THE CHARACTERIZATION OF TAZ PROTEIN-PROTEIN INTERACTIONS IN MUSCLE

ANASTASIA MACKERACHER

A THESIS SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

AUGUST 2024

Abstract

Hippo signaling serves as a critical regulator of cell proliferation, differentiation, and apoptosis. Recent studies have characterized the Hippo signaling downstream target protein TAZ as a repressor of myogenic differentiation. Since TAZ lacks inherent DNA binding ability itself, its function and activity change depending on its protein interactions. Previous research characterized TAZ interacting proteins in muscle cells. Employing a GFP-nanoTrap affinity purification strategy coupled with LC-MS/MS protein identification a list of both novel and previously characterized TAZ protein interactions in a myogenic context were documented. Notably, the pro-myogenic methyl transferase protein, CARM1, was identified as a TAZ interacting protein. Further investigation into this interaction revealed the functional role of the TAZ/CARM1 interaction on the Hippo signalling pathway. Utilizing a Hippo signaling pathway (HOP/HIP) gene reporter assay we observed that CARM1 represses TAZ transcriptional activation function, promoting TAZ Ser89 phosphorylation, leading to the cytoplasmic sequestration of TAZ. The TAZ and CARM1 protein interaction was further investigated in the dysregulated context of embryonic rhabdomyosarcoma cells (RD) in which the TAZ/CARM1 interaction and function was maintained. Overall, Hippo signaling was found to increase compared to control C2C12 myoblasts in RD cells. Further, mass spectrometry analysis was conducted and revealed that TAZ may be a substrate of CARM1 since methylation at amino acid Arg 77 in a PGPR*LAGG consensus peptide was identified and resulted in enhanced TAZ Ser89 phosphorylation. These findings underscore a possible regulatory role of CARM1 in regulation of TAZ in myogenic cells.

Acknowledgments

I would first like to thank Dr. McDermott for taking me on as a student and giving me the platform to grow as a scientist. Your support, guidance, and knowledge during my two years in this lab has meant a lot to me. Dr. Tetsuaki Miyake, you have not only guided me in my experimental approach, but you have also shown a kindness and constant encouragement for me during my entire master's degree. I will take all that you have taught me as a scientist with me for the rest of my career. Catherine Chan, thank you not only for keeping the lab in perfect shape and ordering all the reagents I needed to make these experiments possible but for being so kind and making me laugh every day. Thank you to my lab mates Fatima, Sydney, and Stephanie for your guidance and friendship. To my former lab mate Jonathan Kelebeev, thank you for training me on all the techniques that made this master's possible. You are now and have always been the best partner and my best friend. This degree would not be possible without your help and constant guidance and support. Finally, I would like to thank my family, my Thea Vicky, Uncle Van, Andreas, and Alexandra for allowing me to stay over when I had an early morning and for your love and support. My Yia Yia and Papou, you are my favourite people, thank you for guiding me my entire life, teaching me how to be kind to others, work hard, and persevere. Thank you to my sister Zoe for always being my biggest cheerleader and my best friend. I could not imagine doing life without you. Finally, thank you to my mom and dad for providing me the opportunities and constant support for me to be successful. Thank you for the countless drives to school on the weekends and early mornings. I love you both so much and I am forever thankful that you are my parents.

Table of Contents

List of Figures

List of Abbreviations

AAB5C- ATP binding cassette subfamily C member 5

ACLY- ATP citrate lyase

AJUBA – AJUBA LIM protein

Akt- mTOR - The phosphoinositide 3-kinase-AKT-mammalian target of rapamycin

AMOT – Angiomotin

AMOTL1/2- Angiomotin-like 1/2 **AP1-** Activator protein 1

ARMS- Alveolar Rhabdomyosarcoma

ARID1A – AT- rich interactive domain 1A

ARP- Address resolution protocol

AXL- AXL - receptor protein-tyrosine kinase

BHLH- Basic Helix-Loop-Helix

BIRC5- Baculoviral inhibitor of apoptosis repeat-containing 5

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

BMD – Becker muscular dystrophy

BMP – Bone morphogenic protein

BMP4- Bone morphogenetic protein 4

C2C12 - mouse myoblast cell lineCARM1- coactivator associated arginine methyltransferase 1

CAMK – Ca2+/calmodulin-dependent protein kinase

cAMP- Cyclic adenosine monophosphate

CAND1- Cullin-associated NEDD8-dissociated protein 1

CCAR2- Cell cycle and apoptosis regulator 2

CCND1- Cyclin D1

CK1 – Casein kinase 1

c-MYC- Cellular – Mushroom Fungus

CNN3- Calponin 3

CRM-1 - Chromosomal Maintenance 1 aka, Exportin 1

CTGF – Connective tissue growth factor

CYR61 – Cysteine-rich angiogenic inducer 61

DM - Differentiation Media

DMD – Duchenne muscular dystrophy

DMSO - Dimethylsulfoxide

DNAJA3 - DnaJ homolog subfamily A member 3

dRASSF - The Ras-association domain family (Drosophila)

DS - Dachsous

DVL – Disheveled

E-Box- collective term for DNA motifs with the consensus sequence CANNTG

ECM- Extra Cellular Matrix

EFTUD2- Elongation Factor Tu GTP Binding Domain Containing 2

EMT – Epithelial-to-mesenchymal transition

ERK - Extracellular signal-regulated kinase

ERMS – Embryonic Rhabdomyosarcoma

EYFP – Genetic mutant of green fluorescent protein

FRZLD – Frizzled Receptor

F-actin – Filamentous actin

FASN- Fatty acid synthase

FGF- Fibroblast growth factors

FGF2 – Fibroblast growth factor 2

FOXO1- Forkhead box protein

FT- Fat

GBP- GFP binding protein

GFP – Green Fluorescent protein

GM- Growth Media

GPCR- G-protein-coupled receptors

GSK3 – Glycogen synthase kinase-3

GSPT1- G1 to S phase transition 1 protein

GTP-ase - nucleotide guanosine triphosphate

HDAC- Histone deacetylase

HEK293T- Human Embryonic Kidney cells

Hpo – MST1/2 in mammals

HSA - human skeletal muscle α-actin gene

HSPH1- Heat shock protein

IMPDH2- inosine monophosphate dehydrogenase gene

LATS1/2 – Large tumor suppressor kinase 1/2

MAPK- mitogen-activated protein kinase

MAP4K – Mitogen-activated protein kinase 4

MCK- Creatine kinase

MCM- Minichromosome maintenance

MDM2- Mouse double minute 2 homolog

MEF2 – Myocyte enhancer factor 2

MOB1 – Mps1-binder-related 1

MRFs – Myogenic regulatory factor

MRF4 – Myogenic regulatory factor 4

MST1/2/3 – Mammalian STE20-like protein kinase 1/2/3

MTA2- Metastasis Associated 1 Family Member 2

mTOR – Mammalian target of rapamycin

MuSCs – Muscle stem cells

Myf5 - Myogenic factor 5

MyHC – Myosin Heavy Chain

MyoD – Myoblast determination protein 1

MyoG – Myogenin

NDR1/2- The nuclear Dbf2-related kinase

NF1- Neurofibromin

NME1 – Nucleotide diphosphate kinase A

NuRD – Nucleosome Remodeling Deacetylase

OMD - Oculopharyngeal muscular dystrophy

PCM - Primary Cardio Myocytes

PDCD6IP- Programmed cell death 6 interacting protein

PD-L1- Programed death-ligand 1

PD1- Programmed cell death protein

PKA- Protein kinase A

PI3K – Phosphoinositide 3 – kinase

PRMT- Protein Arginine Methyltransferase

PP1- Protein phosphatase 1

pTAZ- Phosphorylated TAZ

PTCH1 – Protein patched homolog 1

PTM – Post translational modification

PP2A - Protein Phosphatase 2A

PUM1- Pumilio homolog 1

Pax3/7- Paired pox gene 3/7

RA – Retinoic Acid

RAB1A - member RAS oncogene family

RAD50 - RAD50 double strand break repair protein

RASSF - The Ras-association domain family

RBBP4 - RB binding protein 4, chromatin remodeling

RD -Embryonic Rhabdomyosarcoma

Rho-A – Ras homolog family member A

RH30/18 – Alveolar Rhabdomyosarcoma

RMS – Rhabdomyosarcoma

Runx1/2 – Runt-related transcription factor 1

SARAH – Sav/Rassf/Hpo

SAV1 – Salvador 1

SC – Satellite cell

SCF - Skp, Cullin, F-box containing complex

SEC23IP- SEC23 interacting protein

Shh – Sonic hedgehog

SIK2 - Salt Inducible Kinase 2

SMARCA4 - SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4

SMARCC1 - SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily C Member 1

SMO - Smoothened

SH3 - Src homology 3

MAD1/2/3/7 – Suppressor of mothers against decapentaplegic

SWI/SNF - SWItch/Sucrose Non-Fermentable

TAO – Thousand and one

TAZ – Transcriptional activator with PDZ-binding motif

TEAD1-4 – TEA domain family domain 1-4

TGF- β – Transforming growth factor-β

TP53 - Tumor proteinp53

Wnt – Wingless integration site signaling

YAP – Yes-associated protein

ZO-1/2 – Zonula occludens ½

Chapter I: Literature review

Overview of skeletal muscle

Skeletal muscle constitutes 35% of human body weight and is important for everyday function and aging (Mukund et al., 2020; Karagounis et al., 2010). Beyond facilitating voluntary movement, it also serves crucial functions in maintaining posture, storing nutrients, respiratory mechanics, and stabilizing joints (Mukund et al., 2020). Skeletal muscle is dynamic and carries a remarkable regenerative potential in response to injury. Diseases such as sarcopenia, an age-related muscle wasting disorder, contribute to reduced regenerative potential of skeletal muscle due to the ageing process (Wang et al., 2010). In addition, skeletal muscle is subject to atrophy which is characterized by weakening and thinning of muscle fibers which can occur independently or as a consequence of many diseases such as muscular dystrophies and muscle myopathy (Powers et al., 2016). Among the nine types of muscular dystrophy, which arise from birth to old age, the most characterized are Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). The integrity of our muscle is therefore imperative to our overall health and well-being, warranting the investigation into the development and regeneration potential of skeletal muscle.

Skeletal myogenesis

Myogenesis is the process of generating muscle, which arises from muscle progenitors during embryonic development, or from muscle stem cells in the post-natal muscle (Yu et al., 2021). Development of limb and trunk muscle during embryogenesis arises from progenitors labeled by the transcription factor PAX3 and originate from somites. In adults, when skeletal muscle sustains injury or damage, a regenerative response is triggered by activating muscle stem cells

(MuSCs), which are marked by the transcription factor PAX7. Activation of MuSCs is followed by the proliferation, differentiation, and fusion of these cells to repair the damaged muscle (Asfour et al., 2018). In both embryonic and post-natal settings, PAX3 and PAX7 are upstream of the highly conserved class II basic helix-loop-helix (bHLH) transcription factors myogenic regulatory factors (MRFs): Myf5, MyoG, MyoD, and MRF4. These factors lay the foundation of the genetic hierarchy governing myogenic progression (Asfour et al., 2018; Seale et al., 2000). MRFs are self-regulating and promote the activation of other pro-myogenic genes by binding to E-BOX DNA regions that are found at promoter and enhancer regions (Asfour et al., 2018). Furthermore, Myocyte enhance factor 2 (MEF-2) proteins interact with the bHLH myogenic regulatory factors and are required for myogenesis (Ornatsky et al., 1996). Overall, both embryonic and post-natal myogenesis are controlled by a similar subset of myogenic transcription factors that regulate each other to facilitate the development and/or repair of muscle tissue. A schematic of the sequential interactions of these MRFs is shown below (Figure 1).

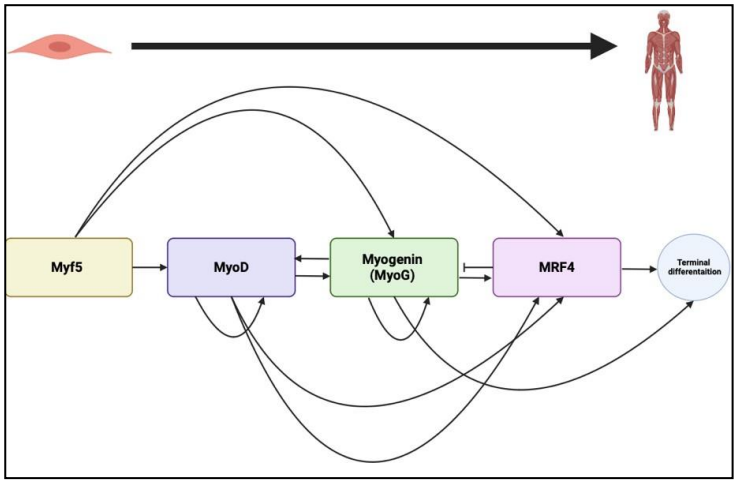


Figure 1. Sequential interactions between myogenic regulatory factors during myogenic differentiation. Cells in a proliferation state begin to express Myf5. Once the cells commit to the muscle lineage, they express MyoD. Cells that start to differentiate express MyoG. Finally, terminally differentiated cells express MRF4 which leads to terminal differentiatio

Embryonic myogenesis

Embryonic myogenesis involves the induction and activation of MRFs in progenitors to orchestrate cell fate determination in the embryo. Embryonic development primarily progresses through gastrulation, a process in which cells migrate and differentiate into the layers of the embryo: ectoderm (outer layer), mesoderm (middle layer), and endoderm (inner layer) (Bentzinger et al., 2012). Muscle development occurs in the mesoderm layer of the embryo, which is divided into three parts: paraxial, intermediate, and lateral mesoderm, with respect to position from the midline (Aulehla et al., 2006).

During embryonic muscle development, a fundamental process in vertebrate species known as somitogenesis occurs in blocks of mesodermal tissue called somites that form across the anterior and posterior axis. This process allows for various tissues such as vertebrae, skeletal muscle, and dermis, to arise (Maroto et al., 2012; Asfour et al., 2018). Somitogenesis involves the expression of genes belonging to multiple signaling pathways including Notch, Wnt, FGF, and Retinoic Acid (RA) (Aulehla et al., 2010; Hofmann et al., 2004). The highest concentrations of RA are at the rostral portion of the embryo while Wnt and FGF remain highest at the caudal portion of the embryo, ensuring these cells remain in an undifferentiated state (Bentzinger et al., 2012). The concentrations of the proteins of these pathways determine the cyclic expression of the Notch pathway genes, leading to the bud like somite structures (Hoffmann et al., 2004).

The ventral-medial region of the somites is under the influence of Wnt, Shh, and BMP signaling, which promote these cells to undergo epithelial mesenchymal transition (EMT) to form the sclerotome containing precursors to cartilage and bone (Johnson et al., 1994; Fan & Tessier-Lavigne., 1994). The remaining dorsal lateral portion of the somite becomes the dermomyotome,

giving rise to non-head skeletal muscle (Bentzinger et al., 2012). This region is distinguishable by the presence of Six1/4, PAX3, and by low levels of Myf5 (Grifone et al., 2007; Giordani et al., 2007). PAX3 expression upregulates Myf5 expression, allowing cells located in the epaxial (dorsomedial) and hypaxial (ventrolateral) regions of the dermomyotome to undergo EMT and delaminate beneath the dermomyotome, forming the myotome (Buckingham, M., & Relaix et al., 2015; Bentzinger et al., 2012). Progenitors committed to the muscle lineage will express MyoD and then subsequent MRFs until multinucleated secondary myofibers are formed (Asfour et al., 2018). A portion of the cells undergoing differentiation will maintain a muscle progenitor phenotype, identifiable by the presence of the PAX7 transcription factor. These cells are known as satellite cells and will reside under the developed muscle tissue once the embryo has developed (Bentzinger et al., 2012).

During embryonic myogenesis, several myogenic transcription factors can compensate for the loss of others. This redundancy, from an evolutionary perspective, is in place to ensure embryos develop properly. For example, mice that contain a *Myf5* knockout display delayed embryonic myogenesis but proceed normally after *MyoD* induction (Braun et al., 1992). Similarly, *MyoD* knockouts compensate by upregulating *Myf5* expression, regaining normal myogenic function (Rudnicki et al., 1992). However, some factors governing myogenesis are required for the proper progression of myogenesis such as PAX3, in which deficiencies lead to offspring with neural crest defects. In human embryonic development, PAX3 deficiencies lead to hearing loss and changes in pigmentation (Waardenburg syndrome) (Pani et al., 2002; Baldwin et al., 1994). Unlike other MRFs, MyoG knockout mice exhibit severe muscle deficiencies and are neonatal lethal (Ganassi et al., 2020). Overall, this demonstrates the importance of the proper expression and regulation of myogenic transcription factors governing embryonic development.

In addition, proper function of signaling gradients is needed for appropriate embryo development. In Wnt signaling, Wnt1 and Wnt 3 are secreted from the dorsal neural tube, while Wnt 4, 6, 7 are expressed from the overlying ectoderm of the embryo (Parr et al., 1993). Mouse embryo mutants lacking Wnt 1,3 exhibit incomplete dermo-myotome formation and reduced expression of PAX3 and Myf5 (Ikeya et al., 1998). Further, Shh signaling positively regulates embryonic muscle development (Bentzinger et al., 2012). The canonical Sonic Hedgehog signaling pathway involves hedgehog proteins binding to the patched receptor, releasing smoothened, and activating gene expression (Lum et al., 2004). Inhibition of this pathway through a smoothened or Sonic Hedgehog knockout decreases the amount of Myf5 and prevents cells from further differentiating into mature muscle fibers (Straface et al., 2009). BMP signaling expression supports initial embryonic myogenesis but can repress muscle differentiation in later fetal developmental stages (Tsumaki et al., 2005). Certain BMP proteins such as BMP4 expression inhibit the expression of later stage differentiation factors Myf5, MyoD, and promote the retention PAX3 expression (Pourquie et al., 1995). Notch signaling acts as a master regulator of cell-cell communication, crucial to specifying cell lineage in a developing embryo (Rios et al., 2011). Active Notch signaling has been known to repress myogenesis, as mutations in proteins such as Delta1 result in excessive differentiated cells and lack of progenitors (Rios et al., 2011; Gioftsidi et al., 2022). This intricate orchestration of multiple signaling pathways to cue the induction of myogenic regulatory factors is crucial for proper embryonic muscle development and the establishment of functional skeletal muscle in the organism. A schematic of embryonic myogenesis is shown below (Figure 2).

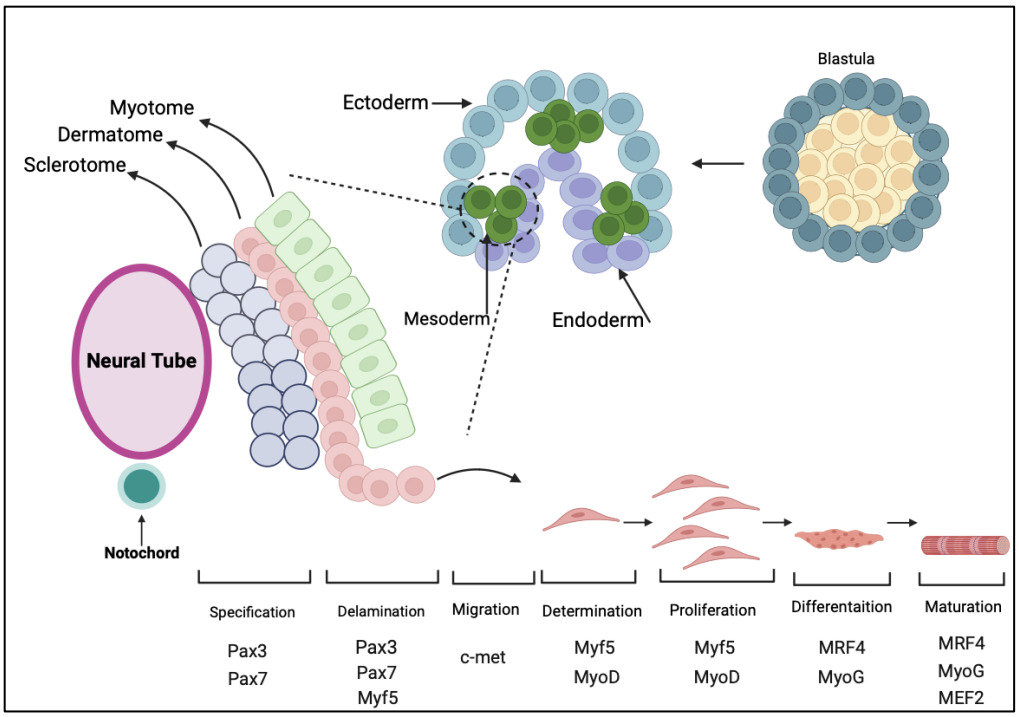


Figure 2. Embryonic myogenesis schematic of somitogenesis and formation in the limb. Cells marked by PAX3, PAX7, Six1/4 expression differentiate and later express Myf5 causing delamination and migration and specification to the limb. Following this there is further differentiation of limb muscle cell populations where cells will begin to express MyoD followed by MyoG and then, in terminally differentiated states, MRF4.

Post-natal myogenesis

Similar to embryonic myogenesis, MuSCs exhibit a hierarchical organization of genetic factors that allow them to have regenerative and self-renewal properties. In the post-natal period, muscle injury can be induced by mechanical damage or daily activity (Laumonier et al., 2016). Consequently, the ability to regenerate muscle in response to injury is essential for maintaining health.

In adults, MuSCs reside as quiescent cells located between the basal lamina and sarcolemma (Yin et al., 2013). These cells remain in a quiescent state during homeostasis but activate and begin to proliferate when induced by stress or injury (Yin et al., 2013). Maintaining the quiescence of the

satellite stem cell pool is crucial for overall muscle health. Several mechanisms allow these cells to maintain their quiescent state, including the upregulation of over 500 genes, such as those that are negative regulators of the cell cycle (cyclin-dependent kinase inhibitors), retinoblastoma tumor suppressors, and fibroblast growth factors (Dumont et al., 2015). Moreover, Notch signaling factors, including the Notch receptor, its co-receptor syndecan 3, and Notch 1 and 3, are enriched in MuSC population to maintain them in a quiescent state (Dumont et al., 2015).

Following muscle injury, these cells can move out of the quiescent state, begin to proliferate, and migrate to the source of injury. This shift includes the expression of mitogenic factors that trigger the cells into an activation state, known as G_{Alert}, where they have a greater regenerative potential (Dumont et al., 2015). Multiple mechanisms control this process, including signals secreted from the injured musculature, such as insulin-like growth factor (Igf1), which leads to the downregulation of the cell cycle inhibitor $p27^{Kip1}$ and results in cell cycle entry (Dumont et al., 2015).

After activation, Myf5-positive cells need to proliferate to generate sufficient nuclei to form mature muscle fibers. This population will then begin to express MyoD, the key MRF that switches cells from a proliferative to a differentiation state. MyoD is activated by its direct targets, including MEF2 and other bHLH transcription factors (Asfour et al., 2018). Both MyoD and MyoG are activated by direct binding of MEF2 family of transcription factors on their gene promoters (Molkentin et al., 1995). As a result, the expression of MyoD leads to the subsequent expression of MyoG. MyoG directly suppresses PAX7 expression, allowing these cells to differentiate further (Asfour et al., 2018). The combined expression of MyoD and MyoG leads to the expression of the late-stage MRF, MRF4, which induces terminal differentiation (Hernández-Hernández et al., 2018). Furthermore, the transition from proliferation to differentiation in MuSCs is achieved by

the switch from Notch gene expression to Wnt signaling protein expression, which in turn promotes the stabilization of β-catenin and subsequent transcription of muscle-specific genes required for differentiation (Brack et al., 2008). Lastly, mature functional muscle fibers will express MyHC and contractile proteins such as muscle creatine kinase (MCK) (Forcina et al., 2020).

In an *in vivo* context, adult muscle regeneration is highly influenced by PAX7 expression, which maintains the self-renewal "stem cell-like phenotype" of MuSCs (Le Grand et al., 2007). Mice deficient in PAX7 expression have a similar amount of satellite stem cells at birth compared to control mice. However, throughout development, this population is lost, leading to a diminished ability to regenerate injured musculature (Le Grand et al., 2007). Furthermore, unlike in embryonic muscle development, MyoD and Myf5 do not compensate for the loss of the other in a post-natal context. (Le Grand et al., 2007). In adult myogenesis, MyoD is required for cell differentiation, while Myf5 regulates the proliferation rate of myogenic cells and maintains homeostasis (Le Grand et al., 2007). Although similar in expression myogenic gene expression patterns, this highlights the important differences between embryonic and post-natal myogenesis.

Signaling gradient defects also affect the effectiveness of adult muscle regeneration. Sonic Hedgehog signaling is important in postnatal development, as its expression is upregulated in response to muscle injury in adult myogenesis (Straface et al., 2009). Inhibition of Sonic Hedgehog reduces myogenic regulatory factors Myf5 and MyoD and decreases myogenic satellite cells at the injury site. This can result in muscle inflammation and decreased motor function after injury

(Straface et al., 2009). A schematic demonstrating musculature repair and transcription factors expressed during differentiation is shown in Figure 3.

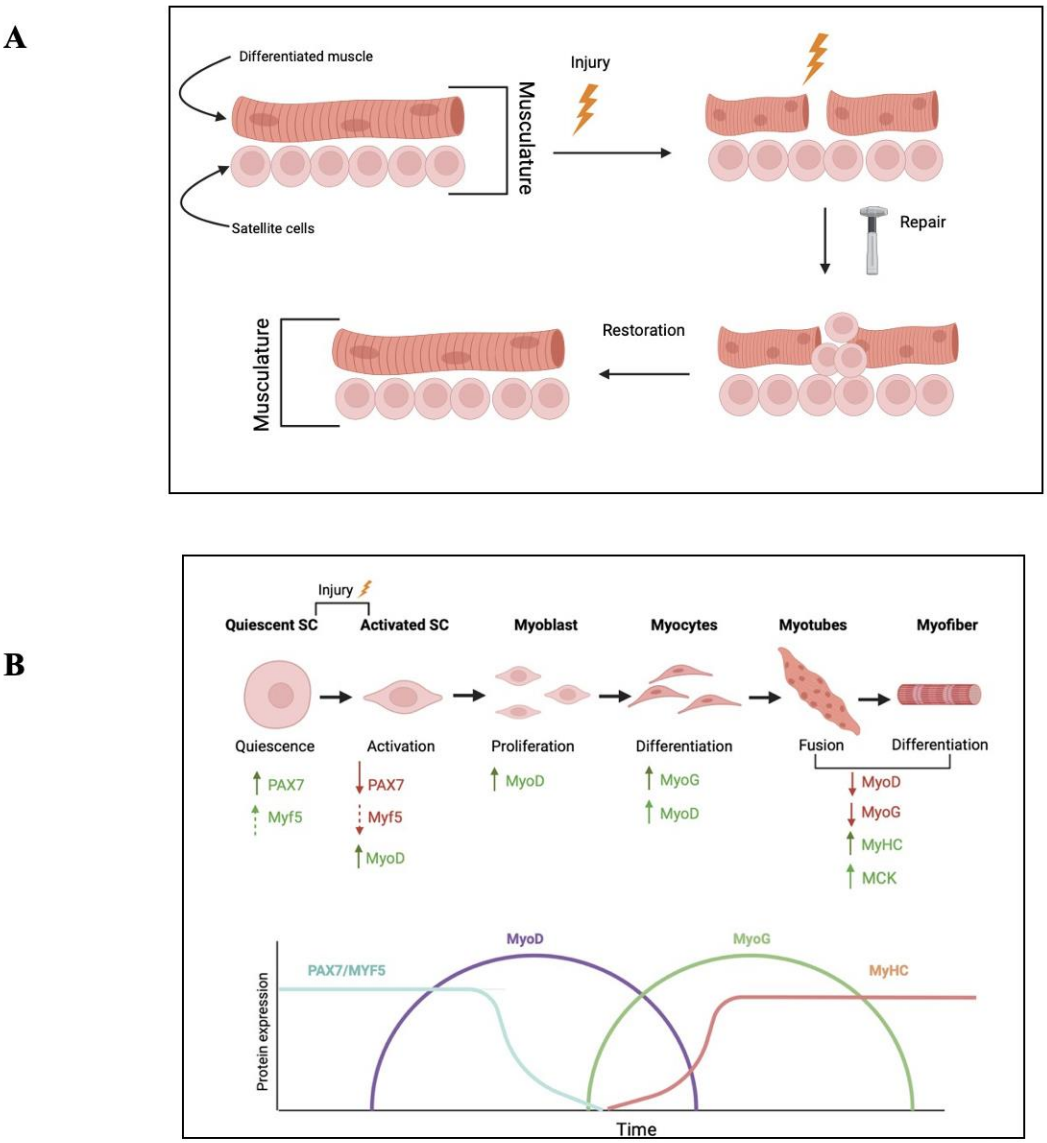


Figure 3. Summary of post-natal myogenesis. (A) Upon muscle injury, quiescent PAX7-positive satellite cells activate, proliferate, and migrate to the injured musculature, where they begin to express muscle regulatory factors (MRFs), leading to muscle differentiation and subsequent repair. **(B)** Schematic of post-natal myogenesis showing the expression of transcription factors during the myogenesis process. Quiescent satellite cells express PAX7, then activate and proliferate into myoblasts under the expression of Myf5 and MyoD. MyoD expression induces the expression of downstream MRFs such as MyoG, followed by transcription factors like MRF4 and contractile proteins such as MyHC and MCK.

The Hippo signaling pathway

Overview of Hippo signaling

Among the signaling pathways responsible for MuSC activation, the Hippo signaling pathway has gained significant attention in recent years for its involvement in proliferation, regeneration, maintaining homeostasis, and its impact on disease development (Zheng et al., 2019). This evolutionarily conserved pathway was first discovered in *Drosophila melanogaster*, where it regulated cell homeostasis and tumor suppressor genes (Staley et al., 2012). Further, this pathway was shown to be conserved in mammals (Zheng et al., 2019). Dysregulation of this pathway both in *Drosophila* and humans contributes to excess growth of organs and tissue, often linking to cancer in humans (Luo et al., 2023; Lei et al., 2008).

In *Drosophila*, the core components of the Hippo signaling pathway include *hippo* (MST1/2 in humans), *salvador* (Sav1 in humans), *warts* (Lats1/2 in humans), and *mats* (MOB1/2, in humans) (Wu et al., 2003; Justice et al., 1995; Meng et al 2016). A serine/threonine kinase signaling cascade regulates downstream transcriptional co-activator Yorkie (YAP/YAZ in humans) (Zhang et al., 2009; Wu et al., 2003). However, when the kinase cascade is inactive, Yorkie (YAP/TAZ) localizes to the nucleus and interacts with Scalloped (TEAD in humans) to activate genes associated with proliferation (Wu et al., 2008). Following these initial discoveries, the Hippo signaling pathway role emerged as a major contributor to organ size control, cancer-related diseases, and autoimmune diseases (Staley et al., 2012).

Upstream regulation of YAP/TAZ: in the cytoplasm

The predominant upstream regulation of YAP/TAZ is mediated by the core kinase cascade of the Hippo signaling pathway (Meng et al., 2016). The cascade initiates at MST1/2 which directly

interacts with and phosphorylates SAV1 (Meng et al., 2016). SAV1 phosphorylation by MST1/2 enhances its interaction with SAV1, subsequently promoting the phosphorylation of downstream LATS1/2. This phosphorylation activates LATS1/2 through the SARAH coiled-coiled domain and MOB1/2 (Zhang et al., 2009; Wu et al., 2003). Further, the interaction between LATS1 with MOB1/2 functions as a positive feedback loop, where the interaction of one component promotes the binding affinity of its upstream components (Bae et al., 2018). The Hippo signaling phosphorylation cascade leads to the direct phosphorylation of downstream transcriptional co-activator YAP/TAZ. Upstream kinases can phosphorylate YAP on five serine residues (Ser61, Ser109, Ser127, Ser164, and Ser381) and phosphorylate TAZ on 4 serine residues (Ser66, Ser89, Ser117, and Ser311) to regulate their function (Wang et al., 2020). LATS 1/2 is the terminal kinase in the cascade that function to directly phosphorylate YAP/TAZ on HXRXXS motif sequences (Zhao et al., 2007; Hao et al., 2008; Yu et al., 2013). Phosphorylation of YAP/TAZ on Ser127 and Ser89, respectively, leads to 14-3-3 binding and in turn its sequestration or degradation (Hong et al., 2012). In addition, the phosphorylation of TAZ (Ser311) and YAP (Ser381) promotes the recruitment of β-TRCP; a subunit of the SCF ubiquitin E3 ligase leading to its degradation (Yu et al., 2013).

The upstream Hippo signaling cascade is regulated through multiple mechanisms. For example, external signals from the extracellular matrix and G-coupled receptors (GPCRs) lead to the activation cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) signaling, thus activating the Hippo signaling pathway (Luo et al., 2019). These signals act on MST1/2 and LATS

1/2 through a Rho-dependent manner to inhibit LATS1/2 function, thus inhibiting phosphorylation of YAP/TAZ (Luo et al., 2019). In addition, components of the core cascade such as MST1/2 and LATS1/2 can undergo autophosphorylation to activate themselves and, consequently, the Hippo signaling pathway (Meng et al., 2016).

Downstream regulation by YAP/TAZ

When Hippo signaling is inactive, YAP/TAZ is no longer phosphorylated by LATS1 and thus allowed to translocate into the nucleus. YAP/TAZ lack an inherent ability to bind to DNA and thus rely on interactions with nuclear transcription factors (Kim et al., 2018). One of the most well characterized interactions with YAP/TAZ is with TEAD transcription factors (TEAD 1-4), most notably TEAD1 and TEAD4 (Pobbati et al., 2020; Hong et al., 2012; Kim et al., 2018). This interaction occurs through the c-terminus of the TEAD protein binding to the transactivation domain of YAP/TAZ, inducing its ability to activate proliferative gene expression such as MYC, Vimentin, CTGF, CYR61, AXL, BIRC5 and CCND1 (Totaro et al., 2018). Furthermore, ChIP sequencing analysis reveals that YAP/TAZ binds to TEAD binding sites through chromatin looping with the AP-1 protein in a breast cancer model (Zanconato et al., 2015). This highlights the significance of TAZ in maintaining a proliferative cellular state, despite its inability to bind to DNA directly. A schematic illustrating both the upstream and downstream regulation of TAZ is shown in Figure 4.

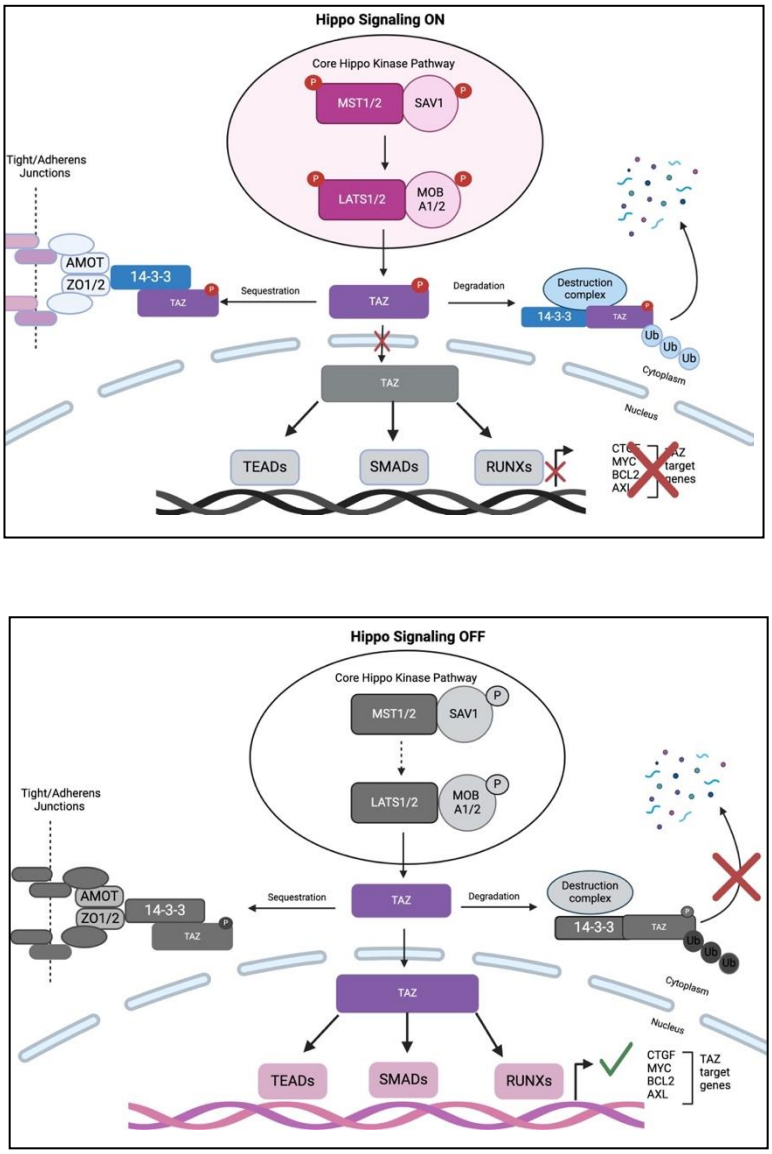


Figure 4. Overview of the Hippo signaling pathway in mammalian cells. When Hippo signaling is active, upstream signals activate the core Hippo kinase cascade, leading to the phosphorylation and activation of LATS1/2 kinases. These kinases then phosphorylate the transcriptional co-regulators YAP/TAZ, which allows for the binding and recruitment of 14-3-3 proteins, resulting in their sequestration and degradation in the cytoplasm. When Hippo signaling is inactive, the kinase cascade remains dormant, preventing the phosphorylation of YAP/TAZ. This enables YAP/TAZ to translocate into the nucleus, where they bind to transcription factors and regulate the expression of proliferative genes such as CTGF, MYC, BCL2, and AXL.

Mechano-regulation of YAP/TAZ

The process by which biophysical forces are converted into biochemical signals is known as mechano-transduction. This includes forces such as proliferation, differentiation, stiffness, and stretch (Pruitt et al., 2014). YAP/TAZ are pivotal mediators of this process and play a key role in mediating responses to mechanical cues, particularly in the context of the Hippo signaling pathway, which is sensitive to many types of external signaling and stress (Dasgupta et al., 2019).

The role of YAP/TAZ in mechano-regulation is mediated by two main mechanisms: ECM stiffness and signal transmission. ECM stiffness and high-rigidity promote the nuclear localization of YAP/TAZ and the upregulation of its target genes. This mechanism is dictated by a GTPase (RhoA), which detects ECM stiffness and responds by promoting actin polymerization into filamentous actin (F-actin) and stress fiber formation (Cai et al., 2021; Li et al., 2022). F-actin polymerization promotes the inhibition of LATS1/2 and MST1/2 upstream kinases from phosphorylating TAZ on Ser89, thus leading to the translocation of TAZ to the nucleus. A known binding partner, ARID1A-SWI/SNF complex plays a large role in mechano-transduction by modifying the chromatin structure to regulate gene transcription (Chen et al., 2023). This complex can respond to the rigidity of the ECM as well as regulating YAP/TAZ function. Conversely, upon cell-cell contact and low ECM rigidity, YAP/TAZ function is inhibited, leading to cytoplasmic retention, and resulting in cell arrest and autophagy (Cai et al., 2021). Further, at high cell density and low ECM stiffness, cells can change shape, leading to the inactivation of RhoA and subsequently of YAP/TAZ (Cai et al., 2021).

Furthermore, mechano-transduction has functional implications on YAP/TAZ in various disease contexts. For example, mechano-transduction is linked to ECM mechanical signaling cues in fibroblasts, leading to fibrosis (Duscher et al., 2014). ECM stiffness activates YAP/TAZ to

promote the production of pro-fibrotic ECM proteins, which may indicate a link between fibrosis and cancer (Duscher et al., 2014). Moreover, YAP/TAZ are known to be hyperactivated in cancer, which can assist in overriding the mechanical checkpoints that regulate and control cancer growth (Cai et al., 2021). ECM-activated YAP/TAZ regulate cytoskeletal regulators associated with cancer, increasing isometric tension, enhancing tumor microenvironment rigidity, promoting cancer growth, metastasis, and angiogenesis (Cai et al., 2021). Therefore, understanding the mechano-regulation of TAZ provides valuable insights into how mechanical signals regulate cell behavior, which can help further our understanding of how cellular mechanics regulate health and disease in a muscle context. A schematic illustrating the mechano-transduction signaling pathway in high ECM conditions is shown in figure 5.

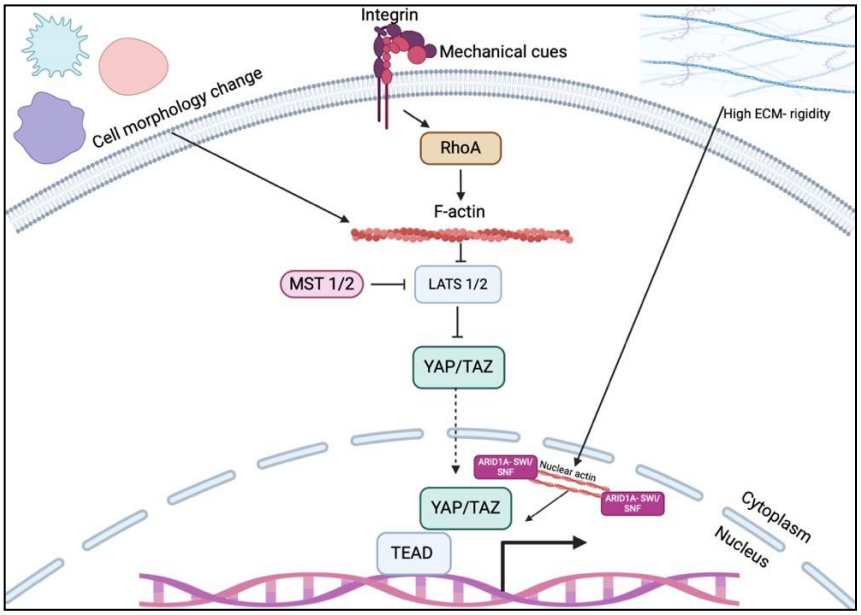


Figure 5. Mechano-regulation of YAP/TAZ. Under high ECM rigidity conditions, integrins signal from the ECM mechanical cues to activate RhoA. This then activates F-actin formation which inhibits LATS1/2 ability to phosphorylate YAP/TAZ. This allows YAP/TAZ to translocate in the nucleus, bind to TEADs and start transcription. In addition, the ARID1A-SWI/SNF complex controls the ability to nuclear actin to act on YAP/TAZ.

Structure of YAP/TAZ

YAP and TAZ are paralogs and share a similar protein structure containing regions such as the TEAD-binding region, WW- domain(s), coiled-coiled domain, transactivation domain, and a PDZ-binding domain (Reggiani et al., 2021). YAP/TAZ are important co-regulators of gene transcription, yet lack the ability to bind to DNA themselves (Totaro et al., 2018). The TEAD-binding regions of these proteins are essential for their function, as TEAD is the most abundant and well-characterized protein that interacts with both YAP/TAZ (Lin et al., 2017). The WW-domain of the proteins mediate protein-protein interactions through its recognitions of serine/threonine rich sites (Ingham et al., 2005). WW domains have been discovered to be highly conserved among many proteins and are found to be frequently mutated in diseases such as cancer (Huang et al., 2020). Furthermore, coiled-coiled domains in both YAP and TAZ function as molecular spacers and influence protein-protein interactions, therefore it is important in mediating protein interactions vital to TAZ and YAP function (Trubestein et al., 2016). The *trans*activation domain is important in the structure of both YAP and TAZ as it allows for transcriptional activation by interacting with general transcription factors (Frietze et al., 2011). Finally, the PDZ domain is present on the C-terminal of both YAP and TAZ as it allows for interactions with PDZ domain containing proteins (Chan et al., 2011), including cytoskeletal components such as ZO-1 (Romero et al., 2011).

Although YAP/TAZ proteins share similar domains, their structure varies in their size and regions of regulation. YAP is a larger protein containing a proline-rich domain, a second WW domain as well as a SH3 binding domain with a total length of 488 amino acids. In contrast, TAZ has a length of 400 amino acids (Chen et al., 2019). An additional distinction between YAP and TAZ is the serine residue responsible for preventing nuclear translocation: Ser127 in YAP and Ser89 in TAZ.

(Hong et al., 2012). Below is a schematic depicting the difference in YAP/TAZ structure and their binding partners (Figure 6).

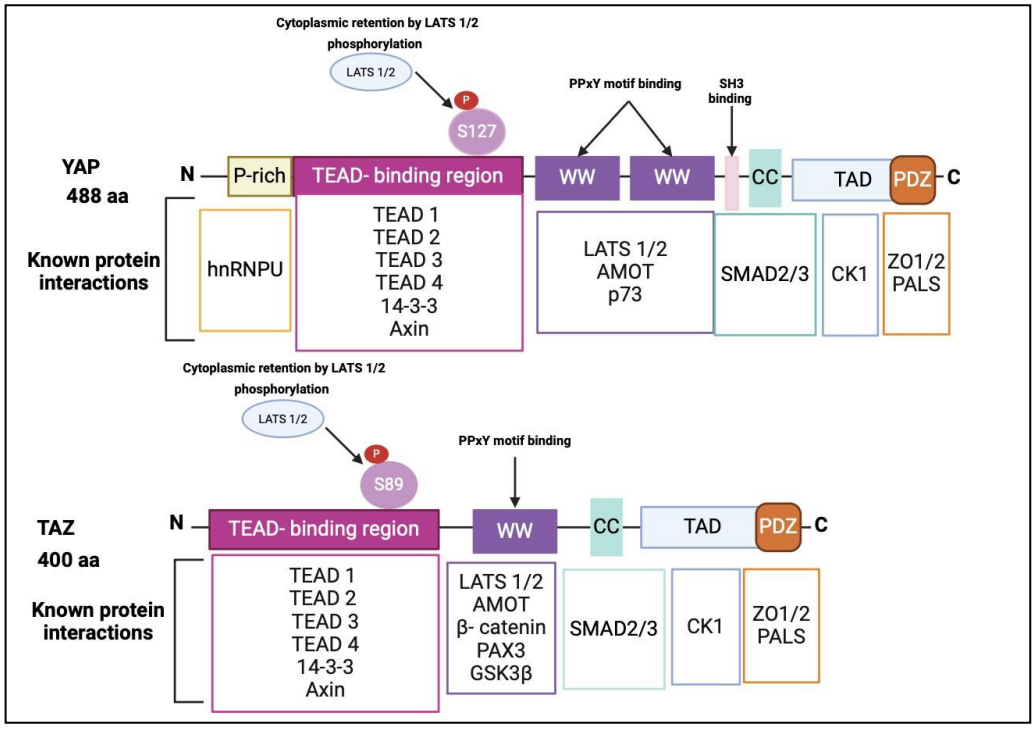


Figure 6. YAP/TAZ domain structures and interacting proteins. Structurally, YAP/TAZ share a TEAD binding region, WW domain, coiled-colied domain, transcriptional activator domain and PDZ domain. These proteins also share similar proteins that bind to each domain respectfully. However, there are domains that differ between the two proteins including P-rich domain, SH3, and an extra WW domain. These proteins also differ in their post translational modifications and regulation as TAZ is phosphorylated by upstream kinases primarily on S89 and YAP on S127.

YAP/TAZ in Chromatin Remodeling

As previously noted, YAP/TAZ do not directly bind to DNA but rely on other binding partners to carry out their functions and mediate transcription. Importantly, several YAP/TAZ protein-protein interactions have been associated with modulating regulatory elements on chromatin (Lopez-Hernandez et al., 2021). The most well-characterized chromatin remodeling interaction is between YAP/TAZ and the SWI/SNF complex. The SWI/SNF complex is composed of ATPase Swi2/Snf2p and actin-related proteins (Arp7 and Arp9), along with the component ARID1A, a potent tumor suppressor, that modulates the nucleosome structure in an ATPase-dependent manner through histone modifications (Hillmer et al., 2019; Tang et al., 2010). This complex maintains a structure to support protein-protein and protein-DNA interactions to mediate cellular regulation, primarily through histone and actin-related proteins at acetylated histone tails on DNA (Tang et al., 2010). Further, dysregulation of the SWI/SNF complex, in concert with increased YAP/TAZ activity, is known to promote cancer (Hillmer et al., 2019).

Additionally, TAZ mediates chromatin remodeling through the recruitment of the ATPase-dependent chromatin remodeling complex known as the nucleosome-remodeling and deacetylase (NuRD) complex (Hillmer et al., 2019). YAP/TAZ proteins are recruited by TEAD proteins to interact with enhancer elements to modify chromatin structure and mediate transcription (Piccolo et al., 2023). This interaction with the NuRD complex is dependent on TEAD binding and forming a complex with YAP/TAZ, which allows for the repression of pro-proliferative genes (Hillmer et al., 2019). YAP/TAZ also interact with the NuRD complex to repress target gene expression during embryonic development, determining cell lineage in portions of the mesoderm. YAP/TAZ recruit the NuRD repressor to switch-enhancer elements bound by TEAD, SMAD2/3, and OCT4, preventing the expression of mesendoderm specification genes and suppressing transcription

(Lopez-Hernandez et al., 2021). Furthermore, YAP itself may independently regulate genome accessibility in cardiomyocytes. Cardiomyocytes expressing constitutively active YAP have more accessible TEAD binding motifs compared to controls, leading to increased transcription in these models (Hillmer et al., 2019). Overall, this highlights how chromatin remodeling mechanisms can influence YAP/TAZ expression in regulating cellular homeostasis.

Hippo signaling in muscle growth and myogenesis

The Hippo signaling pathway has emerged as a significant regulator in the muscle biology of mammals (Sun et al., 2017). Components of the Hippo signaling pathway, including the downstream target protein YAP are at the forefront of this significance. YAP positively regulates muscle mass and protein synthesis in cooperation with TEAD transcription factors (Sun et al., 2017). It also increases in response to injury and muscle mass loss due to nervous system dysfunction (Watt et al., 2015). Further, YAP is highly expressed in fast-growing skeletal muscle, with levels rapidly declining during maturation (Watt et al., 2018). These studies highlight the importance of downstream transcription factors in regulating muscle development.

In mammals, Hippo signaling intersects with multiple signaling pathways to fine-tune muscle development and regeneration, including Mitogen-Activated Protein Kinase (MAPK), Growth Factor- β (TGF-β), and PI3K-Akt-mTOR pathways (Vainshtein et al., 2020). Recent literature highlights a possible mechanism into the repressive role of YAP/TAZ during myogenesis through the crosstalk with Wnt and TGF-.β pathways. Tripathi et. al found that TAZ interacts with SMAD7 and β-catenin to play a proliferative role and repressing pro-differentiation machinery through interactions. Although YAP and TAZ both promote proliferation, their roles differ; YAP is known to suppress differentiation, whereas the role of TAZ in myogenesis is still debated, necessitating further study (Sun et al., 2017; Tripathi et al., 2022). It should be noted that TAZ is more abundant

and less studied than YAP in muscle, therefore there more research into TAZ control of myogenesis is warranted (Tripathi et al., 2022).

Furthermore, components of the Hippo signaling pathway have been implicated in muscle disease deregulation contexts. For example, upstream kinases in the Hippo signaling pathway, including MST1, are differentially regulated and contribute to muscle atrophy upon denervation in mouse models (Wei et al., 2013; Watt et al., 2015). Overall, this evidence suggests that various components of the Hippo signaling pathway are crucial in maintaining our musculature and should be further studied in diseased muscle contexts.

YAP/TAZ in disease contexts

Rhabdomyosarcoma

Rhabdomyosarcoma is a mesenchymal malignancy associated with the skeletal muscle lineage, primarily diagnosed in pediatric patients aged 14 years or younger (Loeb et al., 2008; Yu et al., 2018). Although sarcomas are generally rare cancers occurring in bones and soft tissues, they are relatively more common in children (Loeb et al., 2008). The etiology of RMS remains largely unknown at this point, although a few cases are associated with genetic disorders such as Li-Fraumeni syndrome or NF1 mutations (Kaseb et al., 2022; Loeb et al., 2008). Despite the origin of RMS remaining unknown, the disease exhibits myogenic properties and features of premature muscle development (Keller et al., 2013).

There are two subtypes of rhabdomyosarcoma: embryonic and alveolar rhabdomyosarcoma (Loeb et al., 2008). Embryonic rhabdomyosarcoma is characterized by its embryonic like histology (Pomella et al., 2023) whereas alveolar rhabdomyosarcoma is defined by fusions containing the

gene FKHR and PAX3 or PAX7 (Loeb et al., 2008). Common fusions in alveolar sarcoma include PAX3- FOXO1 or PAX7- FOXO1, as well as MDM2 amplification and its ability to inhibit TP53 among other altercations of the p53 pathway (Marshall et al., 2012). These fusions promote the expression of oncogenes causing cancers to proliferate uncontrollably, leading to poor survival rates (Pomella et al., 2023). Given the strong association of PAX3 and PAX7 in myogenesis, disruption of these genes may be a possible mechanism of how this cancer arises (Keller et al., 2018).

Rhabdomyosarcoma risk factors remain largely unknown, however in some instances such as in-utero radiation, accelerated growth in-utero, low socioeconomic status, and the use of recreational drugs during pregnancy are known to contribute to the likelihood of the onset of rhabdomyosarcoma (Kaseb et al., 2022). In addition, rhabdomyosarcoma is impacted by epigenetic regulatory factors. The expression of DNA methyltransferases involved in histone acetylation, methylation and phosphorylation have been reported in RMS tumors (Megiorni et al., 2020). The clustering methylation profiles of RMS contributes to the genetic heterogeneity of RMS which allows one to distinguish between its two subtypes (Pomella et al., 2023). Further, the methylation status of gene promoters (protooncogenes and tumor suppressor genes) as well as the rarer, direct gene methylation, play are significant role in gene expression patterns. Therefore, they have been noted to play a role in rhabdomyosarcoma development and progression (Sun et al., 2019). An illustration depicting the genetic, environmental, and epigenetic risk factors leading to the formation of cancer is shown in figure 7.

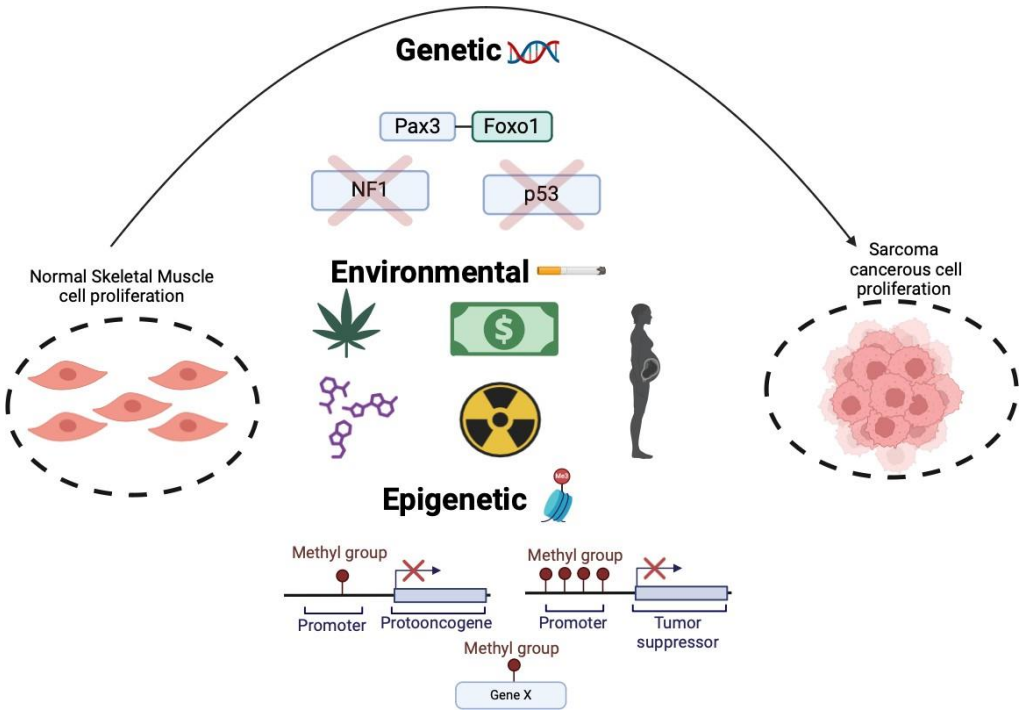


Figure 7. Rhabdomyosarcoma genetic, environmental, and epigenetic risk factors. Key genetic changes can induce a cancerous sarcoma phenotype including PAX3-FOXO1 fusions and deletions of NF1 and p53. Environmental factors contributing to the likelihood of rhabdomyosarcoma development include exposure to in-utero radiation, accelerated in-utero growth, low socioeconomic status, and the use of recreational drugs during pregnancy. Epigenetic modifications, such as hypomethylation of proto-oncogenes, hypermethylation of tumor suppressor genes, and methylation of the protein itself, also play a role. These factors collectively contribute to the likelihood of rhabdomyosarcoma formation.

Myogenesis dysregulation in rhabdomyosarcoma

Although the developmental origin of rhabdomyosarcomas is poorly defined, it is thought to be muscle-like in nature due to the myogenic regulatory factors it expresses. Known markers that are expressed during myogenesis such as MyoD and MyoG are expressed in RMS and have been used

as diagnostic markers for the disease (Yu et al., 2018). As mentioned previously, MyoD plays a large role in the initiation of the myogenesis process in regular muscle cells and maintains cell in a proliferative committed muscle lineage state (Yu et al., 2013). As such, it is thought that these transcription factors are dysregulated in a rhabdomyosarcoma context. In regular muscle cells, MyoD interacts primarily with cofactors such as RUNX1, AP-1 and, MEF2, to regulate myogenesis (Yu et al., 2018). However, in RMS contexts, MyoD cofactor binding is impaired due to changes in chromatin assembly (MacQuarrie et al., 2013; Yu et al., 2018). This leads to the improper binding of MyoD to its target genes in both ARMS and ERMS phenotypes. For example, in ERMS, MyoD preferentially binds to *Myc* consensus sequences leading to *Myc* overexpression. This encourages proliferation rather than differentiation leading to the undifferentiated RMS phenotype (Yu et al., 2018; Kohsaka et al., 2014). This research highlights the importance of maintaining proper regulation of myogenic regulatory factors as well as the consequences of improper protein-protein interactions in proper myogenic progression. Therefore, further research should be conducted on the protein-protein interactions of core myogenic proteins in diseased muscle contexts. A schematic depicting the difference in protein-protein interactions during myogenesis in growth-controlled conditions compare to rhabdomyosarcoma is shown in figure 8.

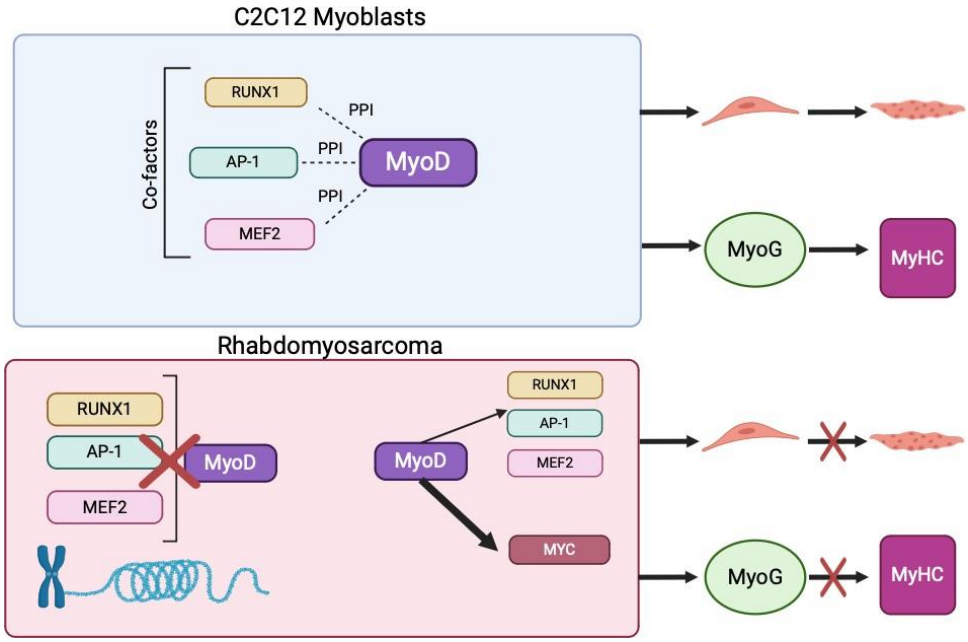


Figure 8. Mutations impacting myogenesis in RMS. MyoD protein-protein interactions are crucial for regulating myogenesis. Under normal C2C12 conditions, MyoD primarily interacts with RUNX1, AP-1, and MEF2, allowing proper progression of myogenesis with the expression of core transcription factors such as MyoG and subsequent MyHC. However, in rhabdomyosarcoma, chromatin remodeling makes interactions between MyoD and RUNX1, AP-1, and MEF2 unlikely, causing MyoD to preferentially bind to MYC. This disrupts myogenesis by preventing the expression of transcription factors MyoG and MyHC, leading to the inability of muscle cells to multinucleate and terminally differentiate.

YAP/TAZ regulation in muscle cancers

YAP/TAZ function in driving proliferation as they are often upregulated in many cancers and are the target of cancer treatments (Mohamed et al., 2016; Luo et al., 2023; Piccolo et al., 2023). Their activity positively correlates with higher risks of metastases, malignancy, chemoresistance, and overall lower survival (Piccolo et al., 2023). Hyperactive YAP/TAZ also has been seen to provide

metabolic competitive advantages in nutrient limiting states through the upregulation of glucose and amino acid transporters. This makes YAP/TAZ desirable candidates for upregulation in nutrient limited environments such as the tumor microenvironment (Cunningham et al., 2022).

Cancer can be driven by YAP/TAZ overexpression in both mouse and human models. This can include hormone-based cancers such as breast cancer (Luo et al., 2023), prostate cancer (Luo et al., 2023), colo-rectal cancer (Piccolo et al., 2023), soft tissue cancer, osteosarcomas (Kovar et al., 2020), and rhabdomyosarcomas (Deel et al., 2018). For example, in rhabdomyosarcoma, RD and RH30 cell lines have increased nuclear TAZ expression in relation to control and have an overall poorer survival rate (Mohamed et al., 2016). In RH18 and RH30 cell lines, the knockout of TAZ induces an upregulation of myogenic regulatory factors including MyoD, and MyoG (Deel et al., 2018). In mouse rhabdomyosarcoma xenografts models, suppression of TAZ delays tumor progression and promotes survival (Deel et al., 2018). Further, TAZ and TAZS89A, a hyperactiveform, overexpression leads to increased cellular proliferation in myoblasts (Mohamed et al., 2016).

Furthermore, Hippo signaling is known to interact and affect other pathways involved in regulation such as the Notch signaling pathway in embryonic rhabdomyosarcoma (Slemmons et al., 2017). In these cell lines both YAP1 and Notch signaling pathways are known to reciprocally activate one another which in turn boosts stem cell gene expression leading to development and disease progression (Slemmons et al., 2017). In summary, this research indicates that YAP/TAZ expression is upregulated in rhabdomyosarcoma models and impacts the ability myogenic regulatory factor expression and function and therefore should be further evaluated for therapeutic implications.

Statement of purpose

The Hippo signaling pathway serves as a key regulator of tissue homeostasis by orchestrating various pathways that shape the transcriptome (Zheng et al., 2019). TAZ, downstream of this highly conserved pathway, undergoes regulation. When the Hippo signaling pathway is off, TAZ remains unphosphorylated allowing it to translocate to the nucleus where it elicits its function on downstream target genes (Zhao et al., 2007; Hao et al., 2008; Yu et al., 2013). When Hippo signaling is activated, TAZ is phosphorylated and remains sequestered in the cytoplasm (Zhao et al., 2007, Hao et al., 2008). Unlike other proteins, TAZ does not bind to DNA; instead, it relies on interactions with other proteins to elicit its function (Kim et al., 2018). Therefore, understanding and classifying the TAZ interactome is essential to unravel TAZ function. Recent findings from the McDermott lab have identified TAZ as a repressor of myogenic differentiation in myoblast cells, highlighting the potential importance of the Hippo pathway in several muscle contexts (Tripathi et al., 2022). Myogenesis is a fundamental process involved in muscle development that involves TAZ specifically enhancing proliferation in myoblasts (Tripathi et al., 2022). TAZ has also been implicated in the formation and progression of various cancers, specifically rhabdomyosarcoma. Thus, understanding its interactions with other proteins in various contexts can help one understand how TAZ is regulated and search for potential targets for its regulation (Kovar et al., 2020). ***The purpose of the study was therefore to identify and characterize protein-protein interactions with TAZ in myogenic cells and further characterize these protein interactions in regulated and deregulated myogenic contexts.***

Chapter II: The TAZ protein interactome in myogenic cells

Anastasia MacKeracher, Jonathan Kelebeev, Tetsuaki Miyake, and John C. McDermott

Author contributions

Experimental Design

Anastasia MacKeracher, Jonathan Kelebeev, Tetsuaki Miyake, and John C. McDermott

Drafting manuscript

Anastasia MacKeracher, Jonathan Kelebeev

Editing manuscript

Anastasia MacKeracher, Jonathan Kelebeev, Tetsuaki Miyake, and John C. McDermott

Conducting experiments

Figure 1: Anastasia Mackeracher and Joanthan Kelebeev

Figure 2: Anastasia MacKeracher

Figure 3: Anastasia MacKeracher

Figure 4: Anastasia MacKeracher

Figure 5: Anastasia MacKeracher

Figure 6: Anastasia MacKeracher

Figure 7: Anastasia MacKeracher

Figure 8: Anastasia MacKeracher

Abstract

Hippo signaling serves as a critical regulator of cell proliferation, differentiation, and apoptosis. Recent studies have characterized the Hippo signaling downstream target protein TAZ as a repressor of myogenic differentiation. Since TAZ lacks inherent DNA binding ability itself, its function and activity change depending on its protein interactions. Previous research characterized TAZ interacting proteins in muscle cells. Employing a GFP-nanoTrap affinity purification strategy coupled with LC-MS/MS protein identification a list of both novel and previously characterized TAZ protein interactions in a myogenic context were documented. Notably, the pro-myogenic methyl transferase protein, CARM1, was identified as a TAZ interacting protein. Further investigation into this interaction revealed the functional role of the TAZ/CARM1 interaction on the Hippo signalling pathway. Utilizing a Hippo pathway (HOP/HIP) gene reporter assay we observed that CARM1 represses TAZ transcriptional activation function, promoting TAZ Ser89 phosphorylation, leading to the cytoplasmic sequestration of TAZ. The TAZ/CARM1 protein interaction was further investigated in the dysregulated context of embryonic rhabdomyosarcoma cells (RD) in which the TAZ/CARM1 interaction and function was maintained. Overall, Hippo signaling was found to increase compared to control C2C12 myoblasts in RD cells. Further, mass spectrometry analysis was conducted and revealed that TAZ may be a substrate of CARM1 since methylation at amino acid Arg 77 in a PGPR*LAGG consensus peptide was identified and resulted in enhanced TAZ Ser89 phosphorylation. These findings underscore a possible regulatory role of CARM1 in regulation of TAZ in myogenic cells.

Introduction

Skeletal muscle constitutes over one third of human body weight (Mukund et al., 2020). Beyond facilitating voluntary movement, it also serves crucial functions in maintaining posture, storing nutrients, respiratory mechanics, and stabilizing joints (Mukund et al., 2020). As the most abundant tissue in the body, skeletal muscle is important for everyday function and is integral in the ageing process (Karagounis et al., 2010). Skeletal muscle is dynamic and undergoes rapid turnover of cells capable of proliferating quickly and responding to injury. However, skeletal muscle is also subject to atrophy which is characterized by the weakening and thinning of muscle fibers which can occur independently or as a consequence of many diseases such as muscular dystrophies and muscle myopathy (Powers et al., 2016).

To combat this, most vertebrates have an evolutionarily conserved mechanism to respond to stress and injury associated with muscle diseases and weakening. This process is known as post-natal myogenesis, where PAX7-expressing satellite cells residing between the basal lamina and sarcolemma activate and begin to proliferate to restore the injured musculature (Yin et al., 2013). As a result, these cells start to proliferate and express early myogenic markers such as MyoD and Myf5, which are required to induce cellular differentiation, regulate the proliferation rate of myogenic cells, and maintain homeostasis, respectively. Once there is sufficient cellular material, cells will begin expressing later stage MRFs such as MyoG and MRF4 that are necessary for the formation of myotubes and more developed muscle fibers (Le Grand et al., 2007). Therefore, the ability for muscle to regenerate is of utmost importance for proper body function and overall well-being.

The Hippo signaling pathway is a key regulator of homeostasis, balancing proliferation, differentiation, and apoptosis (Zheng et al., 2019). It has recently gained attention for its

involvement in muscle regeneration (Sun et al., 2017). Downstream co-activators of the Hippo signaling pathway have been identified to play a role in muscle and their regulation on this pathway is integral to functional muscle regeneration (Sun et al., 2017). When Hippo signaling is activated, cell proliferation is restricted by upstream serine/threonine phosphorylation kinases MST1/2 and LATS1/2 which phosphorylate YAP/TAZ on Ser127 and Ser89 respectively, leading to cytoplasmic sequestration or degradation of YAP/TAZ (Meng et al., 2016). However, when upstream cues promote proliferation, the Hippo signaling pathway is inactivated leading to the reduction or elimination of serine phosphorylation of YAP/TAZ, thus allowing for the translocation of TAZ from the cytoplasm to the nucleus where it can interact with many factors to promote transcriptional activation (Totaro et al., 2018). The most common binding partner for TAZ in the nucleus are TEADs (1-4) which are DNA binding transcription factors that most commonly lead to activation of proliferative target genes (Pobbati et al., 2020; Hong et al., 2012; Kim et al., 2018).

In a muscle context, Hippo signaling has been shown to exert tight control over renewal, differentiation, and muscle regeneration as it was discovered as important in both muscle fiber and satellite cell populations size (Kaya-Çoupur et al., 2021). Components of the Hippo signaling pathway such as the downstream target protein YAP have been known to positively regulate muscle mass and protein synthesis via the TEAD binding transcription factor in muscle cells. YAP has also been reported to increase in response to injury due to the loss of muscle mass because of dysfunction of the nervous system (Watt et al., 2015). Furthermore, YAP/TAZ is thought to regulate both proliferation and differentiation in muscle related diseases such as sarcopenia in which YAP/TAZ may have therapeutic potential (Setiawan et al., 2021). In addition, YAP/TAZ have been reported to be positive regulators of myoblast proliferation, as ectopic expression of

mutated YAP/TAZ (Ser127A and Ser89A) have been shown to have hyperproliferative properties (Mohamed et al., 2016).

Post translational modifications are imperative to the proper function of proteins and dysregulation of these modifications can cause dysfunction and disease (Radivojac et al., 2008). The downstream regulators of Hippo signaling, YAP/TAZ, are heavily regulated by post translational modifications such as phosphorylation and methylation which regulate both the stability and subcellular localization of YAP/TAZ function in various contexts (Oudhoff et al., 2013; Chen et al., 2020). The function of TAZ methylation is not well understood in muscle; however, it has been explored in some cancer models. For example, in multiple myeloma, TAZ hypomethylation upregulates its target gene MYC, which induces proliferation. In contrast, demethylating the TAZ promoter induces apoptosis (Grieve et al., 2019). Furthermore, in cancer patients methylation levels of the TAZ promoter were higher than control samples, suggesting that TAZ overexpression may be driving cancer formation and progression (Han et al., 2021). Therefore, understanding the methylation profile of YAP/TAZ may contribute to the understanding of its regulation. This may be useful in understanding TAZ function in the context of muscle cancer and could be important for therapeutic intervention. Furthermore, dysregulation of the post-translational modifications (PTMs) may be integral to proper function of YAP/TAZ and investigating changes and modifications in these modifications may allow us to gain further insight into their function.

YAP and TAZ are often grouped together due to their similar function, however there are notable differences in their biological roles (Reggiani et al., 2021). In the context of myogenesis, YAP has been shown to be a pro-proliferative factor and inhibit the differentiation of multinucleated myotubes (Sun et al., 2017). Furthermore, TAZ has been shown to change its role as either a co-activator or co-repressor depending on the protein network it associates with (Hau et al., 2013;

Kim et al., 2018). As such, the McDermott lab has previously conducted novel GFP- nanotrap based affinity purification assay followed by a LC MS/MS protein identification analysis to identify a list of TAZ interacting protein in a myogenic context.

From this interactome list, a list of known and novel TAZ interactors was established. One of the novel protein candidates that we found interacting with TAZ through this screen was CARM1, a known an important regulator in muscle. We observed that CARM1 has a repressive role in regulating TAZ function both on the Hippo signaling pathway and in a myogenic context. We further characterized TAZ in the context of rhabdomyosarcoma and documented that some TAZ interactions and function was maintained. Further mass spectrometry analysis revealed that TAZ is a substrate of CARM1 methylation at amino acid Arg 77 within a PGPR*LAGG consensus peptide. This methylation appears to potentiate TAZ Ser 89 phosphorylation. These findings imply a functional interaction between TAZ and CARM1 that may lie at the intersection of proliferation and differentiation signals in myogenic cells.

Materials and methods

Cell line culture

C2C12 myoblasts, HEK293T cells, Neonatal rat cardiomyocytes (NRCMs), and Embryonic Rhabdomyosarcoma cells (RD cells) were obtained from the American Type Culture Collection (ATCC). Cell lines 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). C2C12 myotube formation was induced by replacing GM with differentiation medium (DM), consisting of DMEM supplemented with 2% FBS and 1% penicillin-streptomycin. Cells were maintained in an incubator at 95% humidity, 5% CO_2, and 37 °C and replenished with fresh medium every 48 hours.

Transfections

For ectopic protein expression in HEK293T cells were transfected using polyethyleneimine at a PEI:DNA ratio of three. C2C12 and RD cells were transfected with lipofectamine 2000 (Life Technologies) at a lipofectamine: DNA ratio of three. All cells were re-fed 16 hours post-transfection and harvested the next day.

Cell harvesting

C2C12, RD, HEK293T, and NRCM cells were harvested using NP-40 lysis buffer or, if destined for gene reporter assay, with 1x reporter lysis buffer (Promega, #E4030). Cells were washed three times with cold PBS before adding in lysis buffer. Lysates were collected and vortexed at 4 °C for 15 minutes. Lysates were then centrifuged at 4 °C, 10 000 RPM for 10 minutes, followed by the collection of the supernatant constituting the soluble protein.

Western blot analysis

Protein samples were denatured in 3x SDS loading buffer at 100°C for 10 min. Samples were then loaded onto 10% SDS-PAGE gels and ran at 100V for approximately 1 hr. The gel was transferred to a PVDF membrane (Millipore) in 1X transfer buffer and blocked in 5% blocking buffer (Table 1) for 1 hr on an orbital shaker at room temperature. Primary antibodies were prepared by diluting the indicated antibody (Table 2) in 1% blocking buffer (1:1000). Membranes were probed with primary antibody solutions overnight at 4°C on a rocker. After brief washing with TBS supplemented with 0.1% Tween® 20 (TBS-T), blots were incubated with corresponding HRP-conjugated secondary antibody in 1% blocking buffer (1:2000) for 1 hr at room temperature. After three washes with TBS-T, protein/antibody immune-complexes were detected by incubating blots in HRP substrate (Bio-Rad) and exposing them to an iBright CL1500 Imaging System (Thermo Fisher Scientific).

Immunoprecipitation (IP)

For endogenous IP, Dynabeads Protein G (Invitrogen, #10003D) were washed three times with PBS before incubation with YAP/TAZ antibody (Cell Signaling, #D24E4; diluted to 1:2000 with 1X PBS) or Rabbit IgG (Cell Signaling; #2729; diluted to 1:10000 with 1X PBS) on a rotator at 4°C overnight. Beads were washed three times with PBS to remove unbound antibody. Dynabeads were incubated with 1 mg of C2C12 myoblast extracts on a rotator at 4 °C overnight. Beads were then washed three times with PBS to remove unspecific protein binding. Samples were eluted by heating with SDS loading buffer (Table 1) for 10 mins at 95 °C and analyzed by western blot. For FLAG co-IPs, anti-FLAG M2 magnetic beads (Sigma, #M8823) were washed

three times with PBS before incubation with 500 ug of HEK293T cell extracts on a rotator at 4 °C overnight. Beads were then washed three times with PBS to remove unspecific protein binding. Immunoprecipitants were eluted in 500 μg/ml FLAG 3x peptide solution (Sigma, #4799) on a rotator at room temperature for 30 minutes and analyzed by western blot.

Cell fractionation

C2C12 cells were grown in GM until 90% confluency and then in DM for 4 days. Myotubes were isolated by incubating the cells with 1% cold trypsin for 30 seconds and removing the detached myotubes. Remaining reserve cells were removed using a cell scraper. Nuclear and cytoplasmic fractions of the myotube and reserve cells fractions were harvested using the NE-PER kit (Thermo Scientific, #78833), according to manufacture instructions.

Immunofluorescence

C2C12 cells were seeded on glass-bottom dishes (MatTek) and washed with cold PBS three times before fixation with 4% paraformaldehyde at room temperature for 10 minutes. Cells were washed with PBS and permeabilized with ice-cold 90% methanol on ice for 5 minutes. Cells were washed three times with PBS and incubated with IF blocking buffer (5% FBS in PBS) for 1 hour and 30 minutes at room temperature, then incubated with indicated primary antibody in IF blocking buffer at 4 °C overnight. Cells were washed the next day to remove unbound antibody and incubated with Alexa fluor conjugated secondary antibody (Life Technologies) in IF blocking buffer for 1 hour and 30 minutes. Cells were washed three times with PBS and counter-stained with Hoechst 33342 for 10 minutes. Cells were subjected to imaging with a Zeiss Observer Z1 confocal fluorescent microscope equipped with Yokogawa CSU-XI spinning disk. Images were recorded by AxioCam MRm camera (Zeiss) and processed by Zen 2.5 (blue edition) software (Zeiss).

Reporter gene assays

Transcriptional reporter assays were performed using luciferase reporter plasmids along with expression constructs (indicated in figure legends) and a Renilla plasmid (Promega, pRL-Renilla), as an internal control. Cells were washed with cold PBS and harvested in Luciferase Lysis Buffer with 1X reporter lysis buffer (Promega, #E4030). Enzymatic activity was measured in each sample on a luminometer (Berthold, Lumat LB) using Luciferase assay substrate (Promega, #E1501) or Renilla assay substrate (Promega, #E2820). Luciferase activity values were normalized to Renilla activity in the same cell extracts and expressed as fold activation to the control.

Mass Spectrometry

FLAG-TAZ (addgene plasmid #24809) and HA-CARM1 (addgene plasmid # 81118) plasmids were transfected into HEK293T cells and or C2C12 in both growth media and 48hr differentiation media and harvested the next day with NP40 lysis buffer. 1 mg of protein lysate was incubated with 40 µL of anti-FLAG M2 magnetic beads (Sigma, #M8823) overnight in a rotator at 4 °C. Beads were washed twice the following day for 5 mins with 0.1% acetic acid. The lysate was reduced by 1M DTT (Dithiothreitol) was added to the samples to a final concentration of 10 mM and heated at 60°C for 30 mins. The sample was then alkylated by Idoacetamide was subsequently added to the sample to a final concentration of 40 mM and incubated in the dark at room temperature for 15 mins. 1M DTT was then added to a final concentration of 40 mM. Next the sample was digested by 1 mg/mL of trypsin was added to the sample and incubated overnight at 37 °C. Sample supernatant was then transferred into a new tube processed using the timsTOF Pro 2 mass spectrometry instrument by the MS Facility at YSci core at York University.

LC-MS/MS experiments have been performed on a TimsToF Pro 2 (Bruker) with a nanoElute 2 and the HyStar software suite. Separation has been performed on a C18 column (PepSep Ten

unlimited series) of 10 cm x 150 µm with a particle size of 1.5 µm and a pore size of 100 Å. A trap column was used (PepMap TM C18, Themo Scientific) of 5 mm x 300 µm with a particle size of 5 µm and a pore size of 100 Å. Separation has been performed using a two-column separation method in HyStar including solvent preparation, column equilibration and sample loading steps. Solvents used were water + 0.1 % formic acid (mobile phase A) and acetonitrile + 0.1 % formic acid (mobile phase B). The gradient started at 2 % B to 35 % B in 45 minutes, then to 95 % B in 0.5 min for 1.68 min. The flow rate was 0.5 µL/min throughout the run. Data folder were processed using PaSER 2023 using the "DDA – ProluCID-GPU" search engine and the Uniprot human proteome database. For the GFP nano-trap mass spectrometry protocol please visit Jonathan Kelebeev's thesis manuscript.

Antibodies

Antibodies for YAP/TAZ (rabbit, polyclonal, #D24E24), pTAZ Ser89 (rabbit, monocloncal, #59971) HA (rabbit, polyclonal, #C29F4), Myc (mouse, monoclonal, #9B11), alpha-Tubulin (rabbit, polyclonal, #2144S), c-myc (rabbit, monoclonal, #5605), CARM1 (rabbit, polyclonal, #4438), CARM1 (mouse, monoclonal, #12495), and Histone H3 (rabbit, polyclonal, #9715S) were purchased from Cell Signaling. MyoG (mouse, monoclonal, #F5D) and β-Actin (mouse, monoclonal, #sc-47778) antibodies were purchased from Santa Cruz Biotechnology. Antibodies for FLAG (Mouse, monoclonal, #F3165) were purchased from Sigma-Aldrich, Vimentin (Rabbit, polyclonal Cell signaling #D21H3), and mono and dimethyl arginine antibody (mouse, monoclonal, ab412) were purchased from abcam.

Plasmids

Myc-TEAD4 was a gift from Kunliang Guan (Addgene plasmid #24638). FLAG-TAZ was a gift from Jeff Wrana (Addgene plasmid #24809). HA-TAZ was a gift from Kunliang Guan (Addgene

plasmid #32839). HA-TAZ □ 72-104 was obtained from Addgene (Addgene plasmid #32839) and manually deleted amino acids 72-104 from the plasmid in the McDermott lab. pcDNA3 was a gift from William Sellers (Addgene plasmid #10792). HA-CARM1 was a gift from Sung Hee Baek (Addgene plasmid #81118). HA-TAZS89A was obtained from addgene (Addgene plasmid #32840). HOP- Flash and HIP-Flash (HOP-flash mutant) were gifts from Barry Gumbiner (Addgene plasmid #83467 & #83466). Myogenin core promoter was a gift from Michael Chin (Addgene plasmid #134722). Renilla (pRL-Renilla) plasmid was purchased from Promega.

Results

Affinity purification- LC MS/MS analysis of the TAZ interactome in striated muscle cells

TAZ is an important regulator of gene transcription but lacks the ability to bind to DNA directly. As such, TAZ function is determined by the proteins it interacts with (Kim et al., 2018). Based on previous literature, TAZ is a known repressor of myogenesis (Tripathi et al., 2022), therefore we aimed to characterize protein-protein interactions with TAZ in nuclear enriched fractions of striated muscle. Utilizing a novel GFP- nanoTrap technology we developed, we were able to capture interacting proteins in cell lysates. This consisted of incubating EYFP-TAZ and control EYFP proteins immobilized on ChromoTek GFP-nanotrap beads conjugated with GFP binding protein (GBP) nanobodies. Following this, the immobilized bait constructs were incubated with fractionated myogenic extracts. Unlike traditional immunoprecipitation assays, this approach allows for the identification of myogenic proteins in their endogenous states. A schematic of this process is illustrated in Figure 1A. In addition, a western blot depicting the overexpression of these constructs is shown in supplementary figure 1A.

After completing the GFP- nanoTrap assay, proteins were identified as "of interest" when one of two criteria were met. The first criteria, "unique proteins" included proteins that were identified in the experimental EYFP- TAZ condition but not in the control GFP condition. The second group of proteins were 3-fold enriched using a ($EYFP\text{-}TAZ_{spectra}$ / $GFP_{spectra}$) ratio calculation. This ratio was determined by the threshold of known interactors appearing in each dataset at that enrichment level. In the C2C12 dataset, 249 proteins were identified in the unique and enriched groups. In the cardiomyocyte data sets there were 219 proteins in both groups. In addition, there were 97 overlapping proteins in both datasets (Supplementary Figure 1B). Further, proteins were disregarded when they appeared in the GFP control data set but not in the EYFP- TAZ

experimental condition. The raw data further breaking down the proteins identified in C2C12 myoblasts and neonatal rat cardiomyocytes screens is shown in supplementary figure 1C. A detailed list characterizing the proteins identified in this experiment using these criteria is shown in figure 1B. This list includes categories including known interactors such as components of the Hippo signaling pathways including TEAD 1, TEAD 4, 14-3-3 binding factors, LATS1 kinase, as well as novel categories including chromatin interacting complexes and transcriptional regulators such as CARM1. A full list of all the proteins identified in the mass spectrometry analysis is documented in supplementary figure 1D. Further, a schematic placing the known protein interactors in the context of the Hippo signaling pathway is shown in figure 1C. After identifying a list of novel proteins, a functional assay was conducted to investigate the effect of ectopic expression of novel protein interactors on TAZ function using the Hippo signaling pathway reporter gene assay HOP. These proteins included CCAR2, NME1, PUM1, and CARM1, which were tested with TAZ on the luciferase reporter system. On this reporter system ectopically expressed CARM1 demonstrates repression of TAZ activation on the reporter system (Figure 1D).

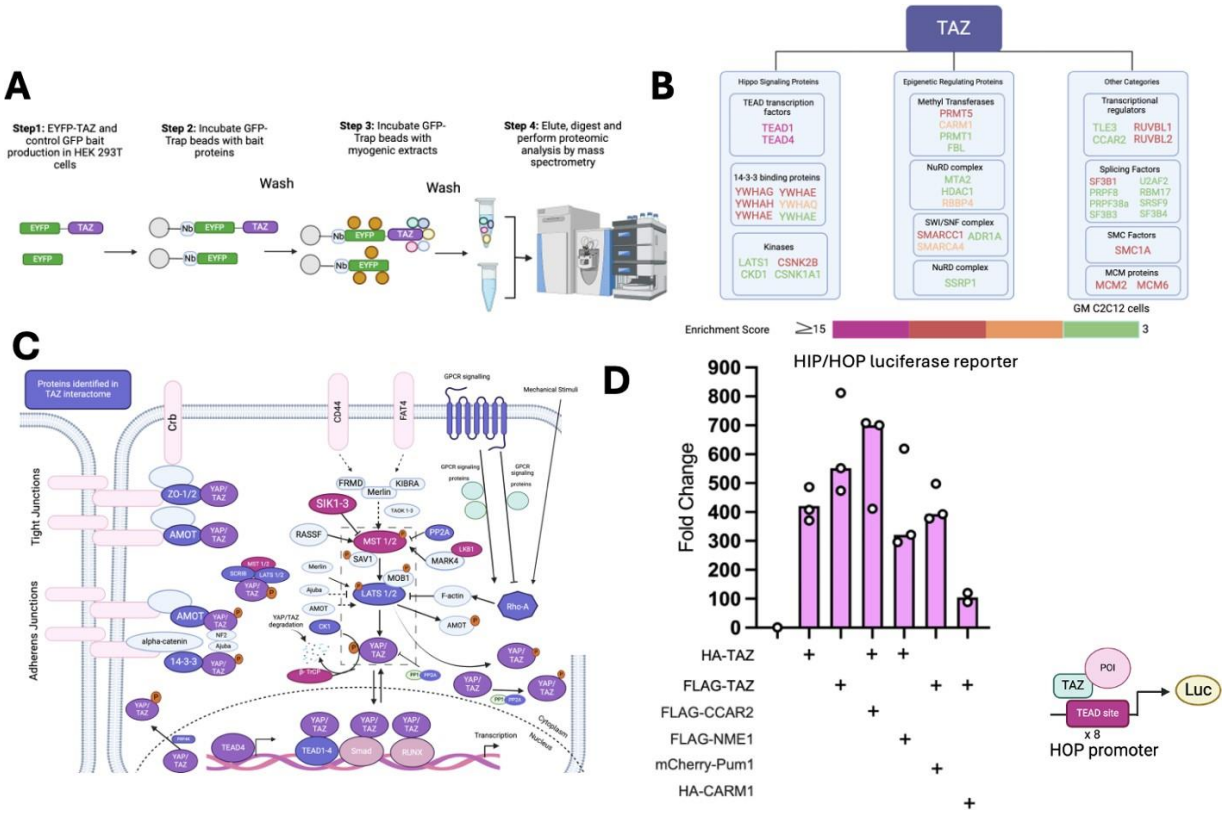


Figure 1. Interactome study of TAZ in striated muscle. (A) Schematic overview of GFP-nanoTrap co-immunoprecipitation method to identify TAZ protein partners with nuclear C2C12 and whole-cell neonatal rat cardiomyocyte (NRCM) extracts. **(B)** Flow chart indicating the number of total and enriched TAZ protein candidates identified in the C2C12 myoblast dataset. Dataset also indicates the enrichment score of each protein in the dataset by a colour coded indicator **(C)** Schematic showing proteins (colored in purple) representing known components of Hippo signaling pathway that were identified in the C2C12 TAZ interactome. **(D)** FLAG-TAZ alone and combined with FLAG-CCAR2, FLAG-NME1, mcherry- PUM1, and HA-CARM1 were ectopically expressed with the HOP luciferase reporter gene. *Renilla* luciferase served as a transfection control. Transfection with an empty vector (pcDNA3) served as a control for endogenous activity (lane 1). The luciferase experiment was conducted n=1 times with 3 technical replicates done indicated by the white points on the graph.

Bioinformatic analysis of the TAZ interactomes in C2C12 myogenic cells

To determine if the CARM1-TAZ interaction warranted further investigation in a skeletal myogenic context, we conducted a bioinformatic analysis on known overlapping CARM1 and TAZ interacting proteins. To do this a dataset was obtained from the Biogrid database containing

known protein interactors of CARM1. Some known protein interactors of CARM1 included pro-myogenic factors such as PAX7, MYOD, MEF2. We compared these known protein interactors with proteins identified in the TAZ GFP-nanotrap screen (C2C12) screen. These proteins include SMARCA4, SMARCC1, ARID1A, DNAJA3, CNN3, FASN, PDCD61P, CAND1, SEC23IP, PRMT5, ACLY, SYNC1H1, CARM1, EFTUD2, GASTA1, GSPT1, HSPH1, IMPDH2, RAB5A, RAD50, RAB1A, RAB5C (22 proteins). There was a total of 306 proteins that were identified in the CARM1 screen and 231 only identified in the C2C12 screen (Figure 2A). From this, the overlapping proteins (22) found in both data sets were put into the G profiler database to determine both the molecular pathways (Figure 2B) and biological processes (Figure 2C) that are associated with TAZ and CARM1 overlapping protein interactions. Using the G profiler database, a -log score was generated for each molecular function and biological process indicating the level of confidence the system predicts that these predicted proteins contribute to the function of that particular function (Supplementary Figure 2A and B). Key molecular functions identified include transcription co-activator and co-regulator activity, protein arginine N-methyltransferase activity, and organic cyclic compound binding. Key biological processes include categories including cell cycle regulation, myogenesis, and immune response and repair.

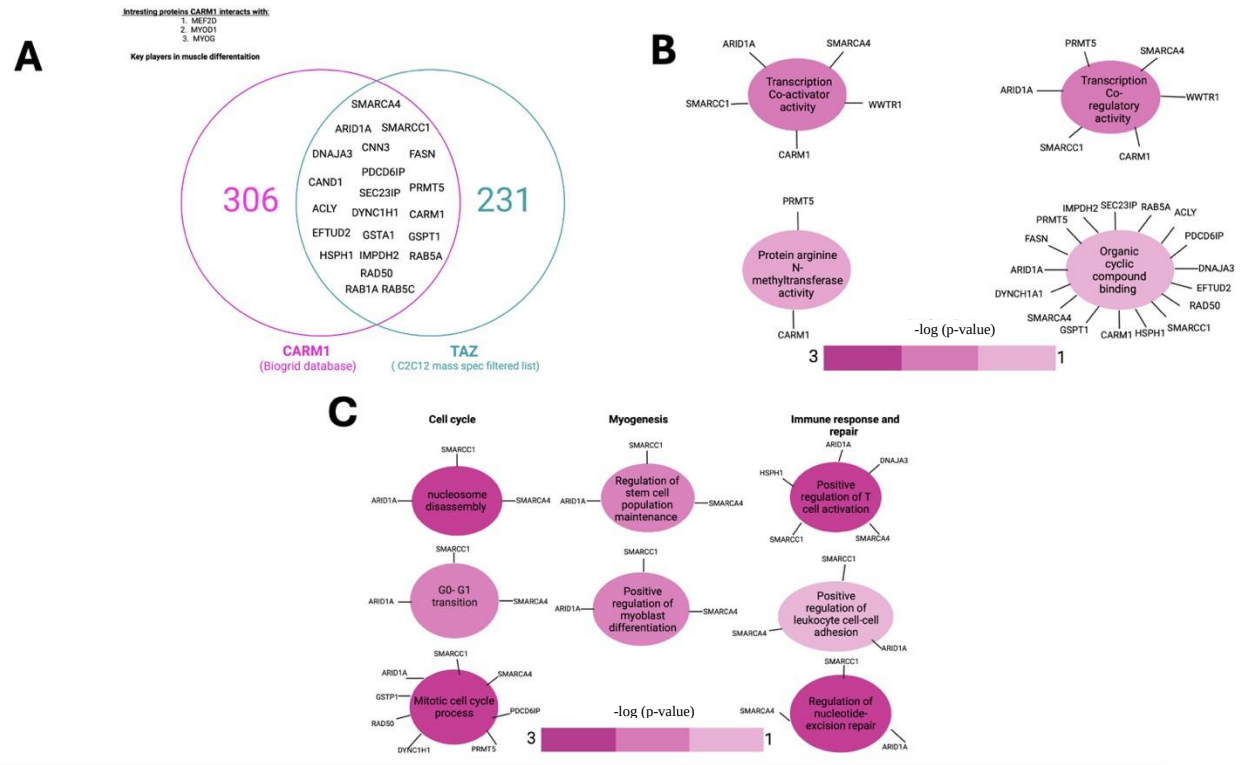


Figure 2. Bioinformatic analysis of the TAZ and CARM1 interacting proteins. (A) Venn diagram depicting the common overlapping proteins identified to interact with both CARM1 from the Biogrid database and TAZ from GFP-nanoTrap screen in C21C12 myoblasts. (B) Molecular functions of CARM1 and TAZ overlapping proteins identified by the G-profiler database accompanied by a -log(p-value) score indicating the likelihood of the molecular function associated with the list of proteins. (C) Biological processes of CARM1 and TAZ overlapping proteins identified by the g-profiler database accompanied by a -log(p-value) score indicating the likelihood of the biological processes associated with the list of proteins.

Biochemical validation of the TAZ interactome and functional relevance of the TAZ/CARM1 interaction

To validate novel protein-protein interactions identified in the interactome we conducted co-immunoprecipitation assays between the identified interacting proteins and FLAG-TAZ in HEK293T cells. A positive control was established using known interactors of TAZ including the

TEAD4 transcription factor (Hau et al., 2013). Conformation of this interaction was displayed by FLAG- coimmunoprecipitation (Figure 3A). Next, the interaction between CARM1 and TAZ was biochemically validated through ectopic expression of FLAG-TAZ and HA-CARM1 constructs (Figure 3B). Furthermore, given the role of TEAD4 as a key DNA binding protein and a tether for TAZ to exert its function on the Hippo signaling pathway we aimed to assess if the TAZ-CARM1 interaction may be mediated in a TEAD dependent manner (Pobbati et al., 2020; Hong et al., 2012; Kim et al., 2018). To investigate this, Myc- TEAD4 was ectopically expressed with FLAG-TAZ alone and with FLAG-TAZ and HA- CARM1 in HEK293T cells. These assays showed an enriched level of TEAD4 in TAZ precipitates when CARM1 is ectopically expressed, indicating that TEAD4 may also be involved in the biochemical interaction between CARM1 and TAZ (Figure 3C). We next explored the functional implications of the TAZ and CARM1 interaction and investigated the function of the CARM1/TAZ interaction on the TEAD dependent reporter HOP/HIP luciferase gene reporter assay. These data indicate that CARM1 represses the TAZ driven activation of this reporter system in HEK293T cells (Figure 3D). We then investigated the endogenous co-localization of both the TAZ and CARM1 protein in C2C12 and rhabdomyosarcoma cell lines in growth media conditions. We found that both TAZ and CARM1 remained enriched in the nucleus in both cell lines (Figure 3E). These findings collectively suggest that the interaction between CARM1 and TAZ most likely occurs in the nucleus and TAZ mediated repression on HOP/HIP luciferase may be mediated through a TEAD4-dependent mechanism.

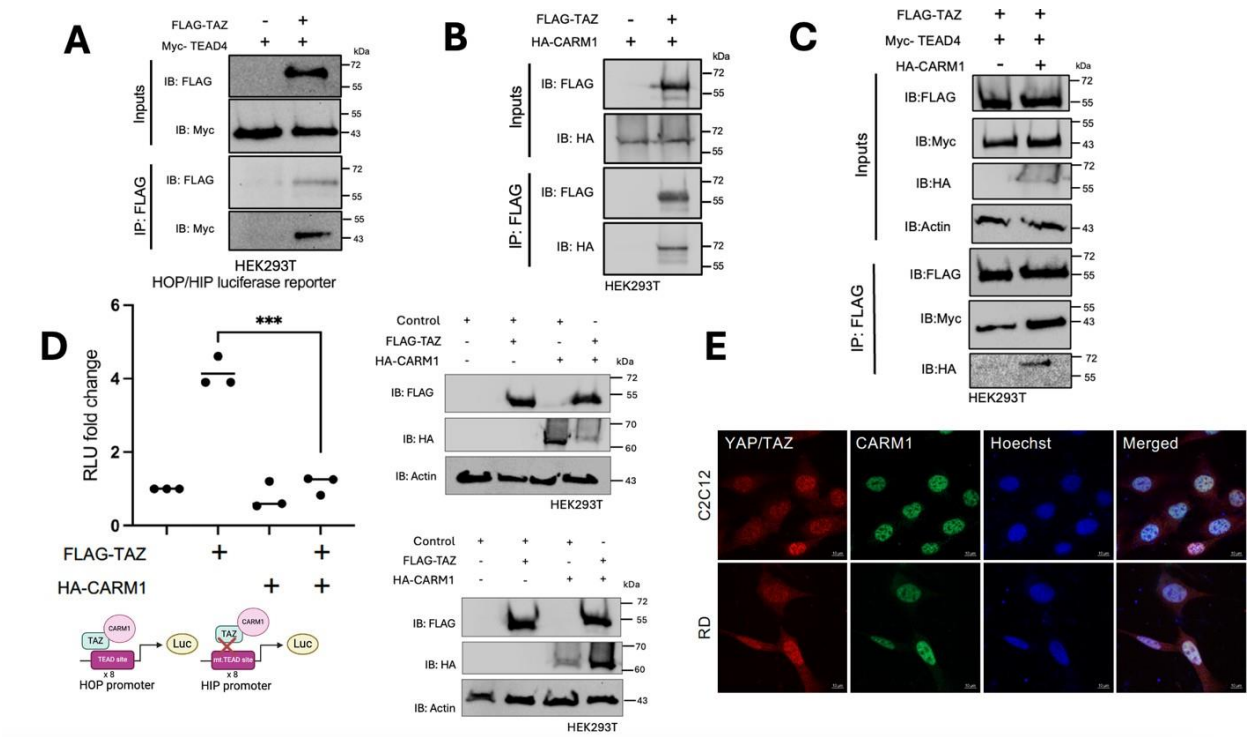


Figure 3. Biochemical validation of the TAZ interactome. (A) HEK293T cells were transfected with Myc-TEAD4 with/without FLAG-TAZ and subject to FLAG immunoprecipitation with anti-FLAG beads. Eluates were analyzed using immunoblotting for contents of co-immunoprecipitated Myc-TEAD4 to serve as positive controls for the co-immunoprecipitation analysis. **(B)** HEK293T cells were transfected with HA-CARM1 with/without FLAG-TAZ, and subject to FLAG immunoprecipitation with anti-FLAG beads. Eluates were analyzed using immunoblotting for contents of co-immunoprecipitation with FLAG-TAZ. **(C)** HEK293T cells were transfected with FLAG-TAZ, and MYC- TEAD4 with/without HA-CARM1 and subject to FLAG immunoprecipitation with anti-FLAG beads. Eluates were analyzed using immunoblotting for contents of co-immunoprecipitation with FLAG-TAZ, HA-CARM1 and, MYC-TEAD4. **(D)** FLAG-TAZ alone and combined with HA-CARM1 were ectopically expressed with the HOP luciferase reporter gene (normalized to HIP reporter as a negative control). Renilla luciferase served as a transfection control. Transfection with an empty vector (pcDNA) served as a control for endogenous activity. This luciferase reporter experiment was conducted n=3 times. Unpaired t-test using GraphPad Prism 10.0 was utilized to test for statistical significance. Adjusted p-value **p<0.01, ***p<0.001, ****<0.0001 are indicated for significance compared to the relevant control condition. **(E)** Immunofluorescence analysis of C2C12 cells during myoblast (GM) stage and embryonic rhabdomyosarcoma (RD) cells. Cells were immuno-stained for YAP/TAZ (red), CARM1 (green) and counterstained with Hoechst 33342 to indicate nuclei (blue).

CARM1 antagonizes TAZ mediated repression of the myogenic program in striated muscle cells

CARM1 promotes myogenesis by interacting with key proteins involved in myogenic progression, such as PAX7 and MEF2C (Kawabe et al., 2012; Chen et al., 2002). Therefore, we aimed to further characterize the role of CARM1 on TAZ regulation within a myogenic context. We investigated the interactions between TAZ and CARM1 using the C2C12 myoblast model of differentiation. We then assessed these interactions by measuring MyoG levels, a well characterized marker of myogenic differentiation commitment (Faralli et al., 2012). First, we explored the endogenous localization of the TAZ and CARM1 protein in differentiation conditions through immunofluorescence imaging. We found that TAZ localization remained enriched in the nucleus in growth media and DM 48hr conditions, with a slight shift in localization during DM 96 hr, becoming less enriched in the nucleus (Figure 4A). However, when investigating the endogenous localization of CARM1 throughout differentiation, CARM1 remained enriched in the nucleus (Figure 4B). Next, we aimed to functionally assess the interaction of TAZ and CARM1 on the *myogenin* promoter-driven luciferase reporter gene assay in C2C12 cells differentiated for 48hrs. Consistent with previous results, we found that TAZ repressed the myogenic reporter assay when ectopically expressed (Tripathi et al., 2022). In addition, we found that when TAZ and CARM1 were co-ectopically expressed CARM1 overexpression significantly counteracted TAZ repression of the *myogenin* gene reporter bringing the activation back to basal levels (Figure 4C). To further characterize the role of CARM1 in myogenesis, C2C12 cells that have been differentiated for 48hr were treated with a selective inhibitor of CARM1 methyltransferase activity. These data indicated a decreased fraction of cells expressed MyoG when treated with an allosteric inhibitor of CARM1

methyltransferase activity compared to the control DMSO condition. Similar trends were observed in protein quantification analysis. (Figure 4D-F). A schematic depicting the overall changes in myogenesis we observe upon the ectopic expression of HA-CARM1 and FLAG-TAZ is illustrated in Figure 4G.

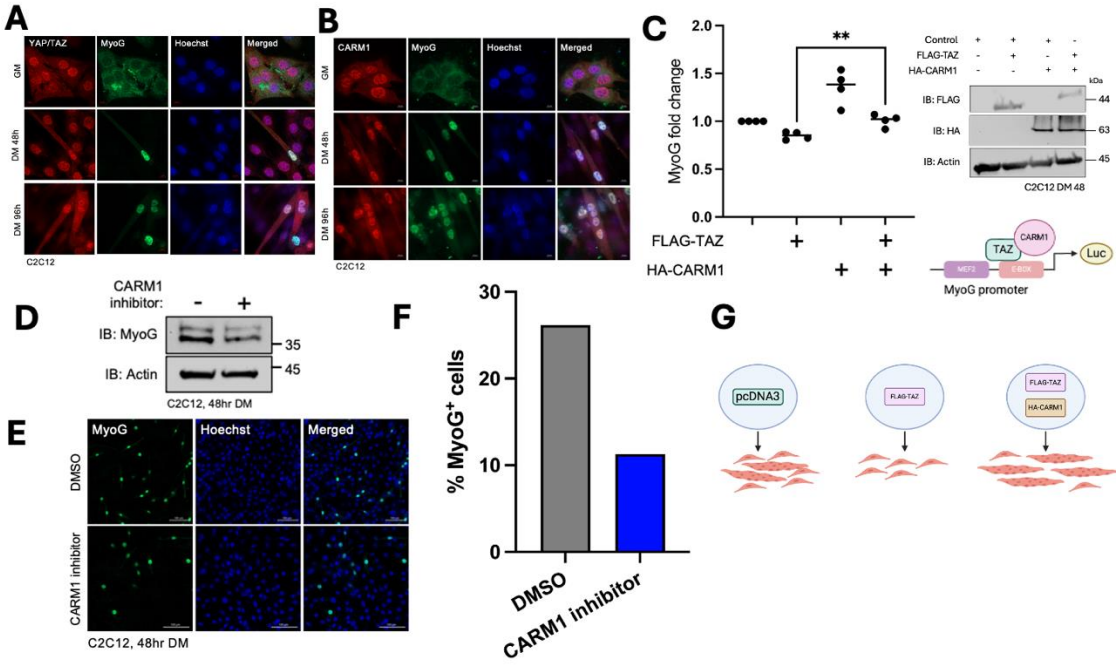


Figure 4. CARM1 antagonizes TAZ-mediated repression of the myogenic program. **(A)** Individual immunofluorescence analysis of endogenous YAP/TAZ and **(B)** CARM1 in C2C12 cells during myoblast (GM), early (DM 48 h), and late myotube (DM 96 h) stages. Cells were immuno-stained for MyoG (green) to label cells entering myogenic differentiation and counterstained with Hoechst 33342 to indicate nuclei (blue). **(C)** FLAG-TAZ alone and combined with HA-CARM1 were ectopically expressed with a *myogenin* promoter reporter gene in C2C12 cells cultured in DM for 48 hours. Renilla luciferase gene served as a transfection control. Transfection with an empty vector (pcDNA) served as a control for endogenous activity. This luciferase reporter experiment was conducted n=4 times. Unpaired t-test using GraphPad Prism 10.0 was utilized to test for statistical significance. Adjusted p-value **p<0.01, ***p<0.001, ****<0.0001 are indicated for significance compared to the relevant control condition. **(D)** FLAG-TAZ alone and combined with HA-CARM1 were ectopically expressed in C2C12 cells cultured in DM for 48 hours. Protein levels of Myogenin were assessed with western blot analysis

using Actin as a loading control. **(E)** C2C12 cells cultured in differentiation media with 5 μM CARM1 inhibitor for 24 hours, followed by a 24-hour recovery period in differentiation media, were immune-stained for MyoG (green) and counterstained with Hoechst 33342 to visualize nuclei (blue). C2C12 cells treated with DMSO served as the control. **(F)** Quantification of the fraction of $MyoG^+$ cells in DMSO- and CARM1 inhibitor-treated C2C12 cells from Figure 4E. This was done using 13 technical replicates and a biological replicate of 1. **(G)** Schematic of showing ectopic expression in C2C12 cells and demonstrating the downstream effects of differentiation under each ectopic expression condition.

Regulation of TAZ and CARM1 in RD cells

In view of our observation that TAZ is pro-proliferative and represses myogenesis we wanted to investigate TAZ expression and regulation by CARM1 in a dysregulated myogenic context such as rhabdomyosarcoma. Initially, we assessed the endogenous levels of TAZ and pTAZ Ser89 to begin to understand the regulation of the Hippo signaling pathway in a variety of cell lines: HEK, proliferative myoblasts (C2C12), and late differentiation stage (DM 96hrs), Embryonic rhabdomyosarcoma (RD), Alveolar rhabdomyosarcoma (RH30), and primary cardiomyocytes (PCM). These results demonstrate that in RD cells, both TAZ protein expression level and pTAZ Ser89 phosphorylation are observed indicating that the Hippo signaling pathway is hyperactive in this cell line (Figure 5A). Next, we conducted a co-immunoprecipitation of TAZ-CARM1 and found that CARM1 and TAZ interact when ectopically expressed in RD cell lines (Figure 5B). We next aimed to investigate differences in the endogenous activation of the HOP reporter system in C2C12 cells and RD cells. We found that, similar to the results in 4A, HOP activation in RD cells is significantly higher than in C2C12 cells. This may indicate that the impaired differentiation machinery may be due to TAZ activation of pro-proliferative genes (Figure 5C). Further investigation of endogenous YAP/TAZ expression in RD cells revealed a pattern similar to that

observed in C2C12 cells during myogenesis. However, RD cells failed to undergo myogenesis and did not form multinucleated myotubes (Figure 5D). Endogenous expression of CARM1 using immunofluorescence analysis indicated that it remained enriched in the nucleus throughout the differentiation period regardless of myogenic expression. Further, a similar trend of inhibited myogenic differentiation like that of (Figure 5D) was observed as these cells also fail to undergo multinucleation even though they are MyoG positive (Figure 5E). To evaluate whether CARM1-mediated repression of TAZ occurs in RD cells we investigated how TAZ modulated the *myogenin* reporter gene activation in RD cells. We reported no significant difference in reporter activation in these reporter assays upon the addition of TAZ (supplementary figure 3A), thus we aimed to further investigate the levels of the c-myc protein, which is a known downstream target gene of TAZ. We observed a reduction in c-myc with the ectopic expression of HA-CARM1 in both RD and C2C12 cells, highlighting that CARM1 driven repression of TAZ may be maintained in both cell types (Figure 5F). In summary, these results show that TAZ is pro-proliferative and represses myogenesis, with its regulation by CARM1 being critical in rhabdomyosarcoma, where CARM1-driven repression of TAZ reduces c-myc levels and contributes to impaired differentiation.

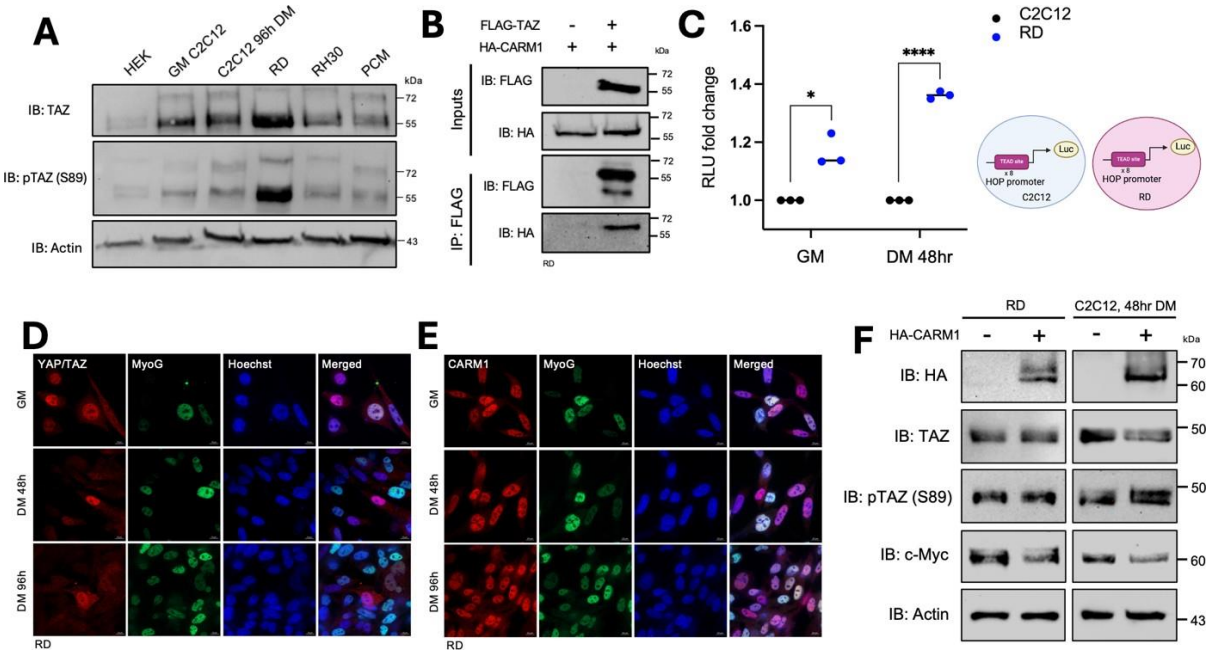
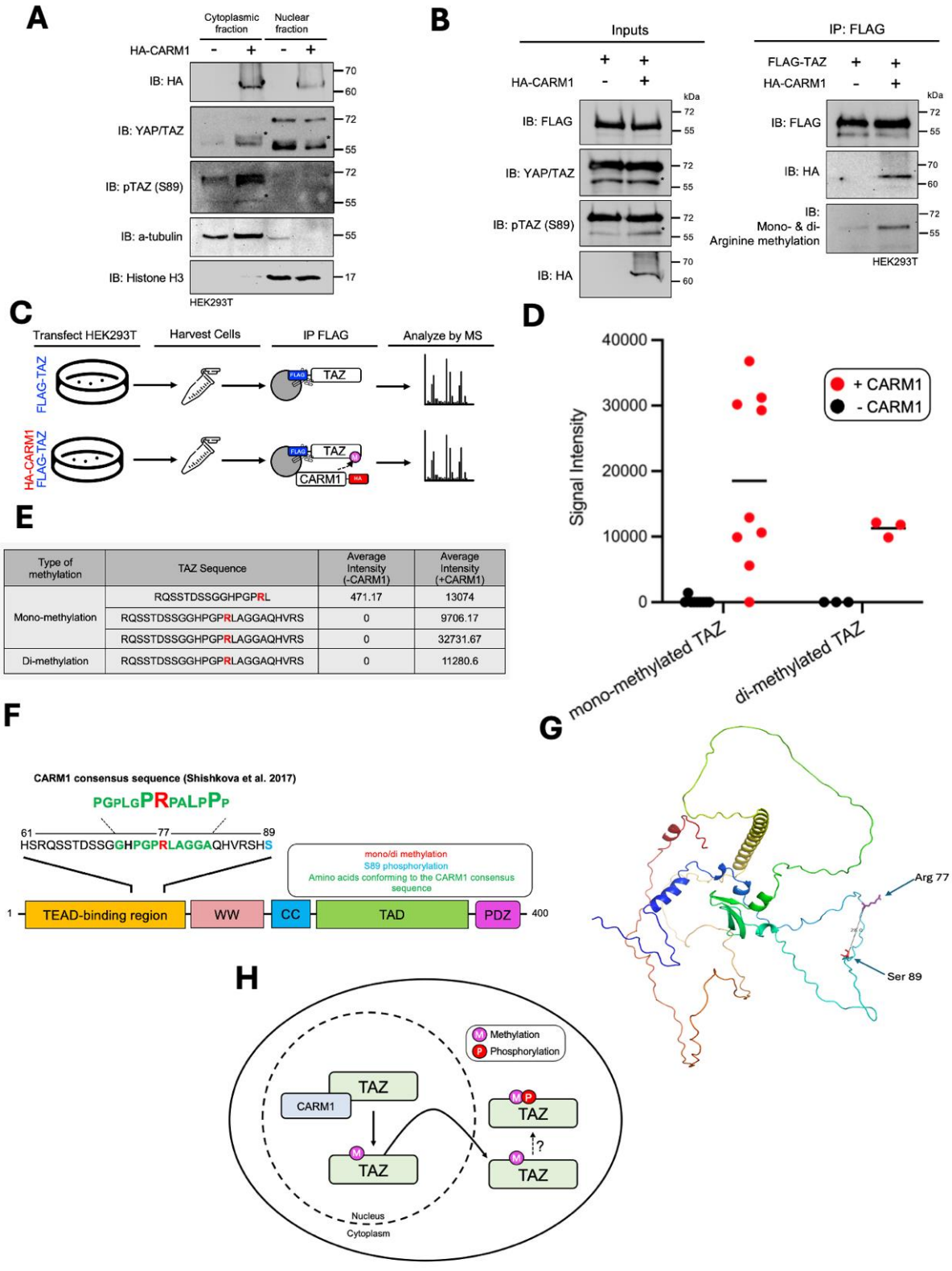


Figure 5. Regulation of TAZ and CARM1 in RD cells. (A) Western blot detecting the endogenous expression of TAZ and phosphorylated TAZ Ser 89 and YAP S127 in HEK293T, C2C12 myoblasts, C2C12 myotubes, Embryonic rhabdomyosarcoma (RD), Alveolar rhabdomyosarcoma (RH30), and primary cardiomyocytes using the actin as a protein loading control. **(B)** RD cells were transfected with HA-CARM1 with/without FLAG-TAZ, and subject to FLAG immunoprecipitation with anti-FLAG beads. Eluates were analyzed using immunoblotting for contents of co-immunoprecipitation with FLAG-TAZ. **(C)** HOP luciferase reporter gene transfected with Renilla luciferase served as a transfection control into both RD and C2C12 cells. This was conducted n=3 times. Unpaired t-test using GraphPad Prism 10.0 was utilized to test for statistical significance. Adjusted p-value **p<0.01, ***p<0.001, ****<0.0001 are indicated for significance compared to the relevant control condition. **(D)** Individual immunofluorescence analysis of endogenous YAP/TAZ and **(E)** CARM1 in RD cells during proliferation (GM), early (DM 48 h), and late stage myogenesis (DM 96 h) stages. Cells were immunostained for MyoG (green) to label cells entering myogenic differentiation and counterstained with Hoechst 33342 to indicate nuclei (blue). **(F)** HA-CARM1 was ectopically expressed in RD and C2C12 cells followed by immunoblot analysis of HA-CARM1, TAZ, phospho-TAZ Ser 89 and c-Myc protein levels. Transfection with an empty vector (pcDNA) served as a control. Actin was used as a western blot loading control.

CARM1 di-methylates TAZ on Arg 77 in HEK293T cells

The Hippo signaling pathway uses a phosphorylation cascade to regulate the subcellular localization of TAZ in the cell (Meng et al., 2016). Ser89 phosphorylation of TAZ is one of the best studied mechanisms in which this pathway maintains homeostasis. In the canonical pathway, Ser89 phosphorylation by LATS1/2 leads to the retention of TAZ in the cytoplasm due to its interaction with binding proteins such as 14-3-3 (Meng et al., 2016). To assess if CARM1 modulates the expression of TAZ we assessed if ectopic expression of HA- CARM1 led to localization changes of TAZ within the cells. We observed that upon ectopic expression of HA-CARM1, there was a reduction of TAZ in the nucleus while a higher abundance TAZ Ser89 phosphorylation in the cytoplasm compared to control (Figure 6A). To further investigate the mechanism into this nuclear-cytoplasmic shift following the ectopic expression of HA-CARM1 we assessed whether CARM1 directly methylates TAZ. This experiment was carried out by co-transfecting HEK293T cells with FLAG-TAZ and HA-CARM1, followed by FLAG-immunoprecipitation and western blotting of the arginine methylation using an antibody that can recognize mono- di- methylated arginine residues. We found that TAZ methylation was more abundant in the condition where HA-CARM1 is ectopically expressed, suggesting that TAZ may be a substrate of CARM1 methylation (Figure 6B). To identify the specific residues in which CARM1 methylated TAZ, we conducted a mass spectrometry analysis on samples containing TAZ immunoprecipitated from cells expressing CARM1 or controls (no ectopic expression of CARM1). A schematic depicting the mass spectrometry protocol is shown in figure 6C. Mass spectrometry analysis of post translational modifications (PTMs) indicated both mono- and di -methylation at the Arg 77 residue via co- expression of HA-CARM1 within the sequence GHPGPR*LAGGA (Figure 6D). Inputs of the mass spectrometry samples are found in

supplementary figure 4A. In addition, table containing the exact amino acid sequences methylated is depicted in figure 6E. Further, figure 6F shows a schematic of where the amino acid sequence identified is present within the TAZ domain structure. This sequence closely aligns with a previous report indicating that CARM1 methylation targets sequences are enriched in prolines, glycines, alanines, and phenylalanines within 5 amino acids of the central methylated arginine (Sishkova et al., 2017). Other proteins found to be differently methylated upon the ectopic expression of HA-CARM1 are shown in supplementary figure 5 A and B. In addition, we used the molecular visualization program PyMOL to analyze a schematic of the TAZ 3-D protein structure, illustrating the locations of Arg77 and Ser89 on the protein. Based on this analysis, we predicted that the distance between these two amino acids is 28.0 Å (Figure 6G). Altogether, these findings demonstrate that TAZ is a substrate of CARM1 methylation. This data also suggests preliminary findings suggesting that the ectopic expression of HA-CARM1 enhances the phosphorylation of TAZ and thus cytoplasmic sequestration of TAZ which may repress TAZ activity. To further validate these conclusions, further experimentation is needed. However, the working model to depict this relationship is demonstrated in schematic (Figure 6H).



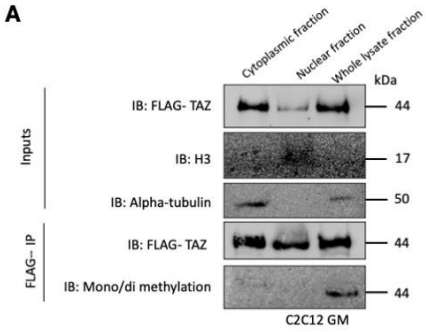
Type of methylation	TAZ Sequence	Average Intensity (-CARM1)	Average Intensity (+CARM1)
Mono-methylation	RQSSTDSSGGHPGPRLAGGAQHVRS	471.17	13074
	RQSSTDSSGGHPGPRLAGGAQHVRS	0	9706.17
	RQSSTDSSGGHPGPRLAGGAQHVRS	0	32731.67
Di-methylation	RQSSTDSSGGHPGPRLAGGAQHVRS	0	11280.6

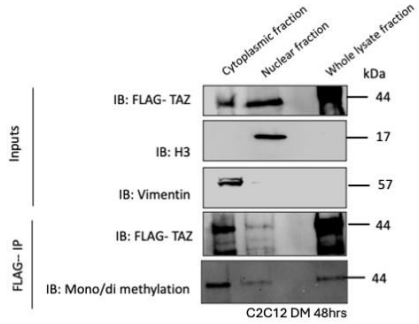
Figure 6. CARM1 di-methylates TAZ on Arg77 in HEK293T cells. (A) HEK293T cells were transfected with HA-CARM1 and subsequently harvested into cytoplasmic and nuclear fractions. Protein levels of HA-CARM1, YAP/TAZ, phospho-YAP Ser 127 and phospho-TAZ Ser89 were assessed by western blot analysis. Alpha-tubulin and Histone H3 were used as cytoplasmic and nuclear markers, respectively. Transfection with an empty vector (pcDNA) served as a control. **(B)** FLAG-TAZ alone and combined with HA-CARM1 were ectopically expressed and immunoprecipitated using FLAG-beads. Eluates were analyzed using western blot analysis for contents of immunoprecipitated mono- and di-methylated FLAG-TAZ in HEK293T cells. **(C)** Schematic illustrating the experimental design of mass spectrometry-based identification of TAZ methylated residues. **(D)** Graph depicting signal intensity mono an di methylated TAZ with and without ectopically expressed CARM1. **(E)** Chart depicting mono and di methylated peptide sequences shown in the mass spectrometry. Methylated arginine's are depicted in Red. **(F)** Illustration of TAZ amino acid sequence highlighting the methylated arginine residue (red) found within the TEAD-binding domain. **(G)** TAZ protein schematic highlighting the various domains of the TAZ protein. This schematic demonstrates where Arg 77 (identified methylated residues) and Ser89 residues on the TAZ protein and their distance (28.0 Å) away from one another within a 3D model. **(H)** Schematic depicting the methylation by CARM1 on TAZ increasing nucleus shuttling TAZ out of the nucleus encouraging TAZ phosphorylation.

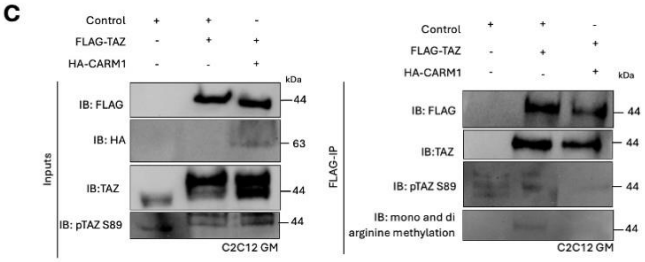
Global methylation changes in Hippo signaling pathway components upon ectopic expression of CARM1

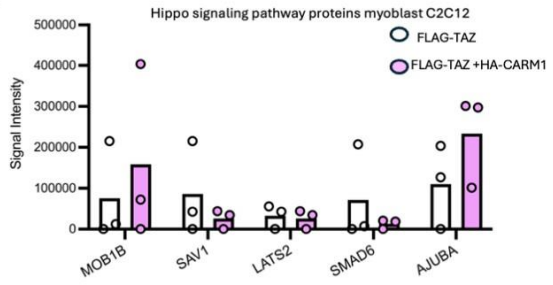
Hippo signaling regulates TAZ throughout myogenesis. In periods of proliferation (growth media conditions) Hippo signaling activity is low, while active TAZ is high (Meng et al., 2016; Misra et al., 2018). However, in periods of high cell density, like in differentiation, Hippo signaling is high, thus, there is increased TAZ phosphorylation (Meng et al., 2016; Misra et al., 2018). Therefore, we aimed to assess if TAZ methylation was different throughout myogenesis. We first assessed where phosphorylation was occurring within the cell during differentiation. To do this we conducted a nuclear cytoplasmic fractionation in growth media (Figure 7A) and in 48 hrs DM (Figure 7B). From this we found that there was an increased amount of methylation in the cytoplasm compared to the nucleus in GM and DM conditions. We also saw increased methylation in the 48hr condition compared to the growth media condition. We then ectopically expressed

pcDNA3, FLAG-TAZ and HA-CARM1 in GM C2C12 cells and then conducted a FLAG-immunoprecipitation. We found under these conditions there was no increase of phosphorylation or methylation when HA-CARM1 was ectopically expressed (Figure 7C). Similarly, we conducted a mass spectrometry analysis on C2C12 GM cells, we ectopically expressed FLAG-TAZ with/without HA-CARM1. In this mass spectrometry analysis, we found arginine methylation variations on components of the Hippo signaling pathway including MOB1B, SAV1, LATS2, SMAD6, and AJUBA. These proteins were assessed by their intensity scores, a graph depicting the difference in intensity score between the conditions is illustrated in figure 7D. We then assessed the methylation profile of proteins in high cell-cell contact conditions such as mid-to late-stage differentiation (48 hr). In this condition, we found Hippo signaling proteins had different mono-di- methylation profiles compared to GM conditions. This included proteins such as TEAD 4 (mono-methylation), SIK2, AMOTL2, and β-catenin. Intensity scores for the peptides with changes in arginine methylated residues is depicted in figure 7E. Inputs of both mass spectrometry analyses is shown in (supplementary figure 4B and 4C). Finally, a schematic depicting the different methylated peptides in both growth media and 48 hr differentiated conditions within the context of the Hippo signaling pathway is illustrated in figure 7F.

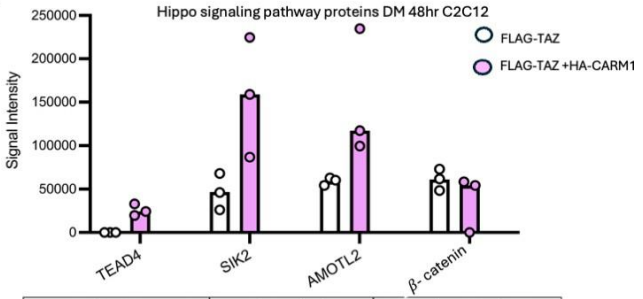








Protein	Intensity 1 (TAZ)	Intensity 2 (TAZ + CARM1)
MOB1B	9,395	134,509
SAV1	75,824	56,458
LATS2	25,631	26,150
SMAD6	35,657	13,050
AJUBA	156,603	201,451



Protein	Intensity 1 (FLAG-TAZ)	Intensity 2 (FLAG-TAZ +HA-CARM1)
TEAD4 (mono-methylation)	0	25,657
SIK2	46,798	156,806
Amot2	39,613	150,564
Beta-catenin	61,097	37,586

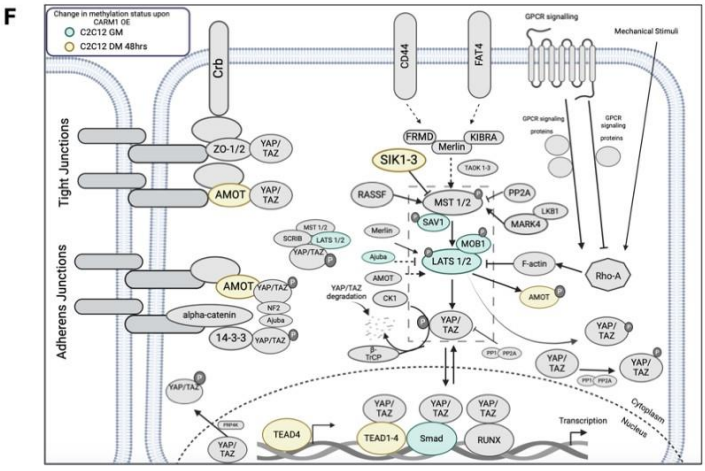


Figure 7. Global methylation changes in Hippo signaling upon ectopic expression of CARM1 **(A)** Myoblast C2C12 cells were transfected with FLAG-TAZ and subsequently harvested into cytoplasmic and nuclear fractions. Protein levels of FLAG, YAP/TAZ, and mono and di-methylation were assessed by western blot analysis. Alpha-tubulin and Histone H3 were used as cytoplasmic and nuclear markers, respectively. **(B)** Differentiated C2C12 cells were transfected with FLAG-TAZ and subsequently harvested into cytoplasmic and nuclear fractions. Protein levels of FLAG, YAP/TAZ, and mono and di-methylation were assessed by western blot analysis. Vimentin and Histone H3 were used as cytoplasmic and nuclear markers. **(C)** Myoblast C2C12 cells were transfected with FLAG-TAZ with and without HA-CARM1. Cells were FLAG-immunoprecipitated and eluates were analyzed using western blot analysis for contents of immunoprecipitated mono- and di-methylated, YAP/TAZ, and S89 TAZ in C2C12 cells. **(D)** Bar graph depicting the intensity scores of di arginine methylation of the Hippo Signaling components affected by ectopic expression of HA-CARM1. **(E)** Bar graph depicting the intensity scores of di arginine methylation of the Hippo Signaling components affected by ectopic expression of HA-CARM1 in 48hr C2C12 differentiated cells. **(F)** Hippo signaling pathway proteins impacted by mono and di-methylation following HA-CARM1 ectopic expression in both growth media (growth media) and differentiation 48hrs (yellow) in C2C12 cells.

Discussion

The Hippo signaling pathway has generated considerable interest for its role in muscle and stem cell regeneration, cancer progression, and immune system homeostasis (Fu et al., 2022). The Hippo signaling pathway consists of a serine/threonine kinase cascade that leads to the downstream inhibition of YAP/TAZ activity. The aim of these studies was to further characterize TAZ function and regulation by identifying and analyzing TAZ interacting protein partners in a muscle context. This was accomplished using a nanobody- mediated two-step affinity purification approach coupled with mass spectrometry in skeletal muscle cells (C2C12) and primary neonatal rat ventricular cardiomyocytes. Among the identified interactome components in muscle cells we further investigated the TAZ interaction with the methyltransferase CARM1. The data presented here indicates an inhibitory role of CARM1 on TAZ function. In addition, we found that in HEK293T cells, the ectopic expression of CARM1 led to the methylation of TAZ on amino acid Arg 77 in a PGPR*LAGG peptide sequence. We noted that this methylation correlated with enhanced Ser89 phosphorylation of TAZ, a marker of active Hippo signaling and TAZ functional repression. We further analyzed this mechanism in a HEK293T cells and found that methylation of TAZ could still occur on mutated TAZS89A. Further characterization of this interaction in a myogenic context indicated that cytoplasmic and overall methylation of TAZ was elevated at the 48-hour differentiation time point condition compared to growth media conditions. We then conducted mass spectrometric analysis which indicated that the methylation profile of several TAZ interactome components of the Hippo signaling pathway exhibited different methylation profiles due to ectopic expression of HA-CARM1. These data raise the possibility that the interaction of TAZ and CARM1 may recruit CARM1 to a number of Hippo pathway components which may then be methylated by CARM1. The consequence of hypermethylation of multiple Hippo pathway

subunits remains to be investigated.

To develop the TAZ interactome list in both cardiomyocyte and skeletal muscle contexts, we used the GFP-nanotrap method. This approach generated a comprehensive list of both well-known and novel protein interactions, including TEADs, components of the 14-3-3 binding complex, and Hippo upstream kinases such as LATS1/2. Consequently, this list provides high confidence that the novel proteins identified are potential TAZ interactors. To conduct the GFP-nanoTrap technique we conducted a 2-step incubation process. We first transfected HEK293T cells with EYFP-TAZ and EYFP alone. These constructs were then incubated the bait HEK293T extracts with the GFP- nanoTrap beads, then the bait-bead complex was incubated with the myogenic extracts from C2C12 cells and PCM cells, which are used for skeletal muscle myogenesis and cardiac myogenesis, respectively. The samples were subsequently analyzed using mass spectrometry where a list of TAZ interactors was generated. This technique offers several advantages over traditional immunoprecipitation methods. Firstly, C2C12 cells and PCM cells are derived from mice, while HEK293T cells are human cells, allowing us to specifically identify mouse proteins as TAZ-interacting myogenic proteins. In addition, the GFP-nanobody is very small, resulting in higher cell entry efficiency compared to traditional antibodies and reduced interference (Chen et al., 2023). Additionally, GFP-nanoTrap has a higher affinity than traditional immunoprecipitation methods (Rothbauer et al., 2007). Thus, there are many benefits to using this new method for identifying protein-protein interactions.

Comparing our interactome findings with previously identified TAZ interacting proteins, revealed the identification of core interactome proteins including TEAD transcription factors, LATS kinases, PDZ proteins, 14-3-3 chaperone proteins, and SWI/SNF complex components (Kohli et al., 2014; Wang et al., 2014; Li et al., 2020). Although, several proteins identified were well

characterized, a number of novel proteins that merit future characterization were identified, including other methyltransferases. Although, the CARM1 methyl transferase was characterized here, it is of note that other methyl transferases such as PRMT5 were also identified in our screen. PRMT5 promotes the progression of cancer and is known to be dysregulated on the Hippo signaling pathway (Wang et al., 2020). In addition, PRMT5 is a downstream target of LATS2 and inhibition of PRMT5 increased both LATS2 and YAP phosphorylation in laryngeal cancer (Wang et al., 2020). PRMT5 has also been shown to promote stem cell expansion in adult mice by inhibiting the p21 inhibitor (Zhang et al., 2015). This therefore suggests that one important aspect of TAZ regulation is recruitment of protein arginine methyltransferase (PRMT) family methyltransferases.

Extensive evidence on TAZ function and regulation has revealed the primary mechanism of TAZ repression due to Hippo pathway activation is by phosphorylation at Ser89 leading to TAZ sequestration and degradation in the cytoplasm (Lei et al., 2008). In this report we propose that CARM1-mediated methylation may serve as a novel repressive regulatory mechanism of TAZ function. This mechanism may contribute to the pro-myogenic function of CARM1 since nuclear TAZ function in myoblasts prevents myogenesis by targeting the myogenic transcription machinery (Tripathi et al., 2022). Several studies have established CARM1 as a positive regulator of the myogenic program, including functional interactions with transcription factors MEF2 (Chen et al., 2002) and Pax7 (Kawabe et al., 2012). In addition, CARM1-depletion by morpholino injection led to reduced *myogenin* expression in the anterior somites of developing zebrafish (Batut et al., 2011), consistent with our observations that CARM1 upregulates a *myogenin* promoter reporter gene. Our fractionation and methylation assay data reveal that, upon the expression of ectopic CARM1, TAZ is methylated at R77 which enhances Ser89 phosphorylation leading to

enhanced cytoplasmic localization, suggesting the possibility that CARM1 represses TAZ during the onset of myogenesis to functionally counteract TAZ repression of the myogenic program. Indeed, mono-methylation of the TAZ paralog YAP at K494 by the SET-domain-containing lysine methyltransferase Set7 has been shown to be necessary for retention of YAP in the cytoplasm, promoting its phosphorylation and subsequent degradation (Oudhoff et al., 2013). These observations in myoblasts could have important implications for the post-natal muscle regeneration program since activated adult muscle stem cells (satellite cells) go through a proliferative phase before committing to the differentiation program. It will be intriguing to assess whether Hippo signaling, and TAZ regulation plays a role in this context.

YAP/TAZ are often referred to in the literature together and have similar functions regarding their roles in proliferation and maintenance of homeostasis and their functions in regeneration (Sun et al., 2017). However, there are notable differences in their biological roles (Reggiani et al., 2021). Methylation of TAZ by CARM1 on Arg 77, may indicate a divergence point in the regulation of YAP and TAZ. In comparing the TAZ and YAP amino acid sequence we find that the peptide sequence between S89 and S127 is not conserved. The consensus sequence for TAZ methylation is R77 PGPR*LAGG in which the R* is methylated. This is a characteristic consensus sequence for CARM1 to methylate its substrates, in which the primary consensus is PGPLGPR*PALPPP along with slight variations in this sequence (Sishkova et al., 2017). Although the serine phosphorylation is conserved between S89 in TAZ and S127 in YAP being QHVRSHS* (Ser89), the consensus sequence of an arginine amino acid at a similar distance to the Ser127 is not apparent. Furthermore, both amino acids are in the unstructured regions the TAZ protein, these regions are often considered to be very important for function of the protein (Dyson et al., 2005). Therefore, modifications in within this domain may be integral to TAZ regulation. Ultimately YAP

must be further investigated to test if it is a substrate of CARM1 methylation. Although the function of TAZ/YAP in myogenic differentiation might be similar, our work suggests that they may be post-translationally modified in different ways to mediate their regulation as alluded to in some literature (Oudhoff et al., 2013). Therefore, it would be interesting to determine if CARM1 methylation is conserved among these paralogs, if conserved this may be an indication of an evolutionarily conserved function maintained to regulate YAP/TAZ localization and function or if this is a modification specific to TAZ regulation in myogenesis, potentially contributing to the difference in function we see between YAP and TAZ in this context. Moreover, the maintenance of TAZ activity by CARM1 ectopic expression, even under dysregulated conditions that we observed in rhabdomyosarcoma suggests that the TAZ-CARM1 interaction may provide a potential therapeutic target in RMS. It is known that nuclear TAZ is enriched in both ERMS and ARMS compared to normal growth regulated muscle cells. In both RD and RH30 cell lines there is increased TAZ expression in relation to normal growth regulated muscle cells (Mohamed et al., 2016). This idea is further supported by the observation that both TAZ and TAZS89A ectopic expression leads to increased cellular proliferation in myoblasts (Mohamed et al., 2016). Furthermore, in RH18 and RH30 cell lines, with silenced TAZ induce an upregulation of myogenic regulatory factors including MyoD, and MyoG (Deel et al., 2018). As well, suppression of TAZ in these cell lines are seen in a mouse xenograft model delayed tumor progression and enhanced survival (Deel et al., 2018). Therefore, characterizing the TAZ/CARM1 interaction in ERMS may serve as a potential pathway for manipulation in cancer treatments.

Overall, these studies provide further evidence of TAZ as a modulator of myogenic gene transcription. Further, upon differentiation, our data implicate CARM1 methylation and repression of TAZ nuclear function to facilitate de-repression of the myogenic differentiation machinery.

These observations may have implications for our understanding of the switch between proliferation and differentiation in muscle cells since this is a crucial determinant of skeletal muscle ontogeny and post-natal muscle regeneration.

Future direction and implications

The dataset generated by this study was able to successfully identify novel and well-characterized TAZ interacting proteins in both cardiac and skeletal muscle. One of the interactions we identified in both datasets was between TAZ and CARM1. We characterized this interaction in both a growth regulated context and in a myogenic dysregulated context. We then further characterized biochemical and functional analysis of this interaction and the mechanism of this interaction. Interestingly, we found that in HEK293T cells, TAZ is a substrate of CARM1 methylation. In addition, in a myogenic context, components of the Hippo signaling pathway had different arginine methylated patterns upon the ectopic expression of HA-CARM1. Although these findings are of interest so far, to further characterize the role of TAZ in myogenic cells, future work should be carried out to determine the potential role of TAZ in recruiting CARM1 methylation activity.

Although this study focused on TAZ interacting proteins in skeletal muscle our study also identified a list of both novel and well-known TAZ interacting proteins in primary cardiomyocytes. TAZ and YAP play an important role in regulation of heart size and overall cardiac homeostasis (Kashihara et al., 2019). Dysfunction of the role of YAP/TAZ play a large role in heart health as their activation plays a role in pulmonary hypertension, myocardial hypertrophy, myocardial fibrosis among others, while knockdown of both YAP/TAZ can lead to aortic aneurysms and aortic dissections (Yu et al., 2020). The Hippo pathway plays a pivotal role in the development, disease, and regeneration of the heart (Chen et al., 2020; Heallen et al., 2011; Heallen et al., 2013). Heart disease is the number one cause of death worldwide, therefore understanding TAZ function in cardiomyocytes is potentially important for understanding heart disease. The only TEAD member appearing in the cardiomyocyte TAZ interactome was TEAD1. The TEAD1 protein is the most abundant TEAD family member in cardiac muscle development (Chen et al., 1994; Liu et al.,

2017). TEAD1 is essential for heart development, remodeling, and maintaining homeostasis (Song et al., 2024). In fact, cardiac fibroblasts, cells responsible for heart remodeling, show elevated levels of TEAD1 (Song et al., 2024). Further, HDAC1 regulates cardiac plasticity while HDAC inhibitors reverse existing hypertrophy (Cao et al., 2011). Therefore, further characterizing the interaction between TAZ and TEAD1 in cardiac muscle may be interesting for further investigation and as a possible therapeutic target for heart disease. In addition, this study was able to generate TAZ protein-protein interactions in myogenic skeletal cells. Therefore, additional protein interactions should be evaluated to further our understanding of TAZ in a skeletal myogenic context. For example, a protein category that should be further explored are components of the NuRD complex. The NuRD complex is composed of an ATPase- dependent chromatin remodelling complex (Hillmer et al., 2019). Although TAZ and components of the NuRD complex have already been reported to interact, further characterization is warranted (Hillmer et al., 2019). This interaction is dependent on TEAD YAP/TAZ complex formation which allows for the repression of pro-proliferative genes (Hillmer et al., 2019). In addition, YAP/YAZ interact with the repressive nucleosome-remodeling and deacetylase (NuRD complex) to repress target gene expression during embryonic development to determine cell lineage in portions of the mesoderm. Further, YAP/TAZ recruit the NuRD repressor on switch-enhancer elements bound by TEAD, SMAD2/3, and OCT4, preventing the expression of the mesendoderm specification genes, suppressing transcription (Lopez-Hernandez et al., 2021). However, the interactions with specific components of this complex such as MTA2, HDAC1, RBBP4 have been identified in both skeletal and cardiomyocyte data and it would be of further interest to understand how these components interact with TAZ to regulate myogenesis.

Furthermore, post translational modifications have been shown to influence protein interactions

(Duan et al., 2015). In the context of TAZ, the most common modification is phosphorylation on Ser89. To further understand whether the CARM1 and TAZ interaction is dependent on post-translational modifications of TAZ, further studies need to be conducted. To answer this question, TAZ and CARM1 could be analyzed using phosphomimetics, methylmimetics, and non-modifiable amino acid substitutions on TAZ to determine the proteomic partner landscape of the various states of TAZ. In addition, amino acid substitutions of TAZ Arg 77 into a lysine or alanine following western blot and functional analysis with and without CARM1 ectopic expression would confirm the mass spectrometry finding that CARM1 exclusively targets TAZ on Arg 77. Lysine would be an ideal candidate for an amino acid substitution due to its similar charge to arginine, pKas (10.5 lysine and 13.8 Arg) (Armstrong et al., 2016). Alanine may also be considered as a viable amino acid substitution as it is the most common amino acid of choice to conduct substitution reactions because it does not alter the protein conformation (Yang et al., 2009).

In addition, further experimentation should be conducted into the timing and dynamic changes of TAZ protein-protein interactions over the course of myogenic differentiation. To complete this, a TurboID assay of TAZ can be employed at different stages of differentiation. The TurboID assay allows for enzyme catalyzed proximity labeling which can further our understanding of dynamic protein-protein interactions in living cells (Branon et al., 2018). This assay relies on a Turbo-ID enzyme fused to a protein of interest, which upon the addition of biotin, labels interacting proteins through with biotin (Wei et al., 2023). Biotinylated proteins are then captured by streptavidin beads and assessed by western blot. Strategic addition of biotin at different stages of differentiation will reveal when interactions are most likely to be active, for example, when TAZ is targeted by CARM1 methylation. Similar experiments may also be done using mass spectrometry analysis on the TimsToF machine at various time points during differentiation.

This model can also be applied to rhabdomyosarcoma cell lines at different stages of cancer development to assess dynamic changes in protein – protein interactions in early and late-stage progression of cancer. For example, in rhabdomyosarcoma cells, it may be possible to map protein-protein interactions during different stages of differentiation such as the onset of MyoD or MyoG expression, as well as other rhabdomyosarcoma markers of fusion status including: AP2□, NOS-1, and HMGA2 (Rudzinski et al., 2014). Genetically edited cell lines that express GFP, driven under the same promoter as the above-mentioned genes can then be sorted by Fluorescence activated Cell Sorting (FACS). Combing this approach with Turbo-ID, will allow for understanding of the differences in interactome networks between GFP- positive and -negative cells which can yield insight into cell state dependent interactions. This will enable a comprehensive understanding of protein interactions during the progression of rhabdomyosarcoma.

Future studies should also address the functional significance of the TAZ-CARM1 interaction *in-vivo* in the context of muscle regeneration. To accomplish this, the use of previously generated conditional PAX-7- Cre TAZ and or CARM1 knockouts would be ideal to understand and characterize this interaction further (Santos et al., 2023; Kim et al., 2023; Hwang et al., 2022). Previously generated TAZ knockout mice are reported to have muscle growth defects and satellite specific conditional knockout of YAP produced a muscle regeneration deficit (Sun et al., 2017; Kim et al., 2023). PAX7-Cre conditional knockouts of CARM1 have a deficit in their regeneration ability as well as satellite stem cell division (Santos et al., 2023). In either a TAZ or CARM1 conditional knockout model, tamoxifen is injected to induce Cre specific activity prior to sustaining injury (Donocoff et al., 2020). Administration of cardiotoxin into one leg to induce injury can be utilized to study the ability of the leg muscle to recover with and without TAZ

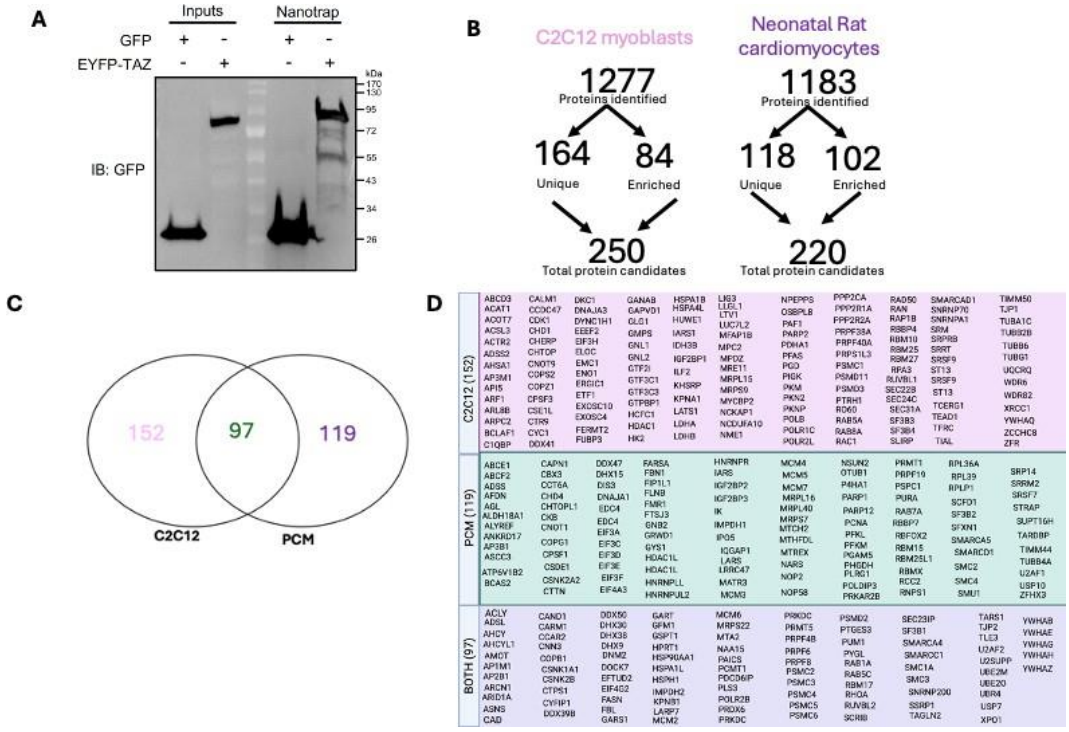
function using the conditional knockout strategy (Fu et al., 2023). This may allow us to further understand the regeneration potential of TAZ and CARM1 in myogenesis.

Overall, this study highlights the crucial role of TAZ in modulating myogenic transcription through its interaction with CARM1. This pivotal interaction lies at the intersection of cell proliferation and differentiation. Our findings reveal that CARM1 counteracts TAZ repression of myogenesis, a function that could be dysregulated in the context of embryonic rhabdomyosarcoma. Additionally, these data demonstrate that TAZ is a substrate of CARM1 methylation in HEK293T cells, also influencing the methylation profile of other Hippo signaling pathway components. Further exploration of this TAZ-CARM1 interaction could uncover potential therapeutic strategies for muscle cancers, such as rhabdomyosarcoma, where TAZ activity is dysregulated. This research also holds promise for advancing our understanding of the role of TAZ in post-natal skeletal muscle regeneration, potentially leading to innovative treatments and improved muscle health.

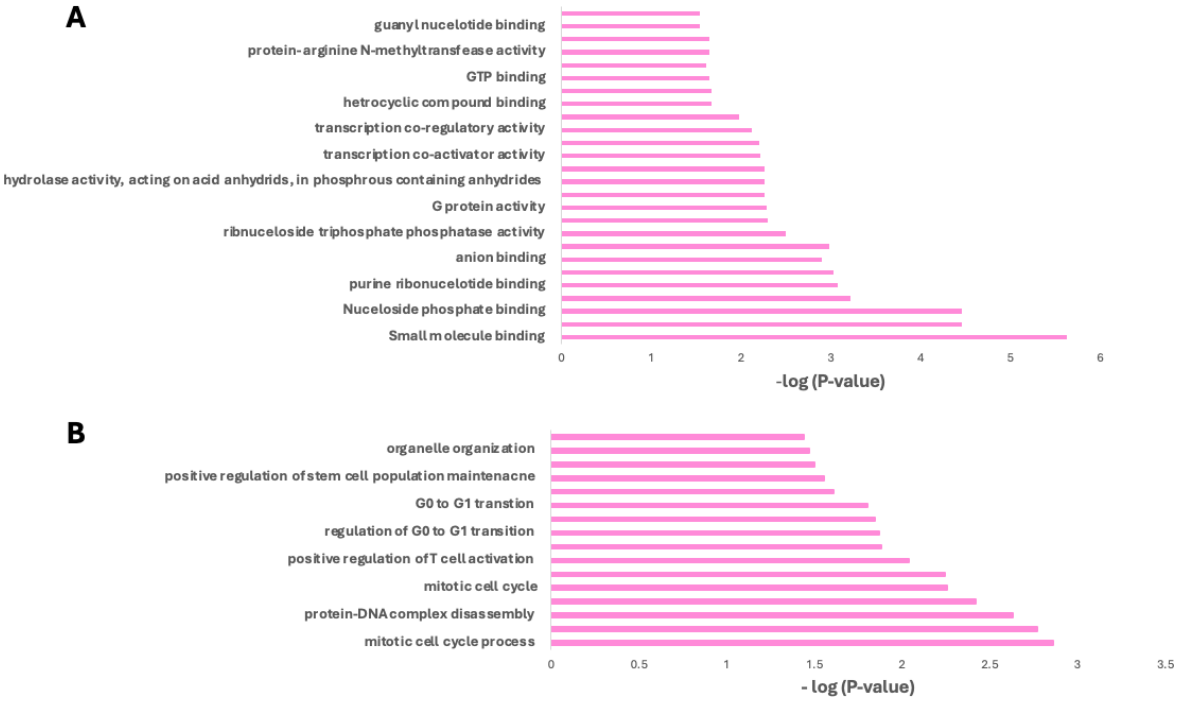
Acknowledgments

I would like to thank Gwillym Declan Williams for assistance with the proteomic data submission. I would also like to acknowledge the SPARC BioCentre, Hospital for Sick Children, Toronto, Canada for performing the mass spectrometry analysis. Lastly, I acknowledge the YSci Core Mass spectrometry Facility, York University, Toronto, Canada for performing mass spectrometry analysis.

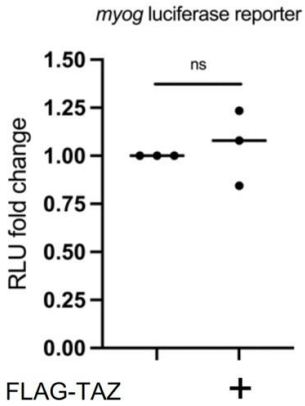
Appendix I



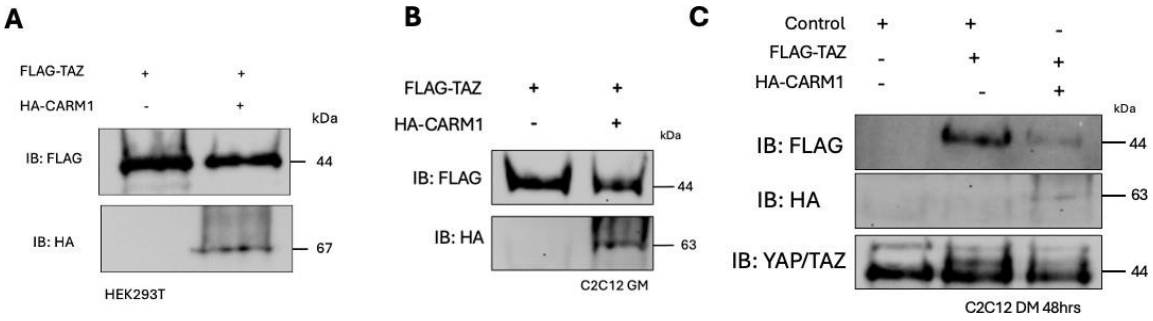
Supplementary Figure 1: The interactome of study of TAZ in striated muscle. **(A)** Validation of GFP-Trap co-IP in HEK293T cells. **(B)** Flow chart indicating the number of total and enriched TAZ protein candidates identified in the C2C12 myoblast and neonatal rat cardiomyocyte datasets.**(C)** Venn diagram indicating the number of unique and overlapping TAZ proteins candidates between the C2C12 myoblast and neonatal rat CM datasets. **(D)** All of the proteins identified in the protein interactome in C2C12, PCM, and in both datasets.



Supplementary Figure 2: Graphs depicting the -log (p-value) of the bioinformatic analysis of TAZ and CARM1 interaction for **(A)** molecular function and **(B)** biological processes.

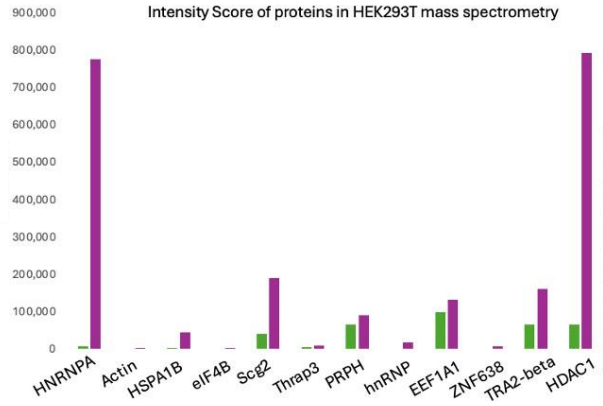


Supplementary Figure 3: Functional analysis of Flag-TAZ ectopic expression on the *Myog* luciferase reporter in embryonic rhabdomyosarcoma cells. *Renilla* plasmid used as a control.



Supplementary Figure 4: Western blot analysis of mass spectrometry inputs for **(A)** HEK 293T, **(B)** GM C2C12 cells and **(C)** DM 48 hrs C2C12 cell lysates

A



B

Legend

Ectopic TAZ

Ectopic TAZ +CARM1

Protein name	Intensity score 1 (TAZ)	Intensity score 2 (TAZ + CARM1)
HNRNPA1	6,855	774001
Actin	0	3077
HSPA1B	1621	44415
eIF4B	0	2486
Scg2	40575	190316
Thrap3	5445	8207
PRPH	64559	88859
hnRNP	0	17597
EEF1A1P5	97540	131900
ZNF638	0	6643
TRA2-beta	64853	161044
HDAC1	64950	791994

Supplementary Figure 5: Hippo signaling proteins modified in HEK293T cells mass spectrometry. **(A)** Graph depiction of other proteins with increase mono- di- arginine methylation with HA-CARM1 overexpression **(B)** Chart showing mono- di- methylation intensity scores of 5A.

References

Armstrong, C. T., Mason, P. E., Anderson, J. L., & Dempsey, C. E. (2016). Arginine side chain interactions and the role of arginine as a gating charge carrier in voltage sensitive ion channels. *Scientific reports*, *6*, 21759. https://doi.org/10.1038/srep21759

Asfour, H. A., Allouh, M. Z., & Said, R. S. (2018). Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery. Experimental biology and medicine (Maywood, N.J.), 243(2), 118–128. https://doi.org/10.1177/1535370217749494

Aulehla, A., & Pourquié, O. (2006). On periodicity and directionality of somitogenesis. Anatomy and embryology, 211 Suppl 1, 3–8. https://doi.org/10.1007/s00429-006-0124-y

Aulehla, A., & Pourquié, O. (2010). Signaling gradients during paraxial mesoderm development. Cold Spring Harbor perspectives in biology, 2(2), a000869. https://doi.org/10.1101/cshperspect.a000869

Bae, S. J., & Luo, X. (2018). Activation mechanisms of the Hippo kinase signaling cascade. Bioscience reports, 38(4), BSR20171469. https://doi.org/10.1042/BSR20171469

Baldwin, C. T., Lipsky, N. R., Hoth, C. F., Cohen, T., Mamuya, W., & Milunsky, A. (1994). Mutations in PAX3 associated with Waardenburg syndrome type I. Human mutation, 3(3), 205– 211. https://doi.org/10.1002/humu.1380030306

Batut, J., Duboé, C., & Vandel, L. (2011). The methyltransferases PRMT4/CARM1 and PRMT5 control differentially myogenesis in zebrafish. *PloS one*, *6*(10), e25427. https://doi.org/10.1371/journal.pone.0025427

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. Cold Spring Harbor perspectives in biology, 4(2), a008342. https://doi.org/10.1101/cshperspect.a008342

Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., & Rando, T. A. (2008). A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell stem cell*, *2*(1), 50–59. https://doi.org/10.1016/j.stem.2007.10.006

Branon, T. C., Bosch, J. A., Sanchez, A. D., Udeshi, N. D., Svinkina, T., Carr, S. A., Feldman, J. L., Perrimon, N., & Ting, A. Y. (2018). Efficient proximity labeling in living cells and organisms with TurboID. *Nature biotechnology*, *36*(9), 880–887. https://doi.org/10.1038/nbt.4201

Braun, T., Rudnicki, M. A., Arnold, H. H., & Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell, 71(3), 369–382. https://doi.org/10.1016/0092-8674(92)90507-9

Buckingham, M., & Relaix, F. (2015). PAX3 and PAX7 as upstream regulators of myogenesis. Seminars in cell & developmental biology, 44, 115–125. https://doi.org/10.1016/j.semcdb.2015.09.017

Cai, X., Wang, K. C., & Meng, Z. (2021). Mechanoregulation of YAP and TAZ in Cellular Homeostasis and Disease Progression. Frontiers in cell and developmental biology, 9, 673599. https://doi.org/10.3389/fcell.2021.673599

Cao, D. J., Wang, Z. V., Battiprolu, P. K., Jiang, N., Morales, C. R., Kong, Y., Rothermel, B. A., Gillette, T. G., & Hill, J. A. (2011). Histone deacetylase (HDAC) inhibitors attenuate cardiac hypertrophy by suppressing autophagy. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(10), 4123–4128.

Chan, S. W., Lim, C. J., Chong, Y. F., Pobbati, A. V., Huang, C., & Hong, W. (2011). Hippo pathway-independent restriction of TAZ and YAP by angiomotin. *The Journal of biological chemistry*, *286*(9), 7018–7026. https://doi.org/10.1074/jbc.C110.212621

Chen, K., Yuan, J., Sia, Y., & Chen, Z. (2023). Mechanism of action of the SWI/SNF family complexes. Nucleus (Austin, Tex.), 14(1), 2165604. https://doi.org/10.1080/19491034.2023.2165604

Chen, S., Sun, S., Moonen, D., Lee, C., Lee, A. Y., Schaffer, D. V., & He, L. (2019). CRISPR- READI: Efficient Generation of Knockin Mice by CRISPR RNP Electroporation and AAV Donor Infection. *Cell reports*, *27*(13), 3780–3789.e4. https://doi.org/10.1016/j.celrep.2019.05.103

Chen, S. L., Loffler, K. A., Chen, D., Stallcup, M. R., & Muscat, G. E. (2002). The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. *The Journal of biological chemistry*, *277*(6), 4324–4333. https://doi.org/10.1074/jbc.M109835200

Chen, X., Li, Y., Luo, J., & Hou, N. (2020). Molecular Mechanism of Hippo-YAP1/TAZ Pathway in Heart Development, Disease, and Regeneration. *Frontiers in physiology*, *11*, 389. https://doi.org/10.3389/fphys.2020.00389

Chen, Y. A., Lu, C. Y., Cheng, T. Y., Pan, S. H., Chen, H. F., & Chang, N. S. (2019). WW

Domain-Containing Proteins YAP and TAZ in the Hippo Pathway as Key Regulators in Stemness Maintenance, Tissue Homeostasis, and Tumorigenesis. Frontiers in oncology, 9, 60. https://doi.org/10.3389/fonc.2019.00060

Chen, Z., Friedrich, G. A., & Soriano, P. (1994). Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes & development, 8(19), 2293–2301. https://doi.org/10.1101/gad.8.19.2293

Cunningham, R., & Hansen, C. G. (2022). The Hippo pathway in cancer: YAP/TAZ and TEAD as therapeutic targets in cancer. Clinical science (London, England : 1979), 136(3), 197–222. https://doi.org/10.1042/CS20201474

Dasgupta, I., & McCollum, D. (2019). Control of cellular responses to mechanical cues through YAP/TAZ regulation. The Journal of biological chemistry, 294(46), 17693–17706. https://doi.org/10.1074/jbc.REV119.007963

Deel, M. D., Slemmons, K. K., Hinson, A. R., Genadry, K. C., Burgess, B. A., Crose, L. E. S., Kuprasertkul, N., Oristian, K. M., Bentley, R. C., & Linardic, C. M. (2018). The Transcriptional Coactivator TAZ Is a Potent Mediator of Alveolar Rhabdomyosarcoma Tumorigenesis. Clinical cancer research : an official journal of the American Association for Cancer Research, 24(11), 2616–2630. https://doi.org/10.1158/1078-0432.CCR-17-1207

Donocoff, R. S., Teteloshvili, N., Chung, H., Shoulson, R., & Creusot, R. J. (2020). Optimization of tamoxifen-induced Cre activity and its effect on immune cell populations. *Scientific reports*, *10*(1), 15244. https://doi.org/10.1038/s41598-020-72179-0

Duan, G., & Walther, D. (2015). The roles of post-translational modifications in the context of protein interaction networks. PLoS computational biology, 11(2), e1004049. https://doi.org/10.1371/journal.pcbi.1004049

Dumont, N. A., Wang, Y. X., & Rudnicki, M. A. (2015). Intrinsic and extrinsic mechanisms regulating satellite cell function. Development (Cambridge, England), 142(9), 1572–1581. https://doi.org/10.1242/dev.114223

Duscher, D., Maan, Z. N., Wong, V. W., Rennert, R. C., Januszyk, M., Rodrigues, M., Hu, M., Whitmore, A. J., Whittam, A. J., Longaker, M. T., & Gurtner, G. C. (2014).

Mechanotransduction and fibrosis. *Journal of biomechanics*, *47*(9), 1997–2005. https://doi.org/10.1016/j.jbiomech.2014.03.031

Dyson, H. J., & Wright, P. E. (2005). Intrinsically unstructured proteins and their functions. *Nature reviews. Molecular cell biology*, *6*(3), 197–208. https://doi.org/10.1038/nrm1589

Fan, C. M., & Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. Cell, 79(7), 1175–1186. https://doi.org/10.1016/0092-8674(94)90009-4

Faralli, H., & Dilworth, F. J. (2012). Turning on myogenin in muscle: a paradigm for understanding mechanisms of tissue-specific gene expression. *Comparative and functional genomics*, *2012*, 836374. https://doi.org/10.1155/2012/836374

Forcina, L., Cosentino, M., & Musarò, A. (2020). Mechanisms Regulating Muscle Regeneration: Insights into the Interrelated and Time-Dependent Phases of Tissue Healing. Cells, 9(5), 1297. https://doi.org/10.3390/cells9051297

Frietze, S., & Farnham, P. J. (2011). Transcription factor effector domains. Sub-cellular biochemistry, 52, 261–277. https://doi.org/10.1007/978-90-481-9069-0_12

Heallen, T., Morikawa, Y., Leach, J., Tao, G., Willerson, J. T., Johnson, R. L., & Martin, J. F. (2013). Hippo signaling impedes adult heart regeneration. Development (Cambridge, England), 140(23), 4683–4690. https://doi.org/10.1242/dev.102798

Heallen, T., Zhang, M., Wang, J., Bonilla-Claudio, M., Klysik, E., Johnson, R. L., & Martin, J.

F. (2011). Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. Science (New York, N.Y.), 332(6028), 458–461. https://doi.org/10.1126/science.1199010

Hernández-Hernández, J. M., García-González, E. G., Brun, C. E., & Rudnicki, M. A. (2017). The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. Seminars in cell & developmental biology, 72, 10–18. https://doi.org/10.1016/j.semcdb.2017.11.010

Hillmer, R. E., & Link, B. A. (2019). The Roles of Hippo Signaling Transducers Yap and Taz in Chromatin Remodeling. Cells, 8(5), 502. https://doi.org/10.3390/cells8050502

Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B. G., & Gossler, A. (2004). WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomitic mesoderm of mouse embryos. Genes & development, 18(22), 2712–2717. https://doi.org/10.1101/gad.1248604

Hong, W., & Guan, K. L. (2012). The YAP and TAZ transcription co-activators: key downstream effectors of the mammalian Hippo pathway. Seminars in cell & developmental biology, 23(7), 785–793. https://doi.org/10.1016/j.semcdb.2012.05.004

Huang, S. S., Hsu, L. J., & Chang, N. S. (2020). Functional role of WW domain-containing proteins in tumor biology and diseases: Insight into the role in ubiquitin-proteasome system. FASEB bioAdvances, 2(4), 234–253. https://doi.org/10.1096/fba.2019-00060

Hwang, J. H., Kim, K. M., Oh, H. T., Yoo, G. D., Jeong, M. G., Lee, H., Park, J., Jeong, K., Kim, Y. K., Ko, Y. G., Hwang, E. S., & Hong, J. H. (2022). TAZ links exercise to mitochondrial biogenesis via mitochondrial transcription factor A. *Nature communications*, *13*(1), 653. https://doi.org/10.1038/s41467-022-28247-2

Ikeya, M., & Takada, S. (1998). Wnt signaling from the dorsal neural tube is required for the formation of the medial dermomyotome. Development (Cambridge, England), 125(24), 4969– 4976. https://doi.org/10.1242/dev.125.24.4969

Ingham, R. J., Colwill, K., Howard, C., Dettwiler, S., Lim, C. S., Yu, J., Hersi, K., Raaijmakers, J., Gish, G., Mbamalu, G., Taylor, L., Yeung, B., Vassilovski, G., Amin, M., Chen, F., Matskova, L., Winberg, G., Ernberg, I., Linding, R., O'donnell, P., … Pawson, T. (2005). WW domains provide a platform for the assembly of multiprotein networks. Molecular and cellular biology, 25(16), 7092–7106. https://doi.org/10.1128/MCB.25.16.7092-7106.2005

Johnson, R. L., Laufer, E., Riddle, R. D., & Tabin, C. (1994). Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. Cell, 79(7), 1165–1173. https://doi.org/10.1016/0092-8674(94)90008-6

Justice, R. W., Zilian, O., Woods, D. F., Noll, M., & Bryant, P. J. (1995). The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes & development, 9(5), 534–546. https://doi.org/10.1101/gad.9.5.534

Karagounis, L. G., & Hawley, J. A. (2010). Skeletal muscle: increasing the size of the locomotor cell. The international journal of biochemistry & cell biology, 42(9), 1376–1379. https://doi.org/10.1016/j.biocel.2010.05.013

Kaseb, H., Kuhn, J., Gasalberti, D. P., & Babiker, H. M. (2024). Rhabdomyosarcoma. In *StatPearls*. StatPearls Publishing.

Kaya-Çopur, A., Marchiano, F., Hein, M. Y., Alpern, D., Russeil, J., Luis, N. M., Mann, M., Deplancke, B., Habermann, B. H., & Schnorrer, F. (2021). The Hippo pathway controls myofibril assembly and muscle fiber growth by regulating sarcomeric gene expression. eLife, 10, e63726. https://doi.org/10.7554/eLife.63726

Keller, C., & Guttridge, D. C. (2013). Mechanisms of impaired differentiation in rhabdomyosarcoma. The FEBS journal, 280(17), 4323–4334. https://doi.org/10.1111/febs.12421

Kikuchi, K., Rubin, B. P., & Keller, C. (2011). Developmental origins of fusion-negative rhabdomyosarcomas. *Current topics in developmental biology*, *96*, 33–56. https://doi.org/10.1016/B978-0-12-385940-2.00002-4

Kim, M. K., Jang, J. W., & Bae, S. C. (2018). DNA binding partners of YAP/TAZ. BMB reports, 51(3), 126–133. https://doi.org/10.5483/bmbrep.2018.51.3.015

Kohli, P., Bartram, M. P., Habbig, S., Pahmeyer, C., Lamkemeyer, T., Benzing, T., Schermer, B., & Rinschen, M. M. (2014). Label-free quantitative proteomic analysis of the YAP/TAZ interactome. American journal of physiology. Cell physiology, 306(9), C805–C818. https://doi.org/10.1152/ajpcell.00339.2013

Kim, K. M., Yoo, G. D., Heo, W., Oh, H. T., Park, J., Shin, S., Do, Y., Jeong, M. G., Hwang, E. S., & Hong, J. H. (2023). TAZ stimulates exercise-induced muscle satellite cell activation via Pard3-p38 MAPK-TAZ signalling axis. *Journal of cachexia, sarcopenia and muscle*, *14*(6), 2733–2746. https://doi.org/10.1002/jcsm.13348

Kohsaka, S., Shukla, N., Ameur, N., Ito, T., Ng, C. K., Wang, L., Lim, D., Marchetti, A., Viale,

A., Pirun, M., Socci, N. D., Qin, L. X., Sciot, R., Bridge, J., Singer, S., Meyers, P., Wexler, L. H., Barr, F. G., Dogan, S., Fletcher, J. A., … Ladanyi, M. (2014). A recurrent neomorphic mutation in MYOD1 defines a clinically aggressive subset of embryonal rhabdomyosarcoma associated with PI3K-AKT pathway mutations. Nature genetics, 46(6), 595–600.

Kovar, H., Bierbaumer, L., & Radic-Sarikas, B. (2020). The YAP/TAZ Pathway in Osteogenesis and Bone Sarcoma Pathogenesis. Cells, 9(4), 972. https://doi.org/10.3390/cells9040972

Kashihara, T., & Sadoshima, J. (2019). Role of YAP/TAZ in Energy Metabolism in the Heart. *Journal of cardiovascular pharmacology*, *74*(6), 483–490. https://doi.org/10.1097/FJC.0000000000000736

Kawabe, Y., Wang, Y. X., McKinnell, I. W., Bedford, M. T., & Rudnicki, M. A. (2012). Carm1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions. *Cell stem cell*, *11*(3), 333–345. https://doi.org/10.1016/j.stem.2012.07.

Lakshminarasimhan, R., & Liang, G. (2016). The Role of DNA Methylation in Cancer. Advances in experimental medicine and biology, 945, 151–172. https://doi.org/10.1007/978-3-319-43624-1_7

Laumonier, T., & Menetrey, J. (2016). Muscle injuries and strategies for improving their repair. Journal of experimental orthopaedics, 3(1), 15. https://doi.org/10.1186/s40634-016-0051- 7

Le Grand, F., & Rudnicki, M. A. (2007). Skeletal muscle satellite cells and adult myogenesis. Current opinion in cell biology, 19(6), 628–633. https://doi.org/10.1016/j.ceb.2007.09.012

Lei, Q. Y., Zhang, H., Zhao, B., Zha, Z. Y., Bai, F., Pei, X. H., Zhao, S., Xiong, Y., & Guan, K.

L. (2008). TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. Molecular and cellular biology, 28(7), 2426–2436. https://doi.org/10.1128/MCB.01874-07

Li, H., Raghunathan, V., Stamer, W. D., Ganapathy, P. S., & Herberg, S. (2022). Extracellular Matrix Stiffness and TGFβ2 Regulate YAP/TAZ Activity in Human Trabecular Meshwork Cells. Frontiers in cell and developmental biology, 10, 844342. https://doi.org/10.3389/fcell.2022.844342

Li, Q., Sun, Y., Jarugumilli, G. K., Liu, S., Dang, K., Cotton, J. L., Xiol, J., Chan, P. Y., DeRan, M., Ma, L., Li, R., Zhu, L. J., Li, J. H., Leiter, A. B., Ip, Y. T., Camargo, F. D., Luo, X., Johnson, R. L., Wu, X., & Mao, J. (2020). Lats1/2 Sustain Intestinal Stem Cells and Wnt Activation through TEAD-Dependent and Independent Transcription. *Cell stem cell*, *26*(5), 675–692.e8. https://doi.org/10.1016/j.stem.2020.03.002

Lin, K. C., Park, H. W., & Guan, K. L. (2017). Regulation of the Hippo Pathway Transcription Factor TEAD. Trends in biochemical sciences, 42(11), 862–872. https://doi.org/10.1016/j.tibs.2017.09.003

Liu, R., Lee, J., Kim, B. S., Wang, Q., Buxton, S. K., Balasubramanyam, N., Kim, J. J., Dong, J., Zhang, A., Li, S., Gupte, A. A., Hamilton, D. J., Martin, J. F., Rodney, G. G., Coarfa, C., Wehrens, X. H., Yechoor, V. K., & Moulik, M. (2017). Tead1 is required for maintaining adult cardiomyocyte function, and its loss results in lethal dilated cardiomyopathy. *JCI insight*, *2*(17), e93343. https://doi.org/10.1172/jci.insight.93343

Loeb, D. M., Thornton, K., & Shokek, O. (2008). Pediatric soft tissue sarcomas. The Surgical clinics of North America, 88(3), 615–vii. https://doi.org/10.1016/j.suc.2008.03.008

Lum, L., & Beachy, P. A. (2004). The Hedgehog response network: sensors, switches, and routers. *Science (New York, N.Y.)*, *304*(5678), 1755–1759. https://doi.org/10.1126/science.1098020

Luo, J., Deng, L., Zou, H., Guo, Y., Tong, T., Huang, M., Ling, G., & Li, P. (2023). New insights into the ambivalent role of YAP/TAZ in human cancers. Journal of experimental & clinical cancer research : CR, 42(1), 130. https://doi.org/10.1186/s13046-023-02704-2

Lopez-Hernandez, A., Sberna, S., & Campaner, S. (2021). Emerging Principles in the Transcriptional Control by YAP and TAZ. Cancers, 13(16), 4242. https://doi.org/10.3390/cancers13164242

Luo, J., & Yu, F. X. (2019). GPCR-Hippo Signaling in Cancer. Cells, 8(5), 426. https://doi.org/10.3390/cells8050426

Luo, J., Zou, H., Guo, Y., Tong, T., Chen, Y., Xiao, Y., Pan, Y., & Li, P. (2023). The oncogenic roles and clinical implications of YAP/TAZ in breast cancer. *British journal of cancer*, *128*(9), 1611–1624. https://doi.org/10.1038/s41416-023-02182-5

MacQuarrie, K. L., Yao, Z., Fong, A. P., Diede, S. J., Rudzinski, E. R., Hawkins, D. S., & Tapscott, S. J. (2013). Comparison of genome-wide binding of MyoD in normal human myogenic cells and rhabdomyosarcomas identifies regional and local suppression of promyogenic transcription factors. *Molecular and cellular biology*, *33*(4), 773–784. https://doi.org/10.1128/MCB.00916-12

Maroto, M., Bone, R. A., & Dale, J. K. (2012). Somitogenesis. Development (Cambridge, England), 139(14), 2453–2456. https://doi.org/10.1242/dev.069310

Megiorni F. (2020). Epigenetics in rhabdomyosarcoma: cues to new biomarkers and targeted therapies. EBioMedicine, 52, 102673. https://doi.org/10.1016/j.ebiom.2020.102673

Meng, Z., Moroishi, T., & Guan, K. L. (2016). Mechanisms of Hippo pathway regulation. Genes & development, 30(1), 1–17. https://doi.org/10.1101/gad.274027.115

Misra, J. R., & Irvine, K. D. (2018). The Hippo Signaling Network and Its Biological Functions. Annual review of genetics, 52, 65–87. https://doi.org/10.1146/annurev-genet-120417- 031621

Mohamed, A., Sun, C., De Mello, V., Selfe, J., Missiaglia, E., Shipley, J., Murray, G. I., Zammit,

P. S., & Wackerhage, H. (2016). The Hippo effector TAZ (WWTR1) transforms myoblasts and TAZ abundance is associated with reduced survival in embryonal rhabdomyosarcoma. The Journal of pathology, 240(1), 3–14. https://doi.org/10.1002/path.4745

Molkentin, J. D., Black, B. L., Martin, J. F., & Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell, 83(7), 1125–1136. https://doi.org/10.1016/0092-8674(95)90139-6

Mukund, K., & Subramaniam, S. (2020). Skeletal muscle: A review of molecular structure and function, in health and disease. *Wiley interdisciplinary reviews. Systems biology and medicine*, *12*(1), e1462. https://doi.org/10.1002/wsbm.1462

Ornatsky, O. I., & McDermott, J. C. (1996). MEF2 protein expression, DNA binding specificity and complex composition, and transcriptional activity in muscle and non-muscle cells. *The Journal of biological chemistry*, *271*(40), 24927–24933. https://doi.org/10.1074/jbc.271.40.24927

Oudhoff, M. J., Freeman, S. A., Couzens, A. L., Antignano, F., Kuznetsova, E., Min, P. H., Northrop, J. P., Lehnertz, B., Barsyte-Lovejoy, D., Vedadi, M., Arrowsmith, C. H., Nishina, H., Gold, M. R., Rossi, F. M., Gingras, A. C., & Zaph, C. (2013). Control of the hippo pathway by Set7-dependent methylation of Yap. *Developmental cell*, *26*(2), 188–194. https://doi.org/10.1016/j.devcel.2013.05.025

Pani, L., Horal, M., & Loeken, M. R. (2002). Rescue of neural tube defects in Pax-3-deficient embryos by p53 loss of function: implications for Pax-3- dependent development and tumorigenesis. Genes & development, 16(6), 676–680. https://doi.org/10.1101/gad.969302 Parr, B. A., Shea, M. J., Vassileva, G., & McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development (Cambridge, England), 119(1), 247–261. https://doi.org/10.1242/dev.119.1.247

Piccolo, S., Panciera, T., Contessotto, P., & Cordenonsi, M. (2023). YAP/TAZ as master regulators in cancer: modulation, function and therapeutic approaches. *Nature cancer*, *4*(1), 9– 26. https://doi.org/10.1038/s43018-022-00473-z

Pobbati, A. V., & Rubin, B. P. (2020). Protein-Protein Interaction Disruptors of the YAP/TAZ- TEAD Transcriptional Complex. Molecules (Basel, Switzerland), 25(24), 6001. https://doi.org/10.3390/molecules25246001

Pomella, S., Danielli, S. G., Alaggio, R., Breunis, W. B., Hamed, E., Selfe, J., Wachtel, M., Walters, Z. S., Schäfer, B. W., Rota, R., Shipley, J. M., & Hettmer, S. (2023). Genomic and Epigenetic Changes Drive Aberrant Skeletal Muscle Differentiation in Rhabdomyosarcoma. Cancers, 15(10), 2823. https://doi.org/10.3390/cancers15102823

Pourquié, O., Coltey, M., Bréant, C., & Le Douarin, N. M. (1995). Control of somite patterning by signals from the lateral plate. Proceedings of the National Academy of Sciences of the United States of America, 92(8), 3219–3223. https://doi.org/10.1073/pnas.92.8.3219

Powers, S. K., Lynch, G. S., Murphy, K. T., Reid, M. B., & Zijdewind, I. (2016). Disease- Induced Skeletal Muscle Atrophy and Fatigue. *Medicine and science in sports and* exercise, 48(11), 2307–2319. https://doi.org/10.1249/MSS.0000000000000975

Pruitt, B. L., Dunn, A. R., Weis, W. I., & Nelson, W. J. (2014). Mechano-transduction: from molecules to tissues. PLoS biology, 12(11), e1001996. https://doi.org/10.1371/journal.pbio.1001996

Radivojac, P., Baenziger, P. H., Kann, M. G., Mort, M. E., Hahn, M. W., & Mooney, S.

D. (2008). Gain and loss of phosphorylation sites in human cancer. *Bioinformatics (Oxford, England)*, *24*(16), i241–i247. https://doi.org/10.1093/bioinformatics/btn267 Rothbauer, U., Zolghadr, K., Muyldermans, S., Schepers, A., Cardoso, M. C., & Leonhardt, H. (2008). A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Molecular & cellular proteomics : MCP*, *7*(2), 282–289. https://doi.org/10.1074/mcp.M700342-MCP200

Reggiani, F., Gobbi, G., Ciarrocchi, A., & Sancisi, V. (2021). YAP and TAZ Are Not Identical Twins. *Trends in biochemical sciences*, *46*(2), 154–168. https://doi.org/10.1016/j.tibs.2020.08.012

Rios, A. C., Serralbo, O., Salgado, D., & Marcelle, C. (2011). Neural crest regulates myogenesis through the transient activation of NOTCH. *Nature*, *473*(7348), 532–535. https://doi.org/10.1038/nature09970

Romero, G., von Zastrow, M., & Friedman, P. A. (2011). Role of PDZ proteins in regulating trafficking, signaling, and function of GPCRs: means, motif, and opportunity. Advances in pharmacology (San Diego, Calif.), 62, 279–314. https://doi.org/10.1016/B978-0-12-385952- 5.00003-8

Rudnicki, M. A., Braun, T., Hinuma, S., & Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell*, *71*(3), 383–390. https://doi.org/10.1016/0092-8674(92)90508-a

Tang, L., Nogales, E., & Ciferri, C. (2010). Structure and function of SWI/SNF chromatin remodeling complexes and mechanistic implications for transcription. Progress in biophysics and molecular biology, 102(2-3), 122–128. https://doi.org/10.1016/j.pbiomolbio.2010.05.001

Tsumaki, N., & Yoshikawa, H. (2005). The role of bone morphogenetic proteins in endochondral bone formation. *Cytokine & growth factor reviews*, *16*(3), 279–285. https://doi.org/10.1016/j.cytogfr.2005.04.001

Totaro, A., Panciera, T., & Piccolo, S. (2018). YAP/TAZ upstream signals and downstream responses. Nature cell biology, 20(8), 888–899. https://doi.org/10.1038/s41556-018-0142-z Truebestein, L., & Leonard, T. A. (2016). Coiled-coils: The long and short of it. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *38*(9), 903–916. https://doi.org/10.1002/bies.201600062

Santos, M., Hwang, J. W., & Bedford, M. T. (2023). CARM1 arginine methyltransferase as a therapeutic target for cancer. *The Journal of biological chemistry*, *299*(9), 105124. https://doi.org/10.1016/j.jbc.2023.105124

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., & Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell*, *102*(6), 777–786. https://doi.org/10.1016/s0092-8674(00)00066-0

Setiawan, I., Sanjaya, A., Lesmana, R., Yen, P. M., & Goenawan, H. (2021). Hippo pathway effectors YAP and TAZ and their association with skeletal muscle ageing. *Journal of physiology and biochemistry*, *77*(1), 63–73. https://doi.org/10.1007/s13105-021-00787-z

Shishkova, E., Zeng, H., Liu, F., Kwiecien, N. W., Hebert, A. S., Coon, J. J., & Xu, W. (2017).

Global mapping of CARM1 substrates defines enzyme specificity and substrate recognition. *Nature communications*, *8*, 15571. https://doi.org/10.1038/ncomms15571

Slemmons, K. K., Crose, L. E. S., Riedel, S., Sushnitha, M., Belyea, B., & Linardic, C. M. (2017). A Novel Notch-YAP Circuit Drives Stemness and Tumorigenesis in Embryonal Rhabdomyosarcoma. Molecular cancer research : MCR, 15(12), 1777–1791. https://doi.org/10.1158/1541-7786.MCR-17-0004

Song, S., Zhang, X., Huang, Z., Zhao, Y., Lu, S., Zeng, L., Cai, F., Wang, T., Pei, Z., Weng, X., Luo, W., Lu, H., Wei, Z., Wu, J., Yu, P., Shen, L., Zhang, X., Sun, A., & Ge, J. (2024). TEA domain transcription factor 1(TEAD1) induces cardiac fibroblasts cells remodeling through BRD4/Wnt4 pathway. *Signal transduction and targeted therapy*, *9*(1), 45. https://doi.org/10.1038/s41392-023-01732-w

Staley, B. K., & Irvine, K. D. (2012). Hippo signaling in Drosophila: recent advances and insights. Developmental dynamics : an official publication of the American Association of Anatomists, 241(1), 3–15. https://doi.org/10.1002/dvdy.22723

Straface, G., Aprahamian, T., Flex, A., Gaetani, E., Biscetti, F., Smith, R. C., Pecorini, G., Pola, E., Angelini, F., Stigliano, E., Castellot, J. J., Jr, Losordo, D. W., & Pola, R. (2009). Sonic hedgehog regulates angiogenesis and myogenesis during post-natal skeletal muscle regeneration. Journal of cellular and molecular medicine, 13(8B), 2424–2435. https://doi.org/10.1111/j.1582-4934.2008.00440.x

Sun, C., De Mello, V., Mohamed, A., Ortuste Quiroga, H. P., Garcia-Munoz, A., Al Bloshi, A., Tremblay, A. M., von Kriegsheim, A., Collie-Duguid, E., Vargesson, N., Matallanas, D., Wackerhage,

H., & Zammit, P. S. (2017). Common and Distinctive Functions of the Hippo Effectors Taz and Yap in Skeletal Muscle Stem Cell Function. Stem cells (Dayton, Ohio), 35(8), 1958–1972. https://doi.org/10.1002/stem.2652

Sun, W., Chatterjee, B., Shern, J. F., Patidar, R., Song, Y., Wang, Y., Walker, R. L., Pawel, B.R., Linardic, C. M., Houghton, P., Hewitt, S. M., Edelman, D. C., Khan, J., Meltzer, P. S., & Barr, F. G. (2019). Relationship of DNA methylation to mutational changes and transcriptional organization in fusion-positive and fusion-negative rhabdomyosarcoma. *International journal of cancer*, *144*(11), 2707–2717. https://doi.org/10.1002/ijc.32006

Vainshtein, A., & Sandri, M. (2020). Signaling Pathways That Control Muscle Mass. International journal of molecular sciences, 21(13), 4759. https://doi.org/10.3390/ijms21134759

Wang, J., & Conboy, I. (2010). Embryonic vs. adult myogenesis: challenging the 'regeneration recapitulates development' paradigm. Journal of molecular cell biology, 2(1), 1–4. https://doi.org/10.1093/jmcb/mjp027

Wang, W., Li, X., Huang, J., Feng, L., Dolinta, K. G., & Chen, J. (2014). Defining the protein- protein interaction network of the human hippo pathway. Molecular & cellular proteomics : MCP, 13(1), 119–131. https://doi.org/10.1074/mcp.M113.030049

Wang, Z., Lu, W., Zhang, Y., Zou, F., Jin, Z., & Zhao, T. (2020). The Hippo Pathway and Viral Infections. *Frontiers in microbiology*, *10*, 3033. https://doi.org/10.3389/fmicb.2019.03033

Watt, K. I., Goodman, C. A., Hornberger, T. A., & Gregorevic, P. (2018). The Hippo Signaling Pathway in the Regulation of Skeletal Muscle Mass and Function. Exercise and sport sciences reviews, 46(2), 92–96. https://doi.org/10.1249/JES.0000000000000142

Watt, K. I., Turner, B. J., Hagg, A., Zhang, X., Davey, J. R., Qian, H., Beyer, C., Winbanks, C. E., Harvey, K. F., & Gregorevic, P. (2015). The Hippo pathway effector YAP is a critical regulator of skeletal muscle fibre size. *Nature communications*, *6*, 6048. https://doi.org/10.1038/ncomms7048

Wei, B., Dui, W., Liu, D., Xing, Y., Yuan, Z., & Ji, G. (2013). MST1, a key player, in enhancing fast skeletal muscle atrophy. BMC biology, 11, 12. https://doi.org/10.1186/1741-7007-11-12

Wei, X. F., Li, S., & Hu, J. L. (2023). A TurboID-based proximity labelling approach for identifying the DNA-binding proteins. *STAR protocols*, *4*(1), 102139. https://doi.org/10.1016/j.xpro.2023.102139

Wu, S., Huang, J., Dong, J., & Pan, D. (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell*, *114*(4), 445–456. https://doi.org/10.1016/s0092-8674(03)00549-x

Wu, S., Liu, Y., Zheng, Y., Dong, J., & Pan, D. (2008). The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. *Developmental cell*, *14*(3), 388–398. https://doi.org/10.1016/j.devcel.2008.01.007

Yang, Y. F., Cheng, K. C., Tsai, P. H., Liu, C. C., Lee, T. R., & Lyu, P. C. (2009). Alanine substitutions of noncysteine residues in the cysteine-stabilized alphabeta motif. *Protein science : a publication of the Protein Society*, *18*(7), 1498–1506. https://doi.org/10.1002/pro.164

Yin, H., Price, F., & Rudnicki, M. A. (2013). Satellite cells and the muscle stem cell niche. Physiological reviews, 93(1), 23–67. https://doi.org/10.1152/physrev.00043.2011

Yu, D., Cai, Z., Li, D., Zhang, Y., He, M., Yang, Y., Liu, D., Xie, W., Li, Y., & Xiao, W. (2021).

Myogenic Differentiation of Stem Cells for Skeletal Muscle Regeneration. Stem cells international, 2021, 8884283. https://doi.org/10.1155/2021/8884283

Yu, F. X., & Guan, K. L. (2013). The Hippo pathway: regulators and regulations. Genes & development, 27(4), 355–371. https://doi.org/10.1101/gad.210773.112

Yu, P. Y., & Guttridge, D. C. (2018). Dysregulated Myogenesis in Rhabdomyosarcoma. *Current topics in developmental biology*, *126*, 285–297. https://doi.org/10.1016/bs.ctdb.2017.10.007

Yu, Y., Su, X., Qin, Q., Hou, Y., Zhang, X., Zhang, H., Jia, M., & Chen, Y. (2020). Yes- associated protein and transcriptional coactivator with PDZ-binding motif as new targets in cardiovascular diseases. *Pharmacological research*, *159*, 105009. https://doi.org/10.1016/j.phrs.2020.105009

Zanconato, F., Forcato, M., Battilana, G., Azzolin, L., Quaranta, E., Bodega, B., Rosato, A., Bicciato, S., Cordenonsi, M., & Piccolo, S. (2015). Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. Nature cell biology, 17(9), 1218–1227. https://doi.org/10.1038/ncb3216

Zhang, T., Günther, S., Looso, M., Künne, C., Krüger, M., Kim, J., Zhou, Y., & Braun, T. (2015). Prmt5 is a regulator of muscle stem cell expansion in adult mice. *Nature communications*, *6*, 7140. https://doi.org/10.1038/ncomms8140

Zhang, X., Milton, C. C., Humbert, P. O., & Harvey, K. F. (2009). Transcriptional output of the Salvador/warts/hippo pathway is controlled in distinct fashions in Drosophila melanogaster and mammalian cell lines. *Cancer research*, *69*(15), 6033–6041. https://doi.org/10.1158/0008- 5472.CAN-08-4592

Zheng, Y., & Pan, D. (2019). The Hippo Signaling Pathway in Development and

Disease. *Developmental cell*, *50*(3), 264–282. https://doi.org/10.1016/j.devcel.2019.06.003

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z. C., & Guan, K. L. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & development*, *21*(21), 2747–2761. https://doi.org/10.1101/gad.1602907

Appendix II

Table 1: List of Reagents

Reagents	Components
NP-40 Lysis Buffer	0.5 % NP-40 50 mM Tris-HCL pH 7.6 150 mM NaCl 100 mM NaF 10 mM Sodium pyrophosphate 2 mM EDTA 1 mM of Na3VO4 1 mM PMSO
3 X SDS Loading Buffer	Per 8 ml buffer: 0.6 ml of 1 M Tris-HCl (pH = 8) 2.4 ml of glycerol 2.4 ml of 10% SDS 0.6 ml of Beta-Mercaptoethanol Drop Bromo blue dye powder
1 X Transfer Buffer	10% 10X Transfer Buffer 30.3g Tris-HCL 144.2 g Glycine 10 g SDS Equilibrated to 1L with ddH20 80% ddH20 10% of methanol
5% Blocking Buffer	5% nonfat dry milk in 1X TBS 0.1% Tween® 20
1% Blocking Buffer	1% nonfat dry milk in 1X TBS 0.1% Tween® 20
Anti-FLAG M2 magnetic beads	Sigma Aldrich (#M8823)
Dynabeads protein G	ThermoFisher Scientific Invitrogen Dynabeads Protein G (#10003D)
FLAG- 3X peptide	Sigma Aldrich (#SAE0194) (#4799)
Luciferase assay substrate	Promega (#E1910)
Renilla assay reagent	Promega (#E2820)
Mass Spectrometry (per individual replicate)	100 µl of 50 mM NH4HCO3 5.12µL OF 1M Sigma 3483-12-3 DTT 2.69 µL of Thermo-scientific iodoacetamide 1µL of 1mg/mL Thermo-scientific trypsin (20µg of trypsin + 50 mM acetic acid)
SDS gels	Resolving gel: 20mL ddH_2O 16.6mL acrylamide

	12.5mL of pH 8.8 Tris 500µL 10X SDS 200 µL 10% APS 20 µL TMED® <u>Stacking gel:</u> 6.8mL ddH_2O 1.7mL acrylamide 1.25mL of pH 6.8 Tris 100µL 10X SDS 100µL 10% APS 12µL TMED®

Table 2: List of antibodies

Reagents	**Source**
YAP/TAZ	Cell Signaling (#D24E24)
pTAZ Ser89 **Wwtr1 Ser89**	Cell Signaling (#E1X9C) Thermofisher (#PA5-105066)
FLAG	Sigma-Aldrich (#F3165)
HA	Cell Signaling (#C29F4)
CARM1	Cell signaling (#C31G9) Cell signaling (3H2) (#12495)
Myc	Cell Signaling (#9B11)
c-Myc	Cell Signaling (#D84C12)
MyoG	DSHB (#F5D)
MyoD	DSHB (#2A5)
β-Actin	Santa Cruz (#sc-47778)
Histone H3	Cell Signaling (#9715S)
Alpha-tubulin	Cell Signaling (#2144S)
Vimentin	Cell signaling (#D21H3)