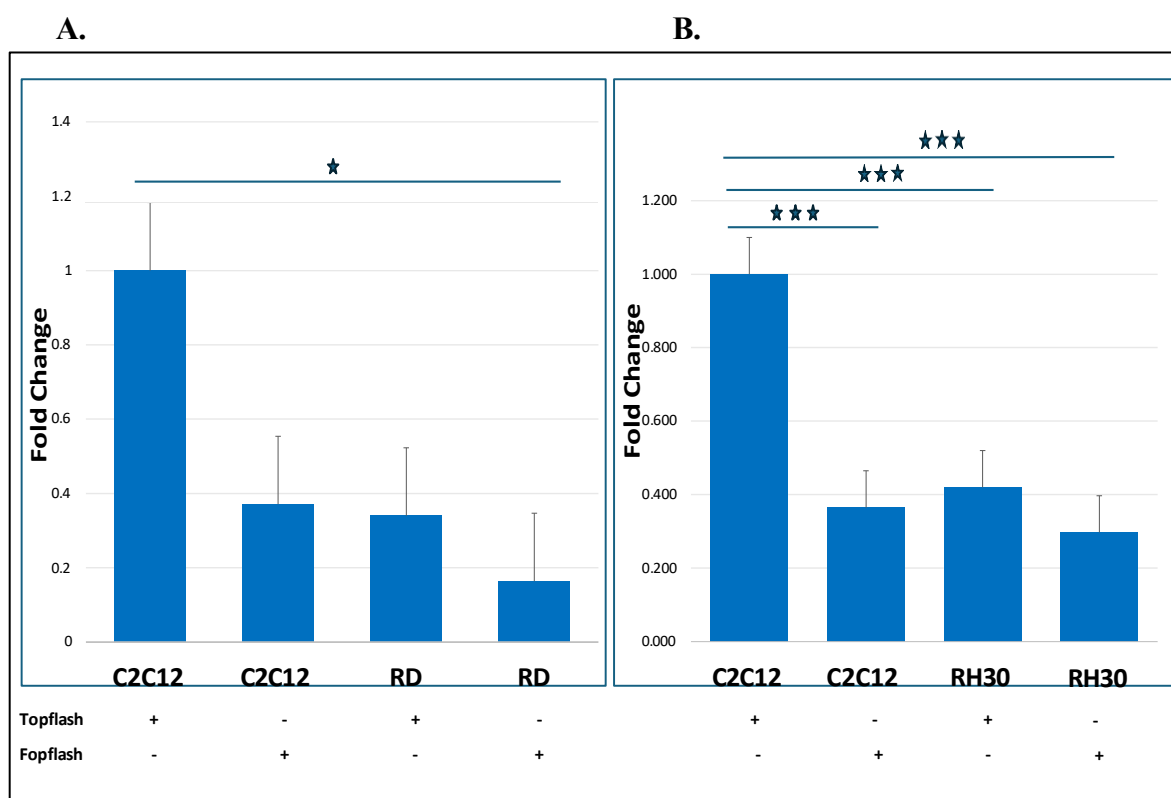


Fig 10: β -catenin activity level measurement by reporter assay in Rhabdomyosarcoma cell lines (RD and RH 30). A) Topflash +PMT2 in C2 cells shows a significant increase in reporter activity, indicating that PMT2 may enhance pathway activation in these cells. Fopflash +PMT2 in C2 cells shows minimal or no change in reporter activity compared to Topflash, suggesting the specificity of the PMT2 effect on the pathway. Topflash +PMT2 in RD cells shows a moderate increase in reporter activity, though not as pronounced as in C2 cells. Fopflash +PMT2 in RD cells demonstrates very little reporter activity, reinforcing the pathway-specific action of PMT2. B) Effects in C2 and RH30 cell lines: Topflash +PMT2 in C2 cells shows a significant increase in reporter activity, similar to Panel A, indicating consistent results across experiments. Fopflash +PMT2 in C2 cells again shows very low activity, emphasizing the pathway-specific effects of PMT2. Topflash +PMT2 in RH30 cells displays very low reporter activity, suggesting that PMT2 may not significantly activate the pathway in these cells or that RH30 cells are less responsive to PMT2. Fopflash +PMT2 in RH30 cells also shows low activity, which is consistent with the expectation that Fopflash should not produce significant reporter activity if the pathway is not specifically activated. The data is derived from 4 independent experiments (N=4), performed in triplicates. Each bar represents the mean of four technical replicates. The error bars represent standard error of the mean $\pm$ (SEM). Luciferase activity values obtained were normalized to Renilla activity in the same cell extracts and expressed as fold activation to the control. Statistical analysis was conducted with the Tukey's multiple comparisons test in one-way ANOVA using Microsoft excel was utilized to test for statistical significance; *** P < 0.001, ** P < 0.01, * P < 0.05.

The procedure for the affinity purification mass spectrometry (AP-LC MS/MS) method is depicted in Figure 4. The datasets were refined by eliminating non-specific interactions that did not achieve at least a threefold enrichment (EYFP-candidate protein/GFP spectra), using a cutoff based on the identification of known, well-characterized interacting proteins near this threshold in the study.

3.5 Mass Spectrometry Data Analysis

Following affinity purification, eluted protein complexes were subjected to high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) for proteomic profiling. Raw spectral data were processed using the SPARC platform, and protein identification was performed by matching against the UniProt human protein database. Trypsin was specified as the proteolytic enzyme, allowing for standard cleavage parameters. Peptide and protein identifications were filtered using a target-decoy approach with a false discovery rate (FDR) threshold to ensure high confidence in the detected interactions. Functional enrichment analyses, including Gene Ontology (GO) and pathway enrichment, were conducted using tools such as DAVID and Enrichr to interpret the biological significance of the identified protein networks.

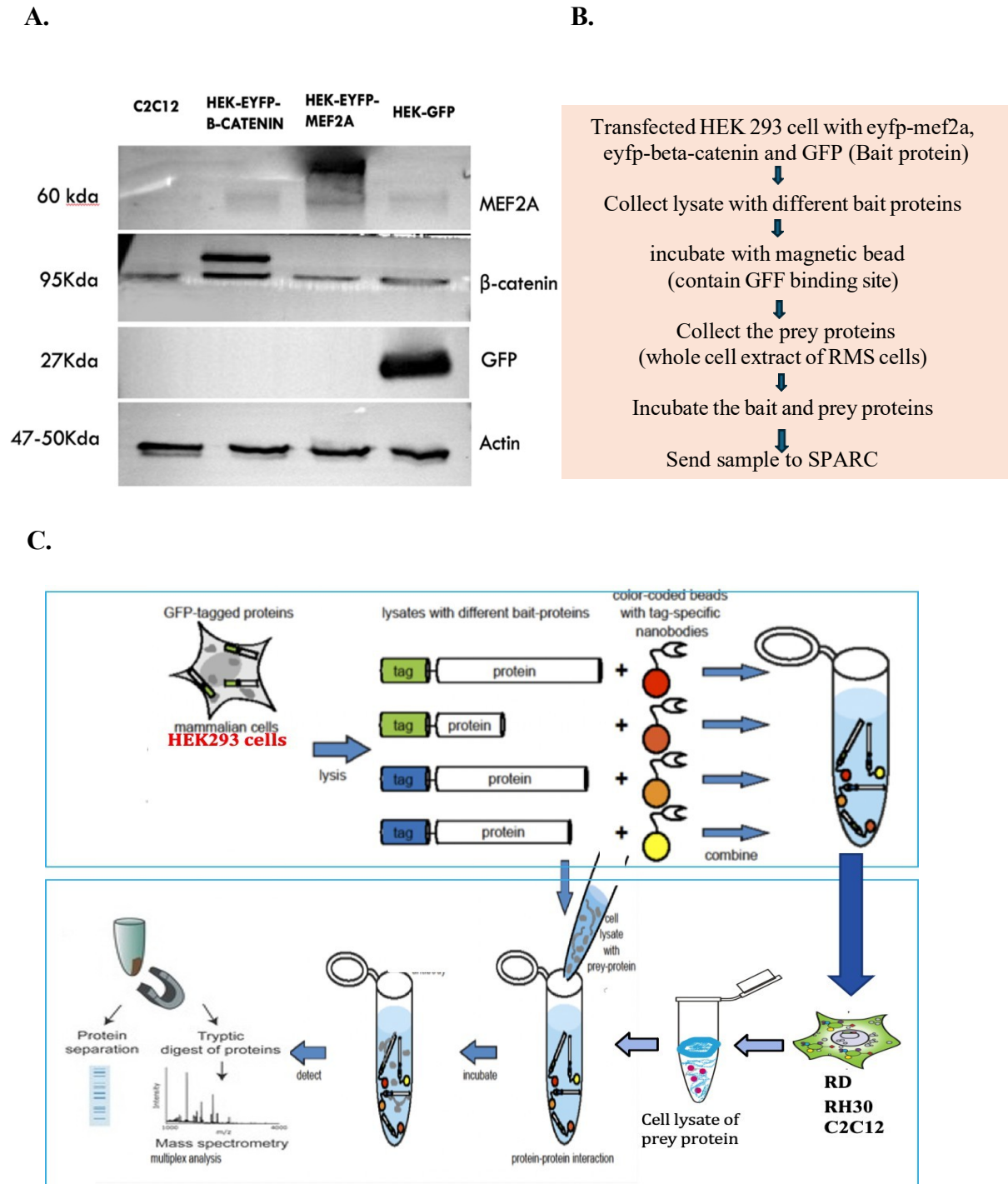


Figure 11: Confirmation of bait protein overexpression in HEK 293 cell by western blot and Affinity purification-LC MS/MS analysis. A.) The overexpression of bait proteins in HEK293 cells were confirmed by western blot analysis and this experiment was repeated for 3 times with consistent results. Actin served as a loading control, exhibiting consistent expression across all cell lines, ensuring equal protein loading and transfer. Double β -catenin band confirmed eyfp- β -catenin overexpression which has slightly higher molecular mass than only β -catenin itself. Similarly, in the 3rd lane MEF2A double band confirms eyfp-MEF2A overexpression in HEK 293 cells. B, C) LC-MS mass-spectrometry procedure.

From the raw SPARC output, we identified over 3,000 candidate protein interactions for both MEF2A and β -catenin in RD and RH30 cell lines. The data were converted into Excel files for downstream analysis. To refine these large datasets, we filtered out non-specific interactions by applying a threefold enrichment cutoff based on spectral count ratios (EYFP-fusion/GFP control). This threshold was validated by the consistent identification of known interacting partners near this cutoff. Only proteins that met or exceeded this enrichment criterion were retained as candidate interactors.

After filtering, we identified 54 candidate interactors in RD cells and 163 in RH30 cells for EYFP-MEF2A. Similarly, EYFP- β -catenin interactome analysis revealed 114 candidate interactors in RD cells and 89 in RH30 cells. In addition, we identified 250 (RD) and 280 (RH30) unique proteins that were exclusively detected in the MEF2A condition and not present in the GFP control. For β -catenin, 186 (RD) and 146 (RH30) unique proteins were identified. Notably, these datasets included well-characterized interactors such as HDACs and STAT proteins for MEF2A, and APC, CK1, GSK3 β , cadherins, Dishevelled (Dvl), FMRP, and α -catenin for β -catenin—consistent with established roles in Wnt signaling and myogenic regulation.

To further investigate the MEF2A and β -catenin interactomes, we employed bioinformatic tools for protein-protein interaction (PPI) network analysis, hub gene identification, and functional annotation. GO enrichment analysis across biological processes, molecular functions, and cellular components was performed using DAVID, ShinyGO, SRPlot, and custom scripts in R and Python. STRING and Cytoscape were used to construct and visualize PPI networks and to identify hub genes using multiple centrality measures.

The statistical significance of enrichment results was quantified using Fisher's exact test, with scores expressed as $-\log_{10}(\text{p-value})$ to estimate the likelihood of false-positive associations within the identified networks.

3.6 Comparison of MEF2A and β -catenin gene set among three cell lines

In our mass spectrometry analysis, we identified over 2000 protein interactions for both MEF2A and β -catenin in RD cells, as well as in RH30 cells. Moreover, we identified 250 and 280 unique genes for EYFP-MEF2A in RD and RH30 cells respectively. Similarly, for EYFP- β -catenin we have found 186 and 146 unique genes in RD and RH30 cells respectively. By applying a cut-off value of 3, we refined these extensive lists, yielding 54 potential interactome in RD cells and 163 in RH30 cells for the MEF2A protein. Unique proteins co-immunoprecipitated with EYFP-MEF2A and EYFP- β -catenin meeting the threshold criteria were automatically included in the list of candidates. Similarly, for the β -catenin protein, we identified 114 potential interactome in RD cells and 89 potential interactome in RH30 cells. Analysis of the interacting proteins of both MEF2A and β -catenin demonstrated the identification of well-known interacting partners of both. For MEF2A we have identified HDACs proteins, STAT proteins. Similarly, for β -catenin well known proteins that form a complex in Wnt signaling pathways such as cadherin, APC, CK-1, Dshlv, FMRP, alpha-catenin were identified. Table 1 illustrate the common genes among RD, RH30 and C2C12 for both MEF2A and β -catenin proteins.

To further elucidate the significance of these interactions, we compared our findings with the well- characterized interactome of MEF2A and β -catenin in C2C12 cells, which serve as our control or normal cell line.

A.

B.

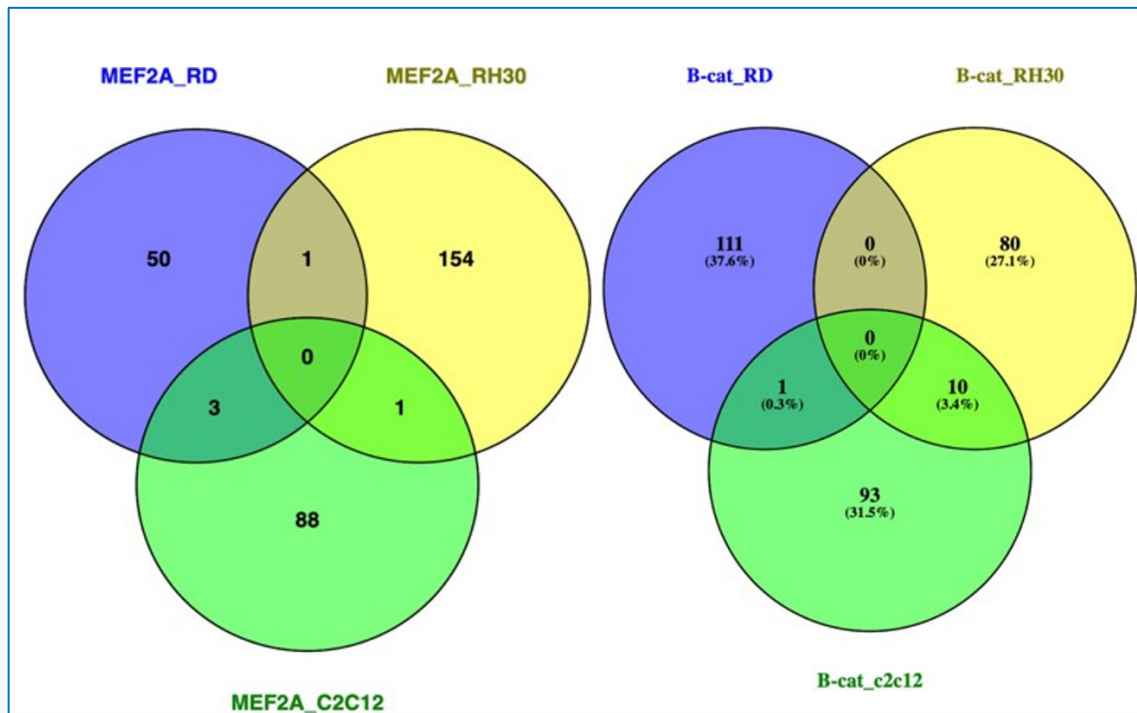


Table1: Common genes among cell lines

MEF2A		β-catenin	
RD&RH30	ERAL1	RD&C2C12	CSE1L
RD&C2C12	SART1, CAD, POLR1E	RH30&C2C12	LRPPRC, CTNNB1, EDC4, MYO18A, CNOT1, DNAJA1, CTNNA1, FLII PPP1R12A, MAP7D1
RH30&C2C12	FERMT2	RD&RH30	No gene

Figure 12: Comparison of MEF2A and β-catenin interactome gene sets across three cell lines. **A.** Venn diagram comparing the MEF2A interactome across RD (list 1), RH30 (list 2), and C2C12 (list 3) cell lines. **B.** Venn diagram comparing the β-catenin interactome across RD (list 1), RH30 (list 2), and C2C12 (list 3) cell lines. The Venn diagrams highlight the shared and unique genes within the interactomes of MEF2A and β-catenin among the three datasets, with C2C12 serving as a control cell line for both analyses. The table elucidates the common genes between data sets. These comparisons underscore the commonalities and differences in protein interactions across these cellular contexts, providing insights into their functional interplay.

Notably, only one protein was shared between RD and RH30 cells for the MEF2A protein, and there were no common proteins identified among the three cell lines. For beta- catenin, no

proteins were found to be common across all three sets of genes. However, one gene is common between RD and C2C12 cells whereas ten genes are common between RH30 and C2C12 cells. This analysis highlights the unique and cell line-specific nature of protein interactions, particularly in cancerous versus normal cellular environments.

3.7 Bioinformatic analysis of MEF2A and β -catenin in Rhabdomyosarcoma cells

To further explore the MEF2A and β -catenin interactome, we employed bioinformatics tools to analyze protein-protein interaction networks, identify hub genes, and uncover relevant annotations and pathways. Gene Ontology (GO) analysis, covering *biological processes*, *cellular components*, and *molecular functions*, was conducted using DAVID, Shiny GO, SR Plot, and programming tools to pinpoint enriched GO functions. The STRING database and Cytoscape software were utilized to visualize the interaction networks and identify hub genes from the protein datasets. The reliability of these analyses was assessed by calculating scores based on the negative logarithm of p-values (Fisher's exact test), which estimate the likelihood of false-positive associations within the networks.

3.7.1 MEF2A Transcription factor interactome in RMS cancer cell

MEF2A PPI network in RD cell

To shed light on the biological and molecular interactions within the MEF2A interactome in cancer cells, we constructed a protein-protein interaction network using the STRING database and the Cytoscape software platform. This network revealed high-confidence interactions among several key proteins, including RACK1, GNL2, GLUD1, HSPA1B, SERBP1, DDX39B, NOL6, SF3A3, and PRPF31. Notably, novel interactions were identified between the MEF2A gene and TRIM32, ACTR3, PRDX5, and SNRNP70 in RD cell. Additionally, strong interactions with several actin-binding proteins were observed. Figure 13 highlights the top enriched network, with high confidence score featuring the connected molecules in RD network.

Includes proteins like PPIH, PRPF31, and SNRNP70. GLUD1 Cluster: Includes proteins like PDHB, PRDX3, and ACLY. The central cluster, which includes proteins like "RACK1," "PSMC2," "SERBP1," and "SF3A3," suggests a core functional group, potentially involved in cellular processes like protein synthesis, RNA splicing, or cell signaling robust interaction networks.

MEF2A Protein-Protein network in RH30

In RH30 cells, we identified a set of 156 MEF2A-associated proteins after applying a specific cutoff threshold to our data. Utilizing this list of interactors, we constructed a protein-protein interaction (PPI) network (fig 14) to uncover how these proteins are functionally interconnected. This network analysis revealed several clusters of proteins that are highly interconnected, indicating potential functional relationships or co-regulation within these groups. One prominent example is the NUP214 cluster, which includes nucleoporins such as NUP98, NUP62, and NUP153. These proteins play vital roles in nucleocytoplasmic transport, a process that governs the movement of molecules between the nucleus and cytoplasm, with NUP98 and NUP62 being key components of the nuclear pore complex responsible for regulating the flow of RNA and proteins in and out of the nucleus.

The PPI network also highlights (fig 14) several central hub proteins that seem to play key roles in maintaining network integrity and connectivity. Among these central nodes are CAS3, SNRPG, CREBBP, NUP214, DDX52, and PSMC5. One protein of particular interest is DDX52, a DEAD- box RNA helicase, which has been implicated in various RNA processing functions, including ribosome biogenesis and RNA degradation. DDX52's involvement in cancer progression is of special interest, as it has been shown to regulate gene expression, influence cell proliferation, and control apoptosis, making it a potential contributor to tumorigenesis through its impact on cellular growth pathways.

In addition to these, several other key proteins in the network are well-documented inhibitors

of myogenic differentiation, which is the primary focus of our study. These include HDAC, RUNX1, YES1, and the CBX family of proteins. Particularly HDAC1, is a well-known interactor of MEF2A and plays a critical role in repressing the transcription of pro-myogenic genes. It achieves this by removing acetyl groups from histones, which results in a condensed chromatin structure that is less accessible to transcriptional machinery, thereby keeping the target genes in a repressive state and blocking myogenic differentiation. RUNX1, another important protein in this network, functions as a transcription factor that inhibits myogenic differentiation by preventing the premature differentiation of myoblasts. RUNX1's ability to regulate gene expression during early muscle development ensures that myoblasts do not differentiate before their proper developmental timepoint. Meanwhile, YES1, a member of the Src family of tyrosine kinases, is also implicated in blocking myogenic differentiation. When overactive, YES1 acts as a negative regulator by phosphorylating key downstream targets that are essential for the initiation of muscle cell differentiation.

Taken together, these findings suggest that the MEF2A interactome in RH30 cells includes a range of proteins that are not only central to critical cellular processes like nucleocytoplasmic transport and RNA processing but also to the inhibition of myogenic differentiation, either through chromatin remodeling or signaling pathways. The involvement of proteins like HDAC1, RUNX1, and YES1 in blocking muscle cell differentiation underscores the complexity of regulatory networks that prevent premature or inappropriate muscle formation, which could be an important consideration in understanding tumor progression in muscle-derived cancers like rhabdomyosarcoma.

3.7.2 Network analysis of β -catenin in RD and RH30 cells

β -catenin Protein-Protein network in RD cell

The multifunctional protein β -catenin operates as a transcriptional coregulator in the nucleus, modulating the activity of DNA-binding transcription factors, and as a component of

adherens junctions in the cytoplasm within the Wnt signaling pathway. Disruptions in this regulatory network are often implicated in tumorigenesis. Our previous findings indicated that β -catenin is downregulated in both RMS cell lines.

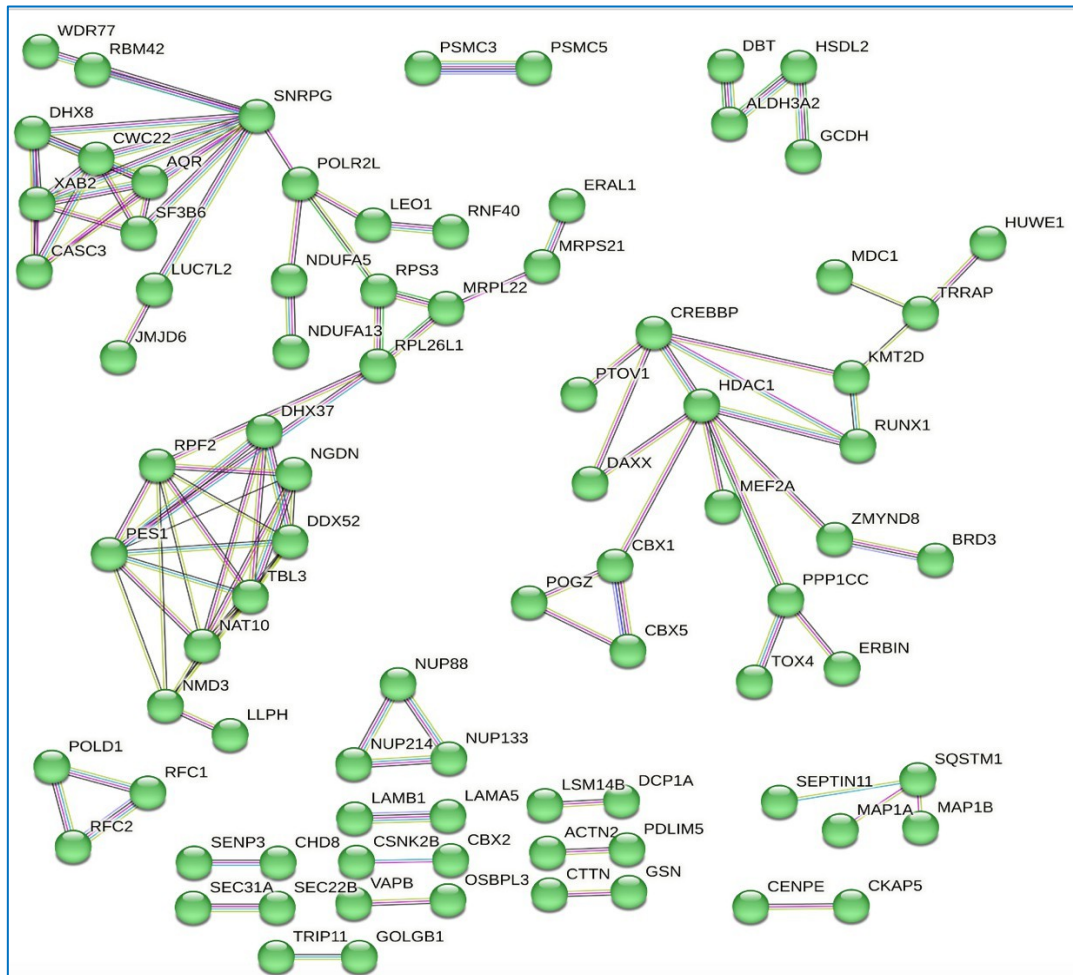


Figure 14: MEF2A protein-protein network in RH30 cell. The network consists of various proteins (nodes) and their interactions (edges). Key central proteins such as SNRPG, POLR2L, HDAC1, and PES1 seem to play significant roles in this network. There are several clusters indicating functional modules, like **PPP2R1A**, **DDX** and **NUP214** cluster. The central nodes exhibit a high degree of connectivity, reflecting robust and intricate interaction networks. The connections visualized in the figure were generated using the STRING database, employing a high confidence threshold (confidence score: 0.700). This threshold ensures the inclusion of interactions supported by reliable evidence while maintaining a comprehensive view of the interactome.

From the analyzed mass spectrometry data and the regulatory network, we constructed, we identified several novel proteins in RD cells that are absent from the β -catenin regulatory network in normal muscle cells. Figure 15 depicts the protein-protein interaction network in RD cells, highlighting novel interactions with highly connected proteins, including BRCA1, DICER, ROCK1, NUP35, CAPZA2, EIF4E, and MRPS15. Most of these proteins are unique when compared to the β -catenin interactome in C2C12 cells.

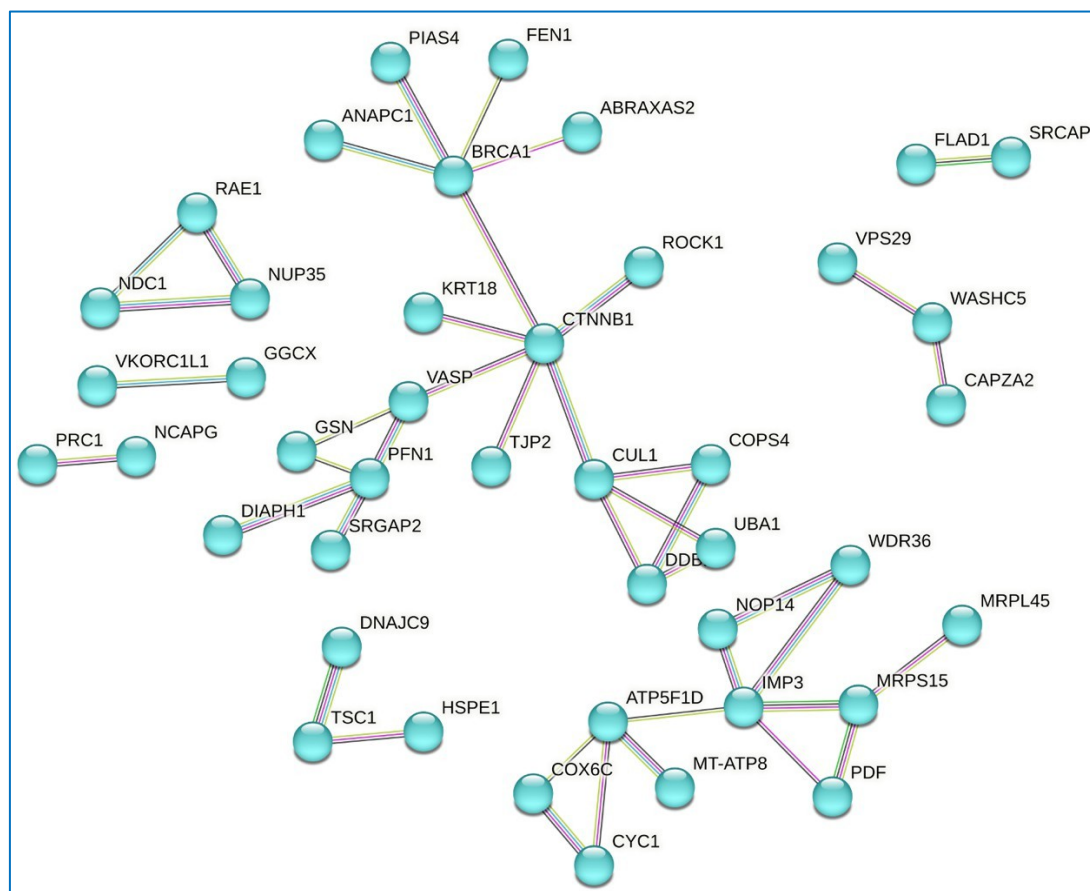


Figure 15: β -catenin PPI network in RD cell. The network consists of various proteins (nodes) and their interactions (edges). Key central proteins such as BRCA1, ROCK1, NUP35, PFN1 and seem to play significant roles in this network. There are several clusters indicating functional modules like **ROCK1**, **CUL1**, and **BRCA1** cluster. The central nodes exhibit a high degree of connectivity, reflecting robust and intricate interaction networks. The connections visualized in the figure were generated using the STRING database, employing a high confidence threshold (confidence score: 0.700). This threshold ensures the inclusion of interactions supported by reliable evidence while maintaining a comprehensive view of the interactome.

Notably, several transcription factors—such as MEIS1, TAF3, and BBX—were identified in the RD network. Both MEIS1 and BBX are involved in regulating gene expression through DNA binding and transcriptional activation, while TAF3 plays a crucial role in initiating transcription by RNA polymerase II, influencing various developmental processes.

In addition to transcription factors, we identified several well-known oncogenes in this network, including PRAME, RRAS2, and BRCA1. These oncogenes are absent in the C2C12 interactome. PRAME is primarily expressed in tumor cells, with limited expression in normal tissues such as the human testis. RRAS2 and BRCA1 are well-documented oncogenes associated with breast and ovarian cancers.

β-catenin Protein-Protein network in RH30 cell

RH30 is a highly aggressive Rhabdomyosarcoma (RMS) cell line. Network analysis of this cell line reveals strong connectivity among central nodes, indicating intricate protein interaction networks with several functional clusters representing distinct cellular processes. Notable central proteins include XPO1, CDH2, RANBP2, and AURKB.

The network features clusters such as the AURKB cluster, which contains proteins associated with cell cycle regulation and mitosis, including MKI67 and UHRF1. RPLP1 Cluster: Contains ribosomal proteins and factors involved in translation (e.g., EIF3E, EIF3B). CCT2 Cluster: Involves chaperonins and proteins associated with protein folding and assembly (e.g., TCP1, CCT3).

Within this network, STAT1—a well-characterized transcription factor involved in cytokine signaling—emerges as a key regulator with known inhibitory effects on myogenic differentiation. Additionally, transcriptional regulators PURB and ZNF768 were identified, further highlighting the altered regulatory landscape.

Several proteins in this network, including XPO1, RANBP2, and FGFR4, exhibit close associations with various cancers. XPO1 functions as an oncogene due to its role in

promoting tumorigenesis and interacting directly with the p53 tumor suppressor. RANBP2 (Ras-binding protein) facilitates cancer progression, while FGFR4 is implicated in both tumor progression and metastasis. These regulatory proteins are distinct from those typically found in the β -catenin interactome of normal muscle cells, underscoring a shift in signaling dynamics within RMS. The altered protein interaction network supports the hypothesis of disrupted β -catenin/Wnt signaling pathways in RH30 cells. Figure 16 illustrates the β -catenin protein interaction network within RH30 cells.

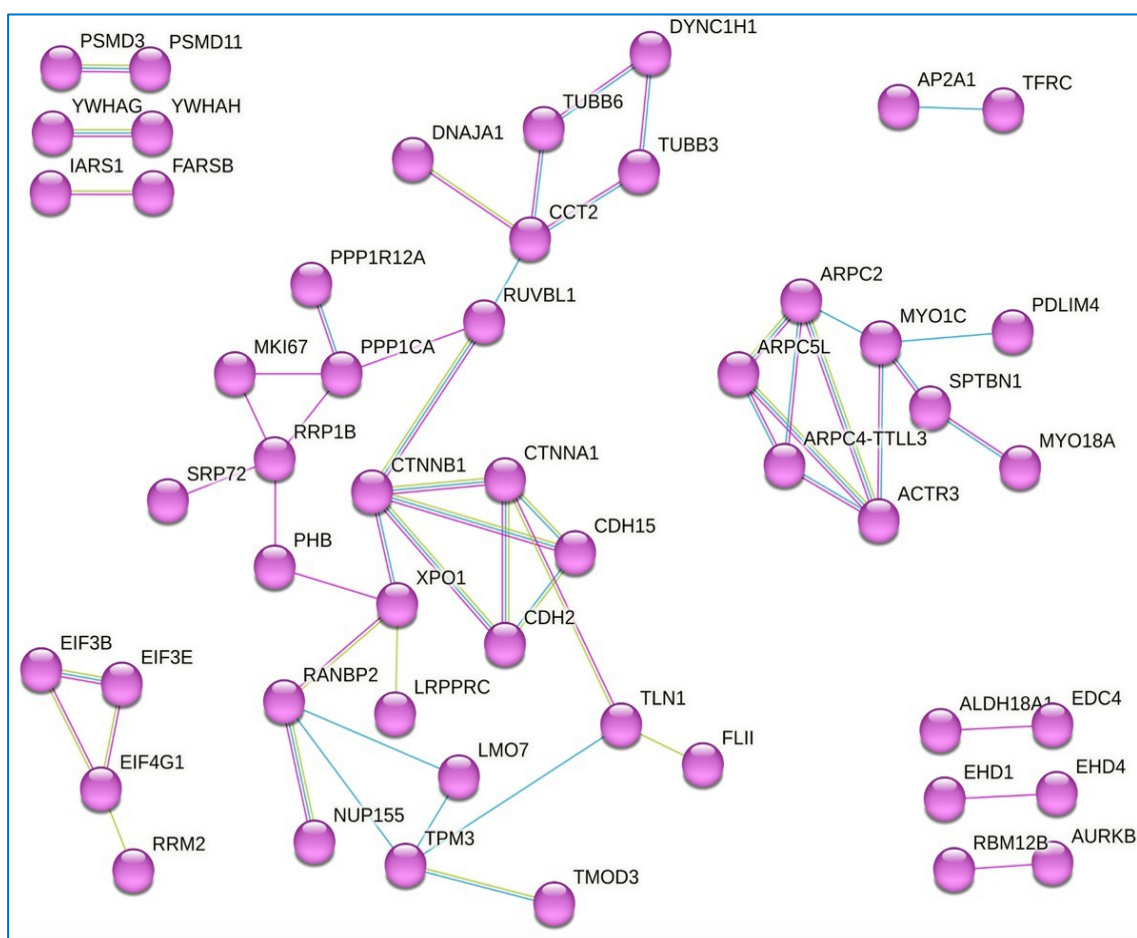


Figure 16: β -catenin PPI network in RH30 cell. The network consists of various proteins (nodes) and their interactions (edges). Key central proteins such as XPO1, RANBP2, CDH2, AURKB and CCT2 seem to play significant roles in this network. There are several clusters indicating functional modules like **ACTR3**, **EIF3E**, **CCT2**, and **AURKB** cluster. The central nodes exhibit a high degree of connectivity, reflecting robust and intricate interaction networks. The connections visualized in the figure were generated using the STRING database, employing a high confidence threshold (confidence score: 0.700). This threshold ensures the inclusion of interactions supported by reliable evidence while maintaining a comprehensive view of the interactome.

3.7.3 HUB genes identification and analysis

Hub genes, which serve as highly interconnected nodes within biological networks, play a pivotal role across diverse pathways and biological processes. Defined by their placement in the upper decile of connectivity metrics, these genes orchestrate the network's functionality. They are often regarded as genetic stabilizers, attenuating the impacts of mutations in other genes and maintaining system robustness. Consequently, hub genes are generally more vital to survival than their lesser- connected counterparts. Disruptions to these critical nodes can precipitate network collapse. In this investigation, we identified hub proteins utilizing five distinct analytical approaches: betweenness, closeness, degree, MCC, and MNC, as illustrated in Figures 17, 18,19 and 20 ((a) through (g)) by using cytohubba plugin and cytoscape software.

3.7.3 Identification of HUB Genes for MEF2A interactome

RD cell

Hub proteins often play crucial roles in cellular functions and signaling pathways. We got the hub genes rank lists for each method from cytoscape and using the hub gene rank lists for 5 distinct method we built a protein-protein interaction network and a Venn diagram to see and compare the common genes. In the network we can see how the top 10 ten genes are interconnected with one another; the color intensity refers the highest scoring gene (red to yellow). From this diagram (fig 17) we were able to see the common proteins among the different methods of hub genes. We found 2 genes are common in all the hub genes and they are GNL2 and DDX39B while RACK1 scores for the most central hub gene.

RH30:

Similarly, for RH30 interactome by using the rank list we build protein networks for top 10 genes in five different methods, and we compared them in a Venn diagram. Figure 18 highlights the hub gene networks for all the methods and the rank list for the hub gene based

on the score. The Venn diagram shows among all five methods only SNRPG is common gene while HDAC1 scores for strongest hub genes among all.

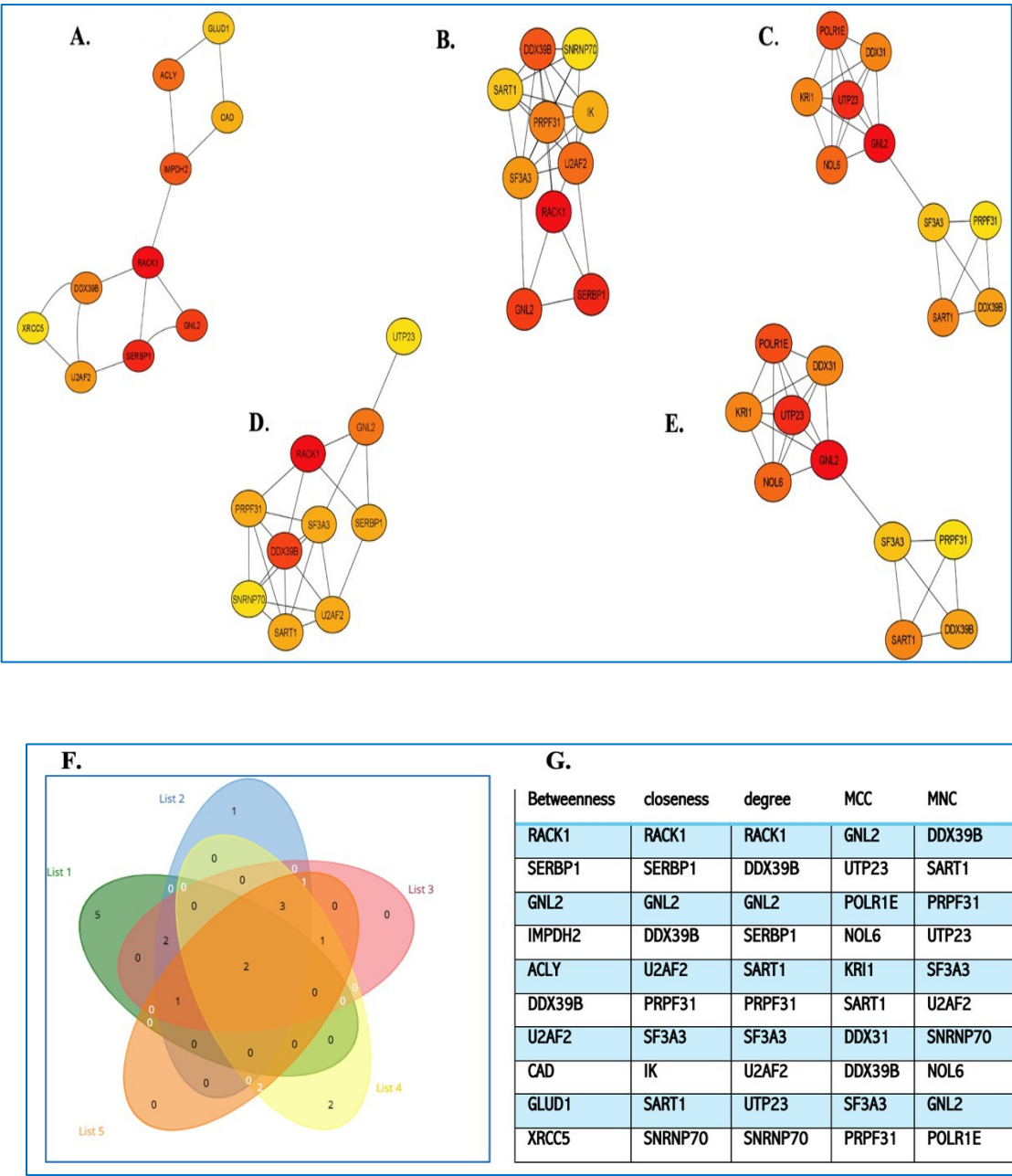


Figure 17: Visualization of the hub genes using five different calculation methods in RD cell for MEF2A protein.: (a) betweenness, (b) closeness, (c) degree, (d) maximal clique centrality (MCC), and (e) maximum neighborhood component (MNC). Here, the PPI network strength depends on color Intensity. Deep Color means the strongest gene. f) Venn diagram to identify significant top 10 hub genes using five intersecting algorithms. Two hub genes were found to be shared in the five methods. g) hub gene rank lists for the five distinct methods.

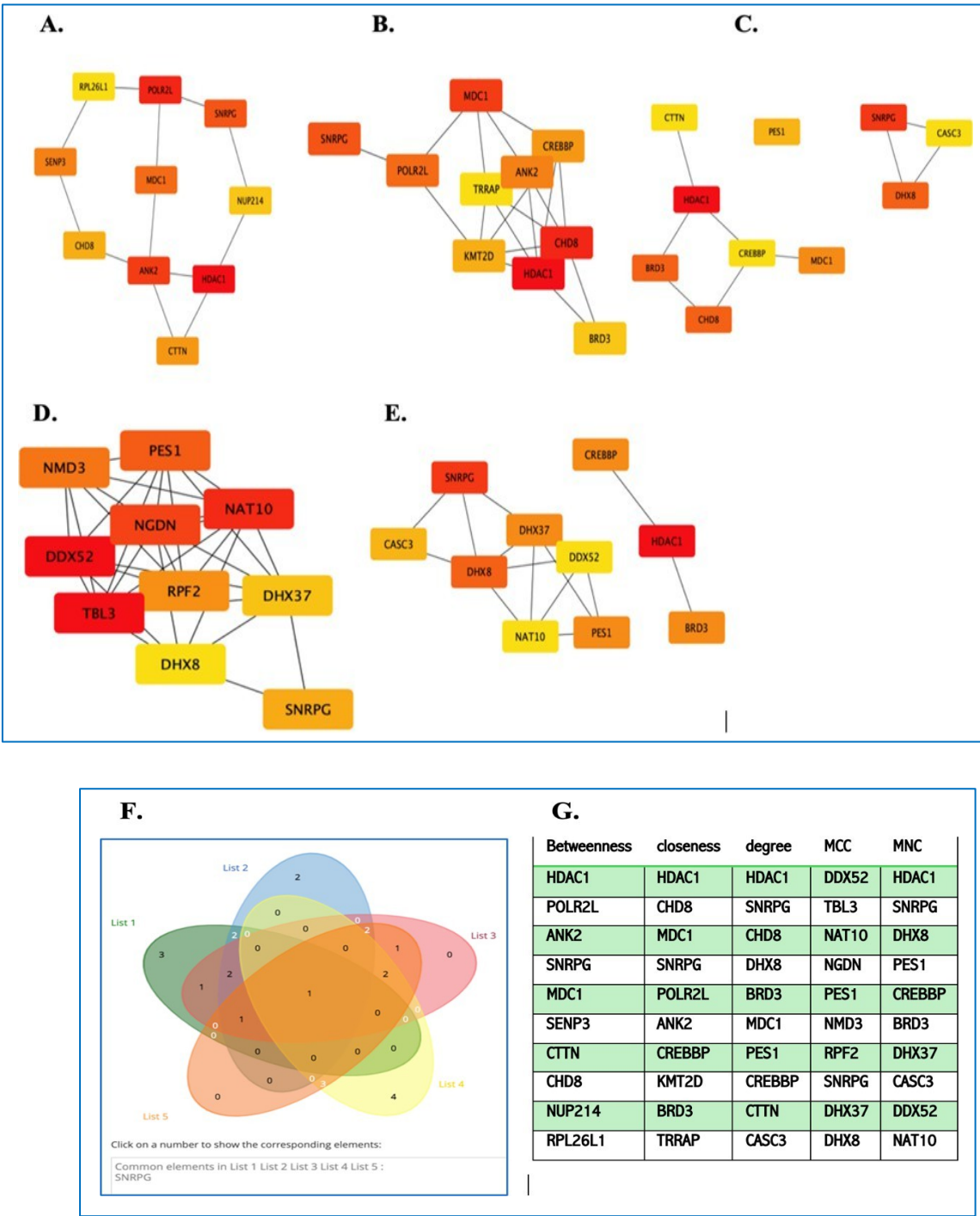


Fig 18: Visualization of the hub genes from the protein-protein interaction (PPI) network MEF2A in RH30 cell using five different calculation methods: (a) betweenness, (b) closeness, (c) degree, (d) maximal clique centrality (MCC), and (e) maximum neighborhood component (MNC). Here, the PPI network strength depends on color Intensity. Deep Color means the strongest gene Visualization of the hub genes using five different calculation methods in RH30 cell for MEF2A protein. Venn diagram to identify significant top 10 hub genes using five intersecting algorithms (betweenness, closeness, degree, MCC, and MNC). One hub gene was found to be shared in the five methods.

3.7.4 Identification of HUB Genes of β -catenin interactome in RD and RH30 cells

RD:

In terms of β -catenin we applied the same methods to find out the top 10 hub genes from the interactome list and found there are two common genes shared by all five individual methods. The genes are BRCA1 and DIAPH1. BRCA1 is a well-known oncogene found in several cancers while DIAPH1 is associated with syndromic microcephaly, blindness, and early onset seizures. However, among all these genes EIF4E scores for the strongest hub gene (fig 19).

RH30:

In RH30 cell 6 genes are common among all five different methods and the genes are: CTNNB1, XPO1, CCT2, PHB, AURKB and PHGDH. Figure 20 represents the hub gene networks and the Venn diagram with the gene rank list for RH30 hub gene analysis. The Strongest gene among the network are CTNNB1, XPO1 and CCT2 whereas they are all linked to several diseases like cancer.

3.8 Gene Ontology

GO annotations are also invaluable for predicting potential functions of lesser-known proteins within a network through association with well-characterized proteins, facilitating a better understanding of each protein's role in the network. Gene Ontology (GO) plays a vital role in analyzing protein-protein interaction (PPI) networks by providing structured annotations that describe the biological processes, molecular functions, and cellular locations of proteins. For our research, GO allows us to group interacting proteins based on shared functions, uncovering the biological processes or pathways that are likely influenced by these interactions.

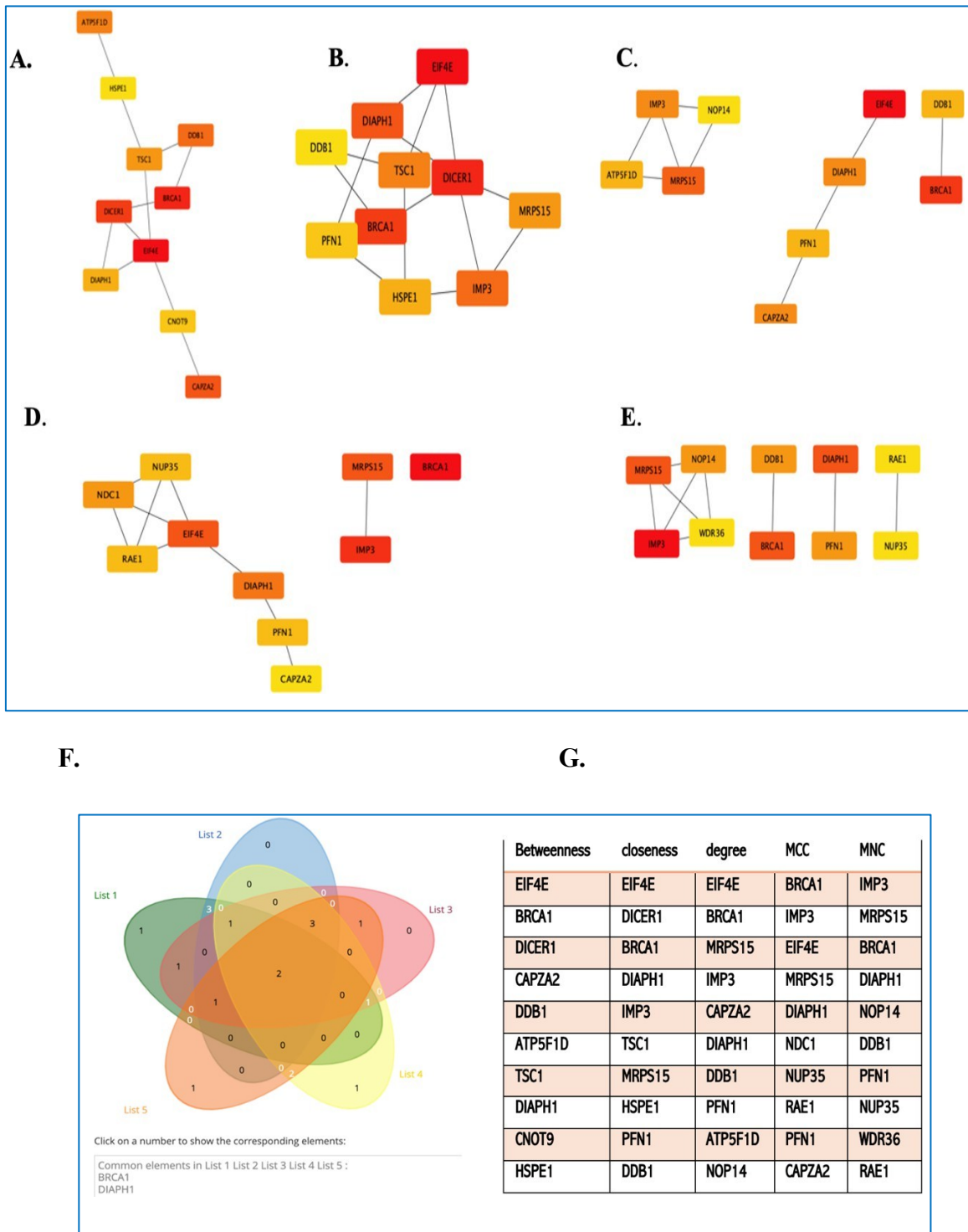


Figure 19: Visualization of the hub genes from the protein-protein interaction (PPI) network of β -catenin in RD cell using five different calculation methods: (a) betweenness, (b) closeness, (c) degree, (d) maximal clique centrality (MCC), and (e) maximum neighborhood component (MNC). Here, the PPI network strength depends on color Intensity. Deep Color means the strongest gene. (f) Visualization of the hub genes using five different calculation methods in RD cell for MEF2A protein. Venn diagram to identify significant top 10 hub genes using five intersecting algorithms (betweenness, closeness, degree, MCC, and MNC). Two hub genes were found to be shared in the five methods.

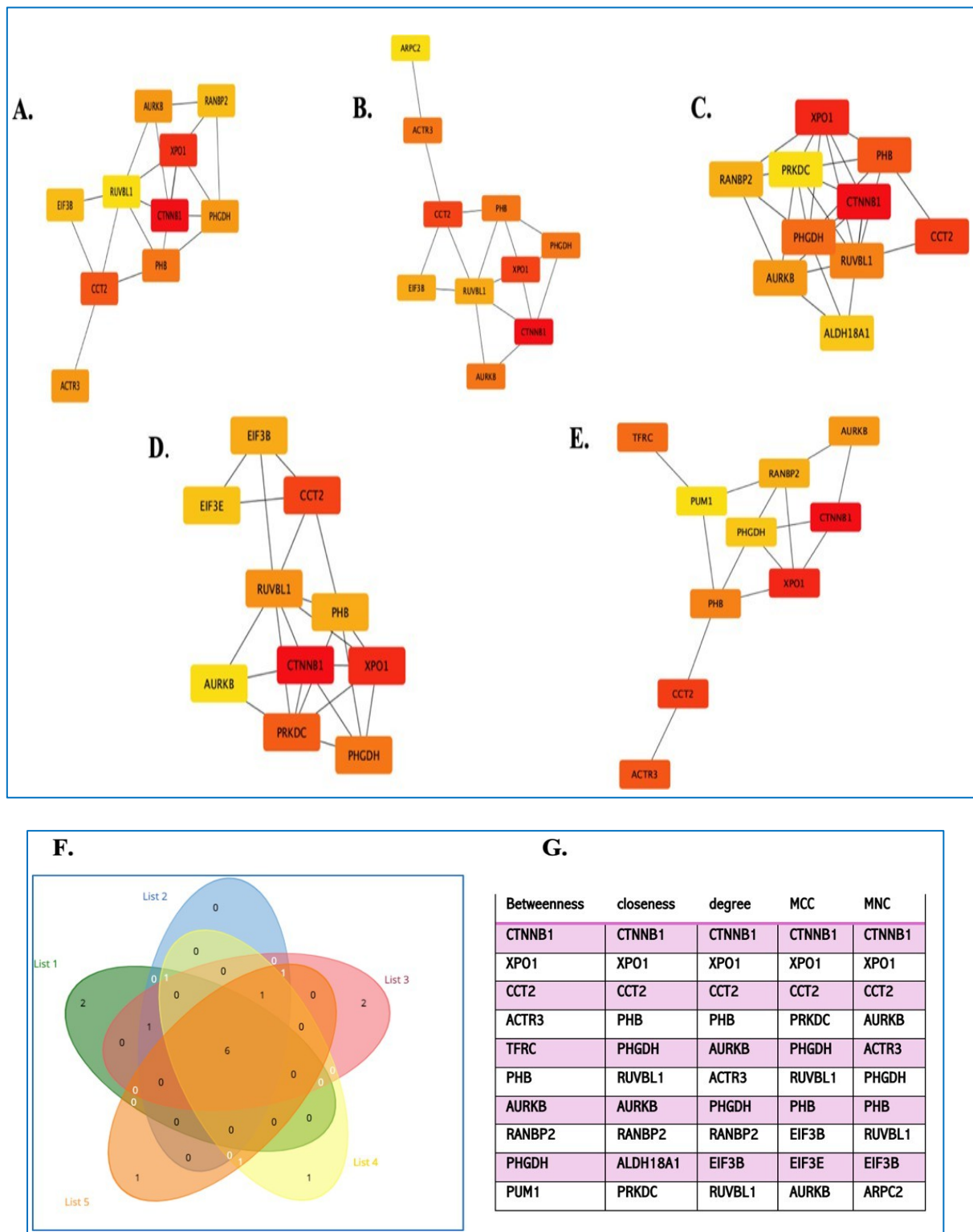


Figure 20: Visualization of the hub genes from the protein-protein interaction (PPI) network of β -catenin in RH30 cell using five different calculation methods: (a) betweenness, (b) closeness, (c) degree, (d) maximal clique centrality (MCC), and (e) maximum neighborhood component (MNC). Here, the PPI network strength depends on color Intensity. Deep Color means the strongest gene Visualization of the hub genes using five different calculation methods in RD cell for β -catenin protein. (f)Venn diagram to identify significant top 10 hub genes using five intersecting algorithms (betweenness, closeness,

degree, MCC, and MNC). Six hub genes were found to be shared in the five methods. (g) Hub gene rank list for all five methods.

Enriched GO terms within our PPI networks, we highlighted key proteins and pathways that may play critical roles in specific cellular processes or disease mechanisms specially cancer formation. There by, we used GO for interpreting the functional context of MEF2A and β -catenin PPI networks and understanding complex biological systems. Our GO bar graph is generated using SR plot based on the enrichment scores and p-values that we got from the David Bioinformatics. The higher Enrichment scores are accompanied by a lower p-value of annotation terms (p-value <0.1). The y- axis shows the significance level of each GO term as $-\log_{10}(\text{p-value})$, indicating the degree of enrichment, with higher bars representing more significant terms. Each bar represents the significance of enrichment for a specific GO term, with the y-axis showing the p-value.

3.8.1 Gene Ontology of the MEF2A interactome in RD cell

To gain deeper insight into the biological functions of the MEF2A interactome, Gene Ontology (GO) analysis was performed to categorize the identified proteins. This analysis highlighted the top ten enriched GO terms across three major categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). These enriched terms offer a clearer understanding of the molecular roles, functional pathways, and subcellular localization associated with MEF2A and are illustrated in Figure 21. This GO analysis highlights that the MEF2A interactome in RD cells is significantly involved in RNA processing, binding, and splicing, with a predominant nuclear localization. The enrichment in RNA-binding and spliceosomal complex-related terms implies that MEF2A may play a role in post-transcriptional regulation, potentially influencing mRNA stability and translation.

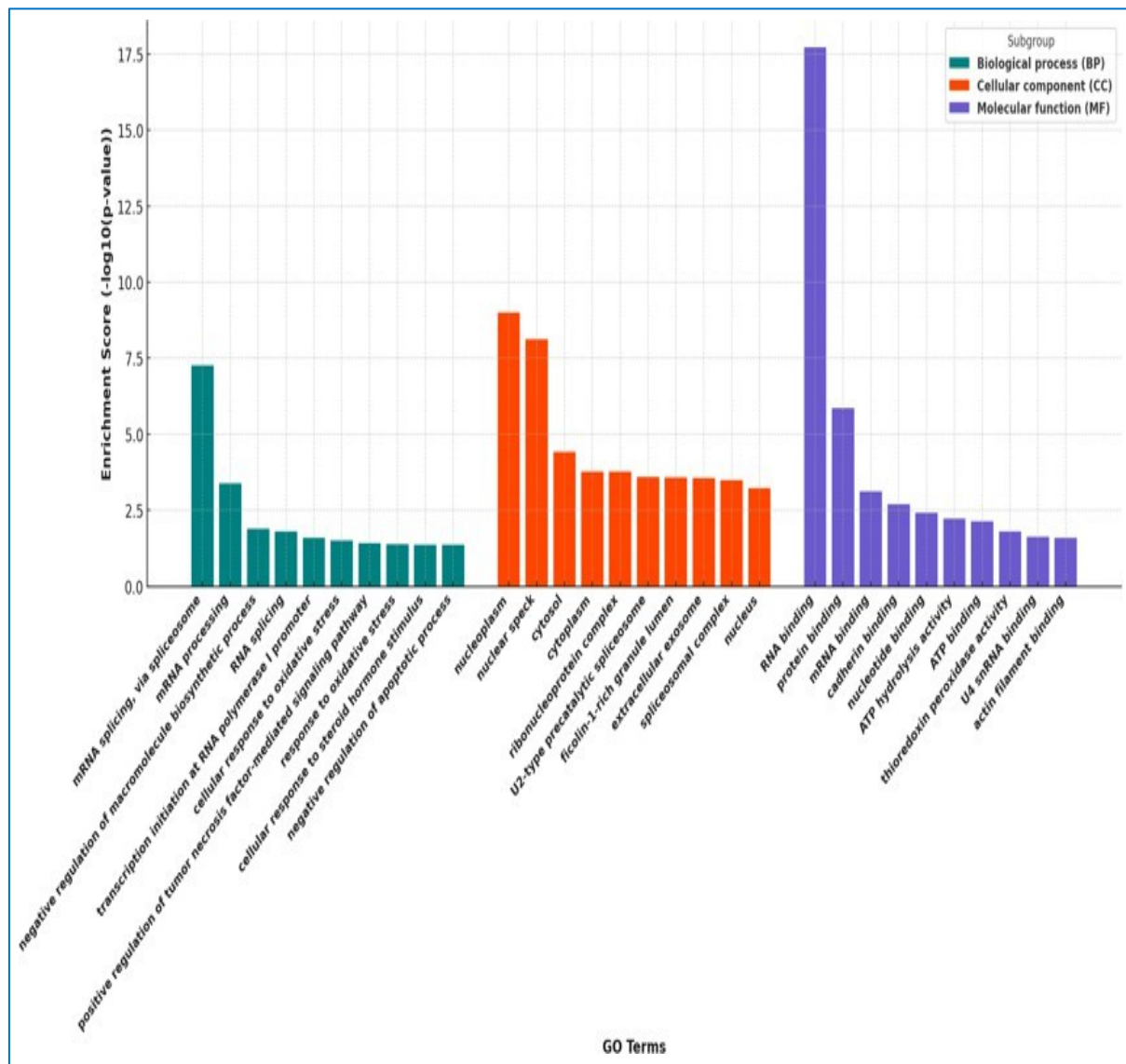


Figure 21: Top ten enriched Gene ontology (biological process, cellular component and molecular function) of MEF2A protein interactomes in RD cell. This image represents the top ten enriched Gene Ontology (GO) terms for MEF2A protein interactomes in RD cells, divided into three categories: Biological Process (BP-green bar), Cellular Component (CC-red bar), and Molecular Function (MF-purple bar). The enrichment score for each GO term is shown on the y- axis as the negative logarithm of the p-value ($-\log_{10}(\text{p-value})$), with higher values indicating greater statistical significance. The enrichment score for each GO term is shown on the y-axis as the negative logarithm of the p-value ($-\log_{10}(\text{p-value})$), with higher values indicating greater statistical significance.

This provides insights into how MEF2A might impact cellular differentiation processes in the RD cell context, particularly through its role in gene expression regulation at the RNA level. The MEF2A interactome's focus on RNA-related functions in both the nucleus and cytoplasm suggests a

multi-layered regulatory role, impacting both gene expression and translation, which aligns with the characteristics of tumor cells that evade differentiation. (Small, E. M. et al., 2011) (Jian Han et al., 2022).

After GO analysis we have also found that some genes like DDX39B, PPIH, PRPF31, SRSF4, SNRNP70, SART1, SF3A3, GRSF1, IK, RBM15B, U2AF2 and ADAR are mainly involved in RNA level processing and post-transcriptional modification. While genes like HSPA1B, PRDX3, PRDX5, TRIM32 are mainly associated with negative regulation of apoptotic process, positive regulation of tumor necrosis factor-mediated signaling pathway and response to oxidative stress. POLR1G, XRCC5, HSPA1B, RACK1, MIF genes are associated with cancer formation.

3.8.2 Gene Ontology of the MEF2A interactome in RH30 cell

This Gene Ontology (GO) analysis of the MEF2A interactome in RH30 cells reveals enriched terms across three main categories: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF), highlighting the roles and localizations of MEF2A-associated proteins illustrated in figure 22. The enrichment analysis of the MEF2A protein interactome in RH30 cells highlights key functional areas where these interactions are concentrated. The top enriched Gene Ontology (GO) terms are categorized into Biological Process (BP), Cellular Component (CC), and Molecular Function (MF), each indicating different aspects of MEF2A's functional network. In the Biological Process category, MEF2A interactors are involved in processes such as mRNA splicing, transcription regulation, and actin cytoskeleton organization, suggesting roles in gene expression and structural organization within the cell.

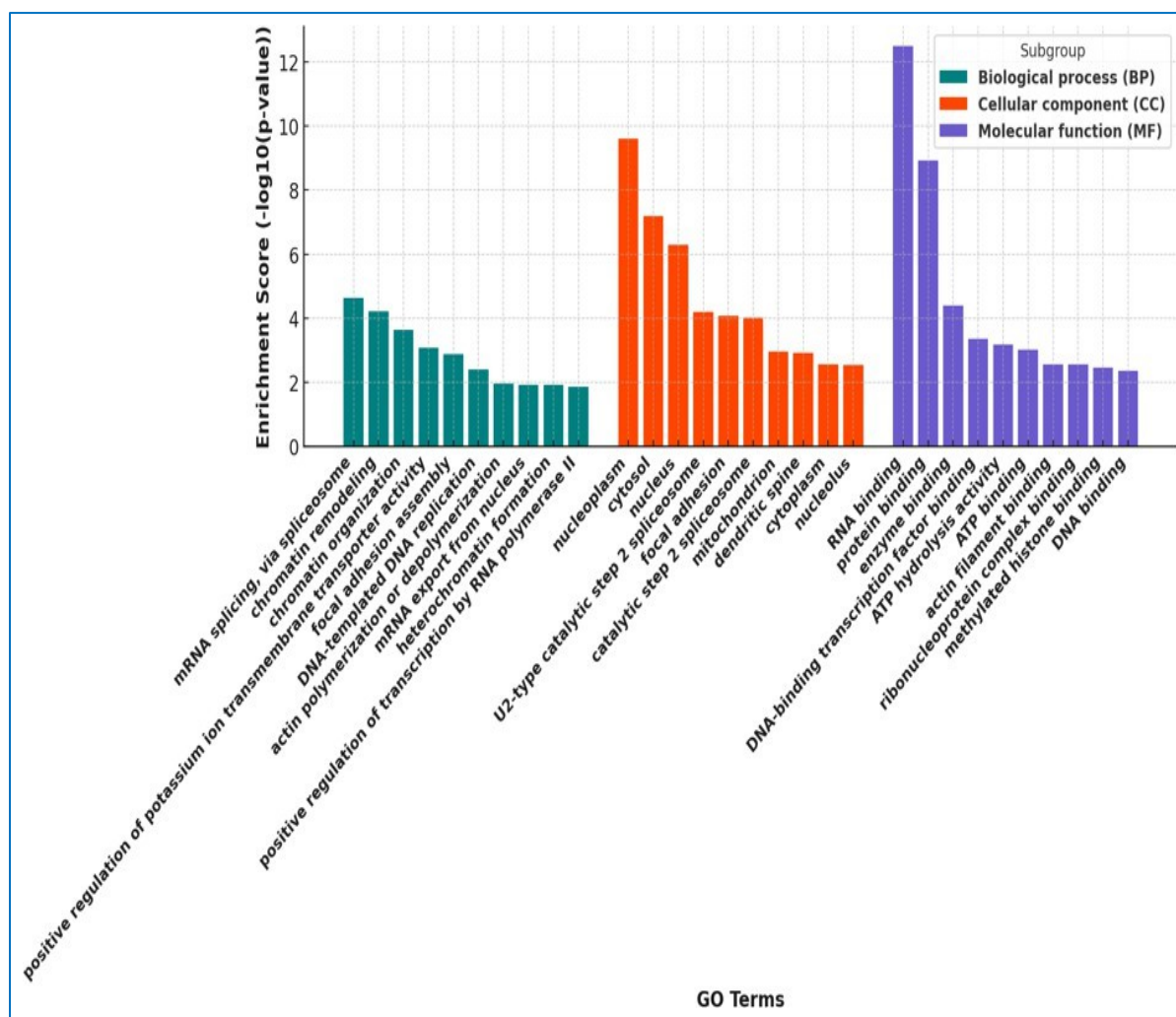


Figure 22: Top ten enriched Gene ontology (biological process, cellular component and molecular function) of MEF2A protein interactomes in RH30 cell. This image represents the top ten enriched Gene Ontology (GO) terms for MEF2A protein interactomes in RH30 cells, divided into three categories: Biological Process (BP-green bar), Cellular Component (CC-red bar), and Molecular Function (MF-purple bar). The enrichment score for each GO term is shown on the y-axis as the negative logarithm of the p-value ($-\log_{10}(\text{p-value})$), with higher values indicating greater statistical significance.

For Cellular Component, the MEF2A interactors are primarily located in the nucleoplasm and spliceosome components, reflecting their involvement in nuclear activities related to RNA processing and transcriptional control, as well as potential roles in focal adhesions. In the Molecular Function category, the interactors exhibit activities such as RNA binding, ribosome structural components, and binding to transcription factors and methylated histones. These

functions are critical for gene expression regulation and epigenetic control. Altogether, this analysis provides a comprehensive view of MEF2A's interactome in RH30 cells, emphasizing its roles in transcriptional regulation, RNA processing, and cellular structure, which may be crucial for MEF2A's regulatory functions in this cellular context.

3.8.3 Gene Ontology of the β -catenin interactome in RD cell

The Gene Ontology (GO) analysis of the β -catenin interactome in RD cells reveals significant enrichment in multiple biological processes, cellular components, and molecular functions, highlighting the diverse roles β -catenin plays in cellular regulation. The GO enrichment analysis of the β -catenin interactome in RD (fig 23) cells provides valuable insights into the multifaceted roles of β -catenin in cancer biology. Within the Biological Process category, the analysis reveals that β -catenin, and its associated proteins are significantly involved in the regulation of critical cellular functions, including the negative regulation of tumor necrosis factor-mediated signaling, the positive regulation of transcription from RNA polymerase II promoters, and processes related to cell cycle control and apoptosis. These findings suggest that β -catenin not only influences gene expression but also plays a role in modulating inflammatory pathways and controlling cell division and cell death, functions that are often dysregulated in cancer cells. The enrichment of these processes highlights the potential impact of β -catenin on tumor progression and immune response modulation in RD cells, which could contribute to the aggressive characteristics of this cancer type. In terms of Cellular Component, the interactome analysis indicates that β -catenin is associated with various cellular structures, including the nucleoplasm, nucleus, PML bodies, proteasome complex, and adherens junctions.

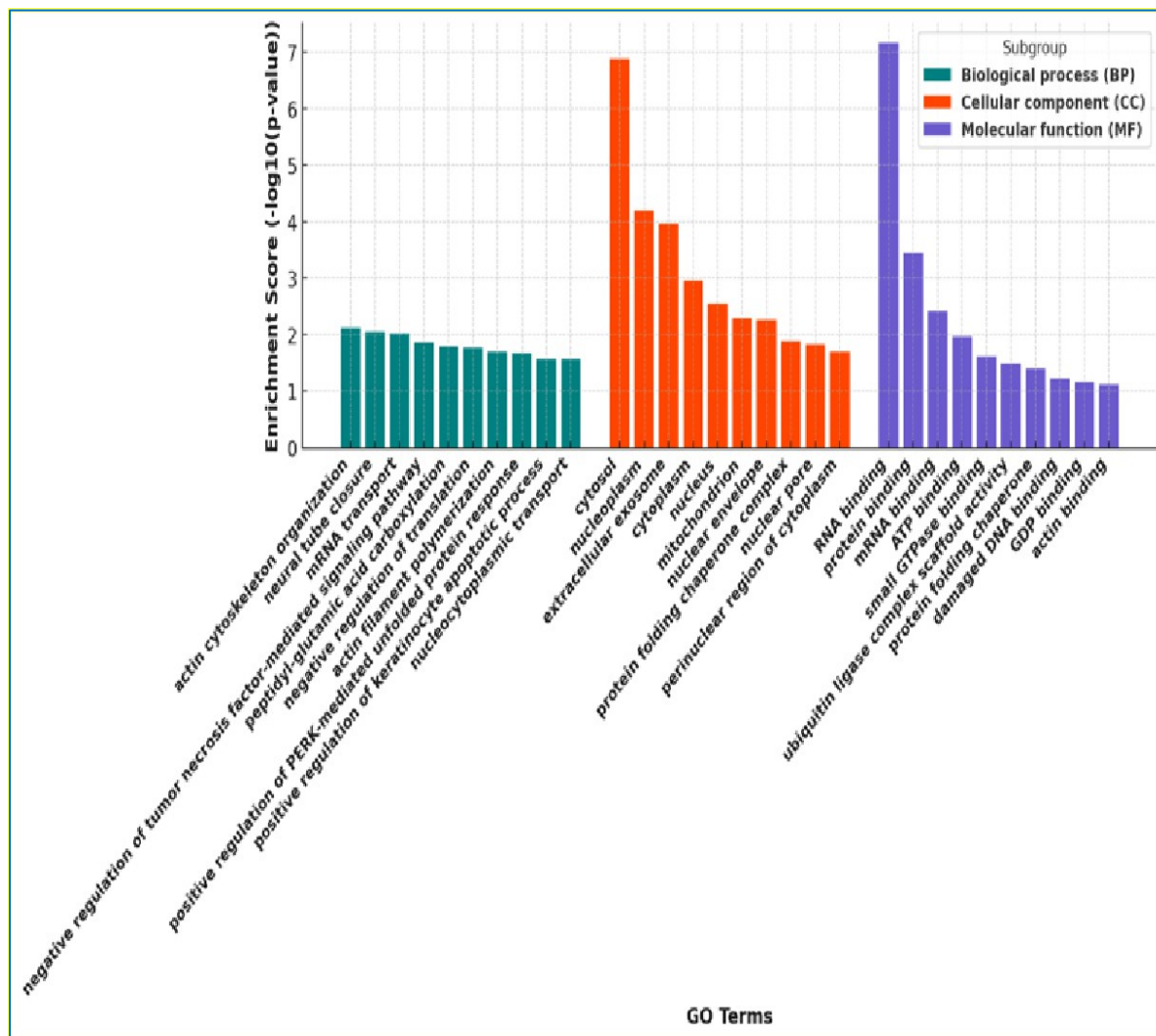


Figure 23: Top enriched Gene ontology (biological process, cellular component and molecular function) of β -catenin protein interactomes in RD cell. Top ten enriched Gene Ontology (GO) terms for β -catenin protein interactomes in RD cells, divided into three categories: Biological Process (BP-green bar), Cellular Component (CC-red bar), and Molecular Function (MF-purple bar). The enrichment score for each GO term is shown on the y-axis as the negative logarithm of the p-value ($-\log_{10}(\text{p-value})$), with higher values indicating greater statistical significance.

The presence of β -catenin and its interacting partners in the nucleus and nucleoplasm aligns with its well-established role as a transcriptional co-activator within the Wnt signaling pathway, where it translocates into the nucleus to drive the expression of target genes. Additionally, its association with PML bodies and the proteasome complex underscores its

involvement in post-translational modifications and protein degradation pathways, essential for regulating protein turnover and signaling dynamics. Notably, the enrichment in adherens junctions reflects β -catenin's classical function in cell-cell adhesion, emphasizing its dual role in both structural cellular integrity and signal transduction, which is especially relevant in the context of tumorigenesis and metastasis.

The Molecular Function analysis further emphasizes β -catenin's versatility, revealing its involvement in protein binding, ubiquitin-like protein ligase binding, DNA binding, and cadherin binding. These functions are indicative of β -catenin's ability to interact with a wide array of proteins, particularly within pathways related to transcriptional regulation and ubiquitination. The role of β -catenin in DNA and RNA polymerase II core promoter binding suggests its direct impact on gene expression, allowing it to control the transcription of genes involved in cellular growth and survival. Furthermore, its ability to bind cadherins reinforces its role in cell-cell adhesion, a critical function for maintaining tissue architecture and preventing uncontrolled cell migration. The enrichment of ubiquitin-like protein ligase binding suggests that β -catenin and its interactome may also play a role in modulating protein stability and degradation, processes that are crucial for maintaining cellular homeostasis and preventing oncogenic signaling.

Together, these enriched GO terms illustrate the diverse and essential functions of β -catenin and its interactome within RD cells. This interactome's involvement in regulating transcription, cell adhesion, and protein degradation pathways highlights β -catenin's central role in both the structural and regulatory dynamics of cancer cells. These findings underscore the potential of targeting β -catenin-associated pathways for therapeutic intervention in cancer, as disrupting these interactions could modulate tumor progression, immune response, and cell survival in RD cells.

3.8.4 Gene Ontology of the β -catenin interactome in RH30 cell

The Gene Ontology (GO) enrichment analysis of the β -catenin interactome in RH30 cells reveals a multifaceted role for β -catenin in essential cellular processes, subcellular localization, and molecular functions. Within the biological processes category, β -catenin and its interacting proteins are prominently involved in pathways regulating cell adhesion, proliferation, apoptosis, and developmental signaling. This indicates that β -catenin plays a crucial role in maintaining cell integrity and tissue structure, largely through its well-established functions in cell adhesion and the Wnt signaling pathway. Furthermore, the enrichment of terms related to negative regulation of cell proliferation and apoptotic signaling suggests that β -catenin's interactome may be integral to cellular growth control and programmed cell death, balancing proliferation and apoptosis to prevent abnormal cell behavior. These biological processes highlight β -catenin's importance in cancer biology, where dysregulation of cell adhesion, proliferation, and apoptotic pathways is a hallmark (fig24).

In the cellular component category, enriched terms like adherens junctions, cell membrane, nucleus, and cytoplasm illustrate the dynamic localization of β -catenin and its interacting proteins. At the adherens junctions, β -catenin acts as a crucial structural component, linking cadherin proteins to the actin cytoskeleton and reinforcing cell-cell adhesion. This structural role is essential for maintaining cellular architecture and tissue organization. Meanwhile, β -catenin's presence in the nucleus points to its involvement in transcriptional regulation, particularly in the Wnt signaling pathway, where it translocates to the nucleus upon pathway activation to co-regulate gene expression critical for cell proliferation and differentiation. The cytoplasmic localization further underscores β -catenin's versatility, as it serves as a bridge between signaling events at the cell membrane and transcriptional outcomes in the nucleus.

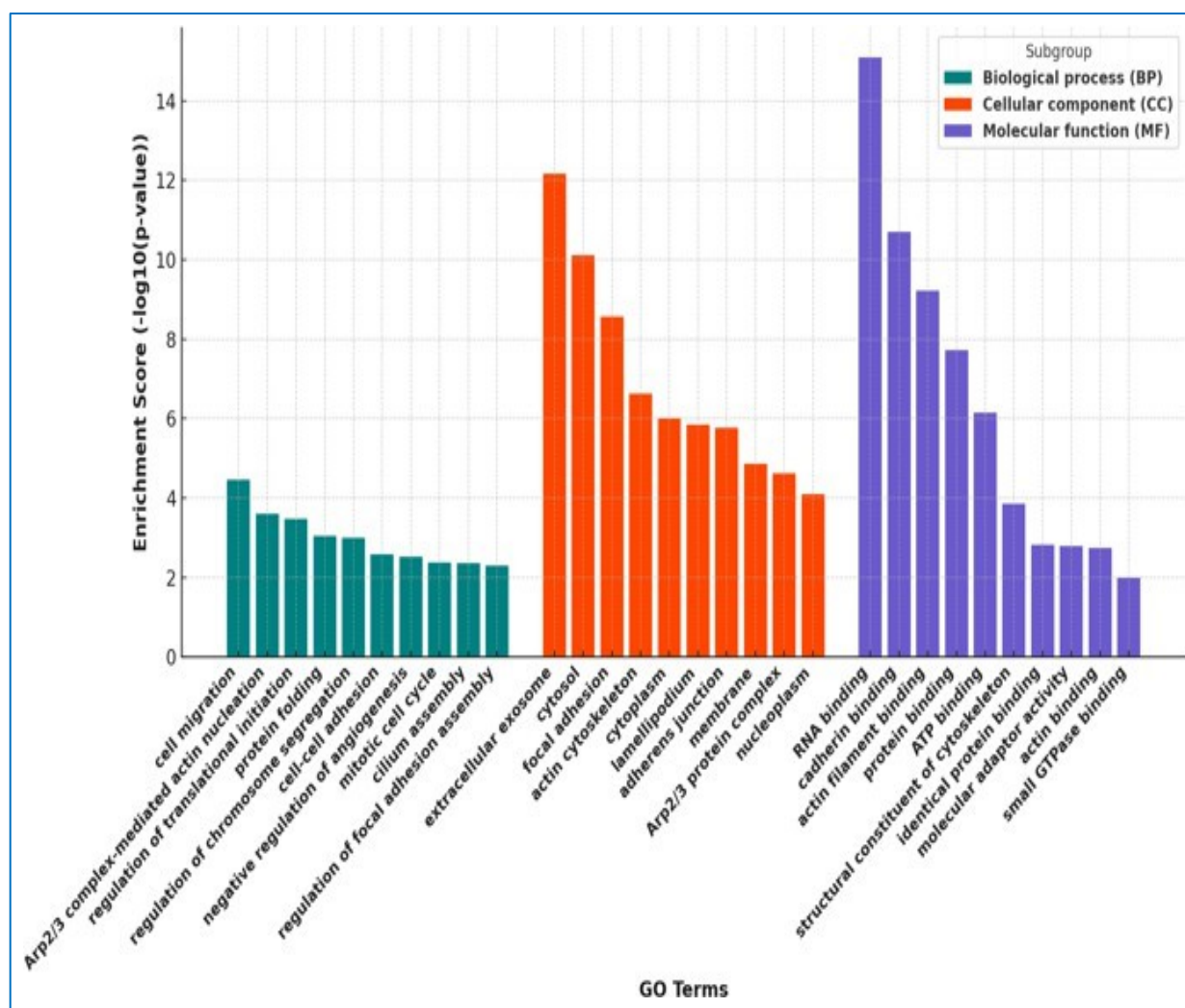


Figure 24: Top 10 enriched Gene ontology of β -catenin protein interactomes in RH30 cell (biological process, cellular component and molecular function). Enriched Gene Ontology (GO) terms for β -catenin protein interactomes in RH30 cells, divided into three categories: Biological Process (BP-green bar), Cellular Component (CC-red bar), and Molecular Function (MF-purple bar). The enrichment score for each GO term is shown on the y-axis as the negative logarithm of the p-value ($-\log_{10}(\text{p-value})$), with higher values indicating greater statistical significance.

In terms of molecular functions, β -catenin's interactome shows strong enrichment in protein binding, transcription coactivator activity, and structural molecule activity. These molecular functions emphasize β -catenin's role as a scaffold protein, facilitating a vast network of interactions that enable it to participate in multiple signaling pathways. Its

transcription coactivator activity is particularly notable in the context of Wnt signaling, where β -catenin partners with transcription factors to drive the expression of genes associated with cellular growth and differentiation. Additionally, its role as a structural molecule at cell junctions further underscores its dual function as both a regulatory and structural entity within cells. This broad spectrum of activities positions β -catenin as a versatile and pivotal protein, capable of switching roles between structural integrity and transcriptional regulation based on cellular needs. Overall, the GO analysis of the β -catenin interactome in RH30 cells highlights its integral role in cellular structure, signaling, and growth control, with profound implications for understanding its function in development and cancer biology.

3.8.5 Diseases associated with MEF2A and β -catenin interactome

GO enrichment analysis allows us for comparative studies between different conditions, such as healthy versus diseased states, by revealing condition-specific changes in protein interactions. After, identifying the hub genes, we sought to explore the diseases associated with the interactome of MEF2A and β -catenin. To achieve this, we utilized DAVID Bioinformatics software and the OMIM database to determine the diseases linked to our four distinct gene sets (MEF2A-RD, RH30 and for β -catenin-RD, RH30). The table 2 below presents the top diseases associated with these interactome.

This table summarizes diseases associated with the MEF2A and β -catenin interactomes in Rhabdomyosarcoma (RMS) cell lines, specifically comparing the RD and RH30 lines. Each cell line is associated with a unique set of diseases for each protein, offering insights into the pathways and broader implications of these interactomes in cancer development, particularly RMS.

From this table, we observe that many interactome partners are linked to tumorigenesis or cancer-related conditions. Notably, the β -catenin interactome shows strong associations with multiple cancers, including lung, ovarian, and breast cancers. This suggests that β -catenin's

role in RMS may involve mechanisms that are also relevant in other types of cancer.

Table 2: Diseases associated with MEF2A and β -catenin interactome in RMS cell

MEF2A in RD	MEF2A in RH30	β -catenin in RD	β -catenin in RH30
Schizophrenia	Acquired Immunodeficiency Syndrome	Ovarian Carcinoma	Adenocarcinoma of Lung
lung cancer	Tobacco Use Disorder	Adenocarcinoma of lung (disorder)	Coloractal cancer
Acquired Immunodeficiency Syndrome Disease Progression	Insulin Resistance	Neoplasm of uncertain or unknown behavior of ovary	Malignant tumor of colon
Acquired Immunodeficiency Syndrome HIV Seropositivity	Prostatic Neoplasms	Ovarian neoplasm	ovarian neoplasm
	Hematologic Neoplasms	Urolithiasis	Malignant neoplasm of ovary
	HIV Infections	Malignant neoplasm of ovary	Polycystic Ovary Syndrome
	Spinal Dysraphism	Isolated ATP synthase deficiency	Breast cancer
	Graft vs Host Disease	Melanoma	Colonic Neoplasms

Therefore, further investigation into these interactomes is warranted to uncover the underlying causes of cancer formation in RMS cells and to explore connections to other cancers associated with these interactome partners. Understanding how MEF2A and β -catenin interactomes function in other cancer types could provide valuable context and help clarify their roles in RMS, ultimately aiding in identifying targeted therapeutic strategies. A gene's upregulation in a particular tissue often indicates its involvement in that tissue's specific processes. Identifying these tissues can help to understand the underlying causes of the disease.

Given that our four distinct sets of interactome are associated with different diseases, we sought to determine in which types of tissue these interactomes are upregulated. To do this, we analyzed each gene set for upregulated tissues using the DAVID bioinformatics tool. This analysis yielded a data sheet containing gene names and their corresponding upregulated tissues, ranked by p-value.

We then visualized this data using pie charts (Fig 25&26) to illustrate the tissue distribution of upregulated genes across our four gene sets. Our analysis revealed that cervical cancer was the most frequent tissue associated with upregulation for all four gene sets, followed by erythroleukemia and liver.

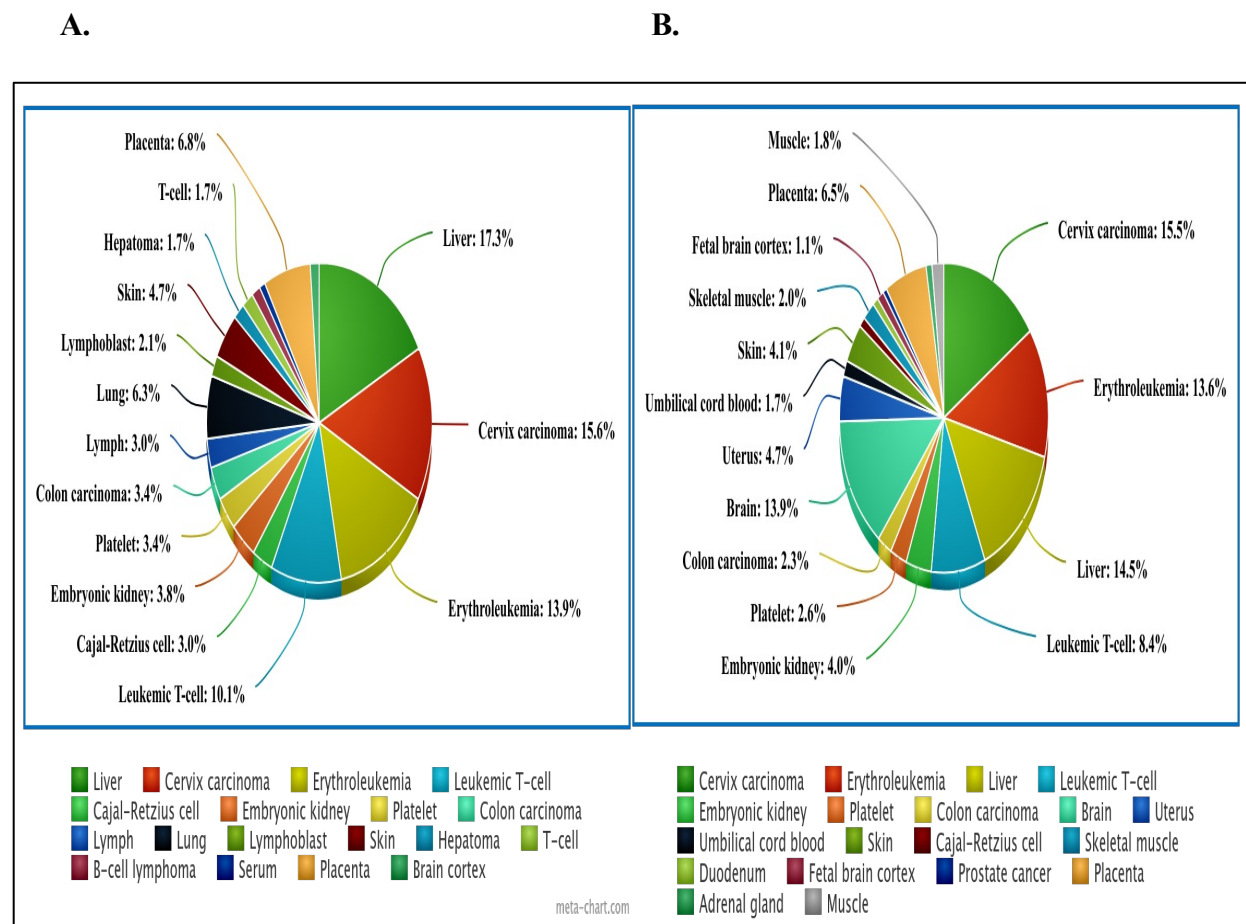


Figure 25: Tissue distribution of upregulated MEF2A-interacting genes in RD and RH30 rhabdomyosarcoma cells. (A) The pie chart illustrates the predicted tissue-specific expression of upregulated MEF2A-interacting genes identified in RD rhabdomyosarcoma cells. In RD cells, the upregulated MEF2A interactome shows predominant enrichment in liver, cervix carcinoma, and erythroleukemia-associated genes, suggesting strong transcriptional activity linked to oncogenic and hematopoietic pathways. (B) In RH30 cells, a similar pattern is observed with strong enrichment in cervix carcinoma, liver, and brain-associated genes, alongside moderate contributions from muscle-related tissues. This shift may reflect the distinct fusion-positive nature and lineage commitment of RH30 cells.

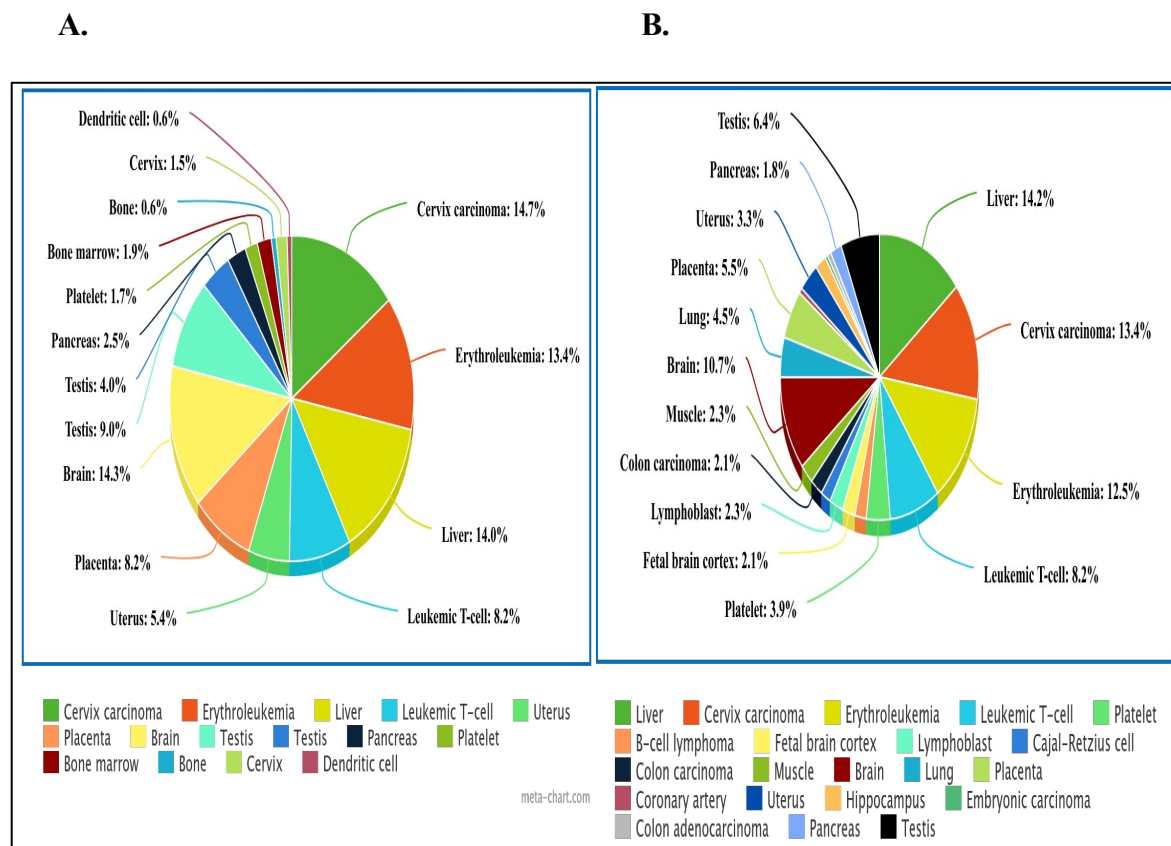


Figure 26: Tissue distribution of β -catenin upregulated-interacting genes in RD and RH30 rhabdomyosarcoma cells. (A) In RD cells, upregulated β -catenin-interacting genes are predominantly enriched in cervix carcinoma, erythroleukemia, liver, and brain-associated tissues, suggesting strong involvement of β -catenin in transcriptional networks related to oncogenesis and neurodevelopment.(B) In RH30 cells, the distribution is similarly dominated by cervix carcinoma, liver, and erythroleukemia, but also includes notable contributions from brain and testis, indicating potential subtype-specific functions of β -catenin linked to reproductive and neural tissue expression

3.9 Prospective Candidate proteins for future analysis:

After performing a comprehensive bioinformatic analysis, we shortlisted the top genes from all four gene sets based on their biological relevance to myogenesis and their roles in cancer development. Each of these genes is equally important, playing a significant role in both myogenesis and cancer progression. Protein-protein interaction (PPI) network analysis revealed that most genes within each set are highly interconnected, with strong confidence scores indicating their functional cooperation, either as a collective group or individually. The PPI network is illustrated below (fig 27) to provide a clearer understanding of their biological and molecular interactions. This resulted in the identification of the top 10 genes for future

analysis. The list is provided below:

Table 3: Prospective Candidate proteins

MEF2A in RD	MEF2A in RH30	β-catenin in RD	β-catenin in RH30
RACK1	HDAC1	BRCA1	STAT1
HSPA1B	CREBBP	DICER1	XPO1
GLUD1	SNRPG	MAP2K1	AURKB
GNL2	CHD8	ROCK1	UHRF1
SF3A3	CASC3	EZH2	YWHAG
XRCC5	RUNX	RRAS2	PRKDC
U2AF2	HUWE1	PRC1	RANBP2
UTP23	MDC1	ASXL2	SPARC
ACLY	POLD	CUL1	ALDOA
NOL6	NUP214	(PRAME)	CTNNA1

Moreover, Gene Ontology (GO) analysis for the MEF2A gene set in RD cells indicates a predominant involvement in ribonucleoprotein complex biogenesis, with major molecular functions centered around RNA binding, heterocyclic compound binding, and organic cyclic compound binding. The key cellular components include the small subunit processome, ribonucleoprotein complex, and nuclear lumen, while the KEGG pathways are primarily associated with the spliceosome. Similarly, GO analysis of the MEF2A gene set in RH30 cells reveals that a primary molecular function is p53 protein binding. The main cellular components identified include the nuclear protein-containing complex, catalytic complex, and nucleoplasm. KEGG pathway analysis highlights involvement in TGF-beta signaling, DNA repair, and the Notch- HLH transcription pathway.

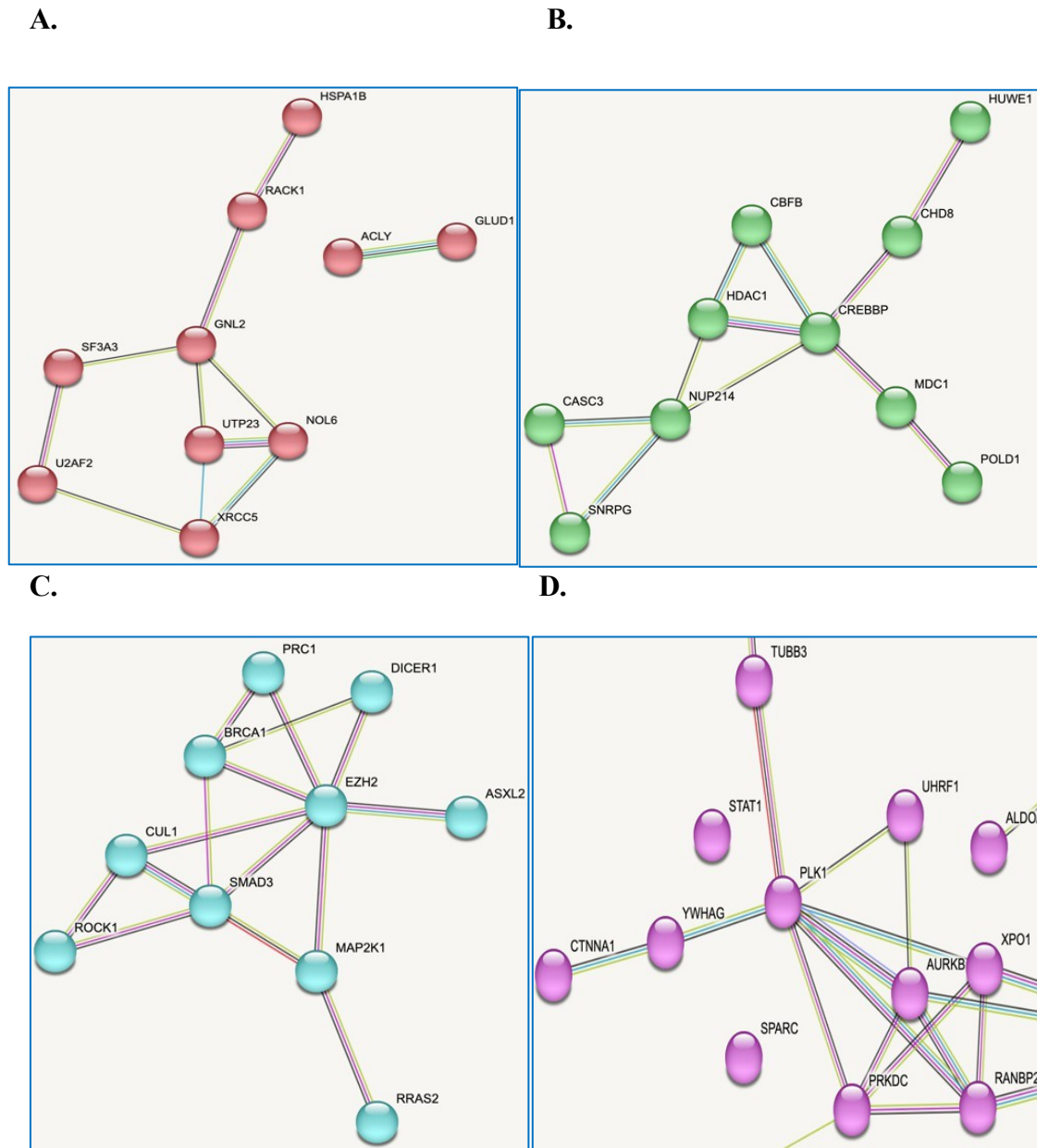


Figure 27: PPI network of prospective protein candidates. a) MEF2A protein interactomes in RD cell. b) MEF2A protein interactomes in RH30 cell. c) β -catenin protein interactomes in RD cell. d) β -catenin protein interactomes in RH30 cell.

For the β -catenin gene set in RD cells, the primary biological process identified is the mitotic cell cycle, with notable KEGG pathways including TGF-beta signaling and MicroRNAs in cancer. In contrast, the RH30 β -catenin gene set emphasizes regulation of epithelial proliferation and the mitotic cell cycle. Key cellular components include the cytoplasmic

periphery of the nuclear pore complex and annulate lamellae. Major molecular functions involve receptor binding, DNA/RNA binding, and protein kinase C inhibition. The primary KEGG pathways for this set encompass cell cycle regulation, RNA transport, and the Hippo signaling pathway.

4. Discussion

MEF2A and β -catenin are well-established positive regulators of myogenesis. MEF2A, a transcription factor, governs myoblast proliferation and differentiation, while β -catenin serves as the principal downstream effector of the canonical Wnt signaling pathway, which is essential for development and muscle formation. Consequently, dysregulation of either protein can lead to muscle-related disorders. One such disorder is rhabdomyosarcoma (RMS), a pediatric malignancy in which muscle progenitor cells fail to undergo terminal differentiation, leading to the accumulation of undifferentiated cells that ultimately form malignant tumors.

Over the years, MEF2A and β -catenin have garnered significant attention due to their critical roles in human development and disease pathogenesis. Transcription factors such as MEF2A typically function within multiprotein complexes rather than acting independently, highlighting the necessity of their integration with other regulatory elements to modulate transcription. Conversely, β -catenin, a transcriptional co-activator, does not directly interact with DNA but instead relies on its extensive protein-protein interaction network to execute its regulatory functions. Given these interdependencies, investigating the protein interactome of MEF2A and β -catenin provides a valuable approach to elucidating their roles in RMS cells and understanding the molecular mechanisms by which they influence gene expression.

In this thesis, we have systematically examined the protein-protein interaction networks of MEF2A and β -catenin in RMS cell lines, recognizing their dual roles in skeletal myogenesis and tumorigenesis. To achieve this, we employed an unbiased high-throughput proteomic approach—nanobody-mediated two-step affinity purification coupled with tandem mass spectrometry (AP-MS)—to identify novel protein interactions using MEF2A and β -catenin as bait proteins within the RMS cellular context. This study represents the first comprehensive interactome dataset for both proteins in RMS and provides critical insights

into their functional networks in muscle differentiation and cancer progression.

Moreover, through bioinformatic analysis of these datasets, we identified the top biological Gene Ontology (GO) terms associated with each dataset following hub gene analysis. Based on this information, we determined the top 10 diseases linked to each dataset, as well as the tissues in which these genes are most highly upregulated. Finally, we propose a list of the top 10 prospective protein candidates for each dataset, selected based on their known roles in myogenesis and tumorigenesis, and constructed protein interaction networks to visualize their functional associations.

Divergent MEF2A Activity in RMS: Implications for Myogenic Regulation

MEF2A, a critical transcription factor involved in muscle differentiation, showed significantly reduced protein expression in both RD and RH30 RMS cells compared to normal C2C12 myoblasts. However, luciferase reporter assays revealed a paradoxical increase in MEF2A transcriptional activity in RD cells, suggesting the presence of altered regulatory mechanisms. While RH30 cells exhibited diminished MEF2A-driven transcription, RD cells displayed an unexpectedly heightened response. Even if MEF2A protein levels are low, the reporter system specifically reflects MEF2A's ability to activate transcription from MEF2 binding sites. In RD cells, MEF2A may interact with different co-factors or signaling proteins than in normal muscle cells, altering its transcriptional activity. In cancer cells, post-translational modifications (PTMs) of MEF2A may enhance or repress its activity. These modifications can alter the transcriptional activity of MEF2A without necessarily changing its protein levels. For example, certain kinases or other signaling molecules that are dysregulated in RMS may phosphorylate MEF2A at sites that increase its transcriptional activity in the reporter assay, even though its total protein levels remain low. This indicates that RD cells may retain a partially functional MEF2A regulatory circuit, potentially influenced by unique cofactors or compensatory signaling pathways.

The observation of increased MEF2A activity despite low protein levels suggests the involvement of post-translational modifications or differential protein–protein interactions that enhance MEF2A’s function in RD cells. In RMS cells, dysregulated signaling pathways—such as PI3K/AKT, MAPK, or Wnt/ β -catenin—are likely contributors to this phenomenon. These pathways may create a more permissive cellular environment, allowing MEF2A to act as an active transcription factor even when its endogenous expression is low.

Moreover, cancer cells often exhibit elevated basal activity of signaling cascades that promote proliferation and survival. This heightened signaling may unintentionally amplify the activity of transcription factors like MEF2A, particularly when experimentally overexpressed. Consequently, the MEF2-luciferase reporter may reflect the influence of hyperactive oncogenic pathways in RMS cells, thereby resulting in elevated reporter gene expression.

Differential Regulation of β -Catenin in RMS Subtypes

Our study also demonstrated downregulation of β -catenin in both RD and RH30 cells, with stark differences in its functional activity between these subtypes. Using TOPflash/FOPflash reporter assays, we observed that RD cells maintained residual β -catenin activity, whereas RH30 cells exhibited near-complete suppression of β -catenin-mediated transcriptional activation. This suggests that while β -catenin signaling is disrupted in both subtypes, RH30 cells may engage alternative oncogenic pathways that bypass Wnt signaling. This finding is consistent with the aggressive nature of RH30 cells, where β -catenin suppression might contribute to undifferentiated, proliferative phenotypes. The functional divergence in β -catenin activity between RD and RH30 cells underscores the heterogeneity within RMS subtypes. In RD cells, partial β -catenin activation could provide a growth advantage by retaining some aspects of differentiation signaling, while in RH30 cells, its suppression may

promote a more stem-like or undifferentiated tumor state, which is often associated with poor prognosis and metastasis.

MEF2A and β -Catenin Interactomes in RMS Cells

To better understand how MEF2A and β -catenin function within the RMS cellular landscape, we mapped their interactomes through affinity purification and mass spectrometry-based proteomics. Our findings revealed marked differences in protein interaction networks between RD and RH30 cells, reinforcing the notion that these subtypes leverage distinct molecular pathways.

- **MEF2A interactome:** In RD cells, the MEF2A network was enriched for proteins involved in RNA processing, alternative splicing (DDX39B, SART1, SF3A3), and transcriptional regulation, suggesting a potential role in post-transcriptional modification of muscle-specific genes. Interestingly, TRIM32 and RACK1, known regulators of muscle differentiation and tumor progression, were identified as central nodes in the network. Meanwhile, in RH30 cells, the MEF2A interactome was dominated by epigenetic regulators such as HDAC1, RUNX1, and CREBBP, suggesting that MEF2A function is more tightly controlled by chromatin remodeling and histone modification in this aggressive RMS subtype. Furthermore, SF3A3 (Splicing Factor 3a Subunit 3) and U2AF2 (U2 Small Nuclear RNA Auxiliary Factor 2): Both are components of the spliceosome, essential for pre-mRNA splicing. Alterations in splicing can lead to the production of different MEF2A isoforms, potentially affecting its function. Since MEF2A reporter assay displays divergent activity across RMS subtypes which has linked with post-translational medications therefore, these could also play critical role in cancer formation.

- **β-Catenin interactome:** In RD cells, β-catenin was linked to BRCA1, DICER, ROCK1, and EIF4E, which are involved in DNA repair, RNA metabolism, and cell signaling. This suggests that β-catenin may retain some context-dependent tumor-suppressive roles in RD cells. In contrast, RH30 cells displayed a β-catenin interactome highly enriched for oncogenic regulators such as XPO1, RANBP2, and AURKB, which are associated with nuclear export, mitotic control, and chromatin regulation, reinforcing its oncogenic potential in this aggressive tumor subtype.

These interactome findings provide a functional framework for understanding how MEF2A and β-catenin exert distinct biological effects in different RMS subtypes, likely contributing to tumor heterogeneity and progression.

Pathway and GO Enrichment Analysis: Identifying Functional Disruptions

Using Gene Ontology (GO) and pathway enrichment analysis, we further characterized the biological roles of MEF2A and β-catenin in RMS.

- **MEF2A:** GO analysis highlighted its involvement in RNA binding, transcriptional regulation, and cell cycle control, with key enrichments in spliceosomal complex formation and nucleocytoplasmic transport. This suggests that MEF2A may influence RNA processing and stability, thereby modulating gene expression at multiple levels. To identify novel interactions between MEF2A and proteins from our list, we compared the data with known interactors from the STRING database. MEF2A is strongly associated with several proteins involved in transcriptional regulation, including MAP kinases (e.g., MAPK11, MAPK12), and other MEF2 family members. However, our provided proteins on string database like TRIM32, ACTR3, PRDX5, and SNRNP70 are not listed as canonical MEF2A interactors. This suggests these might represent potentially novel interactions worth further investigation, detected in our experimental data.

- **β-Catenin:** GO analysis revealed its strong association with cell adhesion, nuclear signaling, and Wnt pathway regulation, with notable enrichments in cadherin binding and transcriptional activation of oncogenes. These findings align with β-catenin's well- documented roles in developmental biology and cancer. The central role of RACK1 suggests that signaling pathways are highly regulated in the RD context. This might be critical for understanding diseases or conditions related to signaling dysregulation. **PPP2R1A Cluster:** Encompasses proteins involved in phosphatase regulation and signaling (e.g., PPP2R1B, PPP2R2A). A regulatory subunit of protein phosphatase 2A (PP2A), involved in cell growth and division. The prominence of PPP2R1A indicates a key role in cell cycle control and signaling. PPP2R1A's prominence indicates a strong focus on regulating cell growth and division, which could be vital for understanding cancer biology or developmental processes.

Our disease-association analysis further linked MEF2A and β-catenin interactomes to various cancers, including lung, ovarian, and colorectal cancers, indicating overlapping oncogenic pathways that might be targeted for therapeutic interventions.

Limitations

To identify the MEF2A interactome in RMS cells using LC-MS/MS technology, we utilized whole cell lysates. This approach, while practical, may result in the identification of non-functional protein interactions due to cytoplasmic protein contamination. To mitigate this, future experiments should optimize nuclear fractionation protocols to enrich for nuclear proteins and reduce background noise. Moreover, in our study, we conducted a reporter assay for the MEF2A protein followed by western blot to confirm the transfection. Interestingly, in RD cells, the reporter assay revealed elevated MEF2A transcriptional activity, which contrasts with the results of the western blot analysis indicating reduced protein expression. This discrepancy warrants further investigation to elucidate the underlying regulatory mechanisms responsible for this paradoxical observation. Additionally, we were unable to perform a post reporter assay western blot to confirm the transfection for β -catenin due to unavoidable technical constraints, which represents a significant limitation of our study.

In our study, we observed that the regulatory networks of both MEF2A and β -catenin are dysregulated in RMS cells, as evidenced by western blot analysis, reporter assays, and comparative protein interaction network analyses between normal (C2C12) and cancerous cells. While our findings provide valuable insight, they are largely based on mass spectrometry data, which, although powerful for generating hypotheses and enabling extensive bioinformatic analysis, are not sufficient on their own to fully elucidate the underlying mechanisms of network dysregulation. Our proteomic LC-MS/MS approach uncovered a broad array of potential binding partners for MEF2A and β -catenin, many of which appear to be novel. Among these, we identified several top candidates—such as MYCBP2, HDAC1, STAT1, BRCA1, and FGFR4—that warrant further investigation for their potential roles in RMS pathogenesis.

Future studies should include validation of these interactions by assessing endogenous protein levels in RMS cells using appropriate controls, followed by confirmation of physical interactions via co-immunoprecipitation assays. Additionally, functional studies such as gene knockdown experiments and reporter assays could help elucidate the biological significance of these interactions. Finally, real-time PCR and targeted DNA sequencing should be performed to detect potential mutations in these candidate genes that may contribute to the dysregulated regulatory networks observed in RMS cells.

Potential Therapeutic Targets and Future Directions

Our findings highlight several key candidate proteins that could serve as potential therapeutic targets in RMS. Based on our hub gene analysis, we identified key regulators such as MYCBP2, RACK1, HDAC1, CREBBP, STAT1, XPO1, AURKB, and BRCA1 which are well-known for their roles in cancer progression and transcriptional regulation. Given the observed dysregulation in MEF2A and β -catenin pathways, future studies should focus on:

1. Targeting MEF2A and its cofactors: Since MEF2A activity is altered in RD cells, investigating its interaction with chromatin remodelers (e.g., HDAC1, CREBBP) could reveal new therapeutic opportunities.
2. Modulating β -catenin signaling: The near absence of β -catenin activity in RH30 cells suggests a potential vulnerability to Wnt-modulating agents. In contrast, strategies that restore β -catenin function in RD cells may induce differentiation and limit tumor growth.
3. Functional validation of candidate hub genes: Knockdown or overexpression studies of hub genes (e.g., RACK1, XPO1, BRCA1) will be essential in determining their precise roles in tumor proliferation, differentiation, and metastasis.
4. MEF2A Mutation: While it is well-established that β -catenin is not commonly mutated in rhabdomyosarcoma cells, there is currently no available data regarding the

mutational status of MEF2A in this cancer type. Given MEF2A's role as a transcription factor and its observed dysregulated activity in our study—particularly the discrepancy between its reporter assay and protein expression levels—it is essential to investigate whether genetic alterations in MEF2A contribute to its aberrant function. Understanding the mutational landscape of MEF2A may provide insights into its regulatory mechanisms and potential involvement in Rhabdomyosarcoma pathogenesis.

Concluding Remarks

The findings of this study offer critical insights into the dysregulated protein interactions involving MEF2A and β -catenin in Rhabdomyosarcoma (RMS), shedding light on their roles in impaired myogenic differentiation and cancer progression. Using a multi-faceted approach that combined western blot analysis, luciferase reporter assays, affinity purification followed by LC-MS/MS, and integrative bioinformatics, we characterized key differences in the expression, activity, and interactomes of MEF2A and β -catenin across RMS subtypes, specifically in RD and RH30 cell lines. Our data reveal that aberrant MEF2A and β -catenin signaling contributes to the altered cellular behavior typical of RMS. Importantly, we identified a set of novel and subtype-specific interacting proteins that may modulate these pathways in the tumor context. Given that certain RMS subtypes harbor frequent mutations in genes such as **HSP90AA1**, **DICER**, **CHD7**, **NF1**, **FGFR1/4**, **TP53**, **NRAS**, and **HRAS** (Stewart et al., 2018), it is plausible that some of the top candidate proteins identified in our interactome analysis may also be subject to genetic alterations. Investigating potential mutations or functional disruptions in these candidates could provide deeper insight into their contribution to RMS pathobiology.

This study presents a comprehensive analysis of MEF2A and β -catenin dysregulation in RMS, highlighting their differential activity profiles, unique interaction networks, and

involvement in key signaling pathways. Our findings underscore the heterogeneity of oncogenic mechanisms among RMS subtypes and point to the potential for developing subtype-specific therapeutic strategies. Continued investigation into the molecular underpinnings of MEF2A and β -catenin function will be essential for designing targeted interventions that aim to restore normal myogenic regulation while impeding tumor-promoting process.

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