The Smad7 protein interaction network in skeletal myogenesis

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A dissertation submitted to the faculty of graduate studies in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Program in Biology

York University

Toronto, Ontario

September 2020

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Abstract

Myogenesis or muscle development begins with cell cycle arrest and continues with a differentiation program that converts myoblasts into multi-nucleated cells referred to as myotubes. A complex hierarchy of transcriptional activation events leads to the expression of skeletal muscle associated genes. Our group previously suggested that the repressive effect of myostatin on muscle differentiation is countered by Smad7 but interestingly no such effect was observed with another TGF- β ligand indicating the possibility of variant aspects of the downstream signaling. Moreover, another study from our group demonstrated co-operation of Smad7 with other transcription factors is required in order to enhance differentiation of skeletal muscle. These observations led us to the central purpose of this dissertation which was to further characterize the molecular function of Smad7 in skeletal muscle cells.

The β-catenin protein has several different properties and functions in cell adhesion at the plasma membrane as well as acting as a transcriptional co-activator in the nucleus. In the first study reported herein, a physical interaction between Smad7 and β-catenin in skeletal muscle cells was documented. While interaction of Smad7 and β-catenin had been reported earlier in prostate cancer cells (1), the role of this interaction in muscle cells was unknown. This study demonstrated that the 575-683 amino acid region of β-catenin constitutes the interaction domain of Smad7 (we dubbed this region SID for Smad7 Interaction Domain). Subsequent experiments indicate that Smad7 and β-catenin function co-operatively on the muscle -specific *ckm* promoter. Furthermore, deletion of the SID region of β-catenin greatly reduced the effect of Smad7 on the *ckm* promoter, suggesting its crucial role during muscle differentiation. A dominant negative role of β-catenin-

SID was also demonstrated by its ability to inhibit the function of wild type β-catenin. β-catenin and the Mediator complex subunit (MED) 12 interaction had been previously reported (2), and our study extended that observation, documenting a novel interaction of MED 13 with Smad7 which also requires β-catenin. This study indicates that the Smad7-β-catenin interaction is important for the recruitment of the Mediator complex for gene activation. The subsequent chapter documents a follow up to the first study aimed at characterizing the protein interactome of β-catenin and SID in skeletal muscle cells. Affinity Purification- Mass Spectrometry analysis identified the unique protein interactome of β-catenin and SID in growth and differentiation conditions. Consecutive GO ontology analysis characterized the protein interactors based on their cellular component domain and biological processes.

Hippo signaling pathway components are expressed in C2C12 myoblasts, primary skeletal muscle as well as myotubes (3). The hippo pathway has been documented to be a crucial mediator of tissue growth in a number cell types, but to date our understanding of the role of this pathway in skeletal muscle is incomplete. Thus, the third major study in this dissertation was aimed at understanding the role of the Hippo effector protein, TAZ, in skeletal muscle cells and its possible role in modulating the function of Smad7 and β-catenin. This led to the documentation of a novel interaction between Smad7 and TAZ in cultured skeletal muscle cells. TAZ inhibits the function of Smad7 and concomitantly inhibited the activity of β-catenin. Previous studies have indicated the regulation of TAZ by direct phosphorylation that is controlled by cell density. Our study demonstrated phosphorylation of TAZ in differentiation conditions (high cell density) where phosphorylated TAZ was mainly localized to the cytoplasm.

In summary, studies reported in this dissertation have characterized several novel Smad7 protein interactors and their functional role in the cellular context of muscle specific gene regulation. In future, it will be of considerable interest to further examine the physiologic role of Smad7 and its interacting proteins in post-natal muscle physiology in health and disease.

Acknowledgement

I would like to thank my Ma, Baba for always believing in me and encouraging me to take my own decisions. Papaji and Mummy ji, thank you for the unconditional love that you poured on me for years. Vikas, it was a tough road for us to move to Canada and start a new life here, but we did it anyways! Well, the best part is we get to spend a lot of time (actually too much time to be honest) as a fellow lab mate and husband, it can't be much better. Mistu, thank you for bringing so much joy in my life. You had been the most caring and understanding kid a mom can imagine.

I would like to express my deepest appreciation and gratitude to my supervisor Dr. John C. McDermott for his mentorship, guidance and patience throughout all these years. You had been instrumental in allowing me to have a wonderful balance managing family and labwork, I can't imagine being any better. I would also like to thank my committee members for their valuable guidance over the years.

Thank you to all my past and present lab mates for giving countless memories. Tetsuaki, you know how instrumental you are in guiding me throughout. Catherine, I would miss our lunch stories.

Finally, I would like to thank all my friends for your constant love and support. Einstein (my dog) and Newton (my bird) thank you for always making me smile.

Soma

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Abbreviations

a.a	Amino acid
AKT	Protein Kinase B
APC	Adenomatous polyposis coli
ATP	Adenosine Triphosphate
ASH2L	ASH2 like Histone Lysine Methyltransferase complex subunit
BHLH	Basic Helix Loop Helix
BMP	Bone morphogenic protein
CBP	CREB binding protein
Ccna2	Cyclin A2
Cdc25c	Cell division cycle 25C
CK	Casein kinase
CoIP	Co-immunoprecipitation
CKM	Muscle creatinine kinase
DMD	Duchenne muscular dystrophy
Dsh	Dishevelled
ERK	Extracellular regulated kinase
E-box	Enhancer box
Fermt2	Fermitin family homolog 2
FGF	Fibroblast growth factor
FOX	Forkhead transcription factor
Fzd	Frizzled
GBP	GFP binding protein
GDF	Growth and differentiation factors
GPCR	G protein-coupled receptors
GFP	Green fluorescent protein
Gli	Glioma-associated oncogene
GO	Gene ontology
GSK3	Glycogen synthase kinase
GST	Glutathione-S-transferase
Hpo	Hippo
ICAT	Inhibitor of beta-catenin and TCF4
IP	Immunoprecipitation
kDa	kilodalton
LATS	Large tumor suppressor kinase
LC MS/MS	Liquid chromatography tandem mass spectrometry
LEF	Lymphoid enhancer factor
LRP	Lipoprotein receptor-related protein

MADS	MCM1, Agamous, Deficiens, SRF
MAPK	Mitogen activated protein kinase
MCAT	Muscle-specific cytidine-adenosine-thymidine sequence
MED	Mediator
MEF2	Myocyte enhancer factor 2
MKK	Mitogen activated protein kinase kinase
MRFs	Myogenic regulatory factors
MSTN	Myostatin
mRNA	Mature RNA
mTOR	Mammalian target of rapamycin
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein 1
MHC	Myosin Heavy Chain
NLS	Nuclear localization signal
PARs	Protease-activated receptors
Pax3	Paired Box 3
Pax7	Paired Box 7
PCP	Planar cell polarity
PDGF	Platelet derived growth factor
Pitx2	Paired-like homeodomain factor 2
PP1α	Protein phosphatase 1 α
PPxY	Proline/proline/any amino acid/tyrosine
PP2A	Protein phosphatase 2A
Ptch1	Patched1
RUNX1	Runt-related transcription factor 1
sFRP3	Frizzled-related protein 3
shRNA	Short hairpin RNA
Shh	Sonic hedgehog
SID	Smad Interacting domain
Six protein	Sine oculis homeobox
siRNA	Short interfering RNA
SRF	Serum response factor
SWH	Salvador-Warts-Hippo
SMAD	Mothers against decapentaplegic homolog
Smo	Smoothened
TBP	TATA binding protein
TGFβ	Transforming growth factor
TEF	Transcriptional enhancement factors
TCF	T-cell factor
TAZ	Transcriptional coactivator with PDZ binding motif

TEAD	TEA domain family member 1
Wnt	Wingless-related integration site
YAP	Yes-associated protein
Yki	Yorkie

Chapter 1

Review of literature

1.1 Myogenesis

1.1.1Paradigm of myogenesis

Skeletal muscle is a form of striated muscle constituting the most common tissue of the body that is required for many bodily processes such as breathing, locomotion and maintenance of metabolic homeostasis. Skeletal muscle cells (or fibers) are comprised of striated myofibers encompassed within a basal lamina and cytoskeleton (4). Myogenesis or muscle development is a complex multi-step process that starts with specification of progenitor cells to myoblasts, followed by cell cycle arrest and fusion of myoblasts to multi-nucleated myofibers along with further innervation to form mature myofibers (5-7). Myogenesis can be further divided into several phases – the embryonic, perinatal and maturation phase. During the embryonic phase, the first fibers of the body are formed followed by addition of more fibers along the initial fibers (8). Myogenic progenitor cells initially proliferate at the perinatal stage but later as muscle protein synthesis increases, proliferation is halted (9). Some of the myogenic progenitor cells remain as quiescent "satellite" cells and upon activation retain the ability to divide and differentiate when they are activated in a post-natal context (10). In mature muscle, satellite cells retain the capacity to proliferate and differentiate in order to maintain and repair the tissue when a muscle is injured (11).

There are many pathologies associated with skeletal muscle form and function such as rhabdomyosarcomas (a cancer with muscle phenotype), neuromuscular degenerative diseases, age related Sarcopenia and Cachexia associated with Cancer and Diabetes (12). Muscle loss can severely impact quality of life and a deeper understanding and characterization of the molecular basics of skeletal muscle development is warranted to facilitate possible treatment strategies for muscle related pathologies.

1.1.2 Myogenesis is exquisitely regulated by signaling pathways

Skeletal muscle arises from blocks of mesoderm located on either side (paraxial) of the neural tube during gastrulation. Paraxial mesoderm contains presomitic mesoderm, a transitory tissue located at the posterior tip of the embryo comprised of an immature posterior and a committed anterior region that gives rise to somites, which initiate the bulk of skeletal myogenesis (Fig 1)(4). The initially equivalent somites develop into multiple compartments, primarily the sclerotome, syndetome and dermomyotome. The sclerotomal cells essentially generates vertebrae and ribs. The syndetome is located between the sclerotome and myotome and gives rise to tendons and cartilage. The dermomyotome separates into an external layer of dermatome and internal layer corresponding to the myotome. Dermatome gives rise to dorsal dermis and the myotome generates myogenic precursors that form the skeletal muscle (13,14).

Somitogenesis is exquisitely regulated by a plethora of signaling pathways including Notch and Wnt (15). High levels of Wnt and also FGF maintain the cells in an undifferentiated state in the rear part of the paraxial mesoderm whereas the ventral domain gives rise to the sclerotome (bone

and cartilage progenitors) (16). Apart from some head muscle, the majority of the skeletal muscle originates from the dorsally located dermomyotome that remains mostly epithelial and is characterized by elevated levels of paired box transcription factors Pax3 and Pax7 and low levels of Myf5 (17). It's further regulated by the induction of MRFs by signaling molecules such as bone morphogenetic proteins (BMPs), sonic hedgehog (Shh) and Wnts to specify myogenic differentiation (18,19).

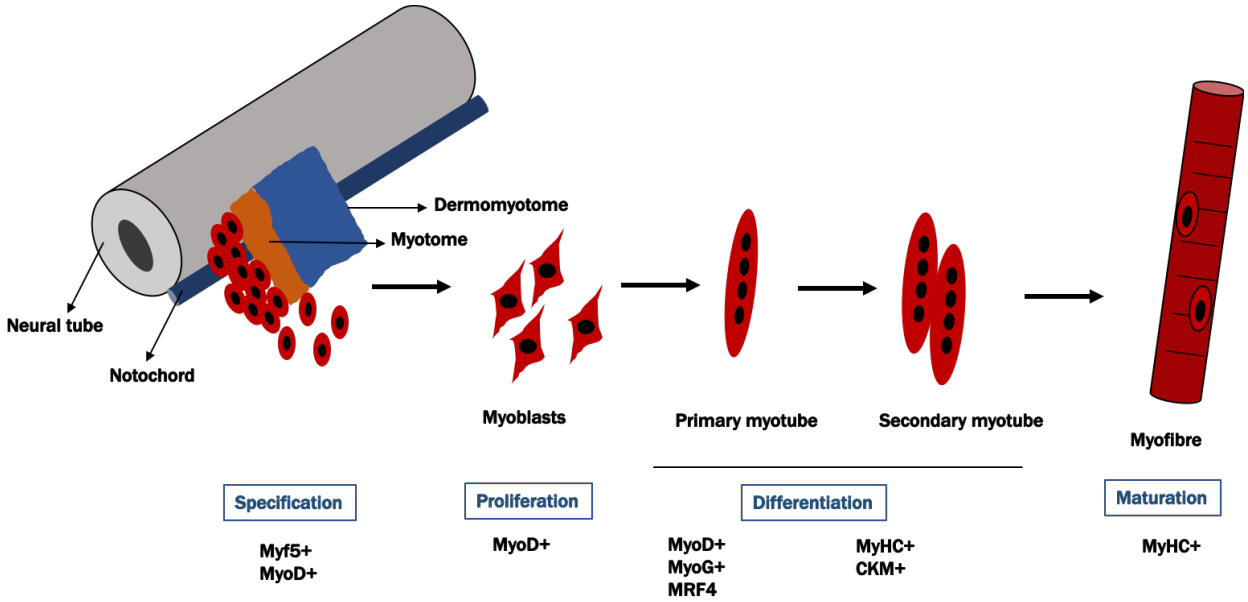

Figure 1: Schematic representation of the steps of skeletal myogenesis in vertebrates. Specification begins with mesodermal precursors aggregation to form somites. Specification ends with the formation of myotome from dermomyotome. Myoblasts originate from the myotome after expression of MyoD and Myf5, enter the cell cycle to proliferate and initiate differentiation upon withdrawal of growth media. Myogenein and MRF4 are expressed during the initiation of differentiation and later fuse with neighboring cells to form multinucleated myotubes expressing skeletal muscle specific proteins such as MHC (myosin heavy chain) and muscle creatine kinase (CKM). The myotubes then mature to myofiber.

1.1.3 Wnt signaling pathway regulates myogenesis

Wnt signaling has been implicated in the control of myogenesis at multiple stages, from regulation of embryonic muscle development to adult skeletal muscle homeostasis. It has been widely established that both canonical and non-canonical Wnt signaling modulate prenatal and postnatal muscle development by regulating the expression of several muscle regulatory factors (MRFs) such as Myf5, MyoD and Myogenin which are critical transcription factors involved in the differentiation program (reviewed in more detail later in this chapter). Both Canonical and non-canonical Wnt signaling also influence adult skeletal myogenesis by regulating the differentiation and self-renewal of satellite cells respectively (20).

Wnt signaling is a highly conserved pathway in species ranging from humans to *Drosophilla,* consisting of 21 secreted glycoproteins related to the *Drosophilla* wingless that can activate β-catenin dependent (canonical) or β-catenin independent (non-canonical) pathways in various physiological conditions (21-23). Canonical Wnt signaling starts with binding of Wnt ligands to the N-terminal cysteine-rich domain of Frizzled receptors (Fzd). FzD receptors are part of the G-protein coupled receptors (GPCRs), characterized by their specific seven transmembrane protein. Binding of Wnt activates a cascade of intracellular responses; wherein the most widely studied is the pathway that involves the activation of β-catenin and it's translocation to the nucleus to activate transcription of specific Wnt target genes (24). β-catenin is a multifunctional protein that consists of an N-terminal domain, a C-terminal *trans*activator domain and a central armadillo repeat region (ARM) composed of 12 armadillo repeats (Fig 2), each comprising approximately 42 residues that form three helices arranged in a triangular shape (25).

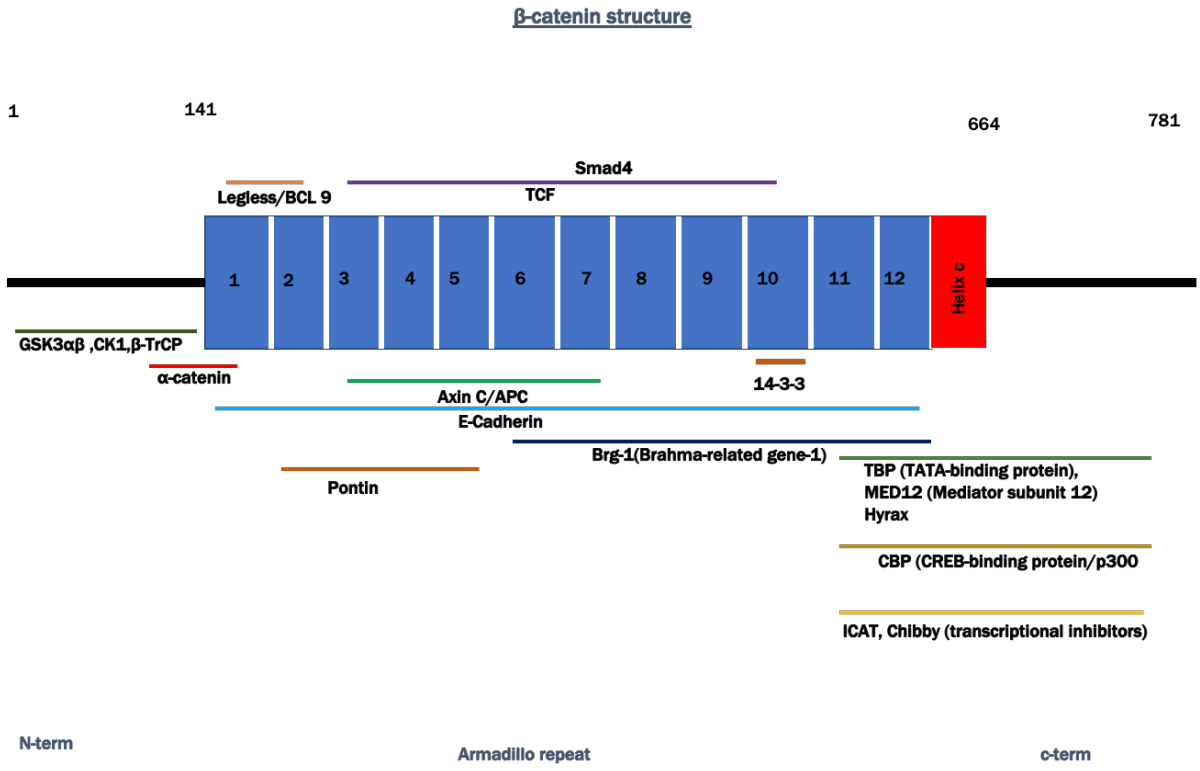

Figure 2: β-catenin protein structure.
β-catenin protein consists of 781 amino acids that comprises of a N-terminal domain for α-catenin binding, GSK3β and CK1 phosphorylation sites for β-TrCP ubiquitin ligase mediated ubiquitination. A central armadillo repeat domain (residues 141-664) comprises of 12 armadillo repeats constituting binding sites of APC, E-Cadherin and TCF and flanked by a conserved Helix-C. The C-terminal domain (residues 664-781) acts as a strong *trans*activator domain.

β-catenin is recruited to its destruction complex composed of Axin, Adenomatosis polyposis coli (APC), Glycogen synthase kinase 3 β (GSK3 β) and casein kinase 1 α (CK1 α) in the absence of

Wnt ligands. β-catenin is phosphorylated by CK1 α that primes it to be phosphorylated by GSK-3β leading to its ubiquitin-dependent degradation. Binding of Wnt ligands to their respective Fzd receptors inactivates the destruction complex by activating G proteins, Dsh and recruiting Axin to the Fzd co-receptor LRP (low density lipoprotein receptor-related protein), thereby inhibiting β-catenin degradation. β-catenin starts accumulating in the cytoplasm, translocates into the nucleus, associates with members of the TCF/LEF family proteins in the nucleus to activate transcription of target genes (26). β-catenin, by acting as a transcriptional co-activator to activate Wnt target genes, regulates various physiological and pathophysiological process (27). Additionally, cytoplasmic β-catenin interacts with E-Cadherin and Actin to form adheren junctions that play a role in cell to cell interactions (28).

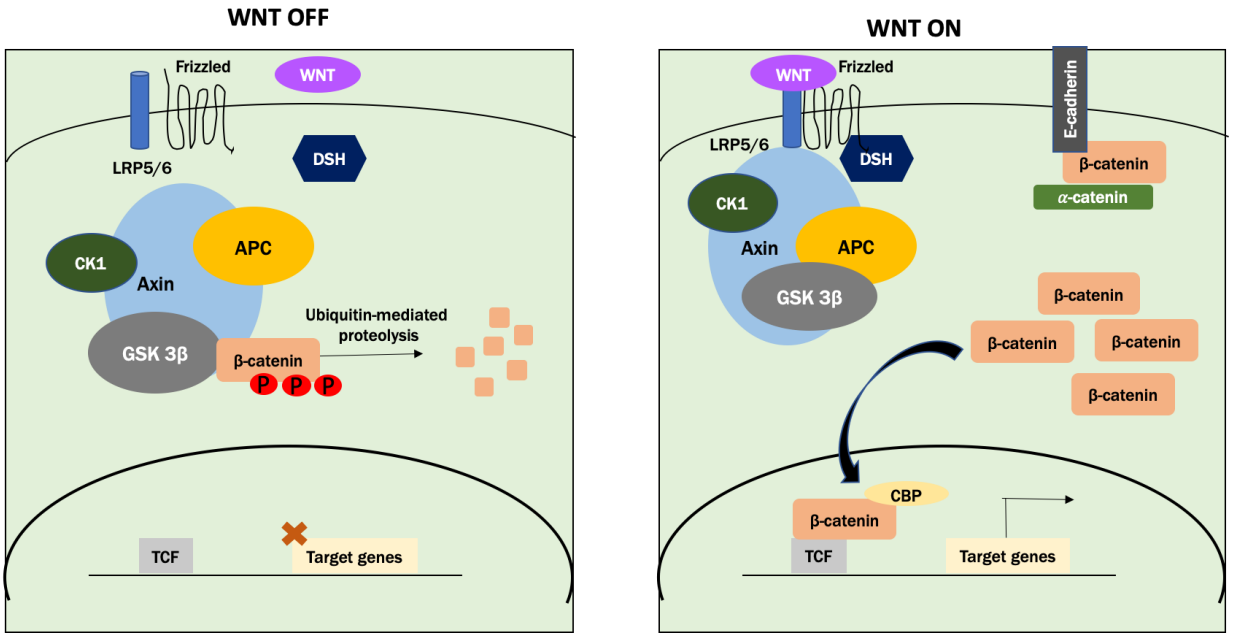

Figure 3: The Wnt signaling pathway.
β-catenin is recruited into a destruction complex comprising of Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3β) in the absence of Wnt ligands. Phosphorylation by CK1 primes for GSK3β phosphorylation resulting in ubiquitination and proteasomal degradation of β-catenin. Binding of Wnt to Frizzled receptor deactivates the destruction complex leading to cytoplasmic accumulation of β-catenin. β-catenin further enters the nucleus and associates with T-cell factor (TCF), lymphoid enhancer binding protein (LEF)-family of transcription factors and transcription coactivators such as CREB binding protein (CBP) to activate transcription of target genes.

Non-canonical Wnt signaling pathways include PCP (planar-cell-polarity), Wnt/Ca2+ and PI3K/AKT/mTOR that function independent of β-catenin's nuclear transcriptional activity, although these signaling pathways function through Fzd receptors, they may or may not require cooperation of LRP. As compared to the canonical signaling, non-canonical signaling has not been exhaustively characterized (29-31).

Wnt ligands such as Wnt1, Wnt3a and Wnt4 cooperate with Shh signaling from the notochord to induce somitic myogenesis during embryonic muscle development (32). Wnt1, 3a, 4 and 6 maintain Pax3 and Pax7 expression in the precursor myogenic cells of the somite through signaling from the surface ectoderm and neural tube (33). Mutation of Wnt genes has been suggested to negatively impact myogenesis. Studies conducted on mice deficient for Wnt 1 and Wnt3a showed a reduction in the expression of skeletal muscle transcription factors Pax3 and Myf5 as well as impaired dermomyotome (34). Additionally, Wnt1 and Wnt3a also induce expression of MyoD in the epaxial myotome suggesting that Wnt signaling plays a crucial role during embryonic myogenesis (35). Interestingly, Wnt 6 expressed in limb ectoderm downregulates MyoD while enhancing Myf5 expression, suggesting a differential role of Wnt's during myogenesis (36). It has been observed that signals from the neural tube and notochord induce MyoD and Myf5 expression in studies done on explant culture of somites from chick embryos (37,38). In vitro, myogenesis can be induced in somite by the addition of purified Wnt 1 and 3 along with low levels of Shh (39). While Wnt 1 activates Myf5 driven epaxial myogenesis, Wnt 7a induces a MyoD driven hypaxial myogenesis in mouse mesoderm explants (40). Wnt 7a enhances the expansion of satellite cells thus regulating the muscle's regenerative potential (41). Satellite cells generate new myofibers by either fusing with themselves or to the damaged myofibers post muscle injury, thus a fine tuning between their differentiation and self-renewal is necessary to maintain a satellite cell pool to generate new muscle fibers. Wnt 3a mediated early activation of canonical Wnt signaling during muscle regeneration can lead to depletion of the satellite cell pool as a result of premature differentiation of progenitor cells. Canonical Wnt signaling controls the differentiation of satellite cells where the progression of signaling from Wnt to Notch plays a crucial role (42).

Various loss of function animal models of Wnt/ β-catenin have been developed that are informative with regard to its role in muscle. β-catenin (Ctnnb1-/-) deficiency is embryonic lethal at E8.5dpc in mice (43). β-catenin conditional depletion in mouse muscle precursors marked by *Pax7*$^{+}$ during fetal myogenesis exhibited a reduction in slow myofibers (44). Interestingly, constitutively activated β-catenin resulted in neonatal lethality associated with a decline in the fiber size of muscle and increased number of motor axons and branching at E18.5 in mice. (45). Thus, a precise regulation of Wnt/β-catenin signaling activity is crucial for Pax7^{+} progenitor cells myogenic ability as observed from studies associated with both loss and gain of β-catenin function.

Muscle specific gene expression of *Myf5* and *Myod1* is induced by Wnt ligands (46). Moreover, Wnt ligands regulate specification, determination and differentiation of skeletal muscle cells through expression of the MRFs. *Ccna2* and *Cdc25c* gene expression is regulated in tongue and hind limb myoblast, C2C12 cells by Wnt/β-catenin signaling (Fig 4). Myoblast fusion mediated by β-catenin induced *Fermt2* expression is essential during differentiation of skeletal muscle (47). Moreover, Wnt/β-catenin signaling connects myofibers to the surrounding extracellular matrix through cadherin/β-catenin/actin complex formation to maintain myofiber structure (47). Altogether, β-catenin has been implicated to be a crucial regulator in various stages of the skeletal muscle differentiation program.

Skeletal myogenesis is inhibited in mouse embryos following treatment of a soluble Wnt antagonist secreted frizzled-related protein 3 (sFRP3) (48). Altogether, Wnt/β-catenin signaling

plays a crucial role in proliferation of myogenic cells during development and regeneration of muscle cells.

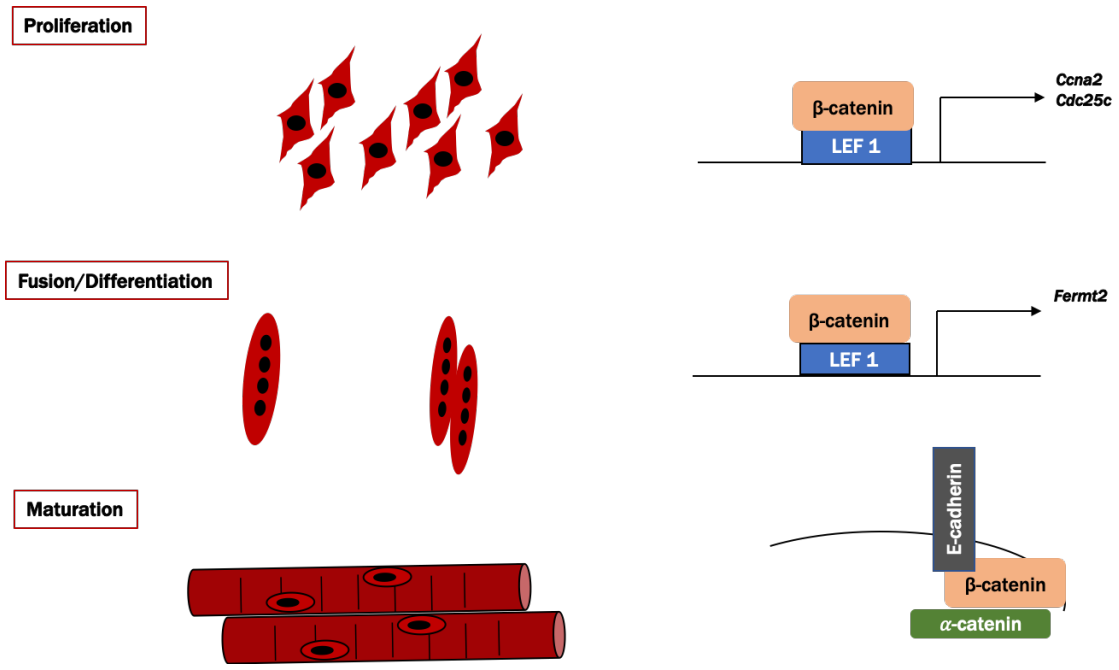

Figure 4: Role of β-catenin during myogenesis.
WNT/β-catenin signaling pathway regulates skeletal myogenesis during proliferation, differentiation and maturation stages. During proliferation, *Ccna2* and *Cdc25c* gene expression is regulated by WNT/β-catenin signaling. 3β-catenin further regulates gene expression of *Fermt2* during differentiation. β-catenin associates with E-cadherin and *α*-catenin to elongate the myofiber. *Adapted from; The temporal specific role of WNT/β-catenin signaling during myogenesis; Akiko Suzuki, Anne Scruggs, and Junichi Iwata. Journal of Nature and Science (JNSCI), Vol.1, No.8, e143, 2015. Copyright permission received from author.*

1.1.4 Regulation by TGF-β

The transforming growth factor-beta (TGF-β) superfamily consists of over 50 structurally related ligands that play a pivotal role in the pathogenesis and physiology of numerous tissues (49). De-regulation of this signaling cascade is associated with various developmental defects and disease as this pathway has been shown to exert versatile effects in a diverse range of tissues (50). Skeletal muscle ontogeny and postnatal physiology in various animals is extensively regulated by this cytokine superfamily (51). Deletion of myostatin has been associated with muscle hypertrophy and hyperplasia (52). TGF-β1and myostatin are the most characterized ligands in skeletal muscle. Signaling molecules such as TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3), BMPs 1-20, activin A activin B, growth and differentiation factors (GDFs), Mullerian inhibiting substance (MIS), leftys1, leftys 2 and nodal constitute the TGF-β superfamily of cytokines (49). TGF-β signaling activates a cascade of Smad proteins, acting as intracellular downstream effectors. Smad refers to the homologies to the c.elegans SMA ("small" worm phenotype) and Drosophilla MAD ("Mothers Against Decapentaplegic") family of genes. The Smad family in humans includes eight members that are categorized into receptor Smads (Smads 1, 2, 3, 5 and 8), Co-Smads (Smad4) and Inhibitory Smads (Smads 6 &7). Smad proteins consist of two globular regions called Mad homology 1 (MH1) at the N-terminus, and MH2 at the C-terminus connected by a linker region (Fig 5).

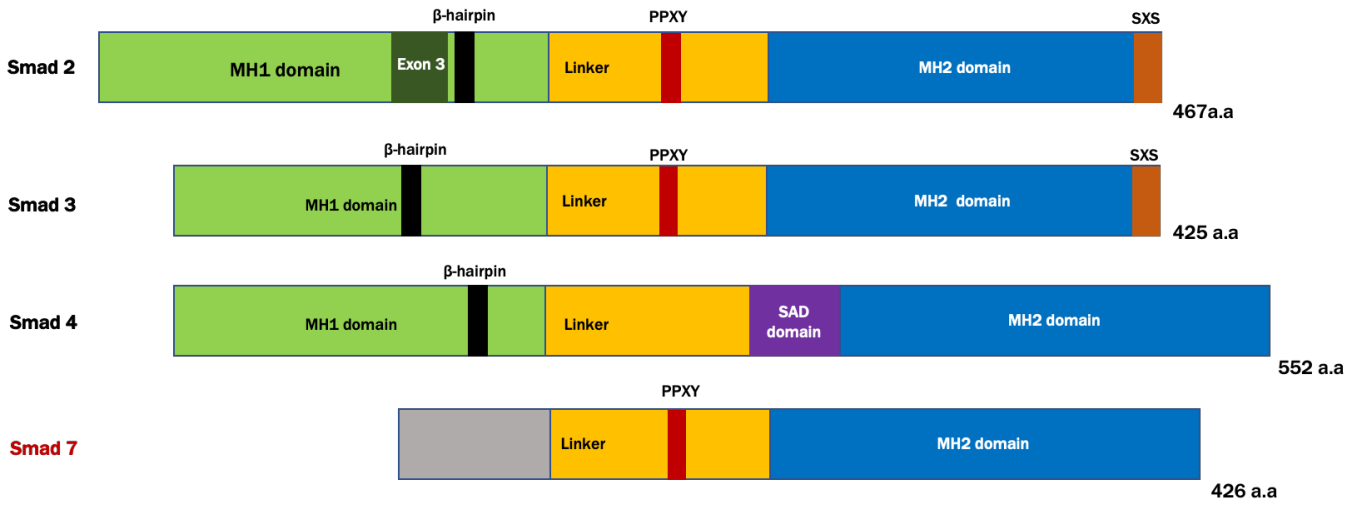

Figure 5: Structure of Smads.

Smads consist of two globular regions, N-terminus Mad homology 1 (MH1) domain and C-terminus MH2 domain, connected by a linker region. The linker region is highly conserved in R-Smads (Smad2, Smad3) and Co-Smad (Smad4). Receptor Smads contain SXS domain within the MH2 domain required for their activation. Smad4 contains a proline rich Smad4 activation domain (SAD). The MH1 domain is not conserved in Smad7 although the MH2 domain is conserved.

This signaling network can be further classified into TGF-β, myostatin (GDF-8) and BMP (BMPs and GDFs) branches depending on the recruitment of receptor regulated Smads (R-Smads); while TGF-β and myostatin signal through R-Smads 2 and 3, BMP pathway signal through R-Smads 1, 5 and 8 (51). Signal transduction is initiated with binding of the ligand to the constitutively active serine/threonine type II receptor (TGF-βRII) kinase that in turn activates its associated type I receptor by forming a heterotetrameric receptor complex containing two type I and two type II receptors. The activated receptor complex can further phosphorylate two serine residues in the conserved "SSXS motif" at the c-terminus of R-Smads and facilitates dimer formation. The R-Smads dimer forms a heteromeric complex with common-Smad (co-Smad), translocates into the nucleus and interacts with other transcription co-factors to regulate transcription (Fig 6). Smad6

and Smad7 (inhibitor Smads) form an autoinhibitory feedback and abrogate this pathway (described further in the sections below) (51).

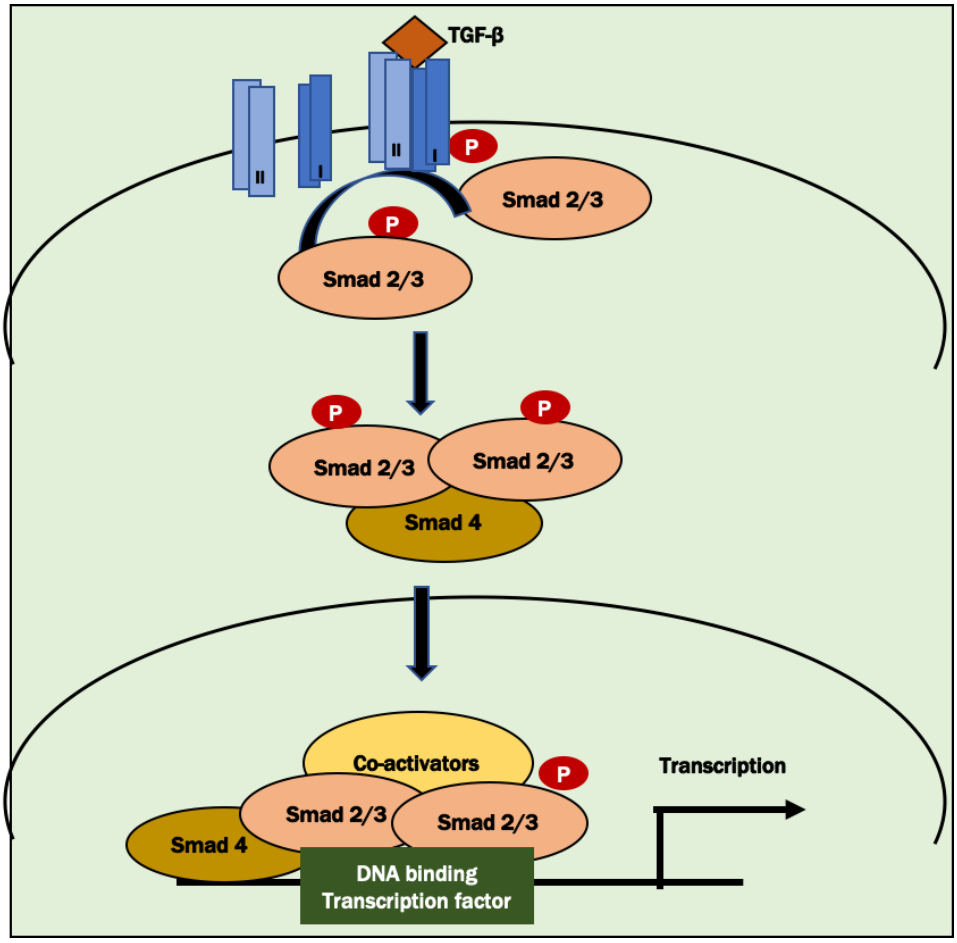

Figure 6: Schematic representation of TGF-β signaling.
TGF-β signaling starts with the ligand (TGF-β) binding to Type II serine/threonine transmembrane receptor kinase that activates and *trans*-phosphorylates Type I receptor kinase. The activated type I-Type II receptor complex further phosphorylate Receptor-regulated Smads (R-Smads; Smad2/3) at the C-terminal serines, the activated R-Smad form complex with common Smad4. The Smad2/3 and Smad4 complex translocates into the nucleus to regulate target gene transcription by associating with DNA binding transcription factors and coactivators.

Slow fibers developing from myotubes are formed before the expression of TGF-β1 whereas fast fibers are formed when myoblasts are adjoining the TGF-β1 expressing connective tissue, suggesting that the precise spatial and temporal expression of TGF-β1 during myogenesis regulates the fiber-type composition of the surrounding myotubes (49,53). Interestingly, TGF-β1 have been shown to inhibit fetal myoblast differentiation but does not impart any inhibitory effect on the differentiation of embryonic myoblasts (54). TGF-β negatively affects skeletal muscle regeneration in mature adult muscle by inhibiting myofiber fusion, skeletal muscle specific gene expression as well as satellite cell proliferation (55). TGF-β1 is implicated to induce fibrosis in myogenic cells (56). A stage specific control of myogenesis was observed by TGF-β and BMP in C2C12 cells. Enhanced BMP activity was observed in undifferentiated myoblasts, in contrast, TGF-β activity was relatively low in undifferentiated myoblasts and gradually increased after differentiation (57). Satellite cell activation and myocyte differentiation is inhibited by increased levels of TGF-β1 and inhibiting the levels of TGF-β1 has proven beneficial in several myopathic conditions (58,59).

Impairment of TGF-β signaling has been associated with inherited and acquired skeletal muscle pathologies (60). TGF-β, MAPK, and/or MSTN levels were reported to be elevated in spinal muscular atrophy (SMA), a neurodegenerative disorder (61,62). TGFβ1 levels were also elevated in Duchenne muscular dystrophy (DMD), a genetic disorder characterized by lack of dystrophin that leads to progressive muscle degeneration (63). Elevated MAPK, TGF-β1, and/or MSTN activation was associated with muscle atrophy caused by hypoxia, suggesting the role of TGF-β

in acquired myopathies (64). Impairment of both TGF-β canonical and non-canonical signaling has been associated with Sarcopenia characterized by age associated loss of skeletal muscle mass and function (65).

1.1.5 Regulation by Shh

The Hedgehog (Hh) signaling pathway is one of the intricate signaling networks governing many crucial events in embryonic development. Impairment in this signaling pathway has been associated with various developmental defects and diseases such as cancer (66). Mammals have three Hedgehog homologues, Sonic (Shh), Indian (Ihh), and Desert (Dhh). In the absence of ligand, Smoothened (Smo), a downstream effector, is inhibited by the cell surface receptor Patched1 (Ptch1). Binding of Shh to Ptch1 activates the signaling pathway via nuclear translocation of Glioma-associated oncogene (Gli) family zinc finger transcription factors comprising of activators (Gli1, Gli2) and repressor (Gli3) to regulate transcriptional activity by Smo (67). Expression of Shh from the notochord and neural floor plate regulates proliferation and differentiation of myoblasts by upregulating the formation and survival of muscle precursor cells in the dorsal myotome (68). Gli activator proteins are specifically expressed in different compartments of the somites; sclerotome predominantly expresses Gli1 whereas dermomyotome and myotome primarily express Gli2 in association with Myf5 and MyoD (69,70). Shh has been implicated to be crucial for expression of MyoD in the somite and it has been shown that Shh -/- mice fail to express epaxial MyoD and Myf5 to form the epaxial myotome (39,69,71,72) and Myf5 and MyoD expression in mouse limb buds (73). Interestingly, Shh or Smo conditional knockout mice were associated with delay in MyoD initiation (74,75). A study conducted in primary cultures of chick myoblasts to understand the regulation of Shh in muscle proliferation and differentiation

reported that mRNA levels of Gli, Shh and Ptch1 are elevated along with myoblast differentiation (76). Myf5 activation in muscle progenitor cells requires Gli2 or Gli3 and in the absence of Shh, Gli3 represses Myf5 transcription (69). Shh regulates myogenic progenitor cell specification by associating with the Gli-binding sites located in the Myf5 enhancer to enhance Myf5 gene expression (75). Since the Myf5 enhancer region contains both Gli and Tcf/Lef binding sites, it can be concluded that Wnt and Shh signaling play synergistic roles in myogenic determination (77,78).

Another study aimed to observe the effects of Smad7 in ocular development found that *Smad7* mutant mice displayed an expanded optic disc with an increase in Shh signaling (79). Expression of MRFs in somites is induced by both Hh and Wnt signaling to regulate embryogenesis and cellular differentiation (80,81). The cross-talk between Hh and Wnt signalling is poorly understood as Hh inhibits Wnt pathway activation via associating with secreted frizzled-related protein 1 (SFRP1). Similarly, Wnt associates with the repressor Gli3 and inhibits transcriptional activity. However, in amphibian limb regeneration, a cross regulation may occur as it was observed that activation of Wnt rescued the inhibitory effect of Hh, thus suggesting that the interplay between these signalling pathways remains to be fully elucidated (82)

1.1.6 Regulation by Hippo Signaling

Hippo/Salvador-Warts-Hippo (SWH) is a highly conserved signaling pathway in *Drosophila* and mammals that controls organ size. The hippo kinase cascade consists of mammalian Ste20-like kinases 1/2 (MST1/2, Hpo ortholog), Salvador (Sav1), large tumor suppressor 1/2 (LATS1/2, Warts ortholog) and Mps One Binder Kinase Activator-Like 1A/B (MOBKL1A /MOBKL1B)

(Mats ortholog) and the downstream transcriptional co-activators YAP and TAZ (Yorkie ortholog) (83). MST1/2 binds and phosphorylates Sav1, the activated complex directly phosphorylates at the T1079 and T1041 kinase domain of Lats 1 and 2 respectively to activate them. Additionally, Mst1/2 phosphorylates MOBKL1A/MOBKL1B that further associates with the Lats1/2 autoinhibitory motif (S909 and S872) to phosphorylate and activate the Lats1/2 activation loop to promote autophosphorylation (84-86). Lats1/2 directly interacts with and phosphorylates YAP/TAZ (87-89), the interaction is mediated by a PPxY motif and WW domain in LATS1/2 and YAP/TAZ respectively (90). Phosphorylated YAP/TAZ associates with 14-3-3 binding proteins that mediates its sequestration in the cytoplasm thus inhibiting the transcription of target genes (91) (Fig 7).

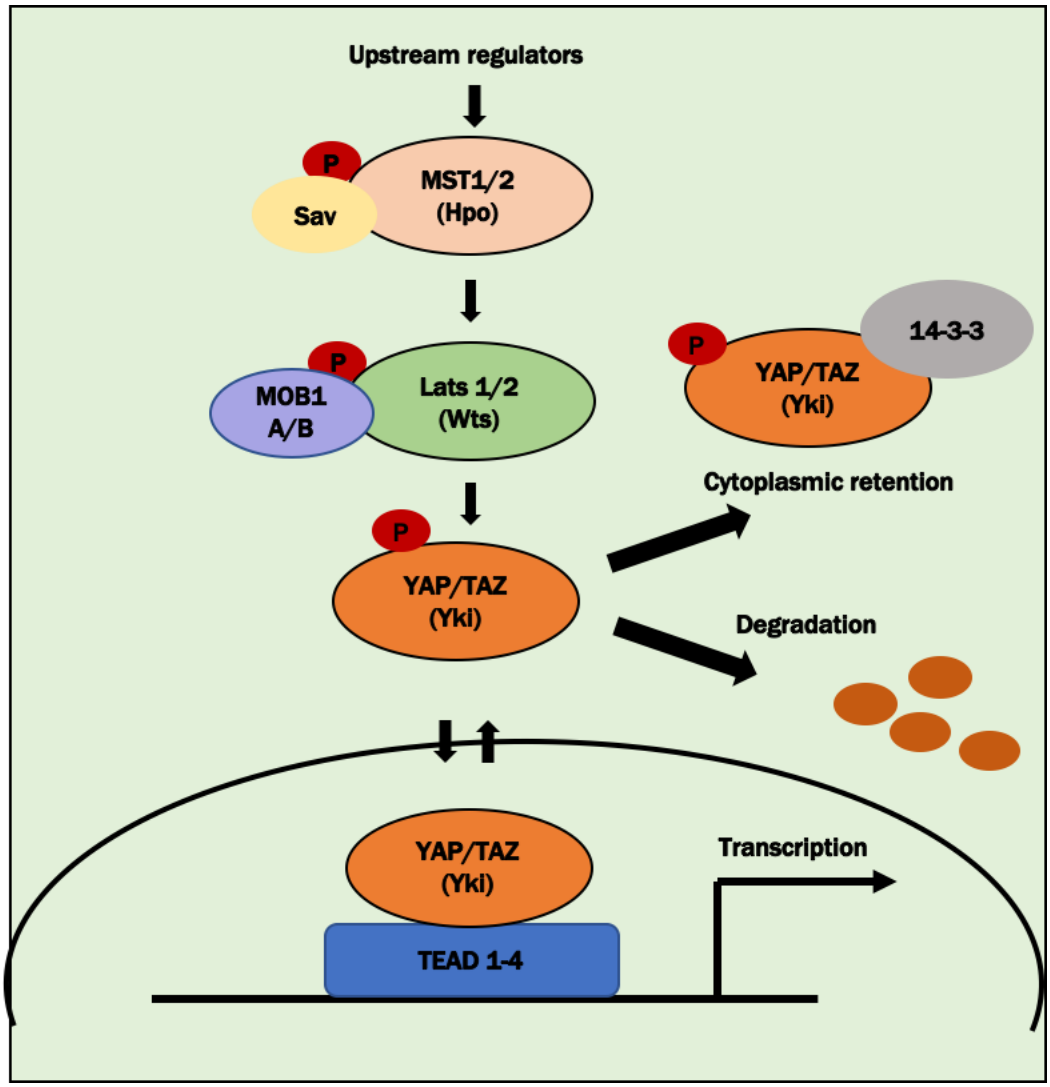

Figure 7: Hippo Signaling pathway.

The sterile 20-like kinases (MST1/ MST2) binds and activate the regulatory protein Salvador (Sav1) that phosphorylates and activates the large tumor suppressor 1/2 (LATS1/2) kinases. MST1/2 also phosphorylate the Mps One Binder Kinase Activator-Like 1A/B (MOBKL1A /MOBKL1B) regulatory subunits of LAST1/2, the activated LATS1/2-MOB1A/B complex further phosphorylates YAP and TAZ, promoting its association with 14-3-3 binding proteins that results in sequestration and/or proteasomal degradation in the cytoplasm.

LATS1/2 regulates TAZ and Yki protein stability by phosphorylation on multiple serine residues in the conserved HXRXXS site (92). LATS1/2 mediated YAP and TAZ phosphorylation at S381 and S311 respectively promotes phosphorylation by casein kinase 1 that further recruits β-TrCP (β-transducin repeat-containing proteins) resulting in degradation of YAP/TAZ (93). YAP/TAZ interacts with DNA-binding transcription factors TEAD1–4 in the promoter region of target genes to regulate the transcription of cell proliferation and death associated genes (94). Interestingly, TAZ/YAP interacts with other DNA-binding transcription factors besides TEADs, such as Smad1/2/3/7 (95-97), RUNX1/2 (98) and p63/p73 (99).

Hippo signaling pathway upstream regulators are identified as components of adherens junctions (AJs), apical–basal polarity protein complexes and tight junctions (TJs) (83). Nf2 (neurofibromatosis-2)/Merlin Ex (Expanded) has emerged as an important mediator of the upstream Hippo pathway regulators (100). Sav and Hpo associates with Mer and Ex whereas Kibra interacts with Wts (101,102), suggesting their possible role in recruiting the Hippo kinases to the plasma membrane. Another Hippo pathway cell surface regulator, Drosophila crumbs (Crb) has been identified in the subapical plasma membrane (103). Epithelial cell polarity and integrity of TJs is maintained by AMOT family of proteins (104) that mediates the recruitment of YAP/TAZ to the actin cytoskeleton or TJs, thus inhibiting YAP/TAZ nuclear localization (105). α-Catenin, another component of AJs, contributes to suppression of tumor formation by inhibiting YAP activity (106). Other proteins such as Scribble enhance TAZ phosphorylation and subsequent degradation by bridging interaction between TAZ, LATS and MST (107). Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (SIP) activates protease-activated receptors (PARs) which were also identified as factors for YAP/TAZ activation (108-110). YAP/TAZ is activated in cells

that have undergone cell spreading whereas it was found inactivated in the round and compact cells suggesting that Hippo signaling is also regulated by cell morphology (111).

TAZ and YAP are paralogs, sharing 46% amino acid sequence identity (112). The distinct domain that confers signaling specificity in TAZ and YAP is the tryptophan residue containing WW domain which recognizes a PPxY (proline/proline/any amino acid/tyrosine) motif. Interestingly, the TAZ isoform in human contains one WW domain as compared to YAP, which has tandem WW domains (Fig 8). The PDZ binding C-terminal domain regulates TAZ/YAP localization by moderating interactions with PDZ domains (113).

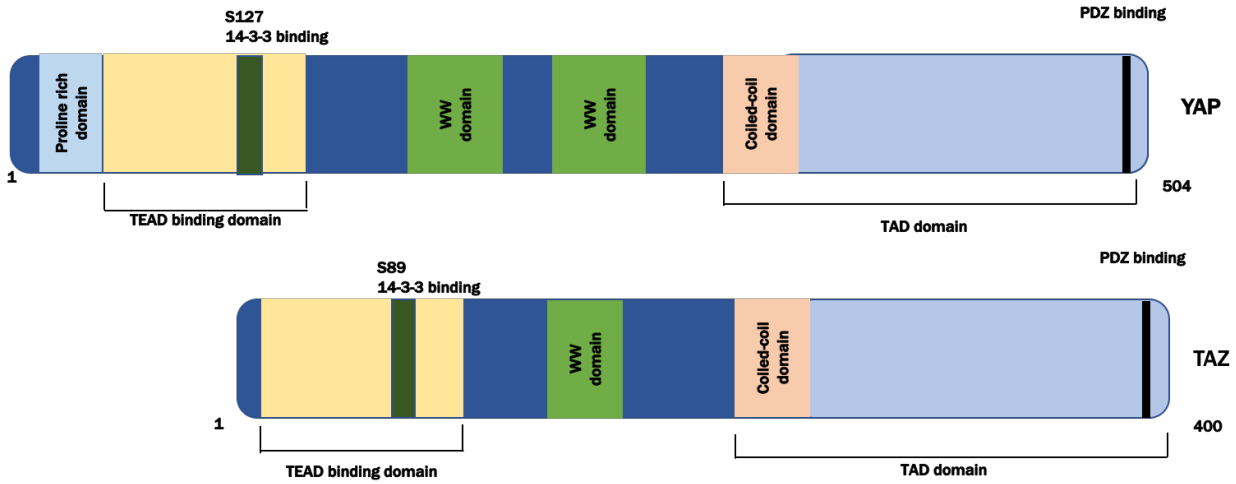

Figure 8: Structure of TAZ/YAP proteins.
TAZ/ YAP protein consists of a N-terminus TEAD binding domain, central WW domain, the coiled-coil (CC) domain and a C-terminal transcriptional activation domain (TAD) and PDZ-binding motif.

Phosphorylation by c-ABL, SRC and YES (114,115) in this residue was suggested to regulate transcriptional activity of TAZ/YAP. Association of TAZ/YAP to TEAD transcription factors is mediated by the N-terminal domain located in close proximity with the 14-3-3 binding region, thus

regulating localization of TAZ/YAP by nuclear exclusion and sequestration to the cytoplasm (91,116). In the absence of Wnt, the β-catenin destruction complex, through phosphorylating β-catenin, maintains TAZ at low levels by bridging TAZ to β-TrCP ubiquitin ligase, thereby promoting the degradation of TAZ. Wnt activation attenuates degradation and promotes nuclear accumulation of both β-catenin and TAZ by rescuing β-catenin from its destruction complex (117). Interestingly, studies suggested that regulation of gene expression by Wnt/β-catenin pathway is not only mediated by β-catenin/TCF complex but also through TAZ-mediated transcription and protein stabilization. Knockdown of TAZ/YAP maintained embryonic stem cells in an undifferentiated state suggesting that the β-catenin destruction complex contains both TAZ and YAP and is crucial for the recruitment of β-TrCP to inactivate β-catenin (118). Moreover, it was also observed that TAZ knockout mice develop polycystic kidneys characterized by accumulation of β-catenin (119). LATS2 has been implicated in the inhibition of Wnt signaling where nuclear LATS2 interacts with β-catenin and interferes with its association with BCL9, suggesting the involvement of hippo kinases in the regulation of Wnt (120). It is interesting to speculate that the divergent role of YAP/TAZ in Wnt signaling may depend on TCF/β-catenin and YAP/TAZ/TEAD (118,121).

Mammalian Hippo pathway components including Yap are all expressed in primary skeletal muscle cells as well as C2C12 myoblasts and myotubes. Yap Ser127 phosphorylation is low in myoblasts and is enhanced during myogenic differentiation promoting its translocation from the nucleus to the cytosol (3). Differentiation of C2C12 myoblasts to myotubes require Yap Ser127 phosphorylation as overexpression of a mutant form of YAP that is inhibitory to phosphorylation by LATS1/2 resulted in the decline of Myogenin and MEF2C (differentiation marker) and

increased expression of Myf 5 (myoblast marker) (3,122). Interestingly, YAP binding to TEAD co-activated muscle specific cytidine adenosine thymidine (MCAT) elements in myoblasts (123). Increased expression of Mst1/2 was observed in postnatal muscle cells followed by a decline in the first three weeks of life suggests a role for hippo components in postnatal muscle development. Moreover, overexpression of constitutively active YAP increases proliferation of neural progenitor cells and reduces the expression of markers of somitic muscle differentiation in *Xenopus laevis* embryos (124).

Both YAP and phosphorylated YAP^{Ser112} were upregulated in muscle hypertrophy in response to myostatin and activin inhibition (125). Moreover, in the Duchenne muscular dystrophy *mdx* mouse model, total YAP and phosphorylation of YAP at Ser 122 was enhanced suggesting that YAP plays a role in the regeneration of skeletal muscle. Increased YAP protein levels were observed in slow-twitch muscle fibers as compared to fast-twitch 2A fibers in human skeletal muscle. Moreover, older patients demonstrated reduced YAP levels as compared to younger controls (126) suggesting its possible role in sarcopenia. Furthermore, YAP activation in satellite cells *in vivo* leads to the formation of embryonic Rhabdomyosarcoma-like tumours (127). YAP is required for skeletal muscle mass maintenance in the post-natal muscle. In the adult myofiber, depletion of endogenous YAP leads to reduction of muscle mass, protein synthesis and myofiber cross section (122) although another study suggested that there was no change in muscle fiber size with YAP depletion in a skeletal muscle-specific YAP knockout model (128). Therefore, YAP and TAZ regulation in skeletal muscle, remains to be fully explored.

TAZ was identified as a mediator of nucleocytoplasmic shuttling of Smad playing a crucial role in human embryonic cells suggesting that components of hippo signaling exert cross talk with TGF-β signalling pathway (129,130). Smads interact with a range of other transcription factors such as the RUNX and p53 that are also regulated by TAZ and YAP, suggesting that perhaps Smads are recruited by TAZ/YAP to modulate the function of these proteins (131). Moreover, the Mediator component ARC105 is suggested to participate in retaining TAZ/YAP–Smad complex in the nucleus (96). TAZ/YAP-Smad2/3 complexes mediate the pluripotent state of Human ESCs by forming a complex with TEAD transcription factors, as well as OCT4, core stem cell regulator (132). The TAZ/YAP-Smad2/3- OCT4 complex further associates with nucleosome remodeling and a deacetylation (NuRD) complex to regulate the expression of pluripotency genes and repress genes that define mesoderm. During specification of mesoderm, Smads dissociate from the complex to activate the transcription factor forkhead box H1 (FOXH1) (133). TAZ mRNA expression was increased in a p38-dependent manner by TGFβ. Induction of TAZ expression by TGFβ was abrogated by myocardin related transcription factor (MRTF) inhibition (134).

1.2 Regulation of myogenic gene expression

1.2.1 Myogenic regulatory factors (MRFs)

The myogenic regulatory factor family (MRFs) is comprised of four muscle-restricted transcriptional regulatory proteins (MyoD, Myf5, Myogenin and MRF4). MRFs are members of a larger family of basic helix-loop-helix proteins that regulate a series of molecular switches determining various cell lineage fates (78). These proteins regulate cell cycle exit of muscle precursor cells, cell proliferation and activation of muscle specific genes thereby governing skeletal muscle phenotype (78). MRFs play indispensable roles during prenatal and postnatal

myogenesis by orchestrating a complex network of transcription factors to regulate selective expression and specific responses to signaling cues (78). Myogenic determination protein 1 (MyoD1) was the first MRF identified by screening a library of C3H 10T1/2 cells possess the ability to convert fibroblasts into myoblast-like cells, the three remaining MRFs were discovered shortly by similar method (135,136). MRFs contain three distinct structural domains, an N-terminal cysteine/histidine domain, a basic helix-loop-helix (bHLH) domain and a C-terminal serine/threonine-rich domain (137). MRFs forms a heterodimer complex with E-protein, can recognize the E-box sequence (CANNTG) in the regulatory sequences of target genes (138).

MyoD and Myf5 are involved in the commitment and proliferation of the specified myogenic directed cells, whereas, Myogenin and MRF4 regulate the terminal differentiation of the committed cells (139). Myf5 is the first MRF to be expressed in somites and maintained in dermomyotome that would further develop into myotome. MRFs, with the exception of Myf5, have the ability to initiate differentiation in the proliferating prenatal skeletal muscle precursors cells (140,141). Transformation of myogenic precursor cells into myoblasts can develop through activation of MyoD achieved through PAX3 mediated Myf5 activation (142) or paired-like homeodomain factor 2 (Pitx2) (143). MyoD expression is induced by the transcription factors FoxO3, Six1/4 and Pax3 and Pax7 in proliferating myoblasts (144,145). MyoD expression is also regulated by various signaling pathways such as Wnt signaling that induce MyoD expression at E10.5 from the dorsal endoderm and BMP4 that induces MyoD expression from the lateral mesoderm in the hypaxial myotome (146). Myf5, MRF4, and MyoD can be directly activated by both Six and Eya proteins in the hypaxial dermomyotome (147). Transcripts for Myogenin and MRF4 are first detected at E8.5 and E9.0, respectively throughout the myotome (10,12).

Interestingly, Myf5 and MyoD expression is not well understood in the context of satellite cells. Adult satellite cells do not express MyoD in resting conditions, a study suggested all the progenitors derived from satellite cells transcribe MyoD prenatally (148), although, both Myf5-positive and Myf5-negative satellite cells have been identified in adult muscles (149). During differentiation, MyoD induces Myogenin expression, and consecutively downregulates Myf5 expression (150). Furthermore, MyoD and Myogenin promote the expression of MRF4 to initiate the multinucleated fibers formation. MyoD and Myogenin are downregulated and MRF4 acts as the predominant MRF in a matured muscle (78).

Interestingly, studies in mice suggested that depletion of functional *MyoD* gene correlated with enhanced Myf5 mRNA but there were no obvious defects in skeletal muscles suggesting that Myf5 can probably circumvent the absence of MyoD in the development of skeletal muscle (151). Similarly, Myf5 deficient animals did not develop any abnormalities in skeletal muscle probably because in those mice, the onset of MyoD expression followed by the expression of Myogenin occurred normally suggesting that MyoD activation is perhaps independent of Myf5 in the development of skeletal muscle. Interestingly, Myf5 and MyoD double knockout model markedly affected the myogenic program, newborn mice were totally devoid of skeletal muscle and died within minutes (152). Interestingly, Myogenin has been suggested to play a crucial role in regulating the later stages of myogenic differentiation as Myogenin homozygous null mice exhibit a distinct phenotype with lack of functional skeletal muscle and perinatal death (153).

1.2.2 Myocyte enhancer factors (MEF2s)

Myogenesis is simultaneously regulated by the myocyte enhancer factor 2 (MEF-2) family of transcription factors. The MEF2 family was originally designated based on the similarity of MCM1, Agamous, Deficiens, and Serum response factor (MADS) box group of transcription factors (154). Muscle development from muscle precursor cells requires collaboration of various muscle specific genes controlled by skeletal muscle-specific transcription factors (155). During skeletal muscle differentiation, the four MEF2 isoforms (A–D) are co-expressed although they do exhibit different temporal patterns of expression (155,156). MEF2 proteins bind to the consensus sequence (*C/T*TA(A/T)$_4$TA *G/A*) within the promoter/regulatory regions of genes. MEF2 proteins comprises of three domains: a N-terminal MADS domain that binds DNA, a central MEF2 domain and a C-terminal transactivation domain (156-158). Although the MADS and the central MEF2 domain is well conserved across MEF2 family, the C-terminal transactivation domain is not very well conserved. Interestingly, it was also observed that during the transformation of fibroblasts to myotubes, MyoD co-operates with MEF2 transcription factors to activate skeletal muscle specific gene expression (159).

In heart, depletion of *Mef2A* results in mitochondrial and contractile defects in the heart (160) and *Mef2C* global deletion is embryonic lethal due to impaired heart morphogenesis (161). MEF2-deficient vertebrate models demonstrated different phenotypes suggesting individual MEF2 isoforms regulate different genes in muscle, for example; *Mef2C* knock-out mice exhibited abnormalities in myofiber structure, some of these mice survived to adulthood with myofiber abnormalities whereas *Mef2A* global deletion had normal skeletal muscle development (162,163). *Mef2a* knockout in injured mice demonstrates impaired myofiber formation (164). Depletion of

Mef2A by miR-155 inhibits myoblast differentiation suggesting that MEF2A confers a critical role in the differentiation of skeletal muscle (165). *Mef2D* deletion in skeletal muscle demonstrated no negative effect on the development of skeletal muscle but interestingly, *Mef2D* knock-out in adult mice showed altered distribution in skeletal myofiber (163). Interestingly, *Mef2A* deletion alone does not affect differentiation in adult satellite cells but cumulative deletion of *Mef2A-C-D* results in impaired differentiation (166). Loss of MEF2A has been suggested to down regulate muscle development in cardiomyocytes (157). Another study aimed to understand the role of the four mammalian MEF2 proteins in muscle observed that knockdown of MEF2B, -C, or -D either individually or in combination regulate numerous nonoverlapping genes in C2C12 cells, did not debilitate C2C12 myotube formation although depletion of MEF2A had a negative effect in the formation and differentiation of myotube (155). An association between Smad2 and MEF2A was observed in C2C12 cells suggesting a cross talk between genes controlled by MEF2 with components of TGF- β signaling (167). Interestingly, it was also observed that once the differentiation program is started and MEF2 is expressed as a co-regulator, TGF-β loses its inhibitory effect and rather promotes the differentiation program.

1.2.3 Six proteins

The sine oculis homeobox (SIX) family of transcription factors have been implicated to play crucial developmental roles. Mammals possess six *SIX* genes and mutations of these genes in flies or mammals adversely affect development of many organs/tissues including muscle. Elevated levels of SIX proteins have been implicated in human genetic disorders and several cancers suggesting their role in cell cycle regulation (168,169). The Six proteins are characterized by two evolutionarily conserved domains; Six domain and the Six-type homeodomain, crucial for DNA

binding and interactions with Eya proteins (170). Mutations within *sine oculis (so*) was associated with impaired visual system in *Drosophila melanogaster* (171,172).

Skeletal myogenesis is coordinated by MRFs, MEF2 and other transcription factors (TF). The MEF3 consensus sequence (TCAGGTTTC) is recognized by TFs of the SIX family of proteins suggesting that SIX proteins could regulate myogenesis in a combinatorial manner by modulating MRFs (173). Specifically, SIX1 and SIX4 have been suggested to regulate development of skeletal muscle *Six1*-null mice exhibits impaired embryonic myogenesis with defects in trunk musculature, develop respiratory problems that leads to death (174-176) Although there was no phenotypical abnormality in *Six4*-null mice (177), it was interesting to observe that abnormalities in skeletal myogenesis was more profound with combined deletion of *Six1* and *Six4* as compared to *Six4*-null mice alone suggesting that SIX1 and SIX4 work concomitantly in skeletal muscle (144). Moreover, enhanced number and the genes associated with fast-twitch (glycolytic) muscle fibers was associated with elevated SIX1 (178). In Zebrafish, *six1a* gene functional loss causes abnormal fast-twitch muscle formation (179). A gene expression profiling to identify potential SIX1 and SIX4 target genes identified *Myog* and *Myf5* as *Six 1* target genes and loss of *Six1* affected expression levels of several muscle specific genes such as *Pax3*, *Myod1*, *MRF4* and *Lbx1* (180-182). Moreover, MyoD expression is depleted in the absence of Myf5 and Six1/4 suggesting a cooperative role played by MRFs and SIX proteins to regulate embryonic skeletal myogenesis (183).

1.2.4 Smad7

Smad (Sma and Mad proteins from Caenorhabditis elegans and Drosophila, respectively) are proteins constituting a family of structurally related transcription factors that are the key signal mediators of the TGF-β superfamily. Smad7, also known as *mothers against decapentaplegic homolog 7* (*MADH7*) and localized on chromosome 18 in both human and mouse and codes a protein with 426 a.a residues. Smad7 was initially identified as TGF-β activated inducible antagonist of TGF-β-mediated signaling. Smad7 associated with the TGF- β receptor complex and blocked TGF-β mediated function in mammalian. Moreover, administration of *Smad7* RNA into Xenopus embryos inhibited activin/TGF-β signaling (184). Perturbation of Smad7 has been associated with a plethora of human diseases such as colon carcinogenesis (185) chronic inflammatory bowel disease (IBD) (186) and muscle wasting (187).

Genetic alterations of Smad proteins characterize gastrointestinal carcinomas (e.g., pancreatic, colorectal) suggesting a context dependent tumor suppressor role in some cancers (188). Smad7 overexpression has been observed in sporadic colorectal cancer (CRC) and colitis-associated CRC where knockdown of Smad7 has the ability to negate CRC cell growth. Interestingly, enhanced expression of Smad7 in the infiltrating immune cells in the patients inflamed gut can attenuate CRC cell growth (189,190) thus suggesting a context dependent role of Smad7 in sporadic and colitis-associated CRC (191). A study conducted in colon cancer identified Smad7 as a direct target of MicroRNA-25 (miR-25) where miR-25 was suggested to function as a suppressor of tumor by targeting Smad7 (192). A protective role of Smad7 in renal injury associated with Diabetes was observed in Smad7 null mice where a heightened TGF-β and nuclear factor-κB (NF-κB) signaling activation was observed that was correlated with increased renal fibrosis and

inflammation. (193). Inflammation associated with Crohn's disease is characterized by high levels of SMAD7. An oral SMAD7 antisense oligonucleotide (Mongersen) was shown to exhibit significantly enhanced remission and clinical response rates as compared to placebo in preclinical and a phase 1 study (194,195). TGF-β signaling was abrogated by Smad7 with the induction of premalignant ductal lesions characterized by pancreatic intraepithelial neoplasia (PanIN), the precursor stage to pancreatic carcinoma (196). Ectopic Smad7 expression was associated with increased gastric cancer cells (SGC7901) survival (197) Interestingly, elevated Smad7 levels was observed in human papilloma and squamous cell carcinoma, (198) although a TGF-β-dependent tumor-suppressive role of Smad7 was observed in metastatic melanoma cells (199,200). In a study conducted in 150 invasive breast carcinoma specimens, size and stage of tumor, and expression of matrix metalloproteinase (MMP)-9 and MMP-14 was positively correlated with Smad7 levels (201). Another study documented that Smad7 can attenuate cell invasion and EMT in carcinoma of the breast (202). Interestingly, in prostate cancer cells, Smad7 associates with APC and facilitates TGF-β-induced accumulation of β-catenin, leading to enhanced migratory responses (203). Another study suggested depletion of Smad7 by microRNA-21 was associated with proliferation and migration of cells in hepatocellular carcinoma (204). Smad7 prevented skeletal muscle wasting by inhibiting Smad2/3 and expression of the atrophy-related ubiquitin ligases suggesting that muscle-directed Smad7 gene delivery could be a promising approach in diseases associated with muscle wasting (187). It can be speculated that a possible explanation of the opposite effects of Smad7 in different cancers could be attributed to different functionality of TGF-β pathway signaling among distinct cancer types (188).

1.3 Regulation of Smad7

1.3.1Canonical role

Smad proteins can be classified into three sub-families- receptor regulated Smads (R-Smads), common co-activator (Co-Smad) and inhibitor Smads (I-Smad). Upon activation of TGF-β signaling, R-Smad is phosphorylated and activated, forms complex with Co-Smad and translocates into nucleus for target gene transcription. On the other hand, inhibitory Smads comprising of Smad6, a relatively specific inhibitor for BMP signaling, and Smad7, a general inhibitor for both TGF-β/activin and BMP signaling is known to negatively regulate this pathway (184,205,206). The Smad binding element (SBE) with the consensus sequence GTCTAGAC is known to bind both Smad2/3 and Smad4. An SBE is localized in the Smad7 promoter and it was observed that this sequence is critical in the transcription of TGF-β regulated genes, as mutation in SBE abrogated TGF-β mediated Smad7 activation in HepG2 cells. Additionally, an adjacent E-box along with an overlapping AP1 site is required for activation of TGF-β-dependent promoter suggesting that transcription of Smad7 is regulated by a specific recognition motif in the SBE (207) .

Smad7 mediated inhibition of TGF-β signaling involves numerous mechanisms (Fig 9). Firstly, Smad7 forms a stable complex with TβRI, thereby attenuating the recruitment and phosphorylation of R-Smads and preventing the assembly of R-Smad–Smad4 complex formation (184,208). Interaction of Smad7 with TβRI is achieved through the Smad7 MH2 domain (209), while and the Smad7 N-terminus domain magnifies this effect (210). Smad7 also associates with BAMBI (BMP and activin membrane-bound inhibitor) for TGF-β signaling inhibition (211).

Secondly, Smad7 can act as an adaptor to recruit WW-HECT-type E3 ubiquitin ligases (Smurf) to participate in TβRI receptor degradation. Smurfs induce ubiquitination and degradation of TβRI through caveolin vesicle mediated endocytosis of TβRI. Smad7 forms a complex with Smurf1/2 in the nucleus through its PY motif, translocate to the cytoplasm, and associate with activated TβRI (212,213). Smad7 can recruit the E2-conjugating enzyme UbcH7 (ubiquitin-conjugating enzyme 7), that further enhances TβRI degradation by Smurf2 (214). Smad7/Smurf2-mediated ubiquitination and degradation of TβRI is further augmented by its association with HSP90 (heat-shock protein 90) (215). Moreover, Smad7 can associate with NEDD4–2 and WWP1/Tiul1 to negatively regulate TGF-β signaling (216,217).

Additionally, Smad7 can also recruit Growth arrest and DNA damage protein (GADD34) to dephosphorylate TβRI (218). Smad7 can also regulate ALK1 (activin receptor like kinase 1) activity by recruiting PP1α (219), and interact with other proteins such as STRAP (serine/threonine kinase receptor-associated protein) (220), SIK (salt-inducible kinase) (221), AIP4 (atrophin 1-interacting protein 4) (222), YAP65 (Yes-associated protein 65) (97), Cas-L (Crk-associated substrate lymphocyte type) (223) and Hic5 (H2O2-inducible clone 5) (224) to modulate TβRI activity or stability.

Lastly, in the nucleus, Smad7 can antagonize TGF-β signaling. Studies conducted in multiple cell lines such as Hela, Hep3B and HPL-1 cells suggested that Smad7 interferes with the R-Smads/Smad4 binding to the promoters of target genes to abrogate TGF-β signaling (225,226). Smad7 can also modulate chromatin epigenetic status by forming complex with histone deacetylases (HDAC1), Sirtuin 1 (SIRT1) and acetyltransferase p300 (221,227-229).

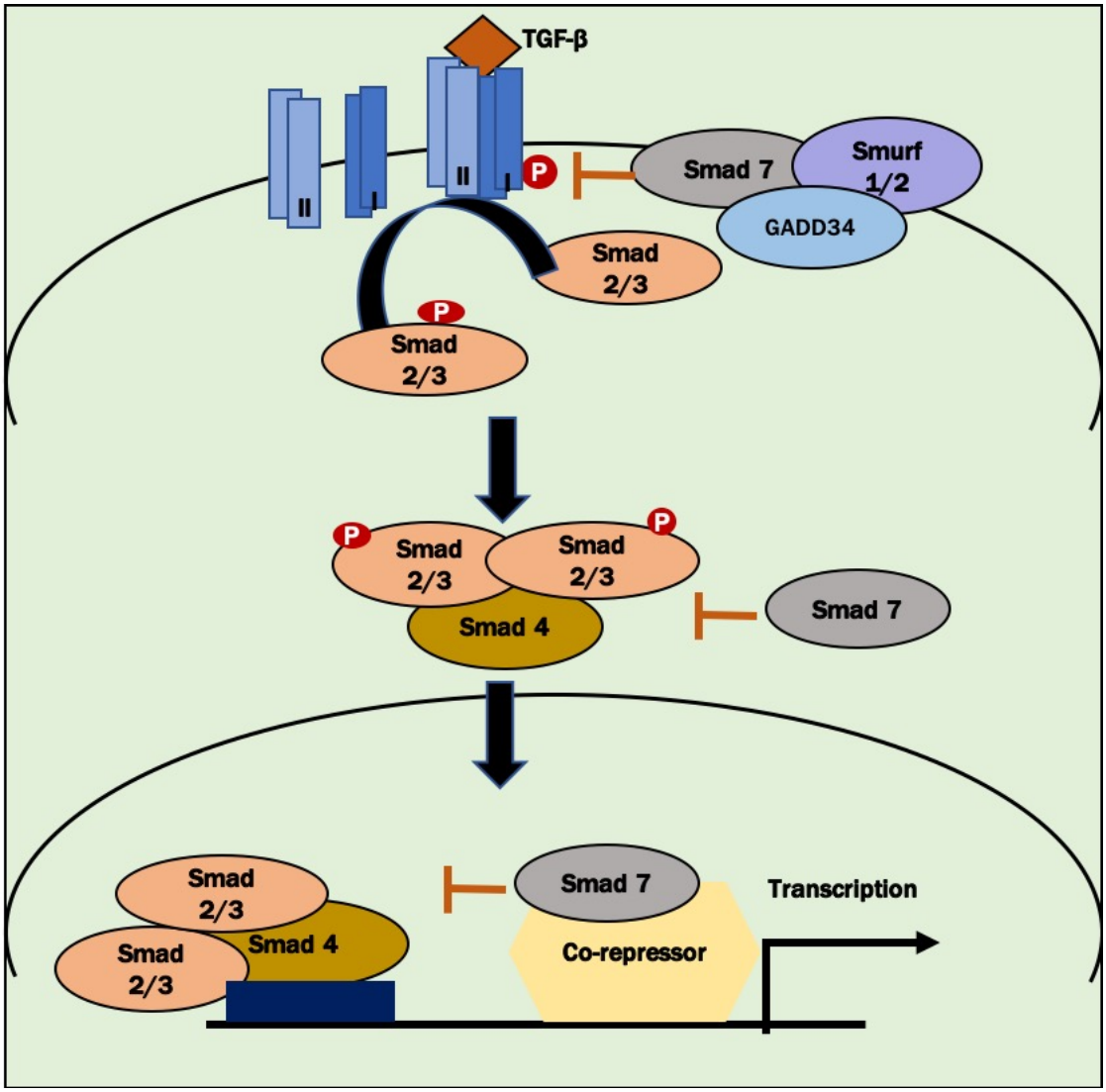

Figure 9: Canonical role of Smad7

Smad7 inhibits the TGF-β signaling pathway through multiple mechanisms. Smad7 can associate with the activated type I receptor and compete with R-Smads which prevents their activation. Smad7 can also promote degradation of type I receptors by recruiting the E3 ubiquitin ligases Smurf1 and Smurf2. Additionally, Smad7 can dephosphorylate the type I receptor by recruiting GADD34 and protein phosphatase 1c (PP1c. Smad7 also inhibits R-Smad and smad4 association in the nucleus. Lastly, Smad7 associates with co-repressors to inactivate transcription of TGF-β target genes.

In muscle, a direct interaction of Smad7 and MyoD was observed that was associated with increased skeletal muscle differentiation. Strikingly, siRNA mediated depletion of endogenous Smad7 inhibited the differentiation of cultured muscle cells suggesting that Smad7 plays a crucial role in the initiation of skeletal myogenesis (230). Interestingly, Smad7 counteracted the repressive effect of myostatin but not TGF-β1, suggesting that non-canonical aspects of TGF-β1 signaling is perhaps not shared with myostatin in a muscle cell context (230). In addition, Smad7 inhibited Smad3/Smad4 activity by reporter gene analysis [(CAGA)9-luc] although TGF-β1 induced growth inhibition was not affected (199).

1.3.2 Nuclear role of Smad7

A previous study from our group suggested that Smad7 can reverse the myostatin inhibition during differentiation of skeletal muscle but does not rescue the inhibitory effect of TGF-β. Smad7 can enhance skeletal muscle differentiation by collaborating with a nuclear transcription factor. Moreover, depletion of Smad7 antagonized myogenic differentiation suggesting it to be a crucial component of the myogenic differentiation program (230,231). A follow up study aimed to bypass Smad7's "canonical" ability to inhibit activation of Smad3 by TGF-β was executed by attaching a nuclear localization signal (NLS) to Smad7 (Smad7-NLS) that would exclusively localize Smad7 in the nucleus. Smad3 activation by TGF-β was not inhibited by Smad7-NLS but retained its ability to enhance myogenic gene activation and phenotypic myogenesis, suggesting that a receptor independent nuclear function of Smad7 is adequate to enhance myogenesis (232). Smad7 and MyoD directly interacted and further relieved the inhibition of MyoD's transcriptional activity by mitogen- activated protein (MAP) kinase kinase (MEK) (230). CT (Cardiotrophin)-1 inhibits

myogenesis through activation of the MEK/ERK, thereby inactivating MyoD activity (233). Smad7 antagonized CT-1's inhibitory effect on myogenesis along with reversing the inhibitory effect of MEK on MyoD indicating that MEK signaling and Smad7 converge on MyoD. Interestingly, a feed-forward loop of Smad7 and MyoD was suggested in skeletal myogenesis whereby MyoD also regulates transcriptional activity of Smad7 through association with the E-box localized in the *smad7* gene promoter (232) (Fig 10).

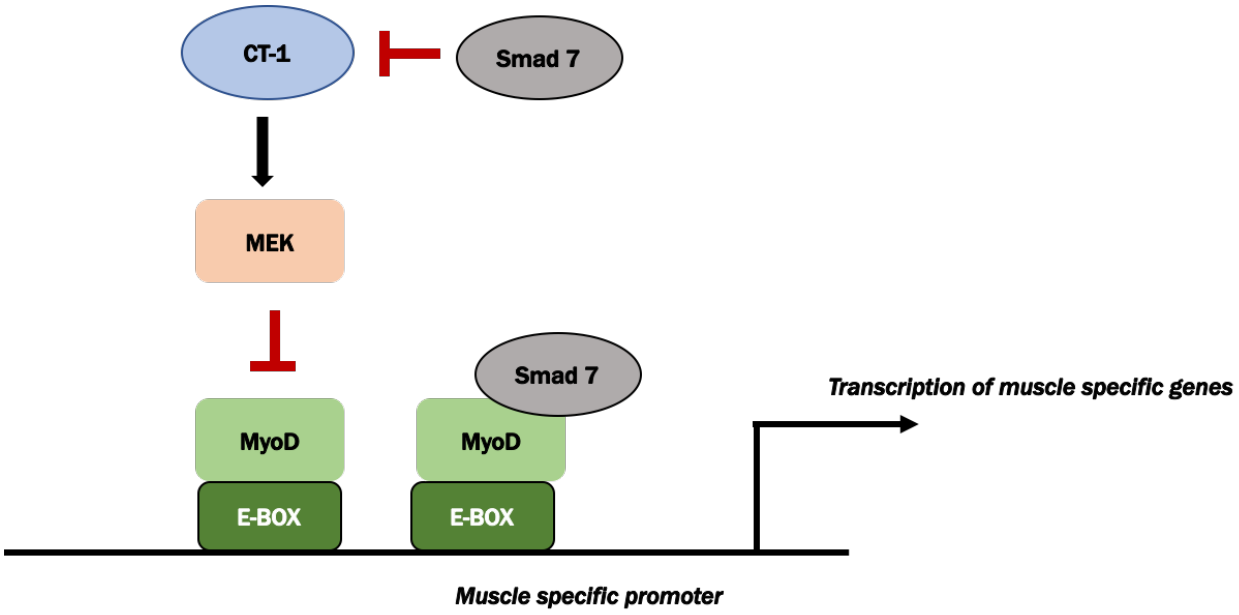

Figure 10: Nuclear role of Smad7.

This schematic represents the non-canonical role of Smad7 independent of its role in inhibiting TGF-b signaling. Smad7 directly interacts with MyoD in the nucleus to enhance transcriptional activity. The MEK/ERK pathway normally represses activation of MyoD by a physical interaction of MEK and MyoD. Additionally, Cardiotrophin-1 (CT-1) inhibits activation of MyoD by activating the MEK/ERK pathway. Smad7 can reverse CT-1's inhibitory effect on MyoD's transcriptional activation properties. Interestingly, MyoD regulates Smad7 expression through association with an E-box located in the Smad7 promoter, suggesting that Smad7 and MyoD establish a feed-forward loop to regulate differentiation of skeletal muscle.

Another study conducted in mesenchymal cells suggested that Smad7 was found to be localized in the nucleus, forming a complex with nuclear factors to exert inhibitory effects in the progression of the cell cycle. Another mechanism of Smad7-induced arrest of cell cycle was suggested in NIH 3T3 mouse fibroblast cells whereby Smad7 associated with endogenous HDAC-1 and HDAC-3, caused binding of HDAC-1 to E2F-1 that lead to repression of E2F target genes (227).

1.3.3 Cross talk with other signaling pathways

Smad7 is a direct target of both TGF-β and BMP signaling pathways. Smad7 transcriptional activity is regulated by various signaling pathway as Smad7 transcription is mediated not only by associating with SBE (207), but a maximal induction of Smad7 transcription needs other transcription factors or cofactors including AP1 (activator protein 1), p300 and FOXH1 (forkhead box H1), Sp1 (stimulating protein-1), TFE3 (transcription factor E3) and ATF2 (activating transcription factor 2) (226,234-237). Smad7 is highly expressed in Inflammatory Bowel disease (IBD) although levels of secreted TGF-β remains elevated suggesting that Smad7 might act through other signaling pathways (238-240). Moreover, Smad7 was also associated with TGF-β-induced activation of Cdc42 and RhoA GTPases to facilitate actin filament rearrangement in human prostate carcinoma cells (241) suggesting that Smad7 can inhibit or promote TGF-β signaling and is context dependent or is regulated by other signaling pathways.

Anti-inflammatory effects of Smad7 are mediated by various mechanisms. Smad7 may induce the expression of IκB, by attenuating IκB phosphorylation and degradation in colon cancer cells (242,243). It can also disrupt the complex formed between the IL-1-receptor-associated kinase (IRAK)1, IRAK4, Pellino-1 and TRAF6, thus abrogating activation of NF-κB (244). Smad7 can

mediate disruption of complex formation between TNF-receptor associated factor 2 (TRAF2)– TGF-β-activated kinase 1 (TAK1)– TAK-binding protein 2/3 (TAB2/3) (245). Interestingly, EGF (epidermal growth factor) also induces Smad7 transcriptional activity along with (246), HER2/Neu (human epidermal growth factor receptor 2) (247), UV irradiation (248), laminar shear stress (249) and PMA (250). Smad7 can interact with TβRI, TAK1, MKKs and JNK/p38 to promote TGF-β-induced activation (251). Furthermore, ERK1/2, JNK1/2 and p38 MAPKs activity was inhibited by Smad7 during erythroid and chondrocyte differentiation (252,253) suggesting MAPK regulation by Smad7. TGF-β and Wnt signaling cross talk with each other at multiple levels and, depending on cell context, Wnt signaling can be activated or repressed by TGF-β. Smad7 has been implicated in TGF-β1 mediated β-catenin accumulation and LEF1 in human prostate cancer (PC-3U) and human keratinocytes (HaCaT cells) (1). Similarly, in Alzheimer's disease, TGF-β mediated complex formation of Smad7 and β-catenin can lead to cortical neuronal cells apoptosis (254), augment β-catenin and E-cadherin expression in breast cancer and hepatocellular carcinoma (255,256). Breast cancer and melanoma cell invasion and metastasis was also inhibited by Smad7 (200). Smad7 can disrupt the β-catenin destruction complex, thereby stabilizing and promoting its association with E-cadherin or N-cadherin (257). Furthermore, Smad7 abrogated β-catenin activation in hepatocellular carcinomas (258) indicating that Smad7 and β-catenin share a context dependent role. Overall, the varied and complex nature of Smad7 function and its particular role in skeletal myogenic cells referred to above led us to propose the following goal of this thesis.

Statement of Purpose

The TGFβ family signaling pathway proteins are associated with diverse cellular functions such as proliferation, migration, differentiation and apoptosis. In skeletal muscle, TGFβ1 signaling represses skeletal muscle cell differentiation [11]. Smad7 has been classically characterized as an inhibitor of the TGFβ Smad2/3 canonical pathway functioning primarily at the receptor level. However, a previous study from our group demonstrated a physical interaction between Smad7 and MyoD in which Smad7 potentiated MyoD-dependent transcription. These data suggested that Smad7 contributes to "non-canonical" aspects of downstream signaling in muscle cells. Depletion of Smad7 expression using small interfering RNA technology in C2C12 cells resulted in decreased expression of Myogenin, MyoD, MEF2A and MyHC, indicating that depleting the level and function of Smad7 inhibits myogenic differentiation (230). A further study from our group confirmed a novel nuclear role of Smad7 that bypasses its "canonical" ability to abrogate TGFβ mediated Smad3 activation. Moreover, Smad7 was shown to antagonize the inhibitory effect of MAP kinase kinase (MEK) on the function of MyoD (232). Homozygous loss of Smad7 in gene targeting experiments in mice corroborated our findings, reporting a reduction in muscle mass and force generation, slow recovery from muscle injury along with fiber type switching from glycolytic to a more oxidative type. Collectively, these studies illustrate a critical role of Smad7 in the context of skeletal muscle (259). A growing theme of work from our group indicates that protein networks serve to dynamically modulate transcriptional activity in differentiating myogenic cells, allowing various permutations of cellular signaling pathways to exert control over the primary regulators. ***The work described in this dissertation was therefore aimed at characterizing and further understanding the protein interactions of Smad7 in myogenic cells.*** Chapter 2 summarizes the

interaction and function of Smad7-MED12/13-β-catenin on a muscle specific promoter. Chapter 3 characterizes the protein interactome of β-catenin in muscle cells. Chapter 4 documents the role of the Hippo pathway effector TAZ in modulating the function of Smad7 and β-catenin in myogenic cells. Collectively these studies further our understanding of the function of Smad7 in skeletal muscle cell biology.

Chapter 2

Smad7:β-catenin Complex Regulates Myogenic Gene Transcription

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Published in

Cell Death and Disease, 2019 May 16; 10(6):387.doi: 10.1038/s41419-019-1615-0

Author contributions

Experimental Design

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Drafting Manuscript

Soma Tripathi and John C. McDermott

Conducting Experiments

Soma Tripathi:	Figure 11A, B, D, E, F
	Figure 12A, B, D, E
	Figure 13A, B, C, D, E, F
	Figure 14A, B
	Figure 15A, B
	Figure 16A, B, C, D
Tetsuaki Miyake:	Figure 11C

2.1 Abstract

Recent reports indicate that Smad7 promotes skeletal muscle differentiation and growth. We previously documented a non-canonical role of nuclear Smad7 during myogenesis, independent of its role in TGF-β signaling. Here, further characterization of the myogenic function of Smad7 revealed β-catenin as a Smad7 interacting protein. Biochemical analysis identified a Smad7 interaction domain (SID) between aa575-683 of β-catenin. Reporter gene analysis and chromatin immunoprecipitation demonstrated that Smad7 and β-catenin are co-operatively recruited to the extensively characterized *ckm* promoter proximal region to facilitate its muscle restricted transcriptional activation in myogenic cells. Depletion of endogenous Smad7 and β-catenin in muscle cells reduced *ckm* promoter activity indicating their role during myogenesis. Deletion of the β-catenin SID substantially reduced the effect of Smad7 on the *ckm* promoter and exogenous expression of SID abolished β-catenin function, indicating that SID functions as a *trans*dominant negative regulator of β-catenin activity. β-catenin interaction with the Mediator kinase complex through Med12 subunit led us to identify MED13 as an additional SMAD7 binding partner. Collectively, these studies document a novel function of a Smad7-MED12/13-β-catenin complex at the *ckm* locus, indicating a key role of this complex in the program of myogenic gene expression underlying skeletal muscle development and regeneration.

2.2 Introduction

Developmental myogenesis, the process of terminal differentiation of skeletal muscle progenitor cells, consists of a series of well characterized highly regulated steps that has become a paradigm for lineage acquisition and cellular differentiation (4,13,260-262). During embryogenesis, pluripotent mesodermal stem cells commit to become myogenic progenitor cells (68). Commitment to the myogenic lineage results in a binary state of either maintenance of proliferative potential and multipotency or, on appropriate cues, withdrawal from the cell-cycle, activation of a battery of structural, contractile and metabolic genes and ultimately formation of multi-nucleated, electrically excitable myofibers (13,263,264). Also, in adult skeletal muscle, resident stem cells (satellite cells) located between the basal lamina and the plasma membrane of mature muscle fibers (265), recapitulate this 'myogenic program' of differentiation in response to injury or for routine maintenance of the muscle tissue (266).

How networks of transcriptional regulators exert molecular control over developmental and adult myogenesis has been a prevalent theme in understanding the molecular control of ontogeny, physiology and pathology of striated muscle (266-268). Extensive biochemical and genetic evidence has implicated a family of DNA binding transcriptional regulatory proteins encoded by the myogenic regulatory factor (MRF) genes, *myf5, myod1, myogenin (myog)*, and *mrf4*, in myogenesis (266,269). In conjunction with the proteins encoded by the *myocyte enhancer factor 2 (mef2a-d)* gene family, the MRF's activate an evolutionarily conserved program of gene expression, which leads to the generation of terminally differentiated skeletal muscle cells (156,270,271). Understanding the 'trans' acting factors contributing to this process has been aided in tandem by extensive analysis of the 'cis' regulatory elements of muscle restricted,

differentiation induced genes such as the *muscle creatine kinase* (*ckm*) gene (272-274).

In addition to the central role played by the MRF/MEF2 axis in myogenesis (155), other transcription factors have been implicated in the control of myogenic differentiation such as Six 1 and 4 (144,174,275), AP-1 (276,277), β-catenin (21,47,278) and Smad7 (230,232,259). Since our initial observations of the pro-myogenic role of Smad7 in cultured muscle cells (230), we have documented a novel nuclear role for Smad7 in muscle that is essentially independent of its 'canonical' role as a repressor of TGF-β signaling (232). In addition, other groups have documented an *in vivo* role for Smad7 in skeletal muscle (259). Understanding the nature of the ancillary role played by Smad7 and other transcriptional regulators at muscle restricted genes is therefore of some importance for our overall understanding of the molecular programming of myogenic identity.

In view of the 'non-canonical' nuclear role of Smad7 alluded to above, the mechanistic basis by which it contributes to the myogenic differentiation program is so far incompletely understood. We were intrigued by a report identifying a protein-protein interaction (PPI) between Smad7 and β-catenin in human prostate cancer cells (1) since both are known, independently, to be key regulators of muscle gene expression in a variety of contexts. We therefore assessed this putative PPI in cultured muscle cells. Our data support a robust interaction between Smad7 and β-catenin that contributes to the transcriptional control of a key myogenic promoter (*ckm*). We further extended these observations in characterizing the recruitment of Mediator components (Med12/13) by the β-catenin/Smad7 complex, thus connecting the muscle transcriptosome assembled on enhancers such as *ckm* to the basal transcription machinery. Integration of the Smad7-β-catenin

complex into the network of proteins regulating the 'myogenic program' expands our understanding of the unique molecular wiring encoding myogenic differentiation, growth and repair.

2.3 Materials and methods

Cell culture

C2C12 myoblasts and C3H10T1/2 cells were obtained from the American Type Culture Collection. Cells were cultured in growth medium (GM) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% fetal bovine serum (FBS) and L-Glutamine (HyClone) supplemented with 1% penicillin-streptomycin (Invitrogen, Thermofisher). Myotube formation was induced by replacing GM with differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum (Atlanta Biologicals) and 1% penicillin-streptomycin. Cells were maintained in an incubator at 95% humidity, 5% CO_2, and 37°C.

Transfections

For ectopic protein expression, cells were transfected using the calcium phosphate precipitation method for transcription reporter assays. Cells were re-fed 16 h post-transfection and harvested. For small interfering RNA (siRNA) experiments, cells were transfected with Lipofectamine 2000 (Life Technologies) using instructions provided by the manufacturer and harvested 48 h later, unless otherwise indicated. For Wnt treatment, murine Wnt-3a (Peprotech) was re-suspended with double distilled water containing 0.1% bovine serum albumin (BSA) and added to the serum free DMEM at a concentration of 100ng/ml. The cells were serum-starved for 8 h prior to drug administration.

Gene silencing

MISSION siRNA (Sigma-Aldrich) for rat and mouse *ctnnb1* siβ-catenin#1 (SASI_Rn01_00099925), siβ-catenin#2 (SASI_Rn01_00099923), siβ-catenin#3 (SASI_Rn01_00099924) siSmad7#1(SASI_Mm02_00290887),siSmad7#2(SASI_Mm02_00290886),siSmad7#3(SASI_Mm02_00290885) and universal scrambled siRNA (SIC001) were used at 75nM concentrations.

Plasmids

Expression plasmids for Myc-His-tagged full-length Smad7, β-catenin-myc, transcription reporter assay constructs *pckm-luc* have been described previously (230,232,279). β-catenin mutant expression plasmids were constructed by the ligation of PCR amplified nucleotides corresponding to the indicated amino acid regions (aa575-683, aa1-574) at Hind III and Xho I sites of pcDNA3-EYFP or pcDNA3-3Xflag-8XHis respectively. Constructs for expression of GST fused β-catenin fragments were described previously (280).

Transcription reporter gene assays

Transcriptional reporter assays were performed using luciferase reporter plasmids along with expression constructs (indicated in figure legends) and a Renilla plasmid (pRL-Renilla, Promega) as an internal control. Cells were washed with 1X PBS and harvested in Luciferase Lysis Buffer (20 mM Tris pH 7.4 and 0.1% Triton X-100). Enzymatic activity was measured in each sample on a luminometer (Lumat LB, Berthold) using Luciferase assay substrate (E1501; Promega) or

Renilla assay substrate (E2820; Promega). Luciferase activity values obtained were normalized to Renilla activity in the same cell extracts and expressed as fold activation to the control.

Nuclear and Cytoplasmic extraction

Nuclear and Cytoplasmic extraction was obtained using the NE-PER Kit (78833; Thermo Scientific), as per the instructions provided by the manufacturer. Immunoblotting of ERK and c-Jun was used as the positive control for cytoplasmic and nuclear fractions, respectively.

Western blot analysis

Total cellular protein extracts were prepared in NP-40 lysis buffer (0.5% (vol/vol)), 50 mM Tris-HCl (pH 8),150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA (pH 8), and 0.1 M NaF supplemented with 1X protease inhibitor cocktail (P-8340; Sigma) and 0.5 mM sodium orthovanadate. Protein concentrations were determined by a standard Bradford assay. Equivalent amounts of protein were denatured in SDS loading buffer at 100°C for 5min and then run in sodium dodecyl sulfate (SDS)-polyacrylamide gels, followed by electrophoretic transfer to an Immobilon-FL Polyvinylidene Difluoride membrane PVDF membrane (Millipore) as directed by the manufacturer. Blots were incubated with blocking buffer that consisted of 5% milk in Tris-buffered saline (TBS)-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) prior to the incubation with primary antibody at 4°C overnight with gentle agitation. After three washes with TBS-T, appropriate HRP-conjugated secondary antibody (BioRad, 1:2000), were added for 2 h at room temperature. Protein/antibody immuno-complexes were detected with enhanced chemiluminescence western blotting substrate (Pierce, ThermoFisher).

Antibodies

Rabbit monoclonal for αSmad7 (ab124890) and polyclonal for αMED13 (ab76923), αMED12 (ab70842) were purchased from Abcam. A Rabbit polyclonal antibody was raised against GST-Smad7 according to the protocol approved by York University of Animal Care Committee. This was used for endogenous Smad7 immunoprecipitation (IP) and detection in cellular and nuclear extract. αβ-catenin (pAb9562) and ChIP grade αFlag antibody (mAb 14793S) were purchased from Cell signaling. Monoclonal αFlag antibody (F1804) was from Sigma. αMyc (9E10), αMyHC (MF20), αMyogenin (F5D) was purchased from DSHB. αActin (Sc1616), αMyoD (sc304) and αCKM (sc-69878) was purchased from Santa Cruz.

Co-immunoprecipitation

For Co-immunoprecipitation (Co-IP), cells were harvested, and proteins were extracted as described above. Immunoprecipitation was performed using an ImmunoCruz Optima kit (Santa Cruz Biotechnology) according to the manufacturer's instructions. Eluates were analyzed by Western blotting as described above.

Live-cell imaging

C2C12 cells were seeded onto the glass-bottom dishes (MatTek Corp). The cells were transfected for expression of fluorescent tagged proteins. Before imaging the cells, the media was replaced for FluoroBrite DMEM (Thermo Fisher Scientific) supplemented with 10%FBS. To mark nuclei, Hoechst 33342 (Sigma-Aldrich) was added to 2.5μM into the media. After 30 min, the stained cells were visualized by a Carl Zeiss Spinning disc system (Zeiss Observer Z1 with Yokogawa

CSU-X1 and AxioCam MRm camera) in the environment chamber (37°C, 5% CO2). The raw images were processed by ZEN (Carl Zeiss) to obtain pseudo-colored micrographs.

Chromatin Immunoprecipitation (ChIP)

C2C12 cells were allowed to grow confluent at approximately $2x10^7$ cells per plate. C2C12 myoblasts or Smad7-Flag ectopically expressing myoblasts were crosslinked with 1% formaldehyde followed by quenching with Glycine. Chromatin was sonicated to shear DNA strands (approx. 500bp fragments) attached to the protein. Smad7/ β-catenin was immunoprecipitated with Protein G Dynabeads (Invitrogen) complexed with Flag or β-catenin antibody to obtain protein-DNA complex. IP with IgG antibody served as controls. The antibody bound chromatin complexes were washed using IP Wash Buffer I (20 mM Tris pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton-X 100, 0.1% SDS) and II (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS). Protein-DNA complexes were eluted from Dynabeads with elution buffer (0.1 M NaHCO3, 1% SDS). Immunoprecipitated DNA was reverse–cross-linked with 5 M NaCl and protein was degraded with Proteinase K, EDTA and Tris pH 6.5. DNA samples were further purified with a Qiagen PCR clean up kit and subjected to ChIP-qPCR analysis of the *ckm* promoter. Primers specific to *gapdh* was used as controls for *ckm* enrichment. Primer sequence: *ckm* Forward: GCTCCTGTCATATTGTGTCCTGCT *ckm* Reverse: TTATAACCAGGCATCTCGGGTGTC; *gapdh* Forward: GCACAGTCAAGGCCGAGAAT *gapdh* Reverse: GCCTTCTCCATGGTGGTGAA

GST pulldown assay

For GST pulldown assays, GST-Smad7 and 6XHis-β-catenin fusion proteins were produced in bacteria using standard protocol. Briefly, GST-Smad7 and 6XHis-β-catenin expressing cells were sonicated and the proteins were purified with glutathione-agarose beads (Sigma-Aldrich G-4510). Protein concentrations were estimated by SDS-PAGE and following Coomassie blue staining, using bovine serum albumin (BSA) for comparative estimation. GST-β-Catenin fragments corresponding to amino acid (aa) (Full length (FL), 1-100, 120-683, 422-683, 575-696, 575-683, 1-574) were utilized for mapping study. 5 µg of GST full length Smad7, β-catenin (or the molar equivalent of the smaller GST, GST-β-catenin fragments), and 25 µl glutathione-agarose beads (50% slurry) were incubated in 600 µl NETN buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8) 0.5 mM EDTA, 0.5% (vol/vol) NP-40) overnight at 4°C. In experiments, where GST-β-catenin was used to co-precipitate Smad7, GST-Smad7 was thrombin (GE Healthcare-27-0846-01) digested to yield the immune-complex without the GST tag (10 µl thrombin per mg fusion protein was incubated at room temperature for 17 h), and eluates were analyzed by Western blotting.

2.4 Results

2.4.1 Smad7 and β-catenin are co-expressed and interact in myogenic cells

A time course analysis of β-catenin and myogenic markers during C2C12 differentiation indicated that β-catenin expression is enhanced along with myosin heavy chain (MyHC), muscle creatinine kinase (CKM) and myogenin levels during differentiation (Fig 11A). Endogenous Smad7 protein levels increased during differentiation as compared to myoblasts in growth conditions (Fig 11B). Immunofluorescence analysis of Smad7 and β-catenin expressing C2C12 cells indicates that both Smad7 and β-catenin are localized in the nucleus, interestingly, two different patterns of

localization were observed. β-catenin exhibited a diffuse pattern of localization within the nucleus in some cells while in others a very defined co-localization occurred in nuclear puncta (Fig 11C). A cytoplasmic and nuclear fractionation demonstrated that Smad7 and β-catenin are abundant in both fractions (Fig 11D). Based on a previous report indicating a PPI between β-catenin and Smad7 in human prostate cancer cells (PC-3U) (1), we assessed whether Smad7 and β-catenin form a complex under these cellular conditions in cultured muscle cells and muscle tissue (mouse tibialis anterior). Total protein lysates from Mouse Tibialis anterior (TA), and C2C12 muscle cells were subjected to co-immunoprecipitaion (Co-IP) with β-catenin and Smad7 antibodies. Interestingly, Smad7 and β-catenin were precipitated together in muscle tissue (TA) and in muscle cells (Fig 11E). The interaction was further confirmed by ectopically expressing Smad7-Flag followed by detection of β-catenin in the Co-IP (Fig 11F).

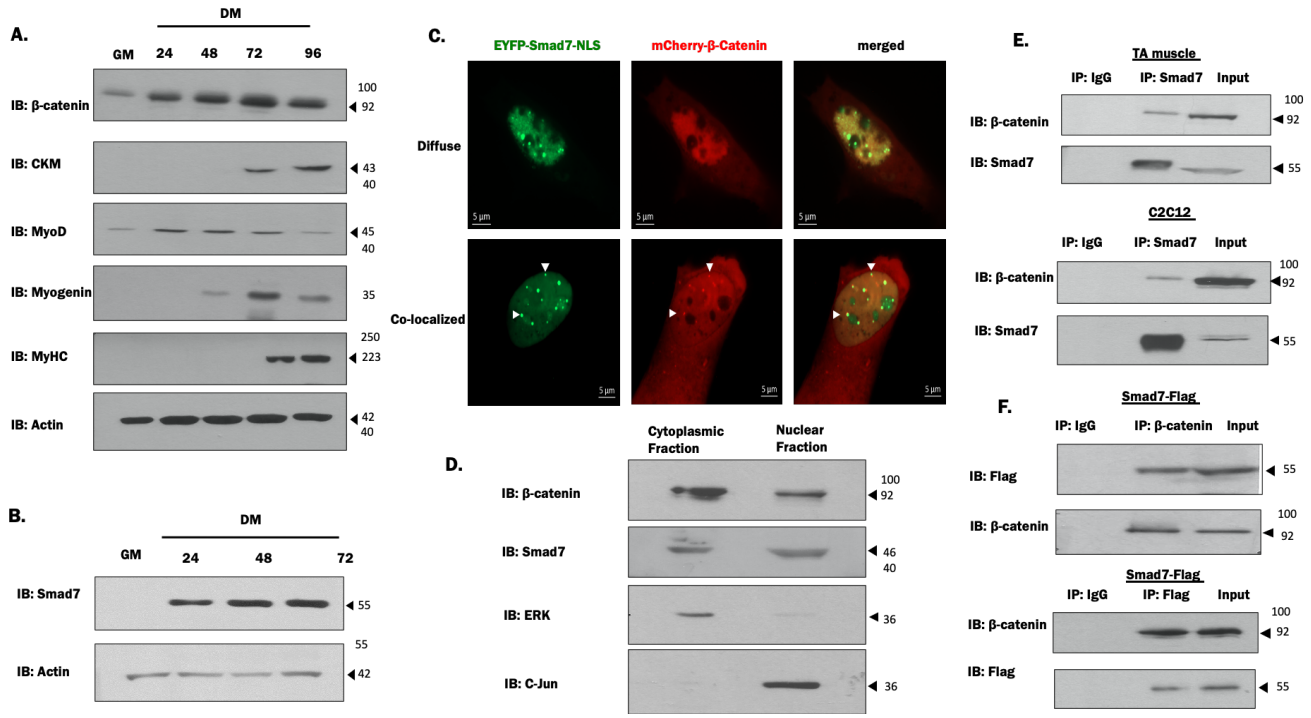


Figure 11. Smad7 and β-catenin interaction and expression during myogenic differentiation. (A) C2C12 myoblasts were cultured in growth media (GM) for 24 h, followed by differentiation media (DM) for designated times. Lysates were collected and assessed for expression of muscle markers by Western blot analysis. (B) Lysates were combined from several plates for immunoblotting detection of Smad7. Actin was used as loading control. (C) Co-localization of Smad7 and β-catenin was analysed by Immunofluorescence in ectopically expressing EYFP-Smad7-NLS and mcherry- β-catenin C2C12 cells. (D) Cytoplasmic and nuclear extraction was done to determine the endogenous localization of Smad7 and β-catenin in C2C12 cells. ERK was utilized as cytoplasmic control and c-Jun as nuclear control. (E) Co-immunoprecipitation (Co-IP) assays were performed using Smad7 antibody to detect an interaction between endogenous β-catenin and Smad7 in extracts from mouse Tibialis Anterior (TA) muscle and C2C12 myoblasts (24 h DM). (F) Alternatively, in another separate experiment, C2C12 myoblasts were transiently transfected with Smad7-Flag. Smad7-Flag lysates were harvested 24 h post changing to DM and utilized for Co-IP with β-catenin or Flag antibody to detect interaction with β-catenin. IP with IgG served as controls.

2.4.2 Smad7 physically interacts with β-catenin

As the Co-IP analysis indicated Smad7 and β-catenin were in the same complex, we next clarified whether this was a direct interaction by utilizing Glutathione-S-transferase (GST) pull down assays with bacterially expressed purified proteins. GST-Smad7 and 6xHistidine (6xHis)-β-catenin fusion protein were produced to conduct the assay. A Coomassie stained blot confirmed the purification of the fusion proteins (Fig 12A). β-catenin was immunoprecipitated with GST-Smad7 but not GST conjugated beads indicated that this interaction was direct (Fig 12B). Next, we determined the Smad7 interaction domain on β-catenin. The 781 amino acid sequence of β-catenin gives rise to a structure consisting of several characteristic repeats, termed armadillo repeats, each approximately 40 amino acids long. N-terminal (NTD) and C-terminal (CTD) domains flank either end of the armadillo repeats. Helix-C, indicates a conserved helix located adjacent to the last ARM repeat proximal to the CTD (281)(Fig 12C). GST-β-catenin protein fragments corresponding to aa's 1-106,120-683,422-683,575-696 and the full length were utilized for determining the interacting region with Smad7. Smad7 interacted with full length β-catenin (as we previously identified) and β-catenin aa's120-683, 422-683 and 575-696. Smad7 did not interact with β-catenin aa1-106 therefore indicating that the region between aa575-683 (Fig 12D) is required for this interaction. To further refine this, we used additional GST-β-catenin fragments, GST-β-catenin aa575-683 and GST- β-catenin aa1-574 and conducted the interaction assay. In agreement with the above results, we observed that Smad7 interacted with β-catenin aa575-683 and not with aa1-574 leading us to conclude that the Smad7 interacting domain (hereafter referred to as SID) on β-catenin lies between aa575-683 (Fig 12E). This region spans the 10th armadillo repeat of β-catenin and a partial region extending into the CTD. Previously, this CTD domain has been associated with β-catenin *trans*activation properties, and has been shown to interact with MED12, TBP

(TATA-binding protein), CBP (CREB-binding protein/p300) and proteins associated with chromatin regulation (281).

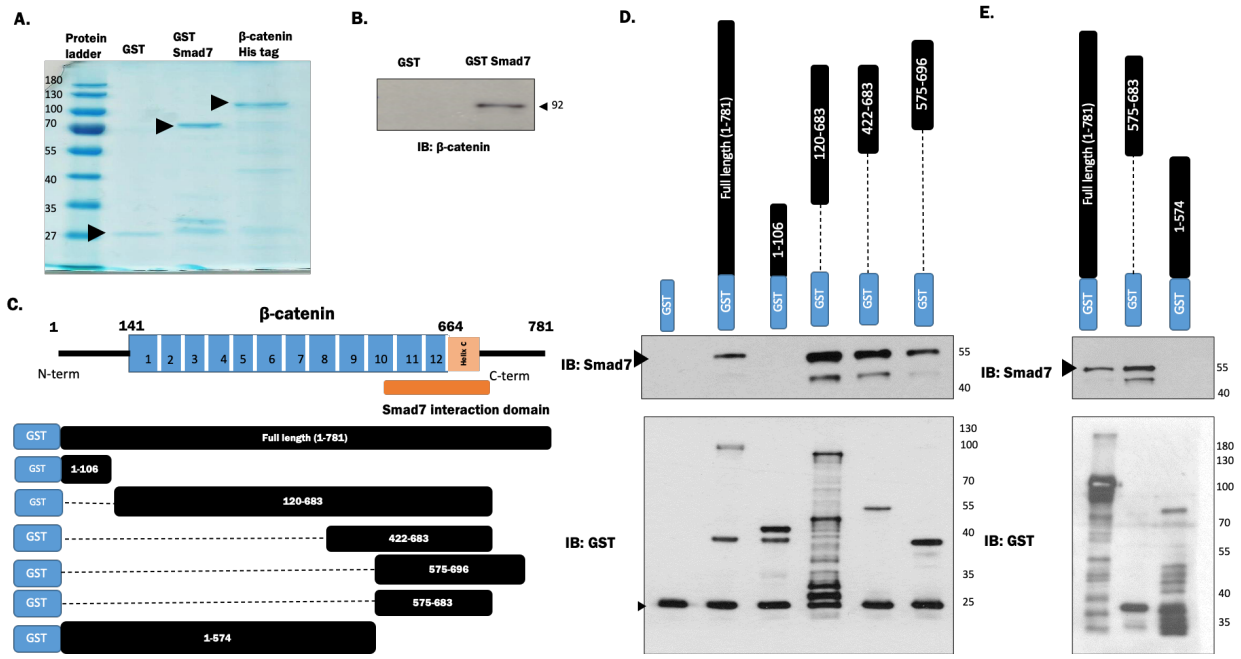


Figure 12. β-catenin C terminal domain (aa575-683) comprises a Smad7 interaction domain (SID)

(A) Coomassie blue staining of the purified proteins GST (26kDa), GST-Smad7 (72kDa) and β-catenin His tag (98kDa). (B) Glutathione-S-transferase (GST) pull down assays were performed with purified GST- Smad7 and 6x Histidine (6xHis)-β-catenin fusion protein expressed in the bacteria. (C) The β-catenin protein (781aa) consists of a central region (aa 141-664) made up of 12 Armadillo repeats that are flanked by distinct N-and C-terminal domains, respectively. A specific conserved helix (Helix-C) is located proximally to the C-terminal domain, adjacent to the last ARM repeat. (D&E) Interaction between Smad7 and β-catenin was assessed by glutathione-S-transferase (GST) pull down assay. Purified Smad7 was incubated with GST or GST-β-catenin fusion protein fragments along with glutathione-agarose beads, detection of Smad7 protein complexed with GST-β-catenin fragments were analysed by immunoblotting with anti-Smad7 polyclonal antibody.

2.4.3 Smad7 and β-catenin were enriched on the muscle specific ckm promoter proximal region

The regulatory region of the muscle creatine kinase gene (*ckm*) has been extensively characterized and has served as a paradigm for tissue restricted transcriptional control during myogenesis (272,282). Our lab previously documented that Smad7 associates with the promoter proximal elements of *ckm* (230). We therefore used this as a test promoter to assess the function of the β-catenin: Smad7 interaction and to interrogate the role of this complex during myogenic differentiation. First, Chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (ChIP-qPCR) for *ckm* and *gapdh* (control) was utilized to assess whether Smad7 and β-catenin are enriched on the *ckm* promoter proximal region. In the absence of a Smad7 antibody efficacious for ChIP, Smad7 enrichment was confirmed by Flag-Smad7 recruitment to the *ckm* promoter (Fig 13A). Additionally, endogenous β-catenin is enriched at the *ckm* promoter in myogenic cells (Fig 13B). Next, we utilized reporter gene assays in which Smad7 and β-catenin were exogenously expressed in C2C12 cells along with a *ckm* (-1082 to -1262) promoter fragment driving a firefly luciferase reporter gene. These data indicate that Smad7 and β-catenin *trans*activate this promoter region both alone and in combination (Fig 13C). Based on the interaction mapping, a mammalian expression vector for β-catenin without the SID (deletion of aa575-683) was constructed. Initially, we performed reporter gene assays of the activity of these β-catenin constructs using the TOP flash luciferase reporter gene, which acts both as a β-catenin reporter and a general Wnt signaling pathway reporter as it contains 7 TCF/LEF consensus sites in which TCF/LEF proteins bind to but cannot activate the reporter gene without β-catenin. Our data indicated that the full length β-catenin activated TOP flash whereas β-catenin 1-574 and β-catenin ΔSID could minimally activate TOP flash compared to the full length β-catenin (supplementary Figure S1). Further, when β-catenin

was ectopically expressed with Smad7, there was an enhancement in TOP flash promoter activity whereas there was no change in TOP flash activity in conditions where Smad7 was expressed in combination with β-catenin 1-574 and β-catenin ΔSID. These data revealed that while Smad7 was able to enhance the function of the intact β-catenin, it did not co-operate with β-catenin 1-574 and β-catenin ΔSID in reporter gene assays (supplementary Figure S1). We next deleted the SID domain to test whether it might prevent the co-operative *trans*activation mediated by Smad7 and β-catenin. To test this idea, Smad7, full length β-catenin (β-catenin-FL), β-catenin (aa1-574, ΔSID) along with a *ckm* luciferase construct were ectopically expressed in C2C12 cells alone or in combination. These data indicate that Smad7 and the β-catenin-FL enhanced *ckm* promoter, this activity was substantially reduced in cells expressing Smad7 in combination with β-catenin aa1-574 or β-catenin ΔSID (Fig 13D). These data support the conclusion that the β-catenin SID is crucial for the co-operative interaction of Smad7 and β-catenin on the *ckm* promoter. To address this co-operativity in a different way, we next investigated the effect of perturbation in Smad7 expression on the function of β-catenin on the *ckm* promoter using siRNA technology. Endogenous Smad7 levels were depleted using three independent siRNA's (supplementary Figure S2) and reporter gene analysis indicated a pronounced decrease in *ckm* promoter activity when compared to the control (Fig 13E). Ectopic expression of β-catenin in Smad7 depleted cells exhibited a reduction in *ckm* promoter induction consistent with the interpretation that β-catenin requires Smad7 in order to affect promoter activation (Fig 13F). To further test this idea, we correspondingly depleted β-catenin expression using three independent siRNAs. Western blot analysis verified a considerable reduction in protein levels of β-catenin using two different siRNAs (si#2 and #3) as compared to controls. Under conditions in which endogenous β-catenin was depleted, we observed that Smad7 mediated *ckm* promoter activation was markedly reduced (Fig

14A). These data indicate that both Smad7 and β-catenin co-operatively activate the *ckm* promoter. Previously, it was shown that MyoD, the archetypal myogenic regulatory factor (283), interacts with β-catenin (284). To test this hypothesis, endogenous β-catenin levels were depleted in C2C12 using two different siRNAs. MyoD and Smad7-myc either alone or in combination were then transfected along with the *ckm* reporter gene in the β-catenin depleted cells. This analysis revealed that *ckm* promoter activity increased by Smad7 or/and MyoD was reduced by depletion of β-catenin (Fig 14B). These results further support the conclusion that β-catenin enhances Smad7 and MyoD driven potentiation of *ckm* promoter activity. These observations in combination with previously reported data indicate that the Smad7: β-catenin interaction may be tethered by MyoD and is required for full *ckm* promoter activation.

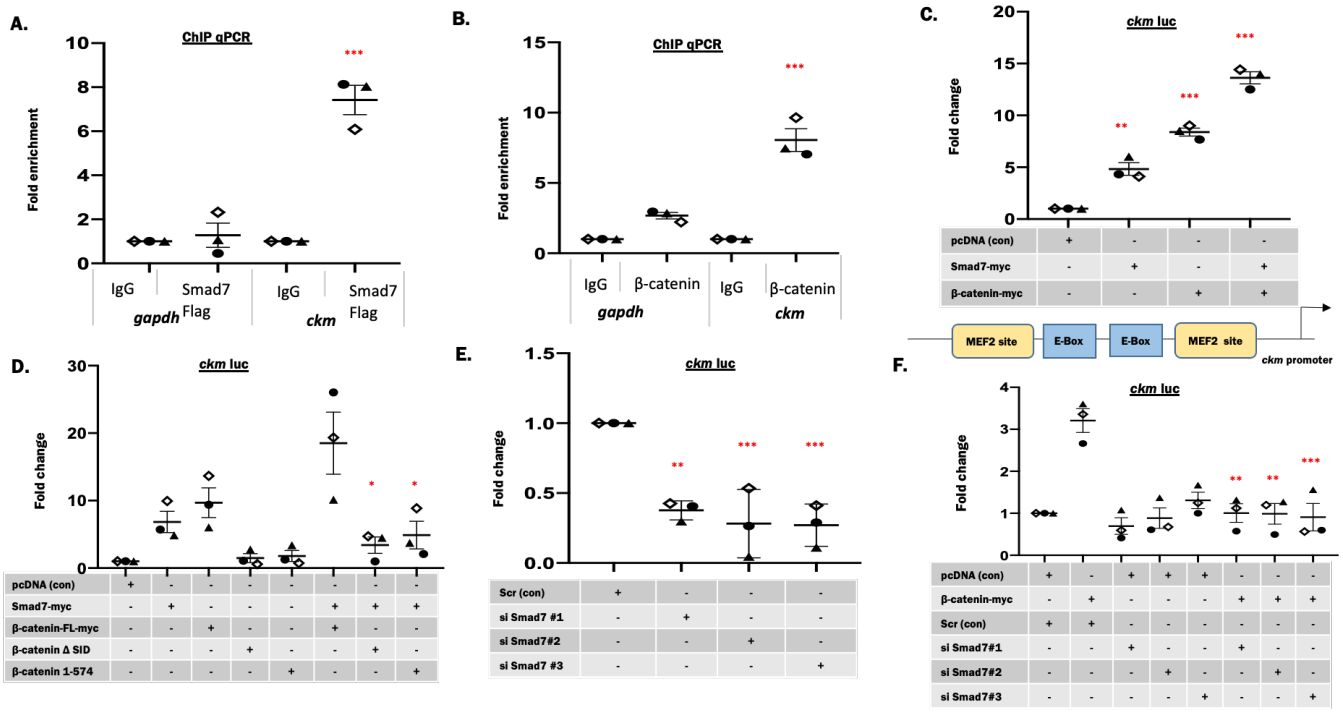


Figure 13: Smad7 and β-catenin enhance *ckm* proximal promoter activity.
(A&B) For chromatin immunoprecipitation, myoblasts expressing Smad7-Flag or un-transfected myoblasts were cross-linked with 1% formaldehyde followed by sonication to shear DNA (Smad7 or β-catenin). IP with anti-Flag or β-catenin antibody was carried out to precipitate protein-DNA complexes. Comparable IP with IgG antibody served as control. Recovered DNA was reverse–cross-linked, purified and subjected to Quantitative PCR (qPCR) using primers specific for the *ckm* promoter to determine enrichment. Primers specific to *gapdh* were used as controls (C) Combinations of Smad7-myc, β-catenin-myc were ectopically expressed in C2C12 cells along with a *ckm* luciferase reporter gene. *Renilla* luciferase served as transfection control. C2C12 transfected with empty vector (pcDNA) and reporter genes served as controls for ectopic expression. Cells were harvested for Luciferase determination at 48 h after changing to DM post transfection. Normalized luciferase activity was compared to the control to determine fold changes. (D) C2C12 cells were transfected with Smad7-myc, β-catenin-FL(full-length)-myc, β-catenin ΔSID and β-catenin 1-574 alone or in combination with *ckm* luciferase reporter construct (E) Three siRNAs specific for Smad7 were used to deplete the endogenous Smad7 levels in C2C12. Unprogrammed Scrambled siRNA was used as controls. (F) β-catenin was transfected along with *ckm* luciferase. Empty vector (pcDNA) was used as a control for ectopic expression. *Renilla* was used as a control reporter to monitor transfection efficiency. Lysates were collected at 48 h after changing to differentiation media (DM) post-transfection. The firefly luciferase activity under each condition was measured independently and normalized to *Renilla* luciferase values. Each condition is compared to the control for the three individually transfected samples to determine fold change. Each dot represents one biological replicate, which corresponds to the mean of 3 technical replicates. N= 3 biological replicates per condition. The error bars represent standard error of the mean (SEM). Dunnett's Multiple comparisons test in one-way ANOVA using GraphPad Prism 8.0 was utilized to test for statistical significance. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

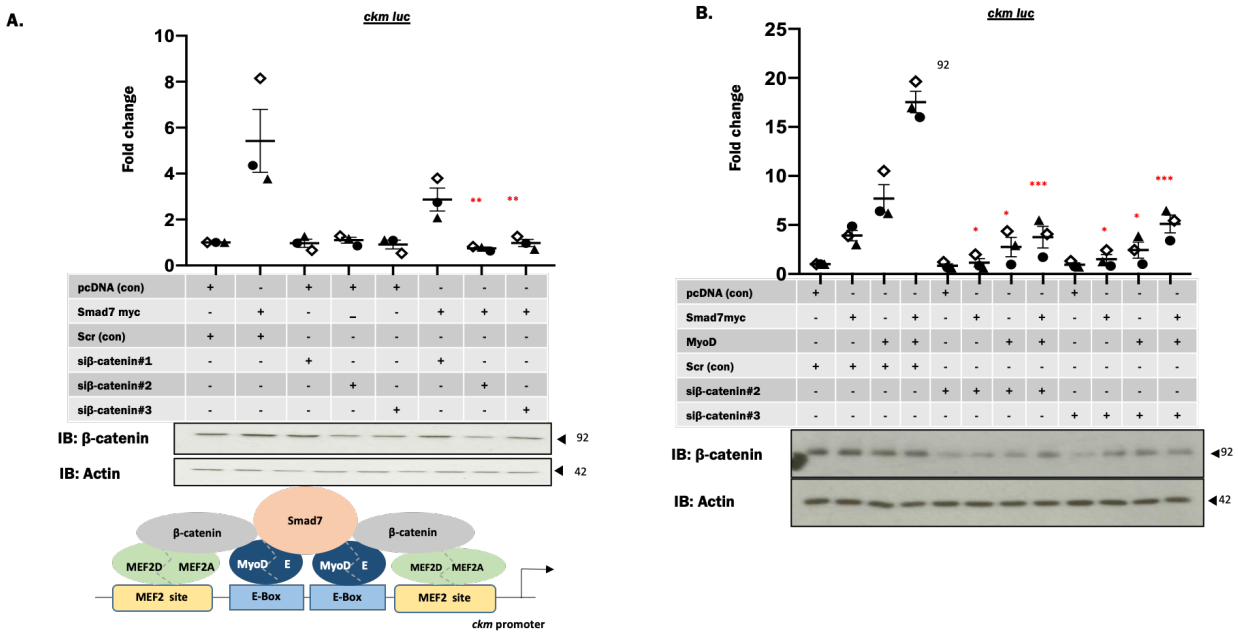


Figure 14: Smad7 and β-catenin co-operativity on *ckm* was mediated through MyoD. (A) C2C12 cells were transfected with siRNAs targeting β-catenin to deplete the endogenous β-Catenin levels. Smad7-myc was transfected along with *ckm* luciferase reporter gene. (B) MyoD, Smad7-myc alone or in combination were transfected along with *ckm* luciferase promoter activity reporter gene in depleted β-catenin condition. Empty vector (pcDNA) was used as a control for ectopic expression, *Renilla* Luciferase was used to normalize for transfection efficiency. Lysates were collected at 48 h after changing to differentiation media (DM) post-transfection. Each condition is compared to the control for the three individually transfected samples to determine fold change. Each dot represents one biological replicate, which corresponds to the mean of 3 technical replicates. N= 3 biological replicates per condition. The error bars represent standard error of the mean (SEM). Dunnett's Multiple comparisons test in one-way ANOVA using GraphPad Prism 8.0 was utilized to test for statistical significance. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

2.4.4. The minimal Smad7 Interaction Domain (SID) on β-catenin functions as a transdominant inhibitor of β-catenin activity

Since our data suggested that the SID is crucial for the function of Smad7 and β-catenin on the *ckm* promoter, we next considered the possibility that this domain of β-catenin might function as a more general *trans*dominant repressor (dominant negative) of endogenous β-catenin activity. To test this idea, we constructed a mammalian expression plasmid for tagged β-catenin aa575-683. Smad7 and β-catenin were ectopically expressed in C2C12 cells along with increasing concentrations of either EYFP-β-catenin aa575-683 (EYFP-SID) or Flag β-catenin aa575-683 (Flag-SID). Reporter gene analysis revealed that *ckm* promoter activity was significantly reduced in Smad7 and β-catenin expressing cells in the presence of either EYFP-SID or Flag-SID (Fig 15A). These data indicate that the β-catenin SID can function as a dominant negative inhibitor of β-catenin function. A similar analysis using a *sprr1a* promoter luciferase reporter gene that is not regulated by MyoD (data not shown) was unaffected by SID expression, suggesting that the effect of SID on *ckm* is specific (supplementary Fig S3). We subsequently assessed the effect of the SID domain on myogenesis by analyzing the protein levels of myogenic markers with and without SID expression. Immunoblot analysis from ectopically expressed SID in C2C12 cells exhibited reduced protein levels of CKM and myogenin as compared to controls indicating an overall repression of myogenesis by exogenous SID expression (Fig 15B). Moreover, transfection of SID was associated with a decreased TOP flash reporter activity as compared to the control (Fig S5) Collectively these data suggested that the β-catenin SID can inhibit the activity of endogenous β-catenin and the myogenic differentiation program.

2.4.5. Smad7: β-catenin complex interacted with the Mediator kinase complex subunits (MED13 and 12)

Previous studies (2) and our unpublished observations confirm that β-catenin directly interacts with the mediator complex subunit 12 (MED12). Since it is established that MED12 and MED13 form an integral part of the mediator kinase module (2,285), we hypothesized that the composite function of the Smad7:β-catenin interaction might be to recruit the MED kinase module. This idea is supported by our Co-IP analysis in which we observed that Smad7 and MED13 are in the same precipitated protein complex (Fig 16A). Furthermore, we also validated the previously reported MED12: β-catenin interaction (Fig 16B). Finally, we observed that the Smad7:MED13 interaction was disrupted when endogenous β-catenin was depleted (Fig 16C, D). The Smad7:MED13 interaction thus constitutes a novel observation in characterizing Smad7 as a component of the transcriptional machinery linking promoter activity to the Mediator kinase complex.

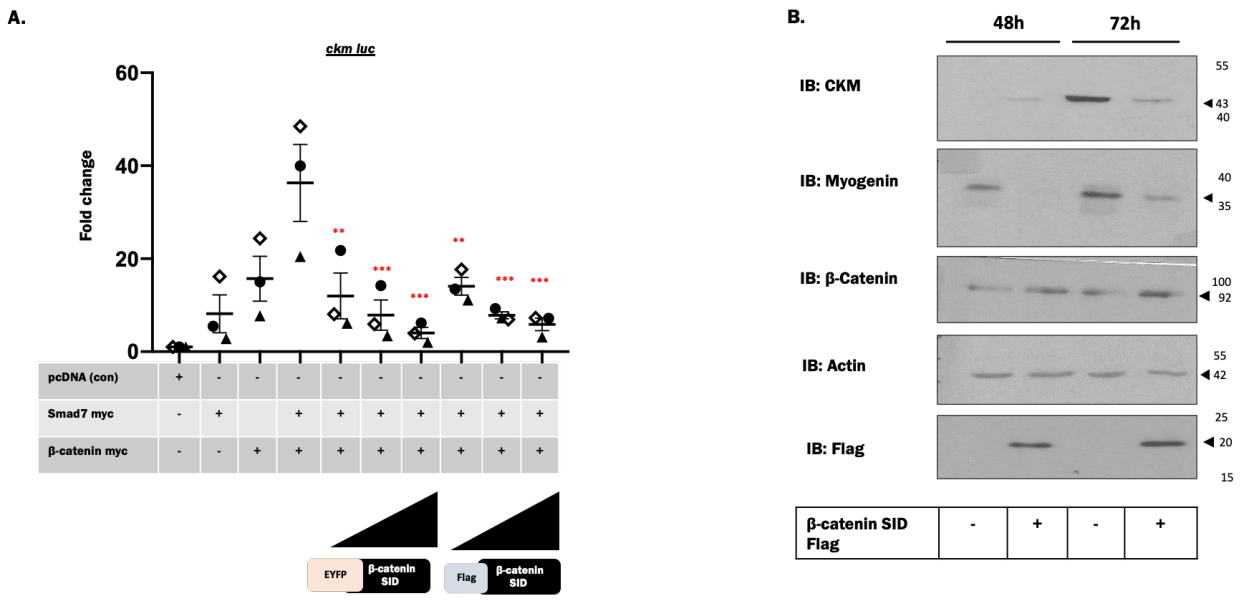


Figure 15: β-catenin aa575-683 (SID) functioned as a dominant negative inhibitor of endogenous β-catenin activity.

(A) Smad7-myc and β-catenin-myc alone or in combination were transfected along with increasing amounts of either EYFP-β-catenin SID or Flag β-catenin SID and *ckm* luciferase construct. Empty vector (pcDNA) was used as a control. Lysates were collected at 48 h after changing to differentiation media (DM) post-transfection. Each condition was compared to the control for the three individually transfected samples to determine fold change. Each dot represents one biological replicate, which corresponds to the mean of 3 technical replicates. N= 3 biological replicates per condition. The error bars represent standard error of the mean (SEM). Dunnett's Multiple comparisons test in one-way ANOVA using GraphPad Prism 8.0 was utilized to test for statistical significance. **$p \leq 0.01$, ***$p \leq 0.001$. (B) Total cell lysates of ectopically expressing Flag β-catenin SID were collected at 48 h and 72 h after changing to differentiation media (DM) post-transfection. Un-transfected cells served as controls. Cell lysates were analyzed for endogenous β-catenin, muscle markers (CKM, Myogenin) and Flag (β-catenin SID) by western blot analysis. An actin blot indicated protein loading of samples.

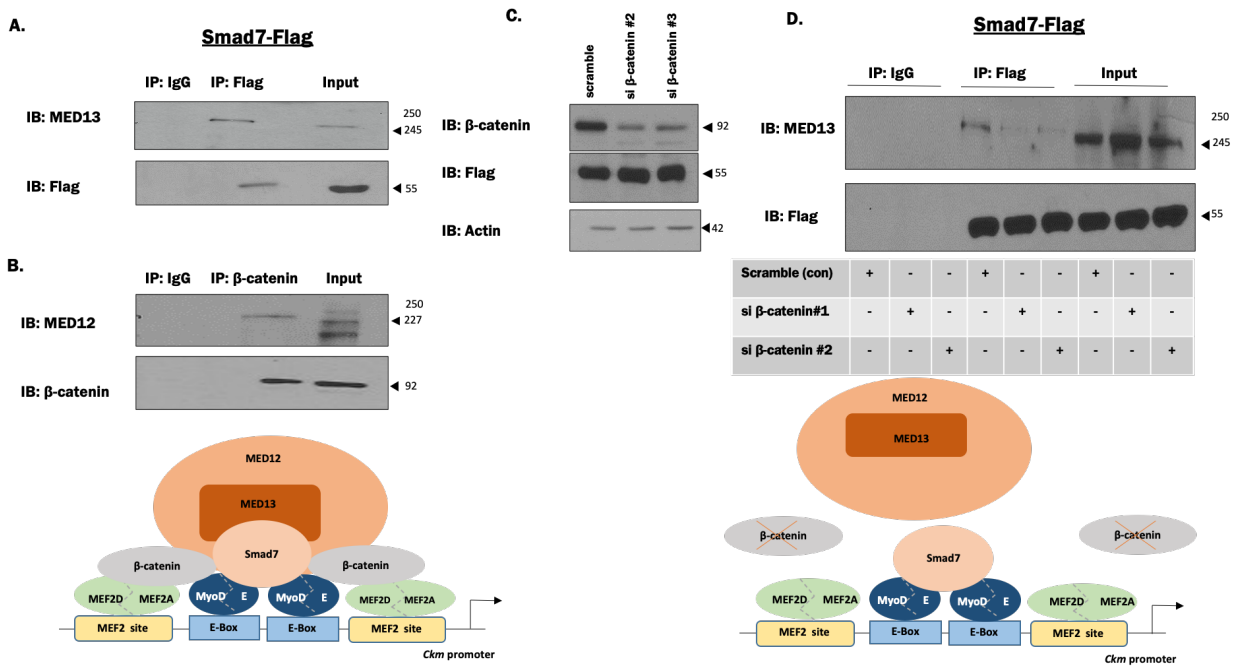


Figure 16: Mediator subunit 13 (MED13) associates with Smad7 in a β-catenin dependent manner.

(A) C2C12 cells were transiently transfected with Smad7-flag. Lysates were collected at 24 h after replenishing media post transfection. Co-IP assays were performed using anti-Flag antibody to test an interaction between endogenous MED13 and Smad7. (B) Co-IP assays were performed using β-catenin antibody to test for an interaction between endogenous MED12 and β-catenin. IP with IgG served as controls. (C) siRNAs specific for β-catenin were used to deplete the endogenous β-catenin levels in C2C12, Scrambled siRNA served as controls. Lysates were collected at 48 h post transfection and immunoblotted for β-catenin, anti-Flag (for Smad7-Flag) and Actin (loading control). (D) Co-IP with anti-Flag was repeated in depleted β-conditions to assess the interaction between endogenous MED13 and Smad7.

2.5 Discussion

2.5.1 Smad7:β-catenin as an essential component of muscle enhanceosomes

Despite a considerable body of literature implicating Wnt-β-catenin signaling in muscle differentiation (47,164,286,287) there is a surprising lack of mechanistic insight into how it fulfills this role. Moreover, recent *in vitro* and *in vivo* data implicating Smad7 in the control of muscle gene expression is also not understood mechanistically. Interestingly, in one study (288) a correlation between TGF-β and Wnt signalling was investigated where TGF-β1 was observed to control the differentiation of fibroblasts to myofibroblasts by upregulating Wnt signalling. Here, we provide evidence of a direct functional interaction between Smad7 and β-catenin that serves a fundamental role in recruiting Mediator components to the well characterized muscle creatine kinase gene (*ckm*). Our data indicate that β-catenin is inefficiently recruited to the *ckm* gene enhancer in muscle cells when Smad7 is depleted and corresponding loss of β-catenin recruitment renders this gene incapable of responding to essential differentiation cues (Fig 17). Here we present evidence indicating that a Smad7:β-catenin complex is a critical part of the transcriptional machinery at a key muscle promoter proximal element, being intrinsically necessary as a co-regulator for the MRFs in the myogenic gene expression program.

2.5.2 Mediator recruitment to ckm by β-catenin /Smad7

The Mediator complex was initially characterized in budding yeast (289) and has since been established as fulfilling an essential function in RNA polymerase II mediated gene transcription from flies to mammals (290). The multi- subunit compositional complexity of the Mediator holocomplex has proved a substantial challenge to define the full extent of its properties but what is apparent is that its fundamental role is to provide a functional bridge between transcriptional

regulatory proteins bound to gene enhancers and the general transcription machinery assembled at core promoters (291-297). The interaction of different Mediator subunits with a variety of transcription factors thus allows a myriad of cellular signaling events that converge on the transcription factors to be subsequently relayed to the transcriptional machinery and ultimately programs of gene transcription (298). Thus, this essential activity in promoting signal dependent transcriptional pre-initiation complex (PIC) assembly and stabilization renders many Mediator subunits essential for life, since gene targeting in mice has revealed that many subunits prove embryonic lethal when deleted and in yeast all Pol II regulated genes are dependent on Mediator (296,299-301). The experimental dissection of Mediator has been aided by the characterization of four relatively stable sub-complexes that have been designated as the head, middle, tail and kinase modules. In our study, we have documented that the Smad7: β-catenin complex specifically associates with the MED12 and 13 subunits of the Mediator kinase module. The kinase domain is speculated to fulfill a transient regulatory function in Mediator by promoting recruitment of all four Mediator domains to enhancers which then transitions to a core promoter bound Mediator complex in which the kinase module is absent. We therefore propose that Mediator recruitment to the *ckm* enhancer is mediated by the Smad7:β-catenin complex in muscle cells (Fig17).

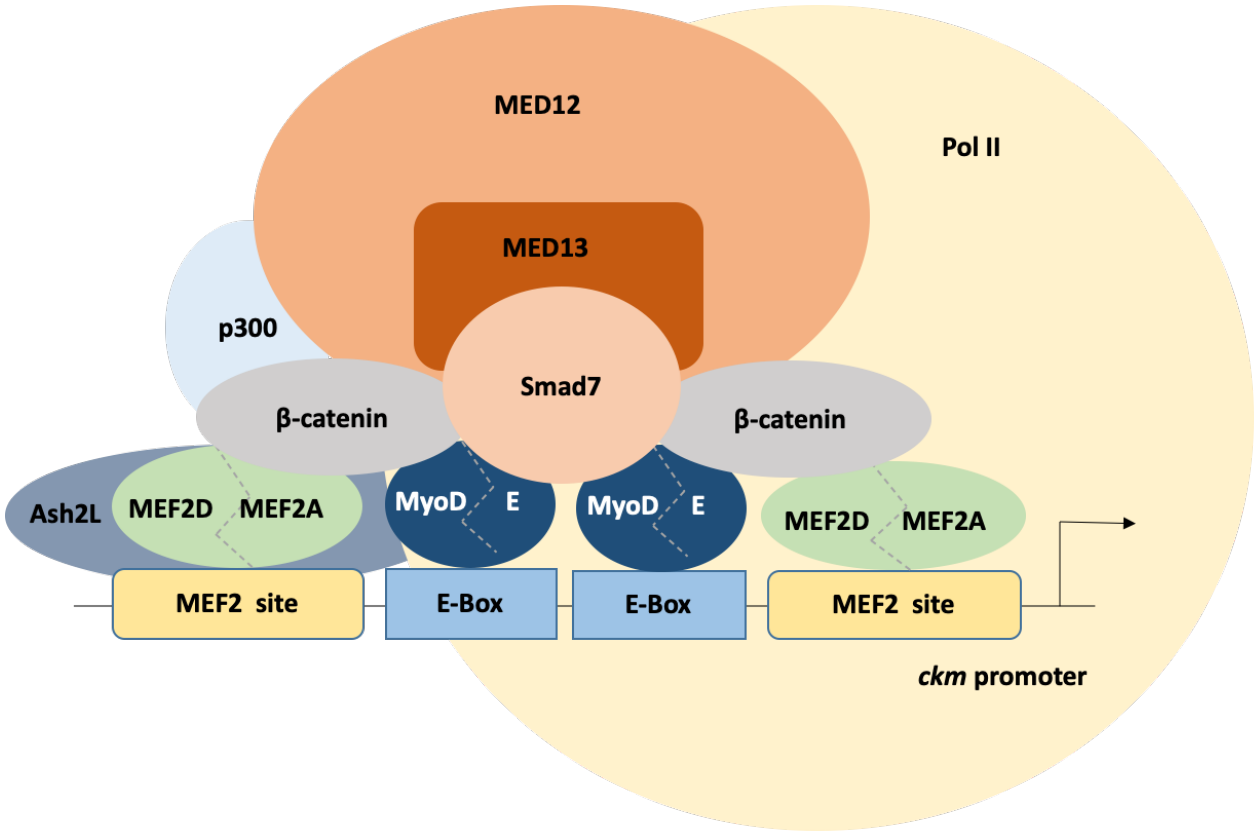


Figure 17: Proposed model of Smad7:β-catenin integration into the transcriptional holocomplex on the *ckm* proximal promoter region.

2.5.3 *Implications of β-catenin: Smad7 interaction in Rhabdomayosarcoma*

In view of the role played by β-catenin: Smad7, it is perhaps worth consideration of the implications of this in the context of the pathology of Rhabdomyosarcoma (RMS), a soft tissue pediatric cancer with features of muscle (302). Previously we reported that constitutive glycogen synthase kinase 3 (GSK3) activity is a feature of the embryonal form of RMS (there are two general categories: alveolar (ARMS) and embryonal (ERMS)). The result of this GSK3 activity is perpetual proteosomal degradation of β-catenin since this is the primary function of the APC-GSK3 complex in canonical Wnt signaling (303). Upon Wnt signalling stimulation, GSK3 activity is substantially reduced and proteosomal degradation of β-catenin ceases, resulting in its cytoplasmic accumulation and translocation to the nucleus where it activates transcription in combination with other transcriptional regulators such as TCF/LEF. Based on these previous observations and the current study, it is worth speculating that the absence of nuclear β-catenin function due to constitutive GSK3 activity contributes to the differentiation defect in ERMS. Promoting terminal differentiation of the myo-like cells in RMS is seen as a potentially effective therapeutic target since differentiation, by nature, results in cell cycle withdrawal and cessation of proliferation which would cause tumour regression. There are several lines of indirect evidence supporting this idea; we recently reported that myogenin is expressed at high levels in RMS but, despite the role of this MRF as a key terminal effector of the myogenic differentiation program, it is functionally inactive in these cells. It is therefore possible that the lack of nuclear β-catenin underlies this functional impairment due to the failure to recruit mediator to connect myogenin function to PIC assembly in these ERMS cells. In support of this possibility, we observed that Smad7:β-catenin is tethered to muscle promoters by its interaction with MRF's (such as MyoD and myogenin) bound to E boxes on muscle promoter/enhancer regions. Another line of

circumstantial evidence supporting this notion is that pharmacological GSK3 inhibitors have been reported to force RMS cells into a more differentiated cellular phenotype, suggesting that re-constitution of β-catenin function in RMS cells promotes their differentiation and could potentially be anti-tumorigenic. Further clarification of the role of β-catenin: Smad7 as a therapeutic target in RMS is therefore warranted.

2.5.4 Further implications of p38 MAPK signalling on ckm regulation

Phospho-dependent protein interactions are a signature of β-catenin function. There is ample evidence of kinase-mediated phosphorylation modulating the affinity of protein-protein interactions in the canonical Wnt pathway, resulting in important outcomes for Wnt dependent target gene activation (279). Specifically, we recently reported a p38 MAPK dependent interaction with the MEF2 transcription factor enhances β-catenin nuclear retention and activity (279). During differentiation, MyoD and MEF2 bind muscle-specific promoters and enhancers, leading to the recruitment of co-activators (including p300) and the basal transcriptional machinery to establish a transcriptionally poised promoter. The previously implicated mechanism is that p38 MAPK activates *ckm* expression by phosphorylation dependent recruitment of the histone methyltransferase Ash2L by MEF2D (304). In view of the current observations, it is also possible that p38 MAPK may promote β-catenin recruitment to *ckm* through its interaction with MEF2.

In summary, we have documented a protein: protein interaction between Smad7 and β-catenin that may serve a fundamental role in the control of myogenic gene transcription. These observations may have implications for our understanding of the molecular control of myogenic differentiation during embryonic development and adult muscle regeneration.

Chapter 3

β-catenin interactome in skeletal muscle

Soma Tripathi and John C. McDermott

Author contributions

Experimental Design

Soma Tripathi and John C. McDermott

Drafting Manuscript

Soma Tripathi and John C. McDermott

Conducting Experiments

Mass spectrometry was conducted in Sick Kids facility, Toronto.

Preparation of samples for Mass spectrometry and analysis of mass spectrometry data was conducted by Soma Tripathi.

3.1 Rationale

Based on the conclusions from the studies depicted in Chapter 2, we sought to identify the protein interaction networks of β-catenin and SID in myogenic cells. β-catenin has a very distinct and well characterized domain structure that determines its specific cellular function. In the absence of a DNA binding domain, β-catenin exerts its effect on gene transcription through various protein-protein interactions. In a skeletal muscle context, a direct interaction of β-catenin with MyoD has been observed that is essential for the function of MyoD thus establishing MyoD as an effector of canonical WNT/ β-catenin signaling (284). Similarly, a p38 dependent β-catenin and MEF2 interaction was observed in various cell types including vascular smooth muscle cells (279). Moreover, β-catenin interacts with MED 12 through its transactivation domain to activate transcription (2). In view of this, we hypothesized that determining the interactome partners of β-catenin and SID may give us more insight into its precise role in skeletal muscle cells. To address this, we employed a unique Co-IP technique using a Nano-trap-affinity purification approach followed by LC-MS/MS mass spectrometric analysis of interacting proteins. The purpose of this study was to identify β-catenin-interacting proteins in myogenic cells that may underlie its tissue specific role.

3.2 Summary

Our previous study demonstrated a Smad7 interaction domain (SID) between a.a 575-683 of β-catenin. Utilizing a unique Mass spectrometry analysis using GFP-Nano-trap, we aimed to categorize the proteins interacting with SID and also wild type β-catenin in growth and differentiation state of skeletal muscle cells. Our data suggested that approx. 30% of total interacting proteins overlapped between wild type β-catenin (Wt- β-catenin) and SID. Strikingly,

the known β-catenin interactors (25) such as α-catenin, cadherin, APC, TCF and GSK3β were identified in the list of interactors, thus validating the efficacy of the experimental approach. We further identified novel protein partners during growth and differentiation stages of skeletal muscle cells. Notably, the known interactors of β-catenin were unchanged in both conditions, although we identified novel interactors that were specific for growth or differentiation conditions. Altogether, our data illustrates common and non-overlapping protein interactome of β-catenin and SID in skeletal myogenesis that paves the way for future understanding of β-catenin's role in myogenic cells.

3.3 Introduction

β-catenin (CTNNB1) is a transcriptional co-activator that has been extensively studied due to its role in cellular adhesion and Wnt regulated gene transcription (305). Vertebrate β-catenin (Armadillo in *Drosophila*) is a multitasking and evolutionary conserved molecule that modulates embryonic development (281). In the presence of Wnt, β-catenin acts as the nuclear effector of canonical Wnt signalling. β-catenin's dual role as an essential component of the adherens junctions and a transducer of Wnt signaling in the nucleus makes it a multifunctional protein regulating a plethora of cellular events. Thus, abnormalities in β-catenin structure and signalling properties can lead to various diseases such as cancer (281). β-catenin levels are enhanced in the nucleus and cytoplasm in many cancers (306). Wnt/ β-catenin activity is associated with poor prognosis for breast cancer patients (307) and activation of β-catenin was associated with tumor development in Hepatoblastoma tissues (308). Accumulation of nuclear β-catenin was also observed in colorectal cancer cells (309) and β-catenin is reported for the activation of T-cell Leukemia (310). Multiple protein-protein interactions thus regulate the localization, stability and transactivation properties

of β-catenin. Characterizing the β-catenin interactome therefore holds great promise for understanding the physiology and pathology of Wnt dependent cancers. In the present study, we characterize the plethora of β-catenin binding partners in a skeletal muscle cell context. Utilizing C2C12 cells as the model system, we performed Co-IP using a GFP- Nano-trap approach. GFP-Nano-trap is an anti-GFP-Nanobody (single peptide chain protein of 13.9 kDa size) covalently bound to magnetic agarose beads that binds to GFP with very high affinity and low background. Nanobodies are the antigen binding domain of heavy chain only IgG antibodies from alpaca and other camelids. Nanobodies only contain heavy chain IgGs as compared to the standard IgG antibodies which have 2 heavy chains and 2 light chains. GFP-Nano-trap beads were incubated with lysates from HEK293T cells that were transiently transfected with EYFP-β-catenin/EYFP-SID or EYFP alone (control). The beads were then incubated with C2C12 whole cell lysates to capture the β-catenin/ SID interacting proteins in a skeletal muscle context. The GFP-Nano-trap approach had been successfully utilized to immunoprecipitate GFP-fusion proteins from cell extracts of various organisms. Co-IP was followed by LC MS/MS to identify the protein interactome. Common interactors between control and β-catenin/ SID were initially subtracted to reveal the β-catenin/ SID interactomes. Further, Gene Ontology (GO) Biological Process enrichment analysis for cellular localization and biological function was performed on the β-catenin/SID interacting proteins.

3.4 Materials and Methods

Cell culture

C2C12 myoblasts and HEK/293T cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in growth medium (GM) consisting of high-glucose Dulbecco's

modified Eagle's medium (DMEM, Gibco), 10% fetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin (Invitrogen, Thermofisher) and L-Glutamine (HyClone). Myotube formation was induced by replacing GM with differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum (Atlanta Biologicals) and 1% penicillin-streptomycin. Cells were maintained in an incubator at 95% humidity, 5% CO_2, and 37°C.

Preparation of Samples for Mass Spectrometry

HEK 293T cells were ectopically transfected with EYFP / β-catenin-EYFP / SID-EYFP. Lysates were collected after 24 h and incubated with GFP-Nano-trap beads for 1h at 4°C. Beads were stringently washed three times with NP-40 lysis buffer and further incubated with C2C12 whole cell lysate for overnight at 4°C. Beads were washed thrice with NP-40 lysis buffer and twice with 1X PBS, re-suspended in 20 μl ddH_20 and send more mass spectrometry analysis.

Gene Ontology enrichment analysis

Enrichment analysis of the β-catenin interacting proteins was performed using the Cytoscape vs 3.7.1. The network specificity criteria were kept at medium and the GO term levels were selected for 2 to 4. The GO term pathway selection was specified for identification of minimum of 3 genes constitutes 2% of a specific GO term. Kappa statistic was used to identify the proportion of GO terms shared proteins with a minimum Kappa score of 0.5. The ClueGO app vs 2.5.4 was used to create visible cluster of the enriched GO terms using the statistically significant proteins.

3.5 Results

3.5.1 Identification of β-catenin and SID interacting partners

The multifunctional protein β-catenin function in the nucleus as a transcriptional coregulator by modulating the function of DNA-binding transcription factors, and in the cytoplasm as component of the adherens junctions (305). Using a unique approach of CO-IP by GFP Nano-trap magnetic beads, the β-catenin containing protein complexes were by identified by LC- MS/MS analysis (Fig 18).

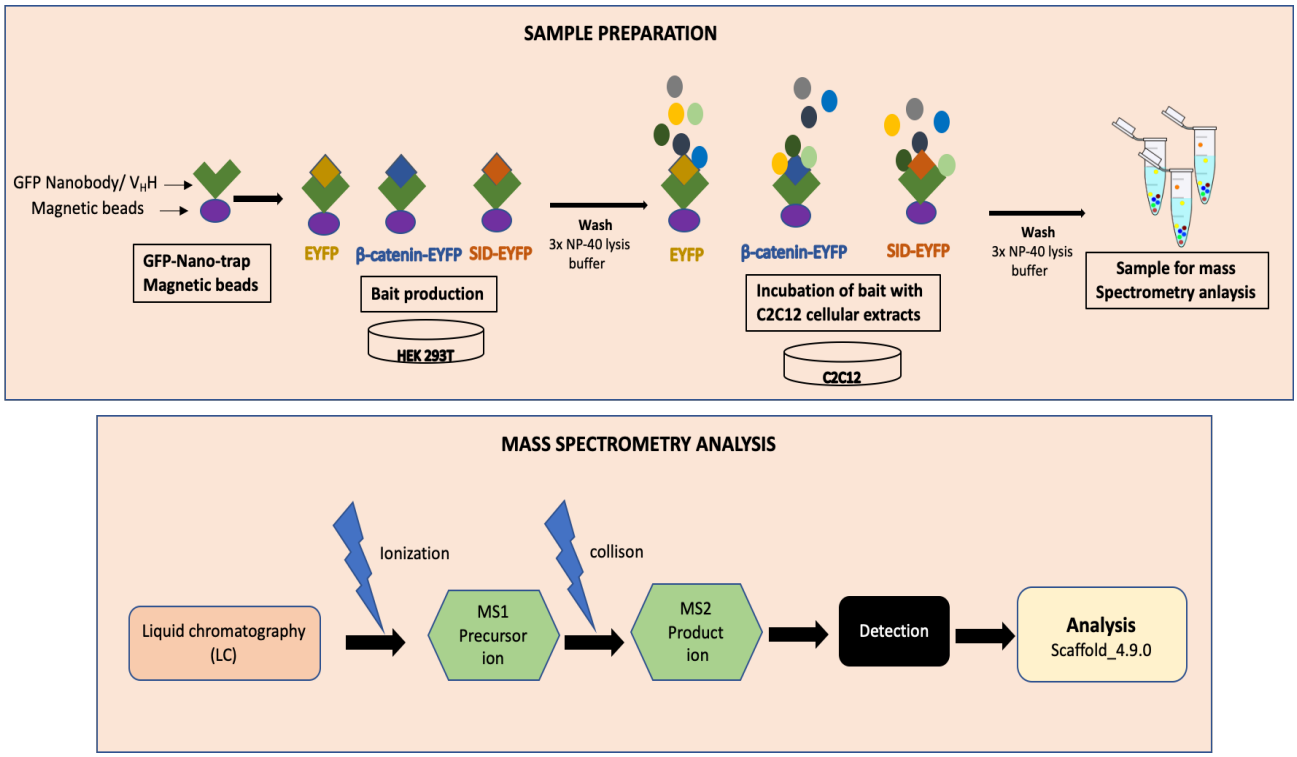
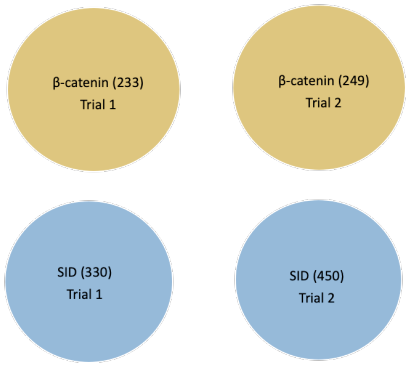


Figure 18: Pipeline for the β-catenin protein interactome screening.
The figure depicts the protocol for the sample preparation for mass spectrometry analysis. GFP-Nano-trap beads were incubated with HEK 293T whole cell lysates that were transiently transfected with EYFP/β-catenin-EYFP/SID-EYFP. Beads were washed and incubated with C2C12 whole cell lysates, elute was send for mass spectrometry analysis.

Mass spectrometry analysis identified a total of 1034 interacting proteins in trial 1 and 1668 proteins in trial 2. The existence of at least a unique single peptide was used as a precedent for identifying a protein as a possible interactor in the β-catenin-EYFP/SID-EYFP IP and none in the EYFP control or the ratio of unique peptide in β-catenin-EYFP/SID-EYFP more than 100 as compared to the controls. Protein threshold was kept at 95% minimum. Using this approach, we identified 233 and 249 potential β-catenin interacting proteins in trial 1 and 2 respectively (Fig 19A). Similarly, 330 potential SID interacting proteins were identified in trial 1 and 450 were

identified in trial 2 (Fig 19A). Analysis of the top β-catenin protein interactors in both trials (Fig 19B&C) demonstrated the identification of known β-catenin interactors (highlighted).

A



B.

β-catenin protein interactome_Trial 1

Identified Proteins	Alternate ID	EYFP (cont)	b-catenin-EYFP
Catenin beta-1 OS=Mus musculus	Ctnnb1	2	399
Catenin alpha-1 OS=Mus musculus	Ctnna1	0	95
Histone H2B type 2-E OS=Mus musculus	Hist2h2be	0	95
Cadherin-2 OS=Mus musculus	Cdh2	0	67
Cadherin-15 OS=Mus musculus	Cdh15	0	48
Junction plakoglobin OS=Mus musculus	Jup	0	47
Adenomatous polyposis coli protein OS=Mus musculus	Apc	0	44
ATPase family AAA domain-containing protein 3 OS=Mus musculus	Atad3	0	22
Alpha-actinin-3 OS=Mus musculus	Actn3	0	20
RNA-binding protein 14 OS=Mus musculus	Rbm14	2	17
Tropomyosin alpha-3 chain OS=Mus musculus	Tpm3	0	16
Beta-catenin-interacting protein 1 OS=Mus musculus	Ctnnbip1	0	15
Moesin OS=Mus musculus	Msn	0	13
AP-2 complex subunit mu OS=Mus musculus	Ap2m1	0	12
Caprin-1 OS=Mus musculus	Caprin1	0	12
Vinculin OS=Mus musculus	Vcl	0	10
Y-box-binding protein 3 OS=Mus musculus	Ybx3	0	10
E3 ubiquitin-protein ligase TRIP12 OS=Mus musculus	Trip12	0	9
60S ribosomal protein L21 OS=Mus musculus	Rpl21	0	9
Keratin, type II cuticular Hb5 OS=Mus musculus	Krt85	0	9
Lamin-B1 OS=Mus musculus	Lmnb1	0	8
Interleukin enhancer-binding factor 2 OS=Mus musculus	Ilf2	0	8
26S proteasome regulatory subunit 6B OS=Mus musculus	Psmc4	0	7
Histone deacetylase complex subunit SAP18 OS=Mus musculus	Sap18	0	7
Keratin, type I cuticular Ha3-II OS=Mus musculus	Krt33b	0	7
Keratin, type II cytoskeletal 8 OS=Mus musculus	Krt8	0	7
Chromodomain-helicase-DNA-binding protein 4 OS=Mus musculus	Chd4	0	6
Nucleolar GTP-binding protein 1 OS=Mus musculus	Gtpbp4	0	6
AP-2 complex subunit beta OS=Mus musculus	Ap2b1	0	6
Myelin expression factor 2 OS=Mus musculus	Myef2	0	6
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochond	Ndufa10	0	6
Neurabin-2 OS=Mus musculus	Ppp1r9b	0	6
cAMP-dependent protein kinase catalytic subunit alpha OS=Mus musculus	Prkaca	0	6
Glycogen synthase kinase-3 beta OS=Mus musculus	Gsk3b	0	6
Fragile X mental retardation syndrome-related protein 2 OS=Mus musculus	Fxr2	0	6
Transforming protein RhoA OS=Mus musculus	Rhoa	0	5
Translationally-controlled tumor protein OS=Mus musculus	Tpt1	0	5
Enhancer of mRNA-decapping protein 4 OS=Mus musculus	Edc4	0	5
Transcription intermediary factor 1-beta OS=Mus musculus	Trim28	0	5
RNA-binding protein Raly OS=Mus musculus	Raly	0	5

Total protein identified = 233

C.

β-catenin protein interactome_Trial 2

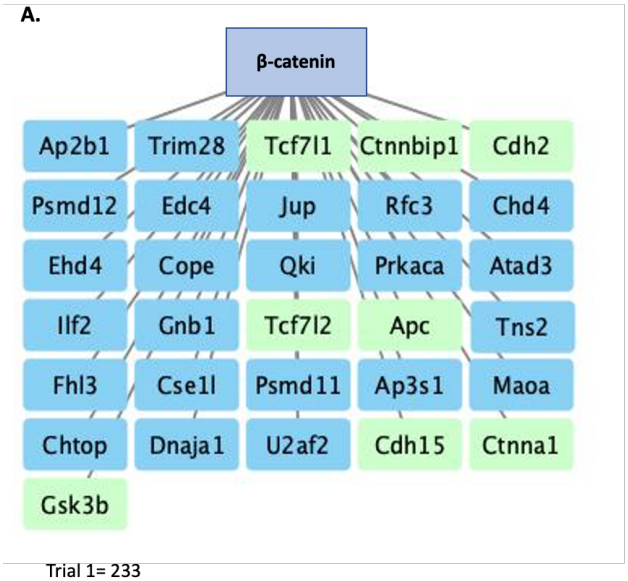
Identified Proteins	Alternate ID	EYFP (cont)	b-catenin-EYF
Catenin beta-1 OS=Mus musculus	Ctnnb1	0	452
Cadherin-2 OS=Mus musculus	Cdh2	0	74
Catenin alpha-1 OS=Mus musculus	Ctnna1	0	68
Cadherin-15 OS=Mus musculus	Cdh15	0	64
Tubulin beta-4A chain OS=Mus musculus	Tubb4a	0	42
Junction plakoglobin OS=Mus musculus	Jup	0	41
Tubulin alpha-1C chain OS=Mus musculus	Tuba1c	0	40
CAD protein OS=Mus musculus	Cad	0	16
Beta-catenin-interacting protein 1 OS=Mus musculus	Ctnnbip1	0	14
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial OS=Mus musculus	Ndufs3	0	13
Adenomatous polyposis coli protein OS=Mus musculus	Apc	0	13
Heat shock-related 70 kDa protein 2 OS=Mus musculus	Hspa2	0	13
Coatomer subunit alpha OS=Mus musculus	Copa	0	10
NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial OS=Mus musculus	Ndufs2	0	10
Cytoplasmic dynein 1 heavy chain 1 OS=Mus musculus	Dync1h1	0	9
tRNA-splicing ligase Rtcb homolog OS=Mus musculus	Rtcb	0	9
Eukaryotic translation initiation factor 3 subunit A OS=Mus musculus	Eif3a	0	7
ATPase family AAA domain-containing protein 3 OS=Mus musculus	Atad3	0	7
E3 ubiquitin-protein ligase TRIM32 OS=Mus musculus	Trim32	0	7
ADP-ribosylation factor 5 OS=Mus musculus	Arf5	0	7
ATP-binding cassette sub-family E member 1 OS=Mus musculus	Abce1	0	6
Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 OS=Mus musculus	Dhx15	0	6
Integrin-linked protein kinase OS=Mus musculus	Ilk	0	5
Pre-mRNA-processing-splicing factor 8 OS=Mus musculus	Prpf8	0	5
PDZ and LIM domain protein 2 OS=Mus musculus	Pdlim2	0	5
Angiopoietin-related protein 2 OS=Mus musculus	Angptl2	0	5
26S proteasome non-ATPase regulatory subunit 3 OS=Mus musculus	Psmd3	0	4
60S ribosomal protein L5 OS=Mus musculus	Rpl5	0	4
U5 small nuclear ribonucleoprotein 200 kDa helicase OS=Mus musculus	Snrnp200	0	4
Sodium/potassium-transporting ATPase subunit alpha-1 OS=Mus musculus	Atp1a1	0	4
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 OS=Mus musculus	Ndufa5	0	4
Transcription factor 7-like 2 OS=Mus musculus	Tcf7l2	0	4
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus	Hspg2	0	3
Unconventional myosin-Ic OS=Mus musculus	Myo1c	0	3
Trifunctional enzyme subunit alpha, mitochondrial OS=Mus musculus	Hadha	0	3
E3 ubiquitin-protein ligase NEDD4 OS=Mus musculus	Nedd4	0	3
26S proteasome regulatory subunit 7 OS=Mus musculus	Psmc2	0	3
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Mus musculus	Rpn1	0	3
Calpain-2 catalytic subunit OS=Mus musculus	Capn2	0	3
E3 ubiquitin-protein ligase SHPRH OS=Mus musculus	Shprh	0	3

Total protein identified = 249

Figure 19: β-catenin protein interactome in C2C12 skeletal myoblasts.
(A) Mass spectrometry analysis identified a total of 233 and 249 potential protein β-catenin interacting partners and 330 and 450 SID interacting proteins in trial 1 and trial 2 respectively. (B&C)A list of top 40 β-catenin protein interactors in C2C12 myoblasts during two trials is indicated, known β-catenin such as α-Catenin, Cadherin and APC are highlighted in yellow.

Further we compared the common β-catenin interacting proteins between both trials and identified 31 potential interactors (Fig 20A). Interestingly, we identified well characterized β-catenin interacting proteins such as α-catenin, cadherin, APC, GSK3β, TCF, FMRP and β-catenin interacting protein 1 (Ctnnip-1) (Fig 20B). It was interesting to observe that this technique successfully identified β-catenin interacting partners in the cytoplasm and cell membrane and few in nucleus thus validating the efficiency of the technique and suggesting the remaining proteins to be novel interactors (Fig 20C).

A.



Trial 1= 233
Trial 2= 249
Common proteins = 31

B.

Identified proteins	Alt ID	KDa	β-catenin Trial 1	β-catenin Trial 2
Catenin beta-1	Ctnnb1	85	399	452
Catenin alpha-1	Ctnna1	100	95	68
Cadherin-2	Cdh2	100	67	74
Cadherin-15	Cdh15	86	48	64
Adenomatous polyposis coli protein (APC)	Apc	311	44	
Beta-catenin-interacting protein 1 (BCIP 1)	Ctnnbip1	9	15	14
Glycogen synthase kinase-3 beta	Gsk3b	47	6	1
Fragile X mental retardation syndrome-related protein 2	Fxr2	74	6	1
Transcription factor 7-like 2	Tcf7l2	51	4	4

C.

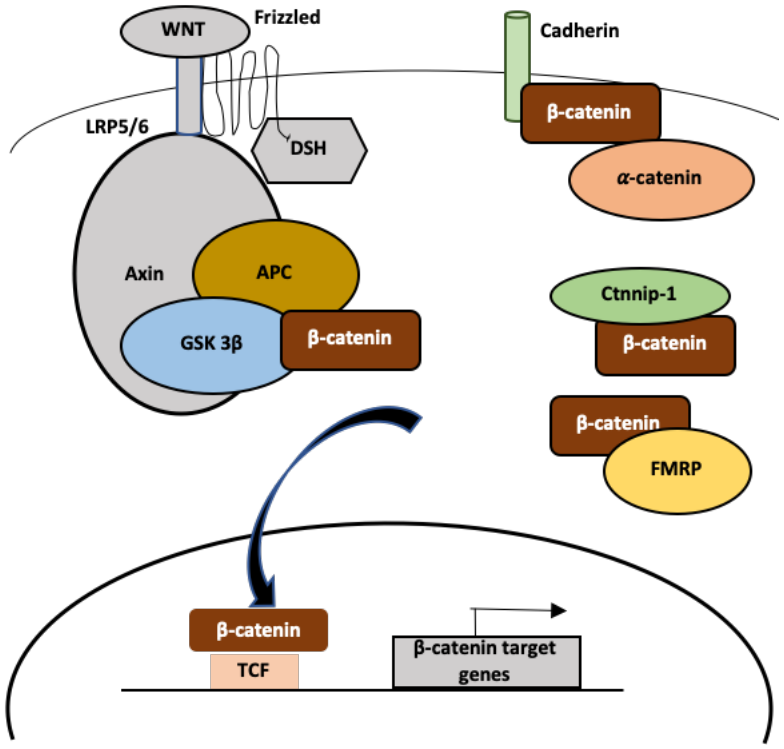
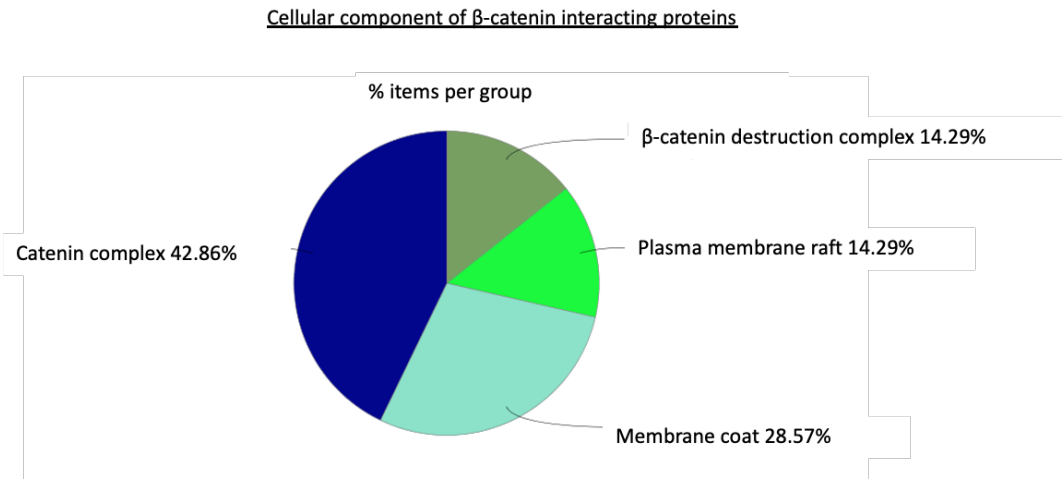


Figure 20: Identification of the known β-catenin interactors in mass spectrometry analysis. (A)A total of 233 and 249 β-catenin interacting partners were identified in trial 1 and 2 where 31 common proteins were identified between trials. A full list of the common interacting proteins

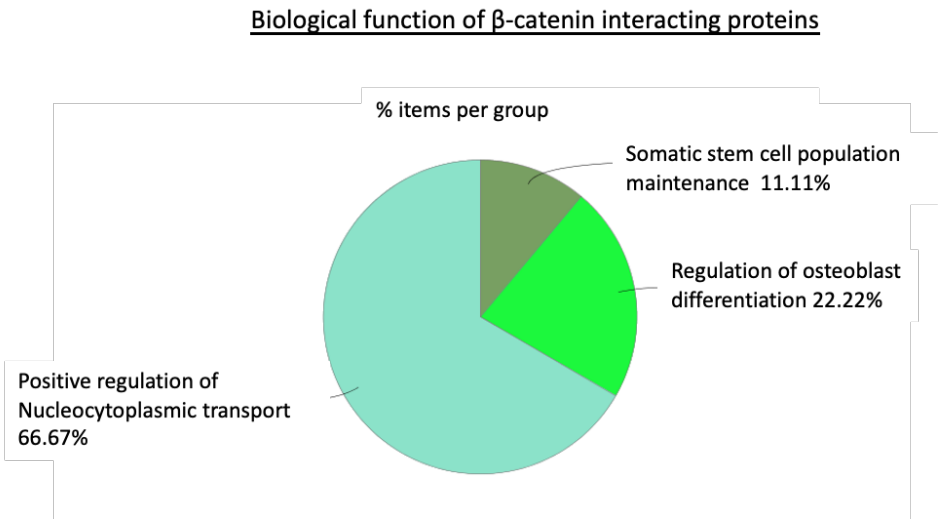
between both trials was created using Cytoscape vs 3.7.1. The known β-catenin interactors are highlighted in green and their peptide scores (B) and localization (C) is indicated.

To further categorize the protein interactome of β-catenin, we carried out a gene ontology (GO) term analysis using ClueGOapp from Cytoscape and analyzed the interactors for the "Cellular Component" and "Biological function" domains. β-catenin interacting partners were identified in multiple cellular compartments, such as with the catenin complex (42.86%), membrane coat (28.57%), plasma membrane raft (14.29%) and β-catenin destruction complex (14.29%) (Fig 21A). The majority of the interactors were associated with regulation of nucleocytoplasmic transport (66.67%) and some were associated with the regulation of osteoblast differentiation (22.22%) and somatic stem cell population maintenance (11.11%) (Fig 21B). SID interactors were primarily associated with membrane coat (25%), oxidoreductase complex (15%), proteasome regulatory particle (15%) and interestingly with eukaryotic translation initiation factor (10%), chromosomal region (7.5%) and nuclear matrix (5%) (Fig 21C). Most of the SID interactors were involved with ribonucleoprotein complex assembly (22.58%) and generation of precursor metabolites (22.58%) (Fig 21D).

A.

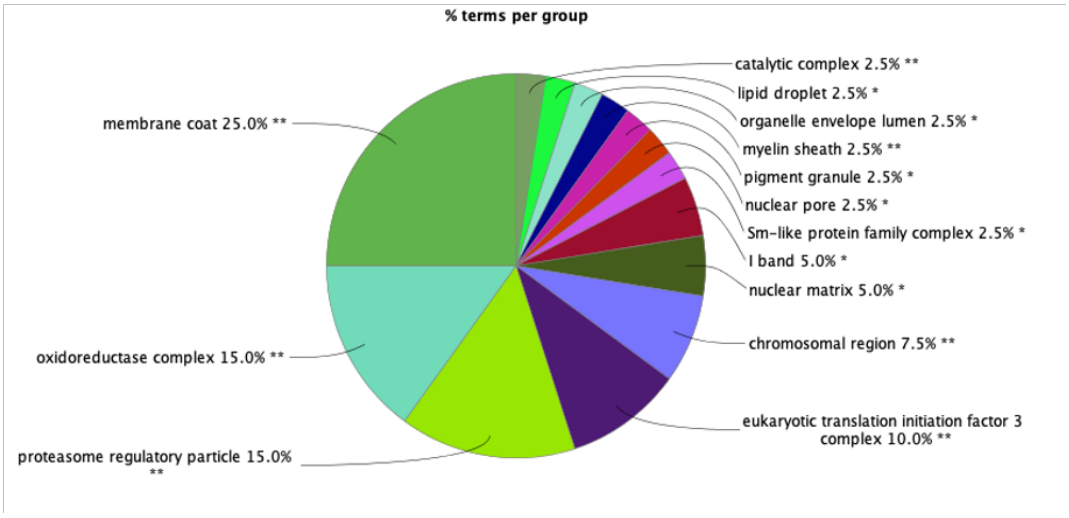

Cellular component of β-catenin interacting proteins
% items per group
β-catenin destruction complex 14.29%
Plasma membrane raft 14.29%
Membrane coat 28.57%
Catenin complex 42.86%

B.


Biological function of β-catenin interacting proteins
% items per group
Somatic stem cell population maintenance 11.11%
Regulation of osteoblast differentiation 22.22%
Positive regulation of Nucleocytoplasmic transport 66.67%

C.

Cellular component of SID interacting proteins



D.

Biological function of SID interacting proteins

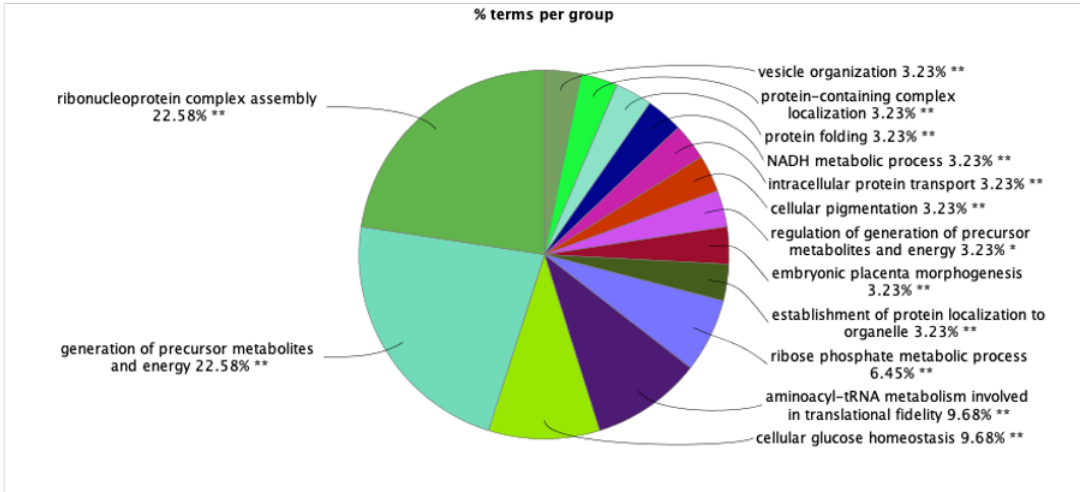


Figure 21: Gene Ontology (GO-term analysis) of β-catenin and SID interacting proteins in skeletal myoblasts.

Gene ontology (GO) term analysis of the β-catenin protein interactors for the (A) "Cellular Component" domain and (B) "Biological function" domain. Similarly, SID protein interactors for the (C) "Cellular Component" domain (D) "Biological function" domain was created using ClueGO vs 3.7.1.

3.5.2 β-catenin and SID interactome in growth and differentiation stages of skeletal myogenesis.

Next, we were intrigued to identify the proteins that exclusively interacted either with β-catenin/ SID during different stages of skeletal myogenesis to provide an insight whether a shift of interacting proteins takes place during different stages of myogenesis. Our results suggested 31 common β-catenin protein interactors in growth media and 42 common protein interactors in differentiation conditions between two trials (Fig 22A&B). Only 10 proteins were identified that were common between growth and differentiation stages of C2C12 (Fig 22C). Similarly, 95 SID interacting proteins were identified during growth stages (Fig 23A) and 98 proteins were identified during differentiation stages (Fig 23B) between two trials. A list of 25 common SID interacting proteins were identified that were common between growth and differentiation stages (Fig 23C).

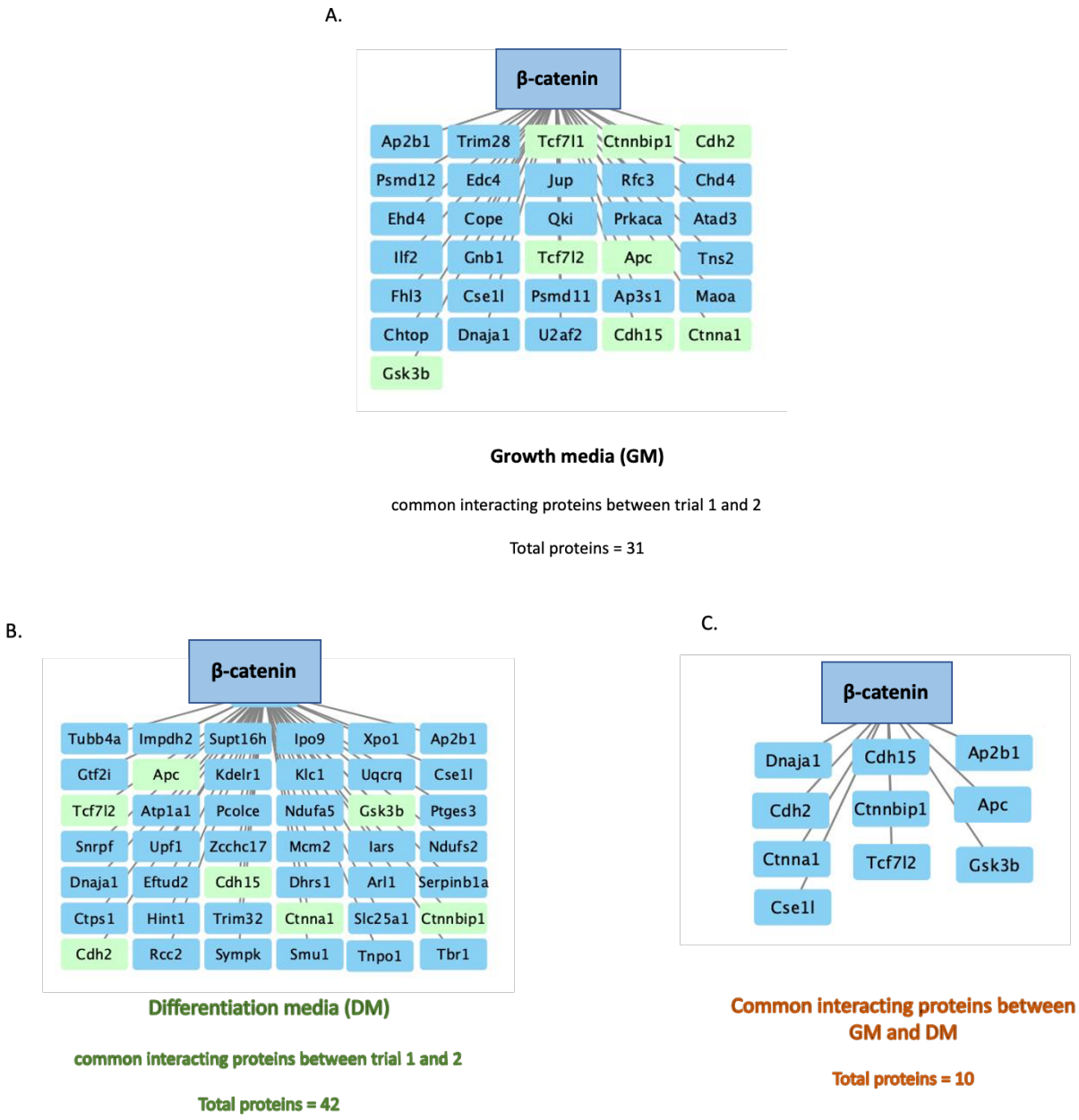
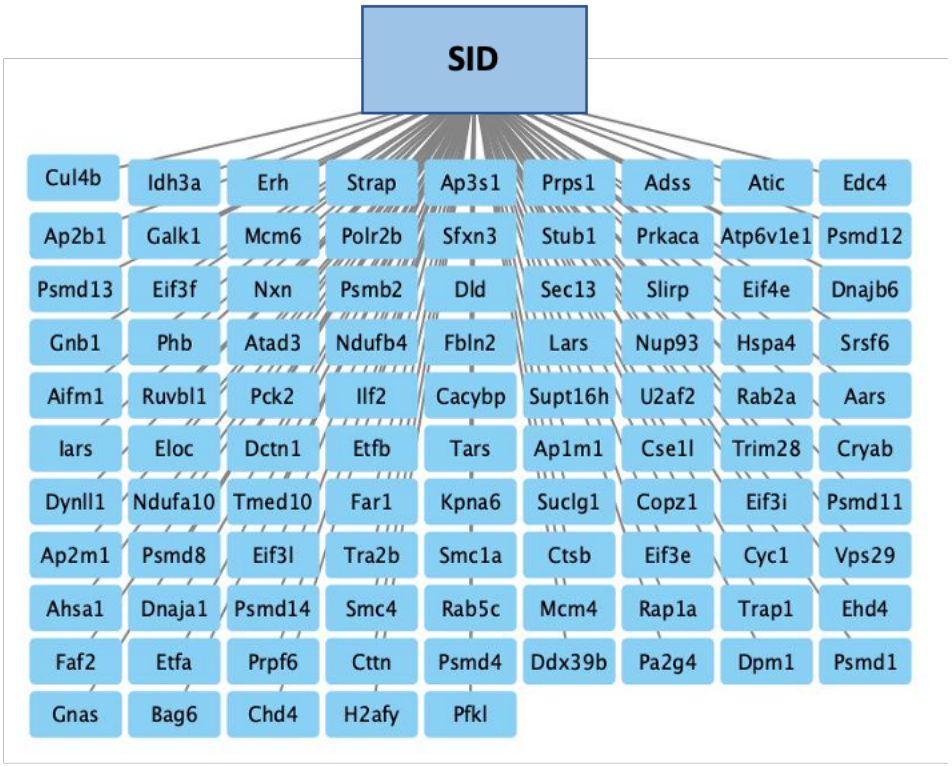


Figure 22: β-catenin common interacting proteins in the growth and differentiation stages of skeletal muscle.
A list of the common protein interactors of β-catenin during (A) growth and (B) differentiation condition of C2C12 skeletal muscle cells between two trials. Known β-catenin interactors are highlighted. (C) The common β-catenin interacting proteins between growth and differentiation condition is indicated. Cytoscape vs 3.7.1 was used to generate the visual clusters.

A.



Growth media (GM)

common interacting proteins between trial 1 and 2

Total proteins = 95

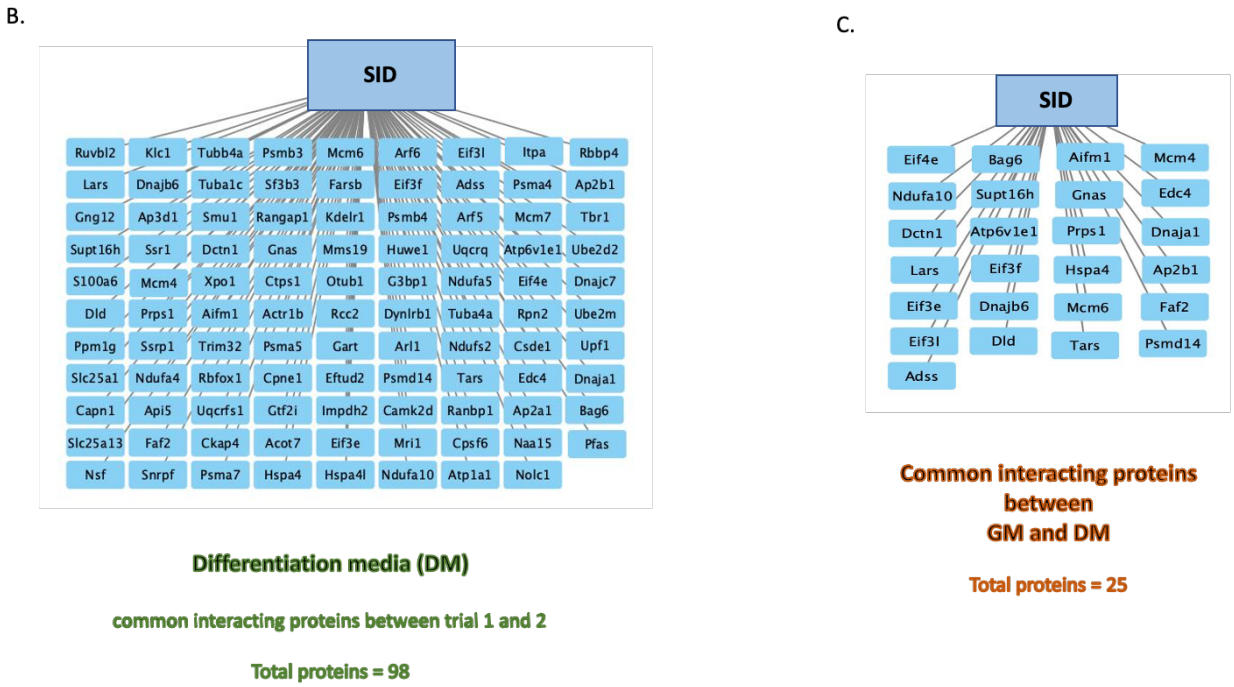


Figure 23: SID common interacting proteins in the growth and differentiation stages of skeletal muscle.

A list of the common protein interactors of SID during (A) growth and (B) differentiation condition of C2C12 skeletal muscle cells between two trials. (C)The common SID interacting proteins between growth and differentiation condition is indicated. Cytoscape vs 3.7.1 was used to generate the visual clusters.

3.6 Discussion

Our previous study (311) identified a direct physical interaction between Smad7 and β-catenin and delineated a domain on β-catenin that we have termed the Smad7 interacting domain (SID) of β-catenin. We sought to understand the protein interactome of both wild type β-catenin and SID to further understand their function. We approached this study with a unique method of pull down using GFP-Nano-trap followed by mass-spectrometry analysis (Fig 18). Our study identified more than 200 proteins that interacted with β-catenin or SID and not with the EYFP control. Remarkably, many known β-catenin interactors previously associated with canonical β-catenin function such as α-catenin, cadherin, GSK3β, APC and TCF were identified indicating the efficacy of the approach and that the remaining proteins would be unique bona fide interactors.

Gene Ontology (GO) for cellular component domain and Biological Process enrichment analysis suggested that the majority of the terms associated with β-catenin were involved in plasma membrane/membrane coat regulation along with the beta-catenin destruction complex. The localized function of the interacting proteins was associated with plasma membrane or cytoplasm. A very similar pattern was observed with the SID interacting proteins where 25% of the proteins localized to the membrane coat. We observed that the majority of β-catenin and SID interactors were primarily localized to the membrane and cytoplasm and the nuclear interacting proteins were minimally detected. This could be attributed to our approach of using whole cell lysate for the pull down assay. Based on these data we now contend that it would be more efficacious to study the β-catenin and SID interactome with nuclear lysates from the cells of interest to enrich for nuclear interacting proteins. Identification of the nuclear interactors can further provide insight on the

various proteins that interact with β-catenin to regulate myogenic gene transcription. Moreover, it would be alluring to identify the specific protein interactome of β-catenin in the cytoplasm and nucleus as this may expand our understanding of the regulation of β-catenin by various signaling pathways.

Further, we were curious to know whether there is a shift in protein interactome during different stages of skeletal myogenesis in which it is known that Wnt signaling and β-catenin can play different roles. Our study identified proteins that interacted with β-catenin/SID in both proliferating and differentiation stages along with proteins that interacted preferentially with either of these stages of myogenic progression. We identified 31 proteins that associated with β-catenin in growth conditions and 42 proteins in differentiation conditions respectively between two trials, among which only 10 proteins were common between growth and differentiation stages. It would be interesting to further analyse the proteins that were distinctively associated with β-catenin in different stages of myogenesis. It was interesting to see that proteins such as Tripartite containing motif 28 (Trim 28) which is involved in transcriptional regulation, cellular differentiation and proliferation associated with β-catenin during the proliferation stage, while this interaction was lost during the differentiation stage. This is particularly interesting as Trim-28 has been suggested to regulate MyoD's function where it associates with MyoD and MEF2 in myoblasts and serves as a scaffold to recruit either co-activators or co-repressors to muscle specific promoters (312). A similar pattern was observed with Serine-Threonine Kinase Receptor-Associated Protein (STRAP) that have been suggested to inhibit β-catenin degradation and promote stabilization in colorectal cancer (313), specifically during the proliferation stage. Interestingly, STRAP also forms a complex with Smad7 and synergizes to inhibit TGF-β signaling (220). Proteins such as Mitogen

activated protein kinase-3 (MAPK-3) and Cyclin dependent kinase 9 (CDK9) interacted during the differentiation stage while no interaction was observed in the growth stage. MAPK3 is a component of the MAPK/ERK signaling cascade that regulates a myriad of biological functions. p38 MAPK regulates myogenic differentiation and activation of satellite cells (314). Moreover, previous studies from our lab have suggested that activation of p38 MAPK increases β-catenin *trans*activation and regulates β-catenin-MEF2 interaction in smooth muscle cells (279). CDK9 is a serine/threonine kinase that associates with CyclinT1/T2 to form the Positive Transcription Elongation Factor Complex (P-TEFb). CDK9 has been implicated to play crucial roles in transcription elongation but recent studies suggested that CDK9 regulates major events of transcription including initiation and termination. During the initiation of transcription, phosphorylation of CDK9 is required for RNA Polymerase II (Pol II) pause release. After the start of transcription, CDK9 is involved in attenuating the elongation block to release Pol II into the elongation phase. CDK9 recruits termination factors at the poly(A) tail during transcription termination (315). Therefore, it would be interesting to further investigate the role of β-catenin and CDK9 in regulating transcription elongation.

A similar list of proteins that interacted with SID specifically during either growth/differentiation stages of muscle cells was generated. We identified more than 90 proteins that interacted with SID during growth and differentiation conditions, respectively. Of these 25 proteins were common between these stages. Interestingly, as observed with β-catenin, Trim-28 and STRAP interacted with SID specifically during growth condition and the interaction was lost during the differentiation stage. Similarly, solute carrier family 25 member 1 (SLC25a1) interacted with both β-catenin and SID during differentiation conditions. Interestingly, other proteins such as KH domain containing RNA binding (Qki) involved in the regulation of mRNA splicing interacted

with β-catenin but not with SID during growth conditions. Likewise, structural maintenance of chromosome 1A (Smc1A) which is involved in the regulation of maintaining chromosomes organization and structure, interacted specifically with SID and not with β-catenin during growth condition. Future to studies will focus on the proteins that interacted specifically with β-catenin/SID during growth or differentiation conditions, allowing insight into their regulation by various signaling pathways.

Altogether, these observations suggest that the protein interactome for β-catenin/SID changes drastically during different stages of skeletal myogenesis and follow up studies could focus on the specific cues that regulate these protein dynamics. To conclude, in this study we have identified interactomes for both β-catenin and SID in growth and differentiation conditions in skeletal myogenic cells. The next phase of this work will be to study these proteins in more depth to understand their specific role in β-catenin function in the context of skeletal myogenesis.

Chapter 4

TAZ exhibits phase separation properties and interacts with Smad7 and β-catenin to repress skeletal myogenesis

Soma Tripathi, Tetsuaki Miyake, and John C. McDermott

Manuscript Submitted for Publication

Author contributions

Experimental Design

Soma Tripathi, Tetsuaki Miyake, and John C. McDermott

Drafting Manuscript

Soma Tripathi, Tetsuaki Miyake, and John C. McDermott

Conducting Experiments

Soma Tripathi:	Figure 24B
	Figure 25A, B, C, D
	Figure 26A
	Figure 27A
Tetsuaki Miyake:	Figure 24A
	Figure 26B, C, D, E
	Figure 27B, C, D, E
	Figure 28A, B

4.1 Abstract

Hippo signaling in Drosophila and mammals is prominent in regulating cell proliferation, death and differentiation. Effectors of Hippo signaling (YAP/TAZ) exhibit crosstalk with transforming growth factor-β (TGF-β) and Wnt-β-catenin pathways. Previously, we implicated Smad7 and β-catenin in myogenesis. A reported interaction between YAP and Smad7 led us to assess a potential role of TAZ on Smad7 and β-catenin in muscle cells. Here, we document functional interactions between Smad7 and TAZ and also Smad7 and β-catenin in myogenic cells. Ectopic TAZ expression resulted in repression of a muscle specific promoter (*ckm*). Furthermore, depletion of endogenous TAZ enhanced *ckm* promoter activation. Ectopic TAZ, while potently active on a multimerized TEAD reporter (Hip-Hop), repressed Myogenin and MyoD enhancer regions. Additionally, a Wnt/β-catenin readout (TOP flash) demonstrated a potent inhibitory effect of TAZ on β-catenin activity. Imaging studies revealed that TAZ is predominantly localized in nuclear speckles in myoblasts, while in differentiation conditions TAZ is hyperphosphorylated at Ser 89 leading to cytoplasmic sequestration. Finally, using Live Cell Imaging, we document that TAZ exhibits robust properties of liquid-liquid phase separation (LLPS). These observations support a role for TAZ as an effector of Hippo signaling that supresses the differentiation machinery in myogenic cells.

4.2 Introduction

Development and maintenance of tissues and organs requires a delicate balance between proliferation, apoptosis and differentiation (133). A search for mutations that cause tissue overgrowth in *D. melanogaster* precipitated the discovery of a conserved kinase cascade comprising of Hippo and Warts kinase and adaptor proteins Salvador and Mob (84,316-320), collectively referred to as Hippo signaling. Activation of Hippo signaling promotes activation of Hippo kinases resulting in phosphorylation of a transcriptional regulator, Yorkie, and its subsequent sequestration in the cytoplasm, thus inhibiting its functional association with another transcription factor (Scalloped) (87). YAP (Yes-associated protein), is the mammalian homolog of Yorkie in vertebrates and MST1/2, LATS1/2 are homologs of the Drosophila Hippo kinases, indicating the evolutionary conservation of Hippo signaling (321,322). TAZ, a paralog of YAP in vertebrates, was subsequently characterized as a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins (91). TAZ phosphorylation on a conserved serine residue (Ser89 in human TAZ; equivalent to Ser127 in human YAP) results in inhibition of TAZ transcriptional co-activation through 14-3-3 protein mediated cytoplasmic retention.

A number of studies have proposed extensive crosstalk between Hippo signaling and other cellular signaling pathways. Documentation of cross-talk between TGF-β and Hippo signaling was first reported in a yeast two-hybrid screen where YAP65 was identified as a novel Smad7-interacting protein (97). In Drosophila, Yorkie interacts with and promotes Mad-dependent transcription in response to DPP signaling (95,323). In another high-throughput screen aimed at identifying novel regulators of TGF-β signaling in human embryonic cells, TAZ was identified as a mediator of Smad (the vertebrate homolog of MAD) nucleocytoplasmic shuttling that is essential for TGF-β

signaling. YAP was also shown to bind BMP-regulated Smad1/5/8 and TGF-β regulated Smad2/3. The YAP/Smad1 complex is regulated by the Mediator-associated kinases CDK8 and CDK9 that phosphorylate the Smad1 linker domain to expose a PPXY motif and promote binding to the YAP WW domains (95). Thus, there is extensive documented cross- talk between the Hippo and TGF-β pathways.

In the absence of Wnt signaling, components of the β-catenin destruction complex have been shown to sequester TAZ, promoting its degradation. β-catenin contributes to this by bridging TAZ to the ubiquitin ligase β-TrCP (324). Receptor activation by Wnt ligands, allows escape of β-catenin from its destruction complex which concomitantly also stabilizes TAZ expression thereby promoting nuclear accumulation of both β-catenin and TAZ (117). TAZ-mediated transcription and protein stabilization are a general feature of the Wnt response in a variety of cellular model systems suggesting that the β-catenin/TCF complex is not the sole modality by which the Wnt/β-catenin pathway can regulate gene expression. Hence, Wnt signaling can promote the nuclear accumulation of both β-catenin and TAZ. The consequences of crosstalk between these signaling pathways is of particular interest for skeletal muscle biology since TGF-β, Wnt and Hippo signaling have all been implicated in regulating various aspects of skeletal muscle development and post-natal physiology (21,325-327).

Therefore, in view of the putative connections between TAZ and both Smad7 and β-catenin, we questioned whether TAZ modulates the function of Smad7 and β-catenin in myogenic cells that we have previously reported (311). Here, we document a protein: protein interaction between Smad7 and TAZ in muscle cells which functions to repress Smad7/ β-catenin function at muscle

specific promoters such as the well characterized Muscle Creatine Kinase (*ckm)* gene. In these studies, we document that TAZ exhibits robust phase separation properties in myogenic cells. Collectively, these observations are consistent with the role of Hippo signaling in myogenic cells which, when off, promotes proliferation due to TAZ nuclear accumulation and activation of genes associated with proliferation. We now propose that, as well as positively enhancing the expression of proliferative genes, nuclear TAZ also suppresses differentiation by acting as a co-repressor of the myogenic transcription complex containing Smad7 and β-catenin. Thus, our data indicate that when Hippo signaling is activated, TAZ is phosphorylated and retained in the cytoplasm which concomitantly de-represses the nuclear activity of Smad7 and β-catenin at muscle promoters. These data support a model in which TAZ acts as a fulcrum to balance the ratio of proliferation and differentiation in myogenic cells. These studies identify TAZ as a potential determinant of muscle differentiation, having implications for control of muscle mass during development, growth and regeneration.

4.3 Materials and methods

Cell culture

C2C12 myoblasts and C3H10T1/2 cells were obtained from the American Type Culture Collection. Cells were cultured in growth medium (GM) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% fetal bovine serum (FBS) and L-Glutamine (HyClone) supplemented with 1% penicillin-streptomycin (Invitrogen, ThermoFisher). Myotube formation was induced by replacing GM with differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum (Atlanta Biologicals) and 1% penicillin-streptomycin. Cells were maintained in an incubator at 95% humidity, 5% CO_2, and 37°C.

Transfections

For ectopic protein expression, cells were transfected using the calcium phosphate precipitation method for transcription reporter assays. Cells were re-fed 16 h post-transfection and harvested. For small interfering RNA (siRNA) experiments, cells were transfected with Lipofectamine 2000 (Life Technologies) using instructions provided by the manufacturer and harvested 48 hr later, unless otherwise indicated.

Gene silencing

Knockdown of target genes was done using mission siRNA (Sigma-Aldrich). siTAZ#1 (SASI Mm01 00107364) and siTAZ#2 (SASI Mm01 00107368) and universal scrambled siRNA (SIC001) were used at 50 nM concentrations.

Plasmids

Expression plasmids for Myc-His-tagged full-length Smad7, FLAG-TAZ, β-catenin-myc, transcription reporter assay constructs *pckm-luc* and TOP/FOP flash have been described previously (230,232,279,328). HIP-Flash (#83466) and HOP-flash were obtained from Addgene (329) . mCherry/EYFP tagged TAZ and β-catenin constructs were described previously (280,328). pMyoD (-2.5/-275)-luc was a kind gift from Dr. S.Tapscott (330). pMyog-luc was described previously (232).

Transcription reporter gene assays

Transcriptional reporter assays were performed using luciferase reporter plasmids along with expression constructs (indicated in figure legends) and a *Renilla* plasmid (pRL-*Renilla*, Promega) as an internal control. Cells were washed with 1X PBS and harvested in Luciferase Lysis Buffer (20 mM Tris pH 7.4 and 0.1% Triton X-100). Enzymatic activity was measured in each sample on a luminometer (Lumat LB, Berthold) using Luciferase assay substrate (E1501; Promega) or Renilla assay substrate (E2820; Promega). Luciferase activity values obtained were normalized to *Renilla* activity in the same cell extracts and expressed as fold activation to the control.

Western blotting

Total cellular protein extracts were prepared in NP-40 lysis buffer (0.5% (vol/vol)), 50 mM Tris-HCl (pH 8),150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA (pH 8), and 0.1 M NaF supplemented with 1X protease inhibitor cocktail (P-8340; Sigma) and 0.5 mM sodium orthovanadate. Protein concentrations were determined by a standard Bradford assay. Equivalent amounts of protein were denatured in SDS loading buffer at 100°C for 5min and then run in sodium

dodecyl sulfate (SDS)-polyacrylamide gels, followed by electrophoretic transfer to Immobilon-FL Polyvinylidene Difluoride membrane PVDF membrane (Millipore) as directed by the manufacturer. Blots were incubated with blocking buffer that consisted of 5% milk in Tris-buffered saline (TBS)-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) prior to the incubation with primary antibody at 4°C overnight with gentle agitation.\ After three washes with TBS-T, appropriate HRP-conjugated secondary antibody (BioRad, 1:2000), were added for 2 h at room temperature. Protein/antibody immuno-complexes were detected with Enhanced Chemiluminescence western blotting substrate (Pierce, ThermoFisher).

Antibodies

Rabbit monoclonal for αTAZ (mAb 8418), αphospho-TAZ (mAb59971) and αβ-catenin (pAb9562) were purchased from Cell signaling. Monoclonal αFlag antibody (F1804) was from Sigma. αMyc (9E10), αMyHC (MF20), αMyogenin (F5D) was purchased from DSHB. αActin (Sc1616), αMyoD (sc304) was purchased from Santa Cruz.

Co-ImmunoPrecipitation

For Co-ImmunoPrecipitation assays (Co-IP), cells were harvested, and proteins were extracted as described above. Immunoprecipitation was performed using anti-flag M2 magnetic beads (Sigma, M8823) according to the manufacturer's instructions. Eluates were analyzed by Western blotting as described above.

Immuonofluorescence Analysis

C2C12 cells were seeded on the Glass-bottom dishes (MatTek) and fixed with 4% PFA. Cells were washed (3 times) with 1XPBS and permeabilized with ice cold 90% Me-OH for 1 min. Cells were washed again (3 times) with 1XPBS and incubated with blocking buffer (5% FBS in 1xPBS) for 2 h at room temperature, and then incubated with an indicated primary antibody in the blocking buffer overnight at 4^0C. After removal of the unbound primary antibody, the cells were incubated with Alexa fluor conjugated secondary antibody (Life Technogies) in the blocking buffer at room temperature for 1.5 h. After washed with 1XPBS three times, the cells were subjected to imaging with a Zeiss Observer Z1 confocal fluorescent microscope equipped with Yokogawa CSU-X1 spinning disk. Images were recorded by AxioCam MRm camera (Zeiss) and processed by Zen 2.5 (blue edition) software (Zeiss).

Live-cell imaging

C2C12 cells were seeded on polymer-coated glass-bottom dishes (#81158, ibidi). The cells were transfected with expression plasmids encoding the indicated fluorescently tagged proteins. The cell culture media was exchanged for FluoBrite (A1896701, ThermoFisher) with 10%FBS (HyClone). The cells were maintained in a moisturized environmental chamber (5% CO2) on the Zeiss Observer Z1 confocal fluorescent microscope platform during live cell imaging.

4.4 Results

4.4.1 Smad7 and TAZ interact in myogenic cells

Previously, the Hippo effector Yap was identified as a novel Smad7 interacting protein in Cos-7 cells (97). We have previously documented that Smad7 is expressed in muscle cells and plays an important role in muscle specific gene transcription (311). Here, we show that TAZ is expressed in both proliferation and differentiation stages of cultured C2C12 muscle cells (Fig 24A, 27C). To initially test for the interaction between TAZ and Smad7 biochemically, Flag- Smad7 was ectopically expressed in C2C12 cells and total protein lysates were subjected to immunoprecipitation (IP) using flag magnetic beads. Lysate from ectopically expressed Flag-Trim 28 was used as a negative control. Immunoblot analysis of the eluates from the Flag-Smad7 IP but not from Flag-Trim28 IP revealed the presence of TAZ indicating that they are potentially constituents of the same protein complex (Fig 24B). Therefore, we hypothesized that TAZ modulates the myogenic transcriptional complex that we and others have previously documented ((304,311), Schematic in 24C).

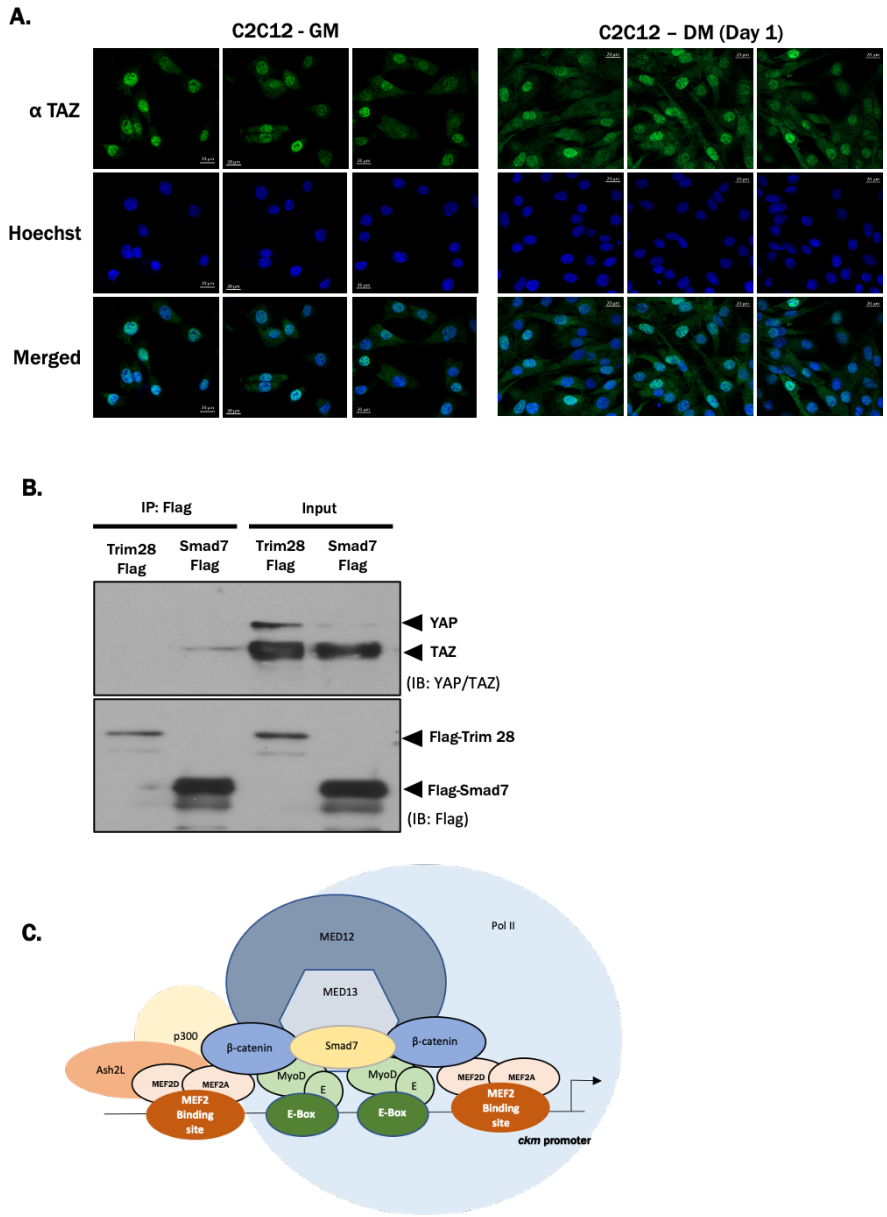


Figure 24: Biochemical interaction between Smad7 and TAZ.
(A) Immunofluorescence analysis of C2C12 cells during myogenic differentiation. C2C12 myoblasts were grown in growth media (GM) and changed to differentiation media (DM) to initiate the differentiation program. Cells were stained for TAZ, Hoechst to detect nuclei (B) C2C12 myoblasts were transiently transfected with Smad7-Flag. Smad7-Flag lysates were utilized for Co-IP with flag-magnetic beads and eluates were probed with TAZ or Flag antibody to detect the possible interaction with Smad7. IP with lysates from Trim28-flag transfected cells served as negative controls. (C) Diagram showing the transcriptional complex in the *ckm* promoter.

4.4.2 TAZ antagonizes the myogenic function of Smad7

Previously, we documented the contribution of Smad7 to the transcriptional control of the muscle creatine kinase (*ckm*) promoter in co-operation with MyoD and β-catenin (Fig 24C, (311)). The *ckm* promoter has been used in many studies in the myogenesis field as a paradigm of muscle specific transcriptional control. We therefore tested whether TAZ could modulate this complex through its interaction with Smad7. Ectopic expression of TAZ alone or in combination with Smad7 demonstrated that TAZ potently inhibits the transcriptional induction of *ckm* by Smad7 (Fig 25A). To further validate these observations, we utilized two independent TAZ directed siRNAs to deplete the levels of endogenous TAZ (Fig 25B) and then performed the *ckm* luciferase reporter assay in TAZ depleted versus normal cells. Depletion of endogenous TAZ enhanced *ckm* transcriptional activity by Smad7 (Fig 25C, D). Moreover, depletion of endogenous TAZ resulted in an overall enhancement of *ckm* transcriptional activity suggesting a repressive function of endogenous TAZ during muscle differentiation (Fig 25C, D).

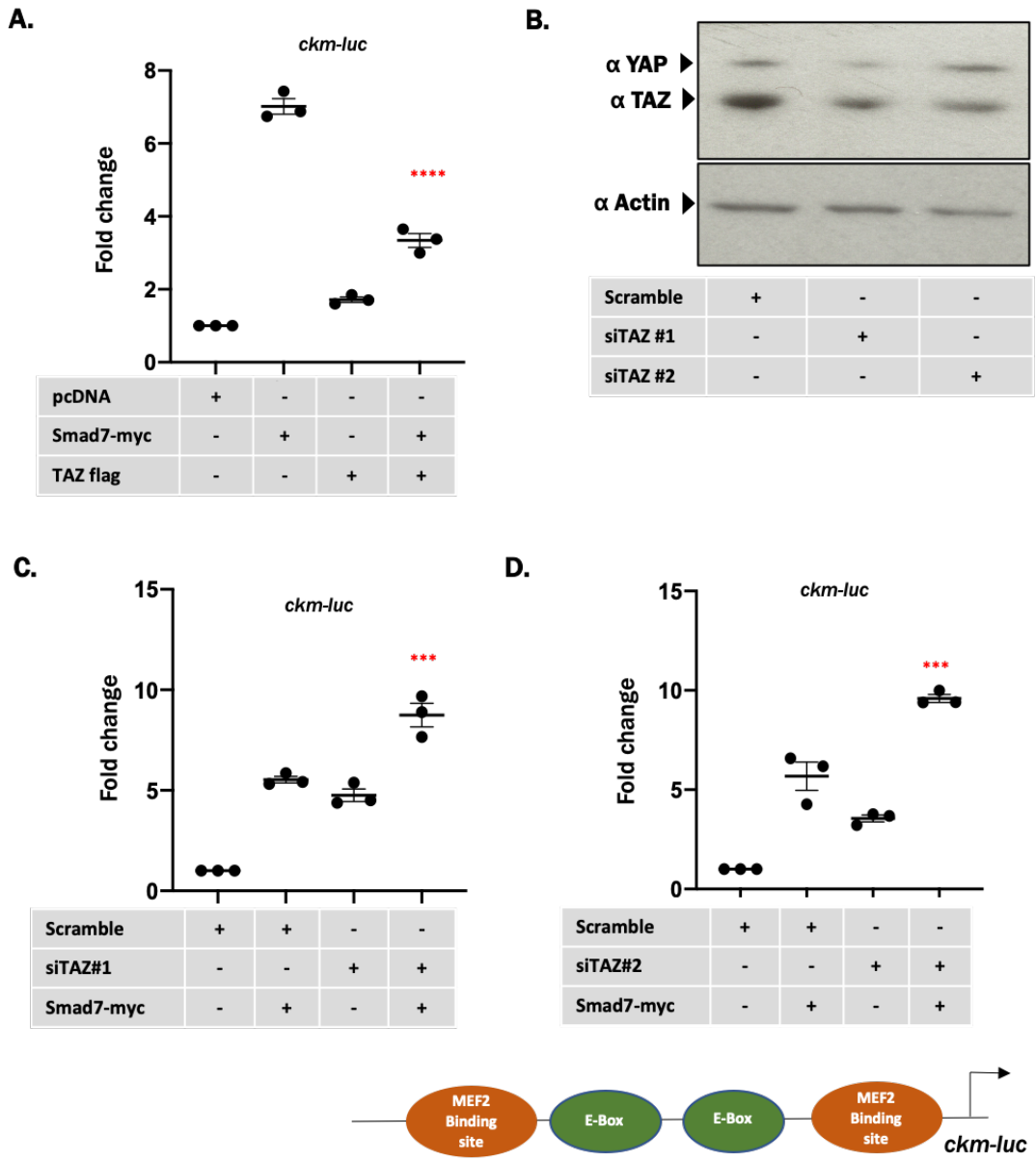


Figure 25: TAZ inhibits the activity of Smad7 on the *ckm* promoter.
(A) Smad7-myc, TAZ-flag alone or in combination were ectopically expressed in C2C12 cells along with a *ckm* luciferase reporter gene. *Renilla* luciferase served as transfection control. C2C12 transfected with empty vector (pcDNA) and reporter genes served as controls for ectopic expression. Cells were harvested for Luciferase determination at 48 h after changing to DM post transfection. Normalized luciferase activity was compared to the control to determine fold changes. (B) Two siRNAs specific for TAZ were used to deplete the endogenous TAZ levels in C2C12. Unprogrammed Scrambled siRNA was used as controls. (C, D) Smad7-myc was transfected along with *ckm* luciferase. *Renilla* was used as a control reporter to monitor and normalize for transfection efficiency. Lysates were collected at 48 h after changing to

differentiation media (DM) post-transfection. Firefly luciferase activity under each condition was measured independently and normalized to *Renilla* luciferase values. Each condition was compared to the control for the three individually transfected samples to determine fold change. N=3 technical replicates. Each bar represents the mean of three technical replicates. The error bars represent standard error of the mean (SEM). Tukey's Multiple comparisons test in one-way ANOVA using GraphPad Prism 8.0 was utilized to test for statistical significance. Adjusted p-value ***$p \leq 0.001$, ****<0.0001.

4.4.3 TAZ inhibits the activity of a Smad7/β-catenin complex

Since our previous study reported a direct interaction between Smad7 and β-catenin and their functional co-operativity is required for their function on the *ckm* promoter, we determined whether TAZ can directly target β-catenin activity using a more specific assay system. For this we utilized the TOP flash reporter gene that contains multimerized binding sites for TCF/LEF which serves as an indicator of β-catenin mediated transcriptional activation. FOP flash contains mutated TCF/LEF binding sites serving as a corresponding negative control in this assay (331). Ectopic expression of TAZ resulted in robust inhibition of β-catenin activity (Fig 26A) while no appreciable effect of TAZ was observed on the FOP flash reporter gene (Supplementary Fig. S4). Protein level expression of exogenous constructs was confirmed by western blot analysis (Fig 26A, lower panels). These data indicate that TAZ specifically represses β-catenin induced transcriptional activation. Next, we tested whether the TAZ S89A mutation inhibits β-catenin activity similar to wild type, which it did (Fig 26B). The logic of using TAZ S89A was based on its property of evading the cytoplasmic degradation machinery that has previously been shown to modulate the protein levels of both β-catenin and TAZ. These data indicate that the inhibitory effect of TAZ on β-catenin transcriptional activation does not appreciably involve the cytoplasmic

degradation machinery affecting β-catenin levels. This is also supported in the protein blots in which ectopic expression of TAZ did not affect endogenous or exogenously transfected β-catenin levels (Fig 26A). In subsequent reporter gene assays, we tested whether TAZ S89A represses some other well characterized myogenic reporter genes. For this we chose to analyze the *myogenin* promoter (*pMyoG-luc*) and the the *myoD* promoter regions (*pMyoD -2.5kb/-275-luc*). In Fig 26C, we document that TAZ S89A inhibits the endogenous activation of the *MyoG* promoter in myogenic cells. In Fig 26D, the ability of MyoD to activate its own promoter was also repressed by TAZ S89A. Lastly, since TAZ is perhaps better known for its role as a transcriptional co-activator, we tested whether TAZ enhances the well characterized HIP/HOP reporter gene system. HOP is comprised of multimerized TEAD binding sites driving a *luc* reporter gene which serves as a readout for YAP/TAZ activity (HIP is the same reporter gene with mutated TEAD sites serving as a negative control) (329). In this assay we observed that both wild type TAZ and TAZS89A robustly activate the HOP reporter gene under the same conditions under which we documented repression of the 'myogenic' promoters (Fig 26E). Collectively, these data indicate that TAZ potently represses a number of myogenic promoters (*ckm, MyoG, MyoD*) while still retaining its potent co-activator potential at a TEAD responsive reporter gene (*Hip*/*Hop* system).

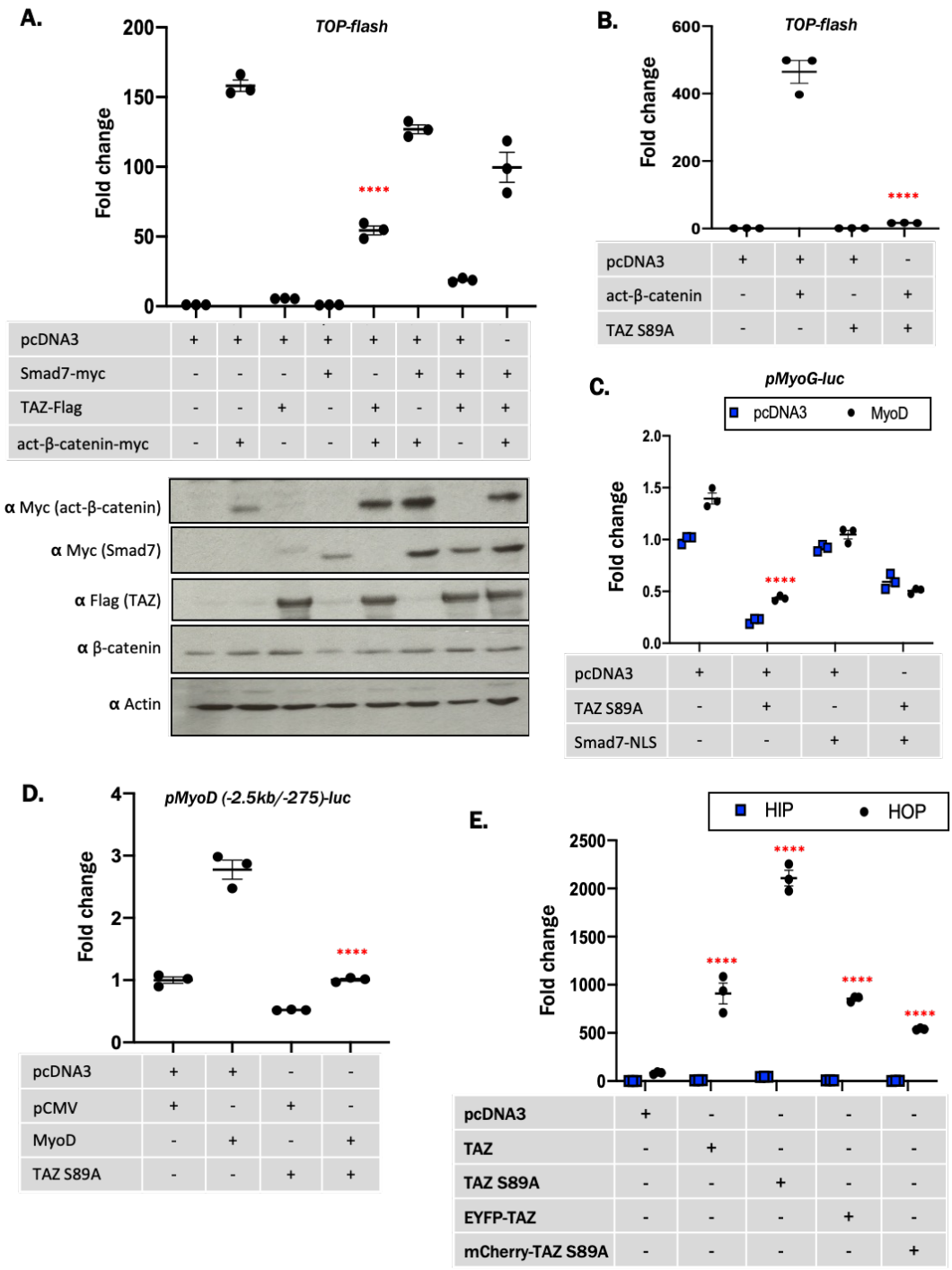


Figure 26: TAZ inhibits β-catenin mediated *trans*activation.
(A) Smad7-myc, TAZ-flag, β-catenin alone or in combination were ectopically expressed along with a TOP flash (FOP flash negative control is in Supplementary Fig 1) luciferase reporter gene. *Renilla* luciferase served as transfection control. Transfection with empty vector (pcDNA) and reporter genes served as controls for ectopic expression. Cells were harvested for Luciferase determination at 16 h post transfection. Normalized luciferase activity was compared to the control to determine fold changes. Lower panels in 3A indicates western blot analysis of the protein levels

derived from transfected plasmids. (B) Indicates the activity of TAZ S89A on β-catenin mediated activation of TOP flash. (C) Displays the activity of TAZ S89A and Smad7, alone or in combination, on the *pMyoG-luc* reporter gene. (D) Shows the effect of TAZ S89A on the *pMyoD (-2.5kb/-275)-Luc* reporter gene. (E) Documents the *trans*activation properties of different TAZ constructs (wt TAZ, TAZ S89A, EYFP-ΔTAZ, EYFP-TAZ, mCherry-ΔTAZ, and mCherry TAZ S89A on the HIP/HOP (HOP is driven by multimerized TEAD binding sites while HIP is the same reporter with mutated TEAD binding sites serving as a negative control) reporter gene system (see methods for further details). N=3 technical replicates. Each bar represents mean of three technical replicates. The error bars represent standard error of the mean (SEM). Tukey's Multiple comparisons test in one-way ANOVA using GraphPad Prism 8.0 was utilized to test for statistical significance. Adjusted p-value ****<0.0001.

4.4.4 Differential TAZ/YAP phosphorylation during skeletal muscle differentiation

Cell-cell contact has been identified as a regulator of Hippo signaling. Activation of Hippo signaling at high cell densities results in a well characterized phosphorylation and cytoplasmic sequestration of TAZ at Ser 89, promoting its binding to 14-3-3 protein in the cytoplasm (111). Therefore, we were interested to determine the levels of endogenous and also Ser 89 phosphorylated TAZ during myogenic differentiation. Time course analysis of total protein lysates from differentiating cells indicate that TAZ levels remain relatively unchanged during differentiation although TAZ is much more abundant than YAP in muscle cells. Importantly, hyperphosphorylation of TAZ was pronounced when the cells were differentiating suggesting activation of Hippo signaling in differentiation conditions (Fig 27A and B). Immunofluorescence analysis of TAZ and phospho-TAZ also indicates that TAZ localization shifts from being predominantly nuclear during the myoblast stage (low cell density) to a more diffuse cytoplasmic localization in differentiating myotubes (high cell density), although some TAZ is still retained in the nucleus (Fig 27C). Phosphorylated TAZ is primarily localized in the cytoplasm in differentiated myotubes (Fig 27D). Taken together these observations are consistent with a model in which the nuclear function of TAZ predominates in proliferating myoblasts. However, under differentiation conditions, activation of Hippo signaling results in TAZ Ser 89 phosphorylation

and increased retention in the cytoplasm. Based on the repressive function of TAZ at the *ckm* gene that we have documented here, we propose that TAZ represses myogenic transcription complexes under proliferative conditions and activation of Hippo signaling during differentiation relieves this repression by the phospho-dependent sequestration of TAZ in the cytoplasm. Analysis of proliferating C2C12 cells by immunofluorescence suggested that TAZ forms a pattern of speckled localization that can be indicative of phase separation properties (Fig 27E). These initial observations of the localization of endogenous TAZ in muscle cells led us to speculate that TAZ might form phase condensates and we therefore subsequently proceeded to test this possibility.

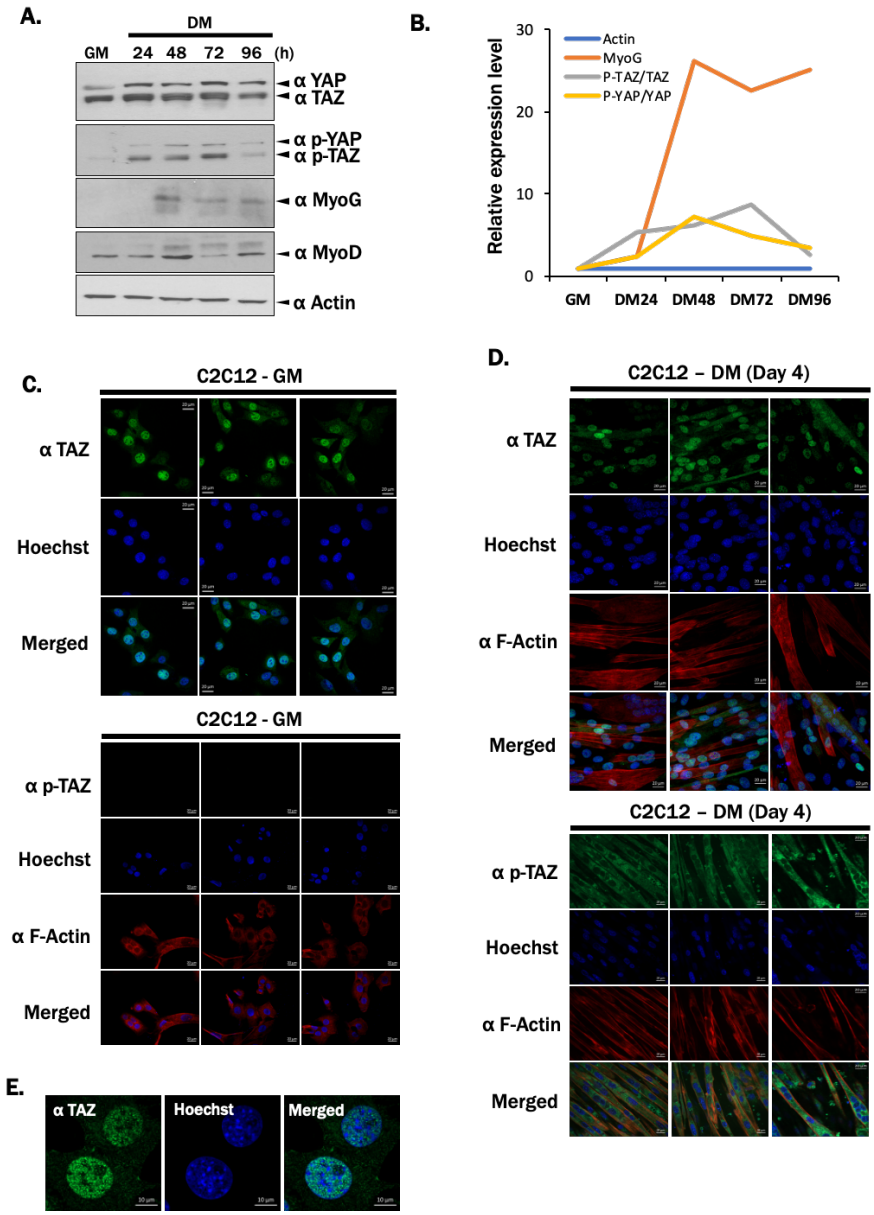


Figure 27: TAZ is differentially phosphorylated and localized in proliferative and differentiating myogenic cells.
(**A**) C2C12 myoblasts were cultured in growth media (GM) for 24 h, followed by differentiation media (DM) for 24 to 96 h. Lysates were collected and assessed for expression of TAZ, phospho-TAZ and muscle markers by Western blot analysis. (B) The relative expression level of indicated proteins was estimated by measuring the band intensity corresponding to the protein captured on the film using ImageJ. Relative intensity of the bands was initially standardized to the Actin loading control at each time point. To establish the relative level of phosphorylation, the ratio of the phosphorylated to total protein was calculated and plotted. (C and D) Immunofluorescence analysis of endogenous TAZ and phospho-TAZ during myoblasts (GM) and myotube (DM day 4) conditions. Staining with Hoechst and F-Actin indicate nuclei and myotubes respectively. (E) At higher resolution, TAZ exhibits a nuclear speckle pattern in GM conditions.

4.4.5 TAZ exhibits robust phase separation properties in muscle cells

Liquid-liquid phase separation of proteins (LLPS) and nucleic acids into condensates (also referred to as nuclear speckles, nuclear bodies or biomolecular condensates) is invoked by weak multivalent interactions between molecules (332,333). Phase separation has recently been implicated in heterochromatin domain formation (334,335), transcriptional control through co-activator condensation (336,337) and the clustering of nuclear enhancer elements and super-enhancers in gene control (338). Our observations indicate that TAZ displays a number of properties associated with phase separation that may underlie its diverse activities in both activation and repression of gene regulation.

As observed in fluorescence live-cell imaging analysis, ectopically expressed EYFP-TAZ wild type (WT) formed a similar localization pattern of spherical structures in the nucleus (Fig 28A and Supplementary Video Files 1 and 2). Ectopic EYFP tagged TAZ expression therefore allowed us to perform more detailed and higher resolution analysis of the TAZ nuclear speckles. EYFP-TAZ (WT) formed dynamic spherical sub-cellular structures. We further observed that TAZ condensates were in some cases juxtaposed with heterochromatin with projections extending into the heterochromatin domain (Fig 28A and Supplementary video file 1). Conversely, we also noted patterns of localization within the same nuclei in which the TAZ condensates are not associated with heterochromatin. These differential associations may therefore reflect variant TAZ containing complexes involved in gene activation or repression. Furthermore, fusion of the spherical structures was observed in time-lapse live cell imaging analysis (Fig 28B and Supplementary video file 2). These observations of the robust capability of TAZ to form condensates was recently reported under *in vitro* conditions and also in cultured MCF-10A cells (339). In addition, YAP has also been shown to exhibit LLPS (340). Collectively, these features of TAZ localization and

dynamic assembly into condensates are consistent with liquid-liquid phase separation properties recently reported for YAP/TAZ and other nuclear regulatory proteins (336-338,341).

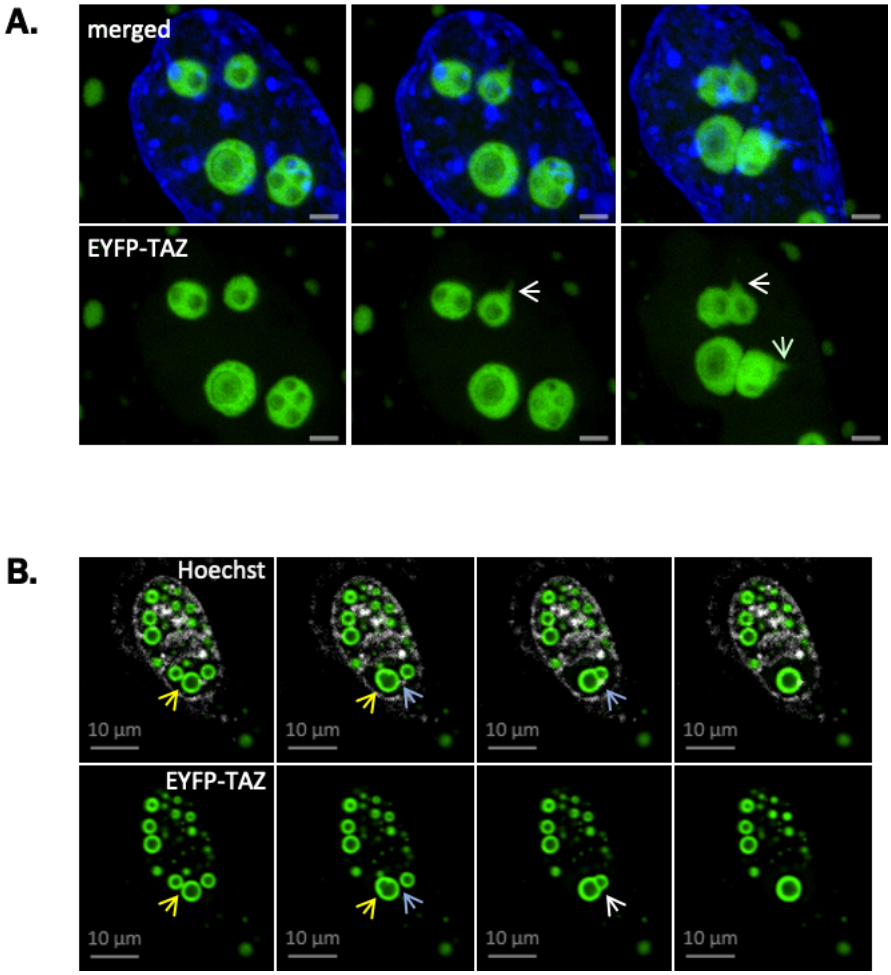


Figure 28: TAZ exhibits robust phase separation properties in myogenic cells.
(A) Live cell spinning disk confocal imaging of ectopically expressed EYFP-TAZ showing spherical condensate structures (B) indicates fusion properties of EYFP-TAZ condensates. Time-lapse demonstration of nuclear condensate formation and fusion of EYFP-TAZ by live-cell imaging (see Supplementary Video Files 1 and 2).

4.5 Discussion

The Hippo pathway has garnered widespread attention for its role in both physiological and pathological conditions in a variety of tissues. It has now been implicated in organ growth and development, stem cell biology, regeneration, and tumorigenesis (118,342,343). The Hippo pathway is composed of a core kinase module and a transcriptional regulatory complex comprised of TAZ/YAP as the main effectors. To date, many cellular studies have documented how TAZ/YAP phosphorylation and nuclear localization are controlled. More recently, there have been efforts to reveal regulatory mechanisms governing how YAP/TAZ can exert selective functions on the transcriptional apparatus assembled at different *cis*-regulatory elements in the genome (344). In this study we document how nuclear TAZ inhibits myogenic differentiation by repressing components of a well characterized muscle-specific transcription complex.

In particular, we characterise components of a pro-myogenic protein complex comprised of Smad7 and β-catenin that is antagonized by TAZ protein interactions, thus serving to supress transcriptional activation of muscle specific genes, maintaining the cells in an undifferentiated, proliferative state. These data support a model for nuclear TAZ function in suppressing the myogenic differentiation program by targeting a key transcriptional regulatory complex assembled on muscle promoters. Phospho-dependent, cytoplasmic sequestration of TAZ by activation of Hippo kinases relieves this repression, providing a potential switch to allow the myogenic differentiation program to proceed. Interestingly, as reported by others recently, we observe TAZ in myonuclear speckles consistent with key features of phase-separated condensates, including high sphericity and fusion behavior (Fig 28B. and Supplementary Video file 2). These studies outline a functional reciprocity between myogenic differentiation and Hippo signaling that may

have important implications for our understanding of the co-ordination of cellular proliferation and differentiation in skeletal muscle growth control.

While YAP and TAZ are often considered functionally redundant, there are structural and functional observations indicating that they might also have non-overlapping roles (345). Despite their high sequence similarity, there are nevertheless some potentially important differences in their structure. Notably, while both contain WW domains, YAP harbors two tandem WW domains while TAZ has one. Also, YAP contains an SH3- binding which is not present in TAZ. Of some interest for our study, in view of the TAZ connection with β-catenin, is the reported observation that GSK3β, a well characterized regulator of β-catenin, can directly phosphorylate TAZ affecting its stability. Several reports in different model systems indicate that TAZ cannot compensate for YAP physiologically, supporting the idea that, while they are both targets of the Hippo kinase pathway, they also retain distinct roles (346-348).

Given the structural and physiological differences between YAP and TAZ it is possible that their interactions with transcriptional complexes may differ. This is particularly highlighted in recent studies documenting differential properties of YAP and TAZ in promoting the formation of liquid-liquid phase separations with different factors being sequestered within the condensates. It is now becoming increasingly more apparent that the sub-cellular assemblage of membrane-less compartments through liquid–liquid phase separation (LLPS) is proving to be an essential feature of temporal and spatial control of various biomolecular processes (336,337,340,341,349,350). Here, we confirm and extend these observations in reporting that TAZ forms liquid-liquid phase like condensates in cultured muscle cells. Moreover, hyperosmotic stress promotes the rapid

assembly and fusion of spherical TAZ condensates leading to the compartmentalization of TAZ and its interacting partners. Our observations confirm a recent report documenting that TAZ forms liquid-like condensates that concentrate transcriptional regulatory proteins such as co-activators and the elongation machinery (351). One of the primary cellular functions of Hippo signaling is to control tissue growth by regulating the proliferative expansion of progenitor pools. Based on our data, we speculate that TAZ nuclear condensates sequester components of the myogenic nuclear machinery involved in cellular differentiation. Thus, TAZ mediated 'hijacking' and repression of the pro-differentiation machinery may be deemed complimentary to its pro-proliferative activities underlying growth.

The best characterized partners for YAP and TAZ in transcriptional control are the TEAD1-4 factors although to date the possibility of differential partners for YAP and TAZ has not been exhaustively tested. Of particular relevance for the discussion of muscle transcription complexes is the previously reported interaction of TEAD factors with the MEF2 proteins that are core regulators of the muscle transcription program (352,353). It is perhaps coincidental but noted that p38MAPK is a potent and well characterized regulator of MEF2 function and it was recently documented that TEAD function is also targeted by p38 MAPK (354). Moreover, our recent studies have documented an interaction between MEF2 and β-catenin that is p38MAPK dependent (279) and a direct physical interaction between β-catenin and Smad7 (311) in a muscle context. Thus, the TAZ/ Smad7 interaction reported here may intersect with these protein interactions. The schematic in Fig 29 documents a working model of these interactions. Collectively, these observations support a model in which a core muscle transcriptional regulatory complex may be targeted by ancillary regulatory subunits, such as TAZ or β-catenin/Smad7, to repress or enhance

its activity, respectively, depending on the cellular conditions. Collectively, these observations place TAZ in the cellular context of muscle specific gene regulation. It will be of considerable interest to further examine the physiologic role of TAZ in post-natal muscle growth in health and disease.

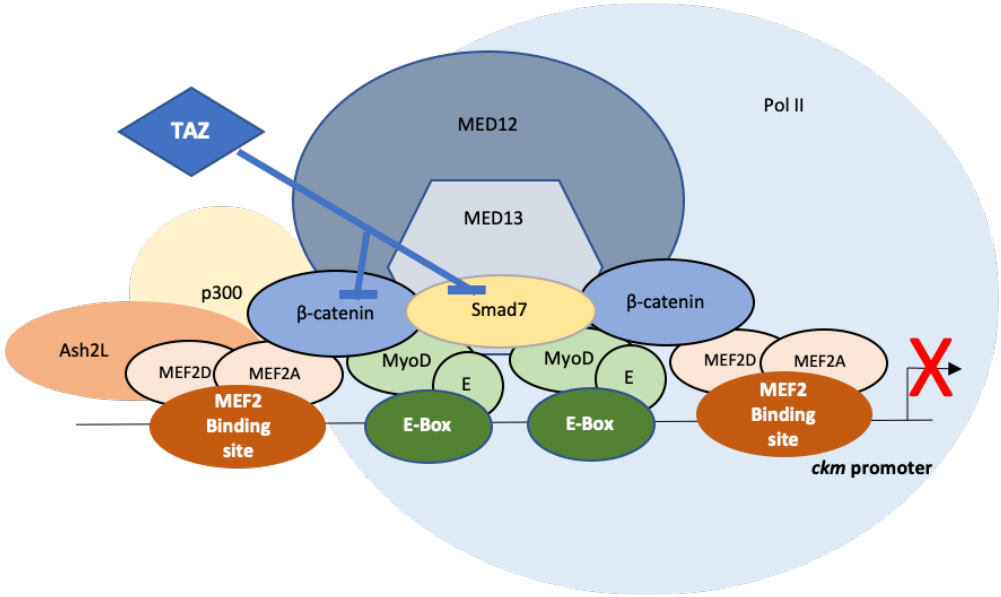


Figure 29: A schematic view of a myogenic 'holocomplex' and the novel role of TAZ.
Based on many previous reports in the literature and our own publications, this schematic document the assembly of a generic myogenic 'holocomplex' on muscle specific promoters. We have added TAZ as an accessory factor to this schematic to indicate its potential role, based on data included herein.

Chapter 5

Summary, Limitations and Future Directions

Smad7 was originally characterized as an intracellular antagonist of transforming growth factor-β signalling having diverse effects in many tissues in both health and disease (184,226). Previous studies from our group have extended these observations by documenting a pivotal role played by Smad7 in regulating muscle cell differentiation (230). Moreover, this work documented a previously unrecognized role of Smad7 in the nucleus, independent of its role in antagonizing TGF-β signaling at the receptor through cytoplasmic recruitment of a proteolytic degradation complex (232). *In vivo* studies from another group confirmed our cell culture based observations on the role of Smad7, reporting that homozygous null mutation of murine Smad7 was associated with decreased muscle mass, force generation and fibre type switching and delayed recovery from injury (259). Interestingly, muscle-directed Smad7 gene delivery also prevented muscle wasting in mice (187). In view of the seemingly critical role of Smad7 in a muscle context, the work described herein aimed to further characterize the molecular mechanisms underlying Smad7 function in skeletal myogenic cells.

Chapter 2 contains the first major study identifying a physical interaction between Smad7 and β-catenin in skeletal muscle cells (311). β-catenin is a multifunctional protein and the key effector of the canonical Wnt signaling pathway. While interaction of Smad7 and β-catenin had been reported earlier in prostate cancer cells (1), the role of this interaction in a muscle context was

unknown. Our study demonstrates a direct interaction of Smad7 and β-catenin and successfully identified 575-683 a.a region of β-catenin as the region that interacts with Smad7, a region we have termed the Smad7 interaction domain (SID). The identification of SID may provide further insights on the specific function of these interacting proteins not only in a muscle context but also in other cell types as both Smad7 and β-catenin are implicated in many other physiological functions. We utilized the *ckm* promoter (a muscle-specific promoter that has been used as a paradigm for muscle specific transcriptional control for several decades) as a primary readout to ascertain the role of this protein:protein interaction in transcriptional control in cultured muscle cells. Smad7 and β-catenin exerted co-operative effects on the *ckm* promoter and depletion of either endogenous Smad7 or β-catenin resulted in a pronounced reduction *ckm* promoter activity, thereby indicating their co-dependent role during myogenesis. In addition, we reported that deletion of the SID region of β-catenin greatly reduced the effect of Smad7 on the *ckm* promoter further suggesting that the interaction of Smad7 and β-catenin is important for muscle specific gene regulation. Subsequently, we determined that ectopic expression of SID functioned as a *trans*-dominant negative of β-catenin function on the *ckm* and also TOP flash (β-catenin dependent) reporter genes. These data indicate great promise for SID as a potential inhibitor of Wnt dependent transcriptional control. Moreover, this could have far reaching implications in therapeutic approaches to various Wnt dependent cancers since, so far, our data support the idea that SID can inhibit canonical Wnt signaling. Whether this will hold in Wnt dependent cancer cells remains to be tested.

Further investigation of the protein complex containing Smad7 and β-catenin revealed a novel interaction of MED 13 with Smad7 and interestingly, perturbation of β-catenin resulted in the loss

of this interaction. Although β-catenin's association with the Mediator complex subunit (MED) 12 interaction had been previously reported (2), our study indicates a similar role for Smad7 in the recruitment of MED13. This study therefore highlights the possibility that the Smad7-β-catenin interaction is perhaps required for the recruitment of the Mediator complex to the basal transcriptional machinery.

A follow up to the first study was aimed at more completely understanding the protein interactome of β-catenin and SID in skeletal muscle cells which is summarized in Chapter 3. Mass Spectrometry analysis identified the protein interactome of β-catenin and SID in growth and differentiation conditions in muscle cells. One prominent feature of this study was the retrieval of so many of the previously well characterized components of the β-catenin interactome. Amongst these were members of the membrane associated cadherin complex (Cadherin 2 and 15), the cytoplasmic APC complex, GSK-3β, Fragile X mental retardation syndrome related protein-2 (FMRP-2), β-catenin interacting protein 1 (BCIP-1). These corroborative data give us considerable confidence that the previously unknown interactors that we have identified in our study will prove to be *bona fide* regulators of β-catenin function. Consecutive GO ontology analysis characterized the protein interactors based on their associated cellular component domain and biological process. Further characterization of the unique protein interacting partners may allow further molecular dissection of β-catenin specific cellular functions.

Chapter 4 summarizes the third study, which was aimed at understanding the role of a TAZ-Smad7 interaction in skeletal muscle. The rationale for this study was based on previous documentation of cross talk between TGF-β and Hippo signaling in which YAP65 (the paralog of TAZ) was

shown to interact with Smad7 (97). In view of the importance of Smad7 in muscle cells and a preliminary observation that TAZ is robustly expressed in muscle cells, we investigated whether TAZ interacts with Smad7. In these studies, we identified a previously uncharacterized interaction between Smad7 and TAZ in skeletal muscle cells. Importantly, TAZ was shown to repress the function of the Smad7-β-catenin interaction in transcriptional control. Previous studies have indicated cell density as one of the factors involved in the regulation of Hippo signaling and TAZ phosphorylation (133). One compelling observation in these experiments was that phosphorylation of TAZ, a hallmark of Hippo signaling, is activated in differentiation conditions (high cell density) suggesting that TAZ is sequestered in the cytoplasm in differentiating cells. This is consistent with our idea that TAZ represses the differentiation machinery through its interaction with Smad7-β-catenin and that this repression is relieved by its phosphorylation dependent cytoplasmic sequestration. Our interpretation of these data is that there is a connection between Hippo signaling and the control of muscle cell differentiation in which the nuclear function of TAZ is operative and repressive to differentiation under proliferative conditions. When differentiation signals are prevalent at high density, the activation of Hippo kinases results in the cytoplasmic accumulation of TAZ and relief of the repression on the muscle transcription machinery. Interestingly, in confocal imaging experiments we observed TAZ localization in nuclear speckles often associated with heterochromatin. The properties of these speckles are consistent with the features of liquid-liquid phase separation that has recently come to the forefront of cell biology. The role of condensate formation in TAZ function in muscle cells will therefore be an interesting avenue of future research in this area. The schematic (Fig 29) attempts to summarize the main findings documented in this thesis in the context of the known regulators of muscle-specific gene regulation and, speculatively, the pathways that converge on these regulators.

Finally, as with all science, it is important to consider the limitations of these studies. In the early phase of these studies there was no reliable commercially available Smad7 antibody and, after trying many commercial antibodies that were not efficacious, we set about making our own Smad7 polyclonal antibody which took considerable time and labour. Because of this we were restricted in being able to visualize the endogenous Smad7 in much of our early experimentation. Eventually we succeeded in making a moderately useful Smad7 antibody that was then used in some later experiments.

One other consideration is that the majority of the experiments carried out in this work have utilized a cell culture model which is, by nature, removed from the *in vivo* context. Therefore, one has to be conservative about making claims that observations made in cell culture will necessarily translate to the *in vivo* situation. It will therefore be important in future to further explore the observations we have reported in an *in vivo* context, possibly using mouse or, even more appropriately, large animal models. Moreover, there are a variety of physiological contexts in which the mechanisms we have described might play a role that are, as yet, undetermined. For example, the Smad7-β-catenin or the Smad7-TAZ complexes could play a role in embryologic muscle development or in post-natal muscle growth or repair from injury. These possibilities all remain to be fully explored based on the fundamental observations reported here.

On a technical note, with regard to the β-catenin interactome study, while we successfully detected several known β-catenin interacting proteins, we realized after carrying out the screen that there was a preponderance of β-catenin interactors that are localized to the cell membrane adhesion complex and the cytoplasm but nuclear interacting proteins were not highly represented in the

dataset. This led us to realize that, since we are primarily interested in the nuclear co-operativity with Smad7, that it would have been more efficient to use nuclear extracts rather than whole cell extracts in the affinity purification phase of the experiment to achieve this experimental goal. While we have uncovered some very interesting β-catenin interactors, it would nevertheless be worthwhile to repeat this study using nuclear lysate to more efficiently identify the nuclear β-catenin interactome.

Lastly, at this point we have made a very preliminary observations that TAZ exhibits features of liquid-liquid phase separations. However, the functional role of this observation it not apparent at this point. Since this phenomenological observation was made towards the end of the studies in this thesis, while of great interest, we have not made further progress in understanding the molecular function of these condensates for TAZ biology in muscle cells.

In summary, we have characterized Smad7 protein interactions with β-catenin and TAZ and their transcriptional function in skeletal myogenesis. To reconcile these observations with previous studies in the literature, we propose a revised model depicted below of the myogenic transcription complex based on the data documented herein (Fig 30). Future work will be required to determine the physiological relevance of these protein interactions in a variety of muscle contexts such as during development and in post-natal growth and muscle regeneration after injury. In addition, it will be of interest to examine potential therapeutic opportunities for Smad7 in counteracting muscle wasting in a variety of pathological myopathies. In considering the Smad7 anchored protein interactions in a wider context, these studies may have potential implications in some cancers in which Smad7 and β-catenin are important players. It is therefore anticipated that Smad7

will continue to be an intriguing molecule that will be vigorously investigated to strengthen our understanding of its role in basic biological mechanisms as well as the pathogenesis of a number of human diseases.

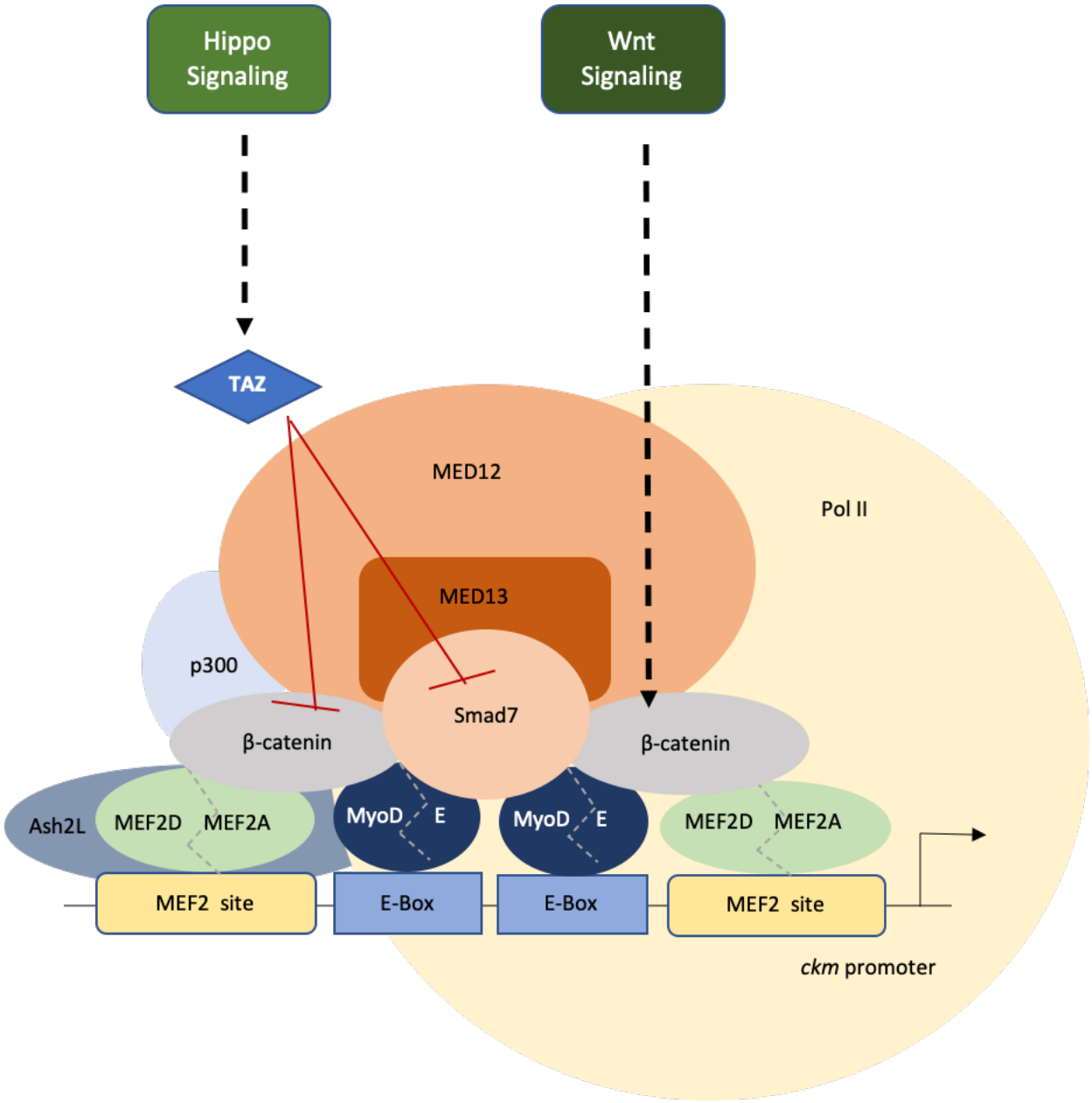


Figure 30: Proposed model indicating the role of Smad7, β-catenin and TAZ in the myogenic transcription complex.

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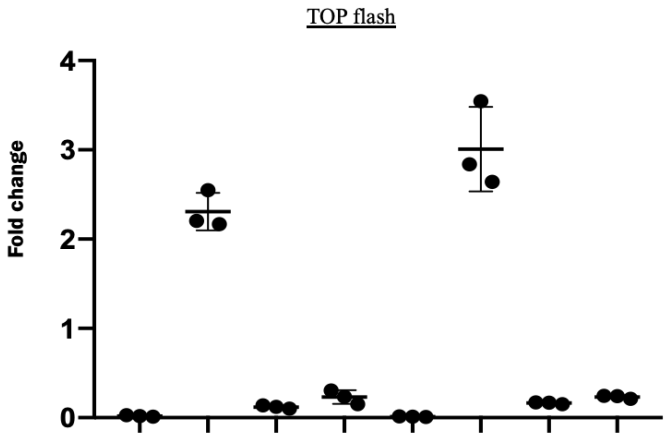
Appendix A: Supplementary Figures



pcDNA	+	-	-	-	-	-	-	-
β-catenin-FL(full-length)-myc	-	+	-	-	-	+	-	-
β-catenin Δ SID	-	-	+	-	-	-	+	-
β-catenin 1-574	-	-	-	+	-	-	-	+
Smad7-myc	-	-	-	-	+	+	+	+

Figure S1. TOP flash reporter assay

Figure S1. Combinations of β-catenin-FL(full-length)-myc, β-catenin Δ SID, β-catenin 1-574, Smad7-myc were ectopically expressed in C2C12 cells along with a TOP flash luciferase reporter gene. Renilla luciferase served as transfection control. C2C12 cells transfected with empty vector (pcDNA) and reporter genes served as controls for ectopic expression. Cells were harvested for Luciferase determination at 12 h after changing to fresh growth media post transfection. Normalized luciferase activity was compared to the control to determine fold changes.

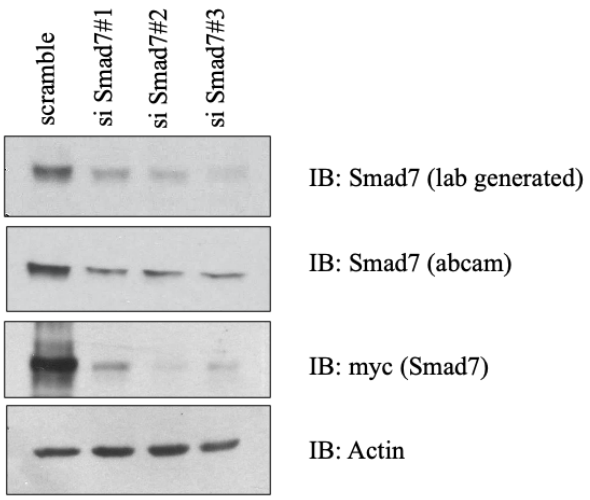


Figure S2. Smad7 depletion by siRNA

Figure S2. Three siRNAs specific for Smad7 were used to deplete the endogenous Smad7 levels in C2C12. Unprogrammed Scrambled siRNA was used as controls. Lysates were collected at 48 h post transfection and immunoblotted with the designated Smad7 antibodies from two different sources (one generated in house, abcam). Lysates were further immunoblotted for myc (Smad7-myc) and actin.

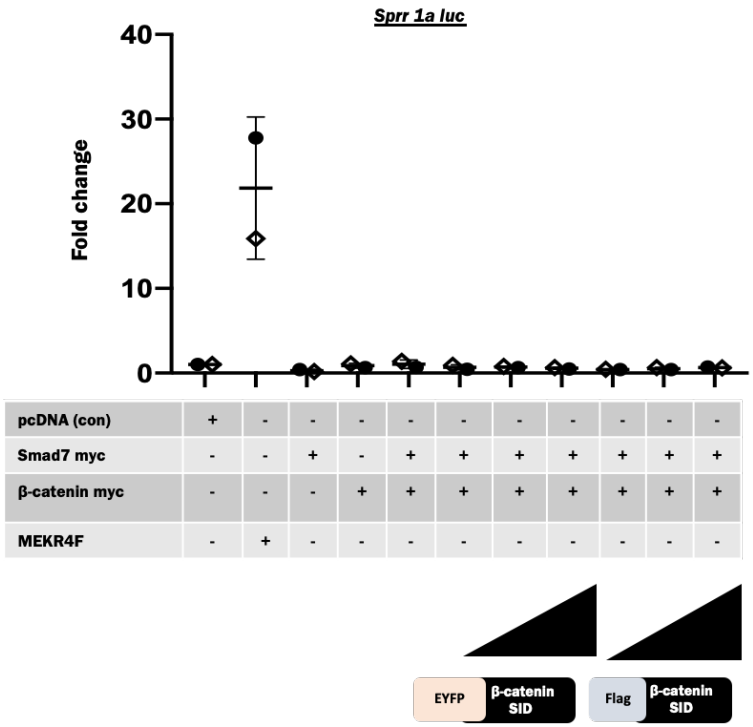


Figure S3. ***sprr1a*** **promoter-luciferase reporter assay**

Figure S3. A previously characterized *sprr1a promoter*-luciferase reporter gene that is not regulated by the myogenic regulators served as a control promoter for general transcriptional effects since it lacks defined MEF2 or MyoD binding sites. Activated MEK (MEK R4F) was used as a positive control for *sprr1a* as previously characterized. Lysates were collected at 48 h after changing to differentiation media (DM) post-transfection. Each condition was compared to the control for the three individually transfected samples to determine fold change. Each dot represents one biological replicate, which corresponds to the mean of 3 technical replicates. N= 2 biological replicates per condition. The error bars represent standard error of the mean (SEM).

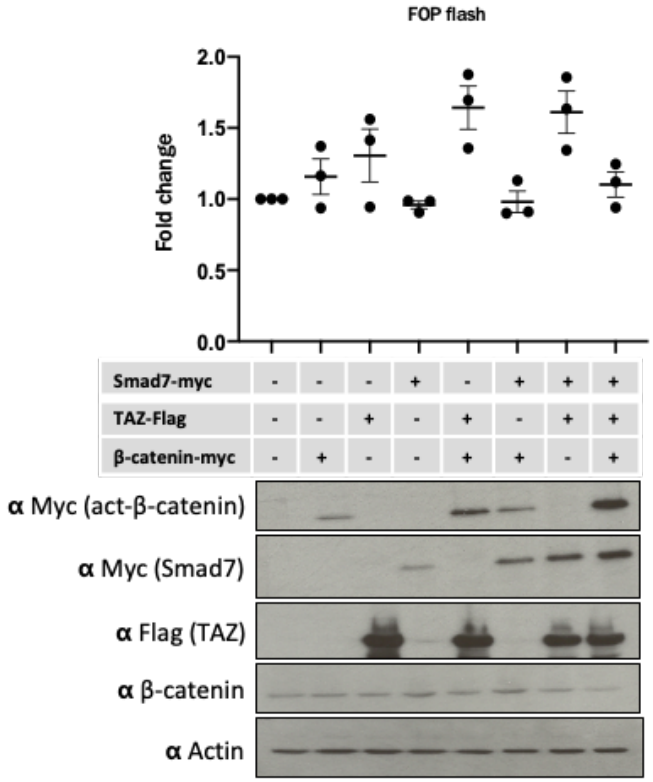
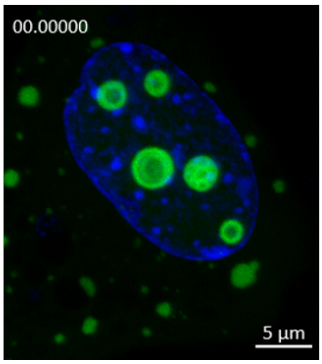
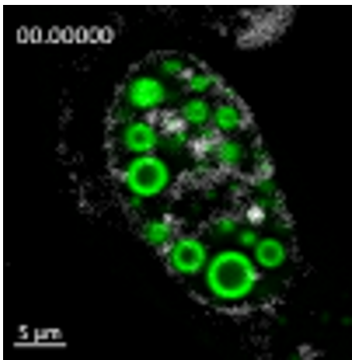


Figure S4: Smad7-myc, TAZ-flag, β-catenin alone or in combination were ectopically expressed in C3H10T1/2 cells along with a FOP flash luciferase reporter gene. *Renilla* luciferase served as transfection control. Transfection with empty vector (pcDNA) and reporter genes served as controls for ectopic expression. FOP flash is the negative control plasmid for TOP flash in which the TCF/LEF binding sites are mutated. Cells were harvested for Luciferase determination at 16 h after post transfection. Normalized luciferase activity was compared to the control to determine fold changes. N=3 technical replicates. Each bar represents mean of three replicates. The error bars represent standard error of the mean (SEM). Western blot analysis showed the protein levels of the represented proteins.

Supplementary Video file 1



Supplementary Video file 2



Supplementary Video Files 1 and 2

Time-lapse demonstration of EYFP-TAZ nuclear condensate formation and fusion of EYFP-TAZ condensates by live-cell imaging. Hoechst 33342 was used for DNA staining and stained DNA was shown in blue (1) and in gray (2) respectively. Video files were generated from the time-lapse images using Zen Blue software (Zeiss).

Dropbox link for video files-

Video file 1: https://www.dropbox.com/s/cnu9k5ubupel66b/A30-e370-Untitled8-Orthogonal%20Projection-04%20adj.avi?dl=0

Video file 2: https://www.dropbox.com/s/donmyi83p5ittko/a17-e321-Untitled5%20adj.avi?dl=0

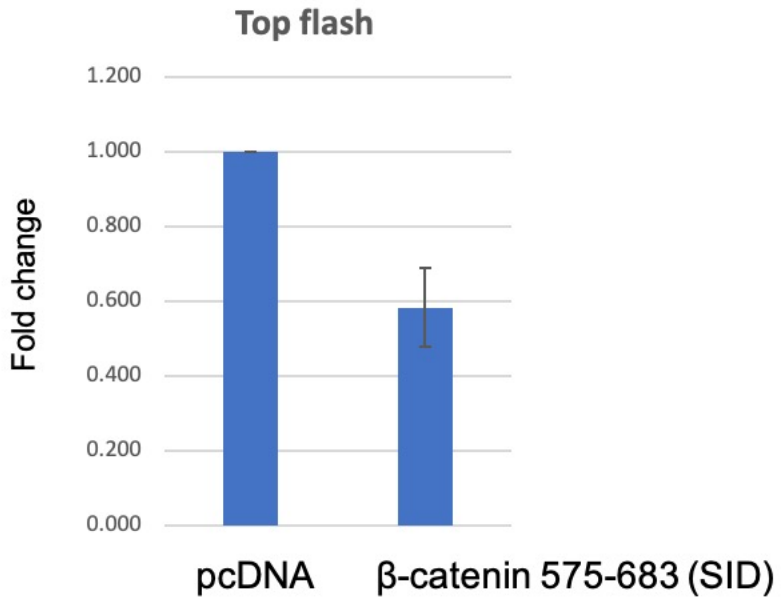


Figure S5: β-catenin 575-683 (SID) was ectopically expressed in C3H10T1/2 cells along with a TOP flash luciferase reporter gene. Transfection with empty vector (pcDNA) was used as control. *Renilla* was used as transfection control. Cells were harvested at 12 h post change to fresh media after transfection. Normalized luciferase activity was compared to the control to determine fold change. N=3 technical replicates. Each bar represents mean of three replicates. The error bars represent standard error of the mean (SEM).

Appendix B: Expanded selected materials and methods

Cell culture:

The following cell lines were utilized in the above studies, C2C12 myoblasts, C3H10T1/2, and primary myoblast cultures.

Reagents:

- DMEM supplemented with Penicillin-Streptomycin (Gibco) and L-glutamine (Gibco) added as required

  Growth Medium: 10% fetal bovine serum (FBS)

  Differentiation Medium: 5% horse serum (HS)

  Freezing Medium: Growth media supplemented with 10% DMSO

- 0.125% Trypsin-EDTA (Gibco) diluted in PBS.

- 1X PBS

Cell Passaging:

Discard cell culture media from the cell culture Petri dish.

Rinse the cell monolayer twice with 10 ml of 1xPBS.

Add warm 0.125% Trypsin-EDTA solution to the cells. (1.0ml to 100mm dish or 0.5ml to 60mm dish), incubate at 37°C for 1-2min.

Add 6.0 ml of DMEM to inactivate Trypsin.

Gently Pipette the cells up and down several times to ensure complete removal of the cells from the dish.

Centrifuge at 1500 g for 5 minutes. Remove media and re-suspend pellet in 5mL of media
161

Count the cells in a haemocytometer (optional) and seed a dilution of cells as per the experiment.

Differentiation:

Replace the growth medium with differentiation media when the cells (C2C12) are 60-80% confluent. Replace every 48 h.

Freezing Cells:

Prepare a cell suspension and pellet the cells by centrifugation at 1500 g.

Resuspend the cells in freezing medium (10%DMSO and 90%DMEM) at a concentration of $1x10^6$ -$8x10^6$ cells/ml

Dispense 1 ml of cell suspension into each freezing vial.

Place vials into polystyrene a box in -80 °C freezer and freeze overnight.

Remove vials from polystyrene box and place in -80 °C freezer for short-term storage, or for long term storage place vials in liquid nitrogen.

Thawing Frozen Cells:

Remove vial from the liquid-nitrogen freezer and thaw in 37 °C water bath.

Resuspend in 5 ml of Growth media. Centrifuge for 5 min at 1500 g; aspirate the supernatant.

Suspend cells in 10ml of growth medium.

Plate cells in 100 mm culture dish.

Luciferase Assay:

Luciferase assays were performed with commercially purchased substrate (Promega).

Reagents:

- Luciferase Lysis buffer: (20 mM Tris, pH 7.4, 0.1% Triton-X 100 in 1xPBS)
- Firefly and Renilla Luciferase substrate (Promega)
- 1XPBS

Protocol:

Wash adherent cells thrice with cold 1x PBS.

Add 400 µL of Luciferase lysis buffer per well of a 6 well culture dish

Incubate for 5 min at room temperature.

Scrape cells off with rubber policeman, collect into labelled tubes, centrifuge at 10,000 rpm for 5min at 4^0C.

Transfer supernatant to new tube. Analyze immediately or and store at -80^0C until analysis.

For analysis, add 50µl of the sample to Luciferase assay tube. Measure readings using Lambat (LB 958) luminometer.

Western Immunoblot analysis:

Reagents:

- 1X cold PBS
- NP-40 Lysis buffer
- 1 mM Sodium vanadate
- 1 mM PMSF (add fresh)
- Protease inhibitor cocktail (add fresh, Sigma, P-8340)
- 3X SDS sample buffer (Biorad)
- β-mercaptoethanol
- 10% Resolving gel (ddH_2O, 1.5M Tris pH8.8, 30% acrylamide, 10% SDS, 10% APS, TEMED)
- Stacking gel (ddH_2O, 1.0M Tris pH6.8, 30% acrylamide, 10% SDS, 10% APS, TEMED)
- 1X Laemmli buffer
- 1XTransfer buffer (100ml) (methanol, glycine, Tris Base)

- 1X TBST (1XTBS buffer+0.1%Tween-20)
- Methanol
- 1xTBS
- Primary and Secondary antibody

Whole Cell Extracts:

The entire protocol is performed on ice.

Remove media from cells and wash thrice with cold 1XPBS.

Add 350 μl cold NP-40 lysis buffer. Incubate for 1 min and gently scrape cells with rubber policeman.

Transfer the lysate to a 1.5ml centrifuge tube new tube.

Vortex cells briefly every 10 min for 40 min.

Centrifuge cell lysate at high speed (>10 000xg), transfer supernatant to new tube.

Determine protein concentration by Bradford assay.

Dilute protein samples with NP-40 lysis buffer to final concentration of 30μg. Add 3X SDS sample buffer (with 1X β-mercaptoethanol) and adjust the volume to 20μl.

Boil samples at 100^{0}C for 5 min and run on SDS-PAGE.

Prepare the Bio-Rad gel apparatus with resolving and stacking gel. Fill with 1X Laemmli buffer.

Load samples on gel. Run gel at 100 V.

Transfer protein to PVDF membrane by wet transfer at 100V for 1h.

Block membranes with 5 % (w/v) skim milk powder in 1XTBST.

Incubate with Primary antibody followed by secondary antibody incubation.

Develop blot with chemiluminescence reagent.

GST protein purification

Reagents:

- PBS+

  1XPBS with 1Xprotease inhibitors + PMSF + Na2VO3

- 100mM IPTG

- 10% Triton X

- STE (Sodium chloride Tris EDTA) buffer

  NaCl (100mM), Tris/HCl (10mM), EDTA (1mM)
  Adjust the pH to 8.

- Elution buffer

  Reduced glutathione (20mM), 1M tris-Hcl (pH-8.0) (20mM), 5M NaCl (120mM)

- Storage buffer
  1M tris-HCl (pH-8.0)(20mM), 5M NaCl (120mM)

- 50% glycerol

Preparation of GST beads:

Weigh 50mg of agarose GST beads (Sigma G-4510).

Add 5ml of dH_20, mix gently and incubate overnight at 4^0C.

Wash the beads thrice with 1ml dH_20 to remove lactose. Re-suspend them in 1ml of PBS+. This is 50%slurry.

Protocol

Wash pellet with 10mls of STE. Centrifuge at max speed (7000rpm for the 50ml tube rotor), 4^0 C for 5min.

Discard the supernatant.

Dissolve the pellet in 5ml of PBS+ (vortex can be used)

Sonicate- Keep it on ice. Use the remote setting, speed 5-6, 15sec (max wattage 10-15). Repeat 3 times.

Aliquot into 1ml tubes. (i.e 1ml in each tube, for 5ml-5tubes).

Add 100ul of 10%Triton-X-100 in each 1ml of bacterial lysate. Gently mix and incubate on ice for 5-10 min.

Centrifuge at max speed, 4^0 C for 5min.

Transfer supernatants to fresh vials. Add 110μl of 50% slurry to each 1ml of supernatant.

Rotate at 4^0 C for 2 h.

Spin down at 3000g for 2min and remove the supernatant.

Re-suspend the beads in 250μl of PBS+. Centrifuge at 3000g for 2min.

Repeat 3 washes with 1ml of PBS+.

Re-suspend the beads in 350 μl of elution buffer. Incubate on ice for 5min.

Centrifuge at 10,000 rpm for 2 mins at 4^0 C.

Transfer supernatant to a fresh vial.

Aliquot 10μl of elution to a fresh vial. Add 10μl of Bradford reagent and 200μl of ddH_20. The mix should turn blue.

Repeat the elution process. Add 250μl of elution buffer to the beads. Incubate on ice for 5min. Centrifuge at 10,000 rpm for 2 mins at 4^0 C.

Combine it to the first elute vial. Repeat the Bradford detection.

Immunofluorescence:

Reagents:

- 1XPBS
- 4%PFA
- 90%methanol
- 5%FBS

Protocol:

Wash cells with 1X cold PBS 3 times.

Add cold 4% PFA (150μl for glass-bottom-dish).

Incubate for 5 min on ice and then 10 min on bench (@room temp).

Wash with 1X ice-cold PBS (1mL) at least 3 times (3 mins each) to remove PFA.

Add cold 90% Methanol (300 μl per glass-bottom dish). Incubate on ice for 1 min.

Wash with 1X cold PBS thrice to remove Methanol.

Incubate with 1X PBS with 5% FBS (or "normal" serum of the species of the secondary antibody), 200 μl / glass-bottom dish) @ room temp for 1-2 h.

Replace blocking solution with the primary antibody incubation solution (primary antibody in the blocking solution; 150 μl / glass-bottom dish).

Incubate @ room temperature for 1h or @4^{0}C over-night.

Wash with 1X ice-cold PBS (1mL) thrice to remove excess primary antibody.

Replace blocking solution for the secondary antibody incubation solution (secondary antibody (conjugated with AF) in the blocking solution; 150 μl / glass-bottom dish).

Incubate @ room temperature for 1h (avoid light).

Wash with 1X cold PBS (1mL) thrice to remove excess secondary antibody.

Counter stain DNA with Hoechst.