FoxP1 represses MEF2A in Striated Muscle

Sydney Steiman

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Abstract

Myocyte Enhancer Factor 2 (MEF2) is a transcription factor complex encoded by genes *mef2a-d*. MEF2 proteins belong to the MADS-box DNA binding protein superfamily involved in many developmental pathways including myogenesis and the survival of cardiomyocytes. MEF2 proteins interact with myogenic regulatory factors and modulate the expression of muscle-specific genes. Thus, unbiased characterization of the MEF2A interactome would expand our understanding of MEF2 functions. A GFP-nanotrap purification followed by LC-MS/MS proteomic analysis was employed to identify MEF2A interacting proteins. Following gene ontology analysis, we focused on an interaction between MEF2A and its novel interacting protein, FOXP1, in striated muscle. FOXP1 was found in the MEF2A-immunocomplex in muscle cells, and ectopic expression of FOXP1 delays myogenic differentiation. FOXP1 inhibited MEF2A activation on myogenic reporter genes driven by the regulatory regions of *creatine kinase muscle* and *myogenin* genes. Additionally, siRNA-mediated deletion of FOXP1 in myoblasts and cardiomyocytes enhances MEF2A *trans*activation properties. Lastly, various biochemical experiments indicate that FOXP1 antagonizes p38 MAPK activation of MEF2A through Threonine-312 phosphorylation. Collectively, we documented a novel repressive interaction of FOXP1 and MEF2A in proliferating striated muscle cells.

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List of Abbreviations

AKT	Protein kinase B
ATP	Adenosine triphosphate
bHLH	Basic helix loop helix
CaMK	calcium/calmodulin-dependent protein kinase
DCM	Dilated cardiomyopathy
DM	Differentiation media
DMD	Duchenne muscular dystrophy
ECM	Extracellular matrix
ERK5	Extracellular signal-regulated kinase 5
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblastic growth factor
FHF	First heart field
FOXP1	Forkhead box protein P1
Gata4	GATA binding protein 4
GFP	Green fluorescent protein
GLUT4	Glucose transporter protein 4
GM	Growth media
GO	Gene ontology
GSK3β	Glycogen synthase 3β
HAT	Histone acetyltransferase
HCM	Hypertonic cardiomyopathy
HDAC	Histone deacetylase
IgG	Immunoglobulin G
IM	intermediate mesoderm
kDA	Kilodalton
KLF2	Krüppel-like factor 2
LC	Liquid chromatography
LPM	Lateral plate mesoderm
MADS	MCM1, agamous, deficiens, serum response factor
MAPK	Mitogen-activated protein kinase
MCK	Muscle creatine kinase
MEF2	Myocyte enhancer factor 2
MRF	Myogenic regulatory factor
MRF4	Myogenic regulatory factor 4
MS	Mass spectrometry
MT	Myotube
Myf5	Myogenic Factor 5

MyHC	Myosin heavy chain
MyoD	Myogenic differentiation
MyoG	Myogenin
NOS	Nitric oxide signaling
NC	Notochord
NT	Neural tube
p53	Tumour protein P53
Pax	Paired box gene
PCM	Primary cardiomyocyte
PE	Phenylephrine
PI3K	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
PPI	Protein-protein interaction
R	Reserve cell
SHF	Secondary heart field
siRNA	Silencing RNA
T	Total
TAD	Transcriptional activation domain
Tbx	T-box protein
TGFβ	Transforming growth factor beta 2
Thr	Threonine
Wnt	Wingless integration site signaling pathway

Chapter 1: Review of Literature

1. General overview of striated muscle

The muscles in our body are responsible for many functions vital to our survival, including movement, blood circulation, and digestion. These muscles are classified into 2 types, striated and non-striated, based on their branching, number of nuclei, banding pattern, and fatigue. This classification is further divided into the 3 types of muscles, smooth, skeletal, and cardiac (Lindskog et al., 2015; Webb, 2003).

Smooth muscle is responsible for involuntary movement and is found in the walls of various organs and structures, including blood vessels and the digestive tract. Smooth muscles are elongated cells with a single nucleus. The contractile proteins actin and myosin, which are responsible for muscle contraction, are organized in a disordered pattern and are arranged in layers or sheets (Webb, 2003).

In contrast, straited muscle is characterized by a highly organized pattern of actin and myosin that gives it a striated or banded appearance. In striated muscle, the actin and myosin filaments are arranged in a highly organized pattern by alternating the placement of light and dark bands. These muscles include both skeletal and cardiac muscles, which are responsible for a variety of functions such as oxygenation and balance (Lindskog et al., 2015; Shadrin et al., 2016). The primary function of the striated muscles is to generate force for movement, aid in respiration through the skeletal muscles, and pump oxygenated and deoxygenated blood to the respiratory system (Shadrin et al., 2016). The composition of the striated muscle group has many physiological similarities. Both are surrounded by an extracellular matrix and have interwoven blood vessels but differ in the number of fibroblasts and the presence of intercalated discs in cardiac cells (Gillies & Lieber, 2011; Shadrin et al., 2016).

Skeletal muscle is extremely versatile in its function in the human body. Skeletal muscle is composed of long striated fibres that are multinucleated, often with nuclei located at the periphery of the cell. Skeletal muscle is responsible for voluntary movement and can regulate temperature by generating heat as a by-product of contraction (Shadrin et al., 2016). Skeletal muscle has a unique ability to regenerate in response to tears or trauma. Resident muscle stem cells, called satellite cells, can be activated to proliferate, and then fuse to repair the area (Dumont et al., 2015; Shadrin et al., 2016).

Cardiac muscle is composed of specialized contractile cells called cardiomyocytes, which, unlike skeletal muscle, often have a central nucleus. These cells have contractile capabilities and are connected through cell junctions called intercalated discs to facilitate synchronistic contraction. These junctions are necessary to withstand the constant contractions required to pump blood around the body at a sustained rate (Severs, 2000; Shadrin et al., 2016). Unlike the regenerative capacity of skeletal muscle satellite cells, cardiac cells do not possess cardiomyogenic stem cell capability and have a much-reduced ability to regenerate damaged areas. Therefore, damage can lead to scarring and impaired heart function over time (Severs, 2000; Tam et al., 1995).

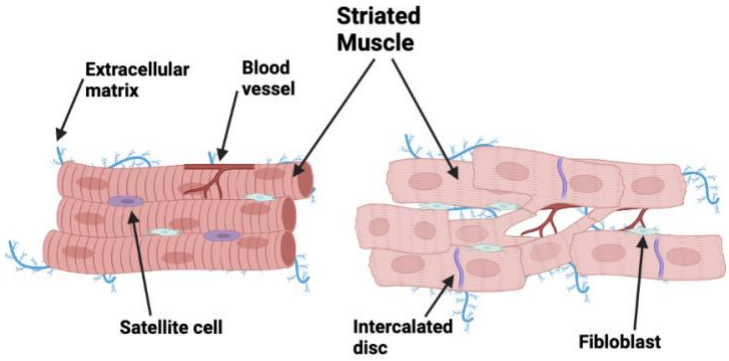

Figure 1: Schematic representation of the two different types of striated muscle. The left is a schematic of skeletal myofibers with satellite cells, muscle stem cells, along the periphery in purple. The right is a schematic of cardiac muscle cells, cardiomyocytes, held together by intercalated discs.

Striated muscles are a necessity of life and require stringent gene expression and signaling to develop properly. Understanding this development and how problems can arise is fundamental to our overall understanding of human physiology and cellular and molecular biology.

1.1 Physiology and formation of striated muscles

The formation of an embryo into a fetus is an extremely complex process involving numerous signaling pathways and the timely activation of a large number of genes. The development of an embryo, embryogenesis, is divided into several steps, beginning with fertilization, followed by cleavage, where the zygote divided and grows, and gastrulation, where the 3 primary germ layers are formed (Amadei et al., 2022; Steventon et al., 2021). The developmental process is influenced by two key components, cytoplasmic determinants such as mRNAs, enzymes, morphogens, and the genome of the zygote. To create a complex organism, cells are given fates early on in embryogenesis to give rise to different organs, tissues, and cell locations along the different axes (Amadei et al., 2022; Gerri et al., 2020). Cells go through three steps to achieve a fate. The first step is specification, in which a cell is instructed what fate it will have and differentiates autonomously or conditionally based on cell-intrinsic or cell-extrinsic factors (Gritz & Hirschi, 2016). The second step is determination, where a fate cannot be reversed and genetic restructuring, such as changes in euchromatin, is expressed (Chen et al., 2010). Finally, there is differentiation, where the specialized cells are produced with selectively expressed genes. While determination does not result in a change in appearance, differentiation involves a change in appearance and function. In human embryos, differentiation can occur at the 16-cell stage; this cluster of cells called a morula, becomes a blastocyst (Gerri et al., 2020; Watson & Barcroft, 2001). A blastocyst consists of two distinct specialized cells, the trophoblast, and the inner cell mass, which is further divided into the hypoblast and epiblast, both of which are pluripotent. This pluripotent property

means that multiple cell lineages can develop from a single progenitor. Unlike the epiblast, the hypoblast does not contribute to the embryo but to the yolk sac. The epiblast undergoes an extremely important event called gastrulation, during which the three primary germ layers, the ectoderm, mesoderm, and endoderm, are formed (Watson & Barcroft, 2001). Each germ layer gives rise to different structures, with the mesoderm and its types responsible for striated muscle formation. The lateral plate mesoderm will form the cardiac muscles, while the paraxial mesoderm will form the skeletal muscles (Amadei et al., 2022; Gerri et al., 2020; Watson et al., 2004).

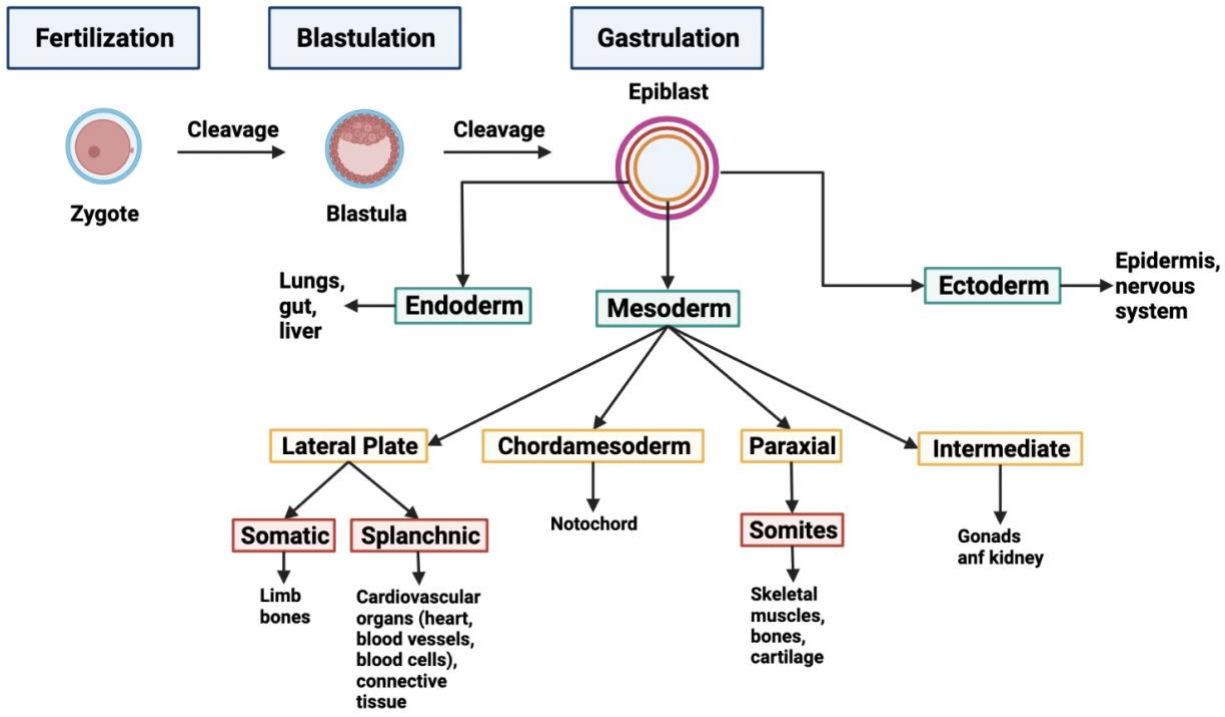

Figure 2: Schematic illustration of the formation of the 3 primary germ layers, endoderm, mesoderm, and ectoderm. The endoderm will give rise to the lungs, gut, and liver. The ectoderm is responsible for the formation of the epidermis and the nervous system. The mesoderm is further subdivided, and each type of mesoderm contributes to distinctive tissues.

The paraxial mesoderm lies along the notochord and neural tube and gives rise to the somites (Maroto et al., 2012). The somites are clusters of progenitor stem cells that give rise to a variety of structures including the vertebrae and skeletal muscles during somitogenesis (Pourquie, 2003; Yamanaka et al., 2023). During this process, many different signaling gradients of

Wingless/Integrated (Wnt), Fibroblastic Growth Factors (FGF), Notch, and Hox transcription factors are activated. These Hox transcription factors specify the somites along the anterior-posterior axis by creating concentration gradients. The first somites specified form the sclerotome, which will give rise to the bones and tendons. The second specified somite's give rise to the dermomyotome, which will then give rise to the skeletal muscles by forming the precursors of the myotome (Aulehla et al., 2003; Pourquie, 2003; Yamanaka et al., 2023).

Over time, these two regions become very distinct and begin to differentiate. The cells that make up the dermomyotome express very specific genes and factors, including Paired box gene 3 (Pax3) and Paired box gene 7 (Pax7). Pax3 is a critical factor present in the lateral mesoderm and muscle satellite cells (Buckingham & Relaix, 2015). In cases where Pax3 is inhibited or lost, heart development can be impaired, resulting in lethality (Conway et al., 1997; Horst et al., 2006). Specific factors expressed in the dermomyotome to form the myotome include a group of transcription factors called Myogenic Regulatory Factors (MRFs). This MRF family of transcription factors consists of *Myogenic Differentiation* (MyoD), *Myogenin* (MyoG), *Myogenic Regulatory Factor 4* (MRF4), and *Myogenic Factor 5* (Myf5). Of these factors, MyoD, MRF4, and Myf5 are the myogenic determinants that ensure that progenitor stem cells undergo myogenesis. MyoG, MyoD, and MRF4 are responsible for myogenic activation (Hernandez-Hernandez et al., 2017; Olson et al., 1996). The protein structure of these factors is very similar, with each having 3 conserved domains. The transcriptional activation domain, a central beta-helix-loop-helix (bHLH) motif, and another transcriptional activation domain at the C-terminus. The factors bind to a conserved sequence, CANNTG, called an E-box, a conserved upstream sequence that regulates the transcription of muscle specific genes, using the basic domain. Other sequences, such as CAGGTG for MyoD, are required for transcriptional activation. These factors are capable

of both homo- and hetero-dimerization with E-proteins (Hernandez-Hernandez et al., 2017; Zammit, 2017). The MRFs Myf5, MyoD, and MRF4 are all required for proper skeletal muscle formation and knockout models of each show lethality or improper muscle formation. Additionally, the expression of MyoD and Myf5 commits cells in the myotome to form a muscle lineage comprised of myoblast cells (Hernandez-Hernandez et al., 2017; Zammit, 2017).

During the myogenic activation, MyoG can induce differentiation to promote myogenesis, resulting in the fusion of mature multinucleated myotubes. MRF binding to E-box sequences can be upregulated by the presence of cofactors such as *Myocyte Enhancer Factor 2* (MEF2) proteins. While many muscle-specific genes have an E-box sequence, they also have a conserved MEF2 site, [(C/T)TA(A/T)$_4$(TA(G/A)], for a tandem binding and gene activation (Gossett et al., 1989; Zammit, 2017).

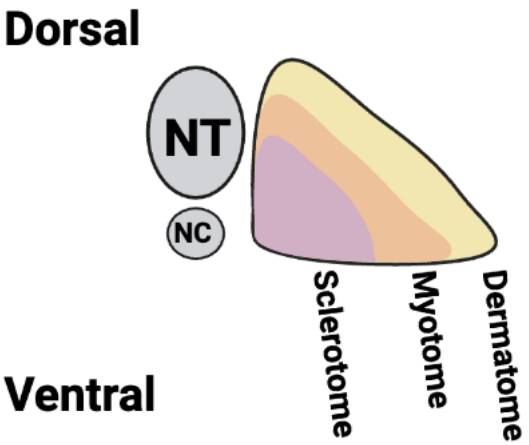

Figure 3: Schematic illustration of the paraxial mesoderm. The cells in the paraxial mesoderm are specified for the sclerotome, which gives rise to the ribs, vertebrae, endothelial cells, and tendons. The dermomyotome gives rise to the skeletal muscles. Cells from the myotome will delaminate and migrate to form proliferating myoblasts when Myf5 and MyoD are upregulated. These myoblasts can undergo differentiation and maturation when MRF4 and MyoG activate the transcription of muscle-specific genes through an E-box consensus sequence. NT is the neural tube and NC is the notochord.

In contrast to skeletal muscle formation, the splanchnic lateral plate mesoderm will form the cardiovascular system. Cardiogenesis is the process of developing the heart, the first functional organ in the developing embryo (Brade et al., 2013). In the heart, there are many specialized cells that form a network for proper functioning. Fibroblasts, endothelial cells, Purkinje fibers, and myocardial cells, are distributed throughout the various chambers and layers of the heart (Brade et al., 2013; Kubalak et al., 1994). Unlike the dermomyotome and sclerotome, the lateral plate mesoderm migrates through the primitive streak at the anterior portion of the epiblast, to the sides of the neural tube. The lateral plate mesoderm is adjacent to the somites (Brade et al., 2013; Yoon et al., 2018).

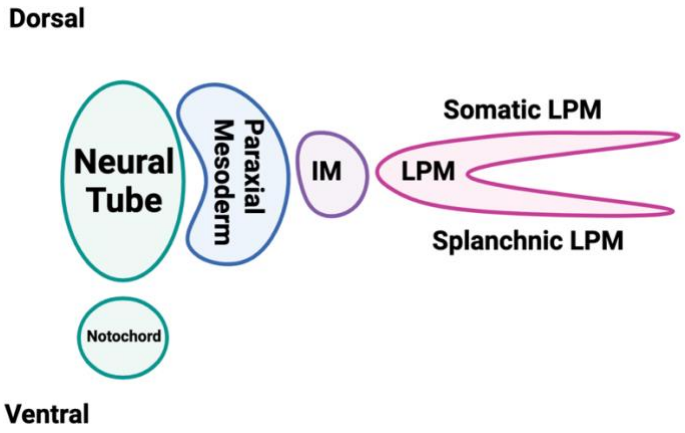

Figure 4: Schematic illustration of the position of the lateral plate mesoderm in relation to the other 2 mesodermal layers. The somatic and splanchnic mesoderm arises from the dorsal and ventral regions of the lateral plate mesoderm, respectively. The splanchnic mesoderm will give rise to the cardiogenic mesoderm in the anterior of the embryo.

Similar to the commitment of the somites during somitogenesis, cells in the lateral plate mesoderm are committed to forming a cardiogenic mesoderm composed of endocardial endothelial cells, atrial myocytes, and ventral myocytes (Yoon et al., 2018). In a region called the First heart field (FHF), the cardiac mesoderm will configure into two tubes that will later form an early heart tube and the left ventricle. A secondary heart field (SHF) is formed adjacent to the FHF, where the right ventricle may arise (Black, 2007). Similar to the MRFs expressed during skeletal myogenesis,

cardiogenesis depends on the expression of cardiac markers. Transcription factors such as Gata4 and Tbx5 drive cardiogenesis in these early progenitors (Watt et al., 2004). Like the impairment of proper skeletal muscle formation in MRF knockout models, inhibition of Gata4 impairs cardiac tube formation. Many other factors are also present and required for heart development, such as MEF2 proteins (Black, 2007; Zlabinger et al., 2019).

Although the two different striated muscles undergo similar formation processes after fertilization and have similar functional units of striated muscle patterning, these two muscles are very different. Skeletal muscles are formed when muscle cells fuse to form multinucleated fibres that are linear (Dumont et al., 2015; Shadrin et al., 2016). In comparison, a syncytium of intercalated discs is required for adhesion. In addition, skeletal muscles are innervated by motor neurons to direct electrical signals for movement, whereas cardiomyocytes are subject to conduction cascades formed by pacemaker cells. However, while voluntary induction differs between these two muscles, both respond to action potentials in a similar manner, requiring ATP to drive actin and myosin filament movement (Severs, 2000; Webb, 2003).

1.2 Skeletal muscle regeneration and differentiation

A unique feature of adult skeletal muscle is its ability to regenerate after muscle trauma and damage. In response to an injury, satellite cells located between the sarcolemma and the basal lamina can become activated (Massenet et al., 2021). Circumstances that trigger this activation include disruption of the sarcolemma by injury, the production of certain signals from damaged muscle cells such as nitric oxide, and fibroblast growth factors released into the extracellular matrix (ECM). Satellite cells express higher levels of Pax7 than the surrounding muscle fibres to maintain an undifferentiated state (von Maltzahn et al., 2013). These satellite cells are generated during embryogenesis from myotome cells that do not undergo differentiation. Depending on the

factor they produce and the abundance of Myf5, these cells can self-renew to replenish the satellite cell population (Hernandez-Hernandez et al., 2017; Schultz & Jaryszak, 1985; Zammit, 2017). When cells do not produce Myf5, they can become quiescent and are referred to as reserve cells that contribute to the stem cell pool. If the cells are expressing Myf5 they undergo myogenesis to repair the injured areas. This homeostasis of satellite cell renewal is critical for maintaining muscle mass and adapting to the levels of injury and formation that muscles undergo (Hernandez-Hernandez et al., 2017; Zammit, 2017).

In response to injury, these satellite cells, which do not self-renew, drive myogenesis by upregulating MRFs in the cells. The first factor to be expressed in these activated satellite cells is Myf5, followed by MyoD during proliferation (Hernandez-Hernandez et al., 2017; Le Grand & Rudnicki, 2007). The expression of MyoD is a critical step in determining whether a cell will continue to express Myf5, MyoD, and MyoG to promote myogenesis and differentiation, or lose MyoD expression to become quiescent (Le Grand & Rudnicki, 2007). Satellite cells knocked out of Myf5 do not undergo the myogenic initiation steps and have reduced expression of MRFs present during differentiation. After activation, many steps take place both in the cells that become myoblasts and in the surrounding ECM where angiogenic factors are activated to revascularize the regenerating tissue. After myoblasts are formed by proliferation, these cells begin to fuse to form mature myofibers. Satellite cells make up approximately 2-7% of the total nuclear content of skeletal muscle, therefore the self-renewal step is critical to maintaining the creation of new muscle mass (Le Grand & Rudnicki, 2007; von Maltzahn et al., 2013).

To maintain quiescence in these satellite cells, genes correlated with cell cycle proteins are repressed for permanent cell cycle exit. A key factor, p53, is required for this cell cycle pause and to balance tumour suppressor factors that, if upregulated, can lead to permanent cell cycle arrest

(Yang et al., 2015). If permanent cell cycle arrest occurs, there will be no activation of satellite cells and therefore no regeneration. Other factors in satellite cells, such as Forkhead box transcription factors, maintain Notch pathways. Notch factors are involved in many cell processes, including proliferation. In addition, these satellite cells have more tightly wound chromatin, resulting in lower protein expression (Wen et al., 2012). Models have shown that these satellite cells have mRNAs for Myf5 and other MRFs but do not undergo translation until needed.

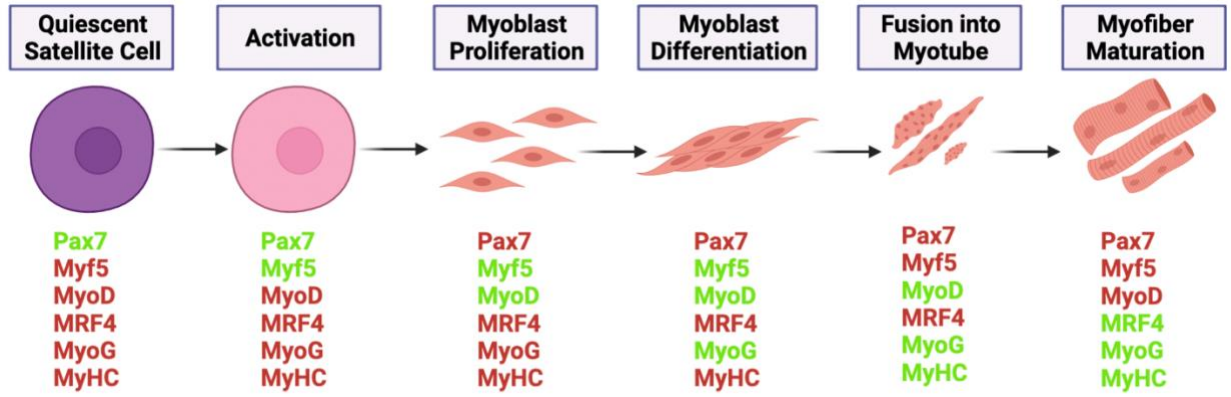

Figure 5: The processes of skeletal muscle regeneration and myogenesis. The activation of muscle satellite cells to undergo myogenesis resulting in mature myofibers is a multi-step process that requires the activation of several factors. The oscillation of these factors from active to inactive drives the differentiation program. Factors that are upregulated are in green while factors downregulated are in red.

1.3 Cardiac hypertrophy and striated muscle dysfunction

While skeletal muscle is capable of regeneration, this replenishment is not infinite. Factors such as an abundance of fibrotic scar in repaired areas, massive muscle loss, advancing age, and sarcopenia will all affect the regenerative capacity and long-term maintenance of new muscle tissue (Neyroud et al., 2021; Shadrin et al., 2016). Certain conditions, such as sarcopenia, the age-related phenomenon of muscle wasting leading to muscle weakness, and cachexia, the muscle wasting associated with disease, contribute to the loss of muscle mass. Both events have been linked to satellite cell depletion and dysfunction (Cornwell & McDermott, 2023). Older mouse

satellite cell samples exhibit impaired fibroblast growth factor 1 (FGFR1) signaling which leads to lower levels of activated p38 mitogen-activated protein kinase (MAPK) signal transduction activity mice (Lassar, 2009; Segales et al., 2016; Shadrin et al., 2016) . These signaling pathways are required for proper myogenesis with both satellite cell proliferation and differentiation. p38/MAPK activation is present in healthy young satellite cells and models with restored levels have shown the potential to rescue satellite cells by restoring the regenerative levels of older cells in aged mice (Lassar, 2009; Segales et al., 2016; Shadrin et al., 2016).

In parallel with diseases that affect skeletal muscle regeneration, cardiac muscle cannot regenerate, but it can be impaired by numerous diseases and factors. A leading heart disease called ischemic heart disease affects adult mortality rates worldwide. In this scenario, atherosclerotic plaques in the coronary arteries increase and cause myocardial infarctions, and heart attacks, when the oxygen supply is interrupted (Severs, 2000; Shadrin et al., 2016; Tam et al., 1995).

Cardiac hypertrophy is a common heart-related disease and manifests as an enlargement of the heart and thickening of the muscle. While this phenomenon can be typical of normal growth induced by exercise, it can also be abnormal when the cardiomyocytes increase in length. This pathological cardiac hypertrophy occurs when the cardiomyocytes lose their elasticity and the thickening of the ventricular walls becomes significant, leading to heart failure. This pathological cardiac hypertrophy can manifest as a result of many different scenarios, such as hypertension and myocardial infarction, ultimately leading to cardiomyocyte death (Cornwell & McDermott, 2023; Shadrin et al., 2016). At the cellular level, these cardiomyocytes undergo sarcomere reorganization with upregulation of certain transcription factors such as c-jun and c-fos. Proteins such as MEF2 can help prevent cardiomyocyte death and promote cardiomyocyte maintenance. However, the expression of MEF2 proteins is low after myocardial infarction. There are many signaling

pathways involved in cardiac hypertrophy including G-protein coupled receptors, MAPK, and calcium ion-dependent pathways (Cornwell & McDermott, 2023; Molkentin & Dorn, 2001; Rose et al., 2010).

While there are a variety of diseases that affect each striated muscle individually, there are many diseases that can affect both simultaneously. Muscle dystrophies caused by mutations in the dystrophin-associated glycoprotein complex and other surrounding proteins, such as laminin, result in damage to all muscles in the body. Duchenne muscular dystrophy (DMD) is a neuromuscular disease caused by dystrophin deficiency and affects one in 5000 boys (Wong et al., 2020). Dystrophin protein is localized in the sarcolemma of both skeletal and cardiac muscle and maintains contraction and relaxation by linking the intercellular skeleton to the extracellular matrix. By the age of 10 years, 81% of DMD patients have hypertonic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), or other cardiac manifestations (Wong et al., 2020).

2. MEF2 transcription factors

The well-structured developmental processes of muscle formation require stringent transcription factor interactions and tight control of gene expression to ensure that satellite cells maintain their stemness and that myoblasts can proliferate and fuse (Le Grand & Rudnicki, 2007). In both skeletal and cardiac muscle, the *Myocyte Enhancer Factor 2* (MEF2) family of transcription factors regulates proper development and function. MEF2 proteins are a MADS (MCM1, Agamous, Deficiens, SRF) box containing family of transcription factors that contain a conserved DNA binding domain to recognize and bind to A/T-rich sequences. These A/T-rich sequences are often found near the E-box sequence of muscle-related genes (Pon & Marra, 2016). The MADS-box is a conserved motif found in different kingdoms and is located within the DNA-binding domains of transcription factors. Minichromosome maintenance 1 (MCM1) is derived

from yeast, Arabidopsis Agamous and Antirrhinum Deficiens are both found in plant species, and the serum response factor (SRF) is found in mammals (Cornwell & McDermott, 2023; Pon & Marra, 2016).

There are four mammalian *Mef2* genes that encode the four different isoforms MEF2A-D, which have the ability to homodimerize with themselves or form heterodimers with MEF2A-D and MEF2B-C. The MEF2 proteins are composed of three distinct regions, the 58 amino acid MADS box for DNA binding at the N-terminus, followed by the 29 amino acid MEF2 domain for dimerization, and the transcriptional activation domain (TAD) at the C-terminus. The N-terminus is highly conserved between isoforms while the C-terminus is highly variable, giving rise to individual isoform function as well as specificity in function when each isoform undergoes post-translational modifications (Cornwell & McDermott, 2023; Pon & Marra, 2016; Potthoff & Olson, 2007).

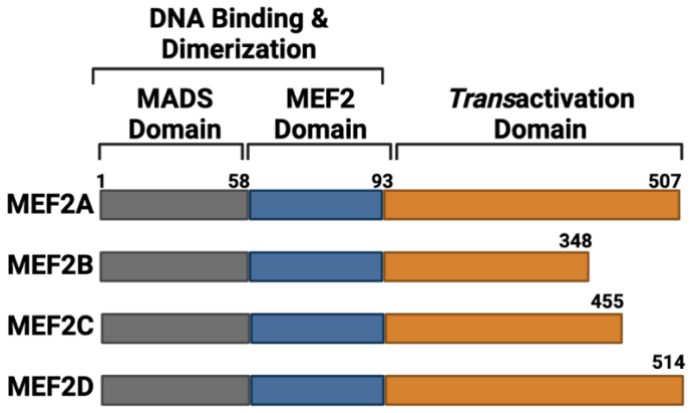

Figure 6: Diagram of the amino acid domains of each MEF2 isoform. MEF2 proteins share a highly conserved N-terminus with a MADS-box and MEF2 domain followed by a transcriptional activation domain that is often the site of post-translational modifications to give isoform and transcript specification.

The MEF2 dimers will form and bind to a specific MEF2 sequence to act as a transcriptional switch, activating or repressing gene expression depending on the presence of

cofactors and post-translational modifications (Gossett et al., 1989). While MEF2 was originally described as a muscle-specific transcription factor, many studies have shown that all isoforms are expressed in many tissues, including the brain. In addition, MEF2 proteins have been implicated in other cellular functions, including apoptosis (Hashemi et al., 2015). The tissue and cell-specific properties make MEF2 proteins an important focus of study. A better understanding of MEF2 and the unique functions of each isoform will provide insight into both our knowledge of protein function specificity and how transcription factors influence a variety of developmental pathways.

2.1 MEF2 Isoforms

In vivo, mouse models have shown that all *Mef2* genes are expressed during embryogenesis in both skeletal and cardiac muscle but decrease in activity postnatally. Many mouse models have been used to determine the expression patterns of each isoform during early myogenesis and cardiogenesis. Mouse knock-out models of *Mef2a-d* have also revealed the phenotypic effects of depleting each isoform (Majidi et al., 2019; Pon & Marra, 2016). In both cardiac and skeletal muscle, *Mef2c* is the first isoform to be expressed. *Mef2c* is expressed before primitive cardiac tube formation at day E7.5 post-coitum and around day E8.5 in the myotome following Myf5 expression in mice. In cardiac muscle, *Mef2c* expression stops at E11.5 but is maintained in skeletal muscle (Pon & Marra, 2016; Potthoff & Olson, 2007). *Mef2a and Mef2d* are expressed shortly after *Mef2c,* and unlike *Mef2c* are maintained in both skeletal and cardiac development. It is important to note that although *Mef2b* mimics the expression patterns of *Mef2c,* it does not appear to have a critical unique input into the developing muscle, unlike the other isoforms. Mouse models with knockouts of *Mef2b* have not shown lethality. Research has suggested that functional redundancy between the isoforms, as well as heterodimerization, may allow *Mef2c* to compensate for the loss of the *Mef2b* isoform (Pon & Marra, 2016).

MEF2A-deficient mice survive through the gestation period but die as neonates due to large heart defects. In contrast, MEF2C-deficient mice will die before birth due to insufficient cardiac looping in the left ventricle and decreased sarcomere organization in skeletal muscle. In addition, MEF2C-deficient mice have impaired brain development as it is required for neuronal differentiation. Both MEF2D and MEF2B null mice models were viable, once again due to the compensation of the other isoforms during embryogenesis (Pon & Marra, 2016; Potthoff & Olson, 2007; Xia et al., 2020). However, while these embryonic MEF2D models are not lethal, adult null MEF2D mice develop cardiac hypertrophy and fibrosis. Adult MEF2A null mice have impaired skeletal muscle regeneration capacity and myoblast differentiation, suggesting that MEF2A is a key factor involved in skeletal myogenesis. In addition, cardiomyocytes lacking MEF2A have myofibrillar defects due to MEF2A regulation of myocardial gene expression (Potthoff & Olson, 2007).

Individual MEF2 isoform activity has been studied in models by Estrella et al. to investigate and compare the target genes of each MEF2 protein. This study used microarray analysis in a C2C12 myoblast culture to determine the number of dysregulated genes in MEF2 single isoform depletion models. While the study found 21 common gene targets among all four isoforms associated with the neural Nitric Oxide (NOS) signaling and Focal Adhesion Kinase (FAK) signaling pathways, it also showed that MEF2A had the largest number of unique target genes with 4,020 dysregulated genes (Estrella et al., 2015).

Table 1: Table summarizing the characteristics of each MEF2 isoform. A table summarizing and comparing different characteristics of each MEF2 isoform, including the time of activation.

Isoform	Time of Activation	Tissue expression	Function	Cardiac Muscle	Skeletal Muscle
MEF2A	E8 in cardiac muscle and E9.5 in skeletal muscle.	Cardiac, skeletal, and smooth muscle. Brain, lung, skin, soft tissue.	Regulate cardiac-related genes, myofibroblast differentiation, skeletal muscle regeneration.	Expressed in the linear heart tube. Required for proper myoblast differentiation.	Satellite cell activation and regeneration.
MEF2B	E7.5 in cardiac muscle to E11.5 and E9 in skeletal muscle.	Cardiac and skeletal muscle, bone marrow, lymph node.	Development of cardiac, smooth, and skeletal muscle.	Expressed in early development.	Expressed in early development.
MEF2C	E7.5 in cardiac muscle and E8.5 in skeletal muscle. Expression halts in cardiac muscle E11.5.	Cardiac and skeletal muscle, nucleoplasm, and brain tissue.	Development of cardiac and skeletal muscle, immune cell production	Expressed in sinus venosus contributing to the cardiac atria and cardiac looping. Essential for murine cardiac development.	Needed for normal fiber composition.
MEF2D	E8 in cardiac muscle and E9.5 in skeletal muscle.	Cardiac and skeletal muscle, endocrine system, and kidneys.	Neuronal development and terminal differentiation of neonatal cardiomyocytes.	Expressed in the linear heart tube.	Satellite cell activation and regeneration.

2.2 Function of MEF2 in striated muscle

As previously mentioned, MEF2 proteins associate with MRFs by recruiting them to the promoter regions of muscle-related genes. While there are four different MEF2 isoforms in mammals, there is only one MEF2 protein in *Drosophila* that drives skeletal muscle formation

(Potthoff & Olson, 2007). Because of this increased complexity in mammals, it is imperative to understand the functions of the different isoforms in striated muscle. MEF2 expression begins as early as myotome formation during the commitment stages by upregulating MRF4 activity and myogenin during differentiation. In addition, the role of MEF2A in the early stages of myoblast differentiation will influence the overall formation of a mature multinucleated myofiber (Gossett et al., 1989; Potthoff & Olson, 2007; Zammit, 2017).

To investigate the role of MEF2 in skeletal differentiation, Estrella et al. used a C2C12 myoblast cell culture to induce differentiation under different isoform null conditions. Their experiments showed that MEF2A null cells have impaired differentiation compared to the other 3 isoforms (Estrella et al., 2015). The null model of MEF2B-, -C, and -D did not affect the fusion of myoblasts into myotubes compared to MEF2A. This makes MEF2A an important transcriptional regulator of myogenesis important to study. While conditional depletion of MEF2A and MEF2D in mice results in phenotypically normal skeletal muscle, *in vitro* models have shown that MEF2A is required for proper differentiation and regeneration (Estrella et al., 2015; Pon & Marra, 2016; Potthoff & Olson, 2007).

MEF2 proteins can control the proper development of the heart, including the differentiation of cells into cardiomyocytes. For example, MEF2 proteins are known regulators of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) and cardiac tropin genes that are required to regulate the intracellular calcium homeostasis of cardiomyocytes (Seth et al., 2004). MEF2 proteins are of great interest to study in the heart due to their sustained expression and function. In contrast to skeletal muscle, MEF2A null models show a negative phenotypic change in the ventricular chamber. MEF2A is required for both atrial and ventricular gene expression, and while

cardiomyocytes cannot regenerate, MEF2A is crucial during cardiogenesis through interactions with MRFs (Black, 2007; Cornwell & McDermott, 2023; Zlabinger et al., 2019).

2.3 MEF2 A-D heterodimerization

Through the unique conserved MEF2 domain, each MEF2 isoform can homodimerize with itself or form a heterodimer between MEF2A-D and MEF2B-C. While the functional redundancy between each isoform makes it difficult to characterize each isoform, recent studies have characterized distinct functions through biochemical experiments such as immunoprecipitation and the presence of specific heterodimer pairs in specific tissues. For example, the MEF2A homodimer is more common in the adult heart (Potthoff & Olson, 2007). In addition, while both MEF2A and MEF2D have persistent expression in skeletal muscle, individual knockouts have shown isoform-dependent, related results (Estrella et al., 2015; Potthoff & Olson, 2007).

Mora et al. studied the MEF2A-D heterodimer during striated muscle-specific expression of the insulin-responsive muscle/adipose-specific glucose transporter (GLUT4) (Mora et al., 2001). Their studies showed that MEF2 dimers affect GLUT4 expression, and through immunoprecipitation experiments, MEF2A homodimers were not present, but as a MEF2A-D heterodimer. Studies such as these suggest that while MEF2A may have an individual function, such as the promotion of myoblast differentiation, its transcriptional switch capabilities may be enhanced through heterodimerization. The overall composition of dimer pairs can influence gene expression through the cofactor relationships of the individual isoforms (Mora et al., 2001).

2.4 MEF2 protein interactions with co-factors and signaling pathways

To function as a transcriptional switch in a variety of tissues, MEF2 proteins are activated and repressed by cofactors and signaling pathways. Protein-protein interactions with MEF2 are

determined by the tissue type, developmental time, and intracellular signaling. During skeletal myogenesis, MEF2 protein interactions with MRFs such as MyoD and myogenin are required to promote proper myoblast differentiation (Gossett et al., 1989; Le Grand & Rudnicki, 2007). In addition, during cardiogenesis, MEF2 will interact with transcription factors such as Gata4 and Tbx (Watt et al., 2004). Protein-protein interactions with MEF2 can be facilitated by cofactor binding to the MADS-MEF2 N-terminus of MEF2, such as histone deacetylases (HDACs), or modifications in the TAD, such as phosphorylation by MAPK p38 pathways (Jayathilaka et al., 2012; Zhao et al., 2005).

Many direct protein interactions with MEF2 have been characterized to understand its role as a transcription factor. HDACs are a family of transcriptional effectors that reduce the binding of transcription factors to chromatin and are divided into 2 classes. Class 1 (1, 2, 3, and 8) and 2 (4, 5, 6, 7, 9, and 10) HDACs differ in their cellular localization, with class 1 HDACs being primarily nuclear (Jayathilaka et al., 2012). Class 2 HDACs can be further characterized as class 2a (4, 5, 7, 9) and class 2b (6 and 10). Class 2a HDACs are highly expressed in muscle and neuronal cells. In order to affect gene expression, class 2a HDACs do not have DNA binding capabilities, but will bind to MEF2 to repress gene transcription (Jayathilaka et al., 2012; Zhao et al., 2005). This interaction is made possible by the binding of an amphipathic helix structure at the N-terminus of the class 2a HDAC to a hydrophobic groove located in the MADS/MEF2 domain of MEF2 (Nebbioso et al., 2009). Subsequent binding between these 2 proteins will prevent the activation of MEF2 activity on the promoter regions of its target genes. The release of the repressor by dephosphorylation of the HDAC protein allows MEF2 to switch from a repressive state to an active state. The repression of MEF2 by class 2a HDACs is essential to maintain a balance of gene expression and prevent the transcription of muscle-specific genes when skeletal muscle cells are

not required to regenerate (Jayathilaka et al., 2012). To balance out the repressive nature of the class 2a HDACs with MEF2, histone acetyltransferases such as p300 can take the place of HDACs when they are phosphorylated to promote gene expression (He et al., 2011). Understanding this balance of MEF2 activity may increase our overall knowledge of satellite cell activation and the promotion of muscle-specific gene activity.

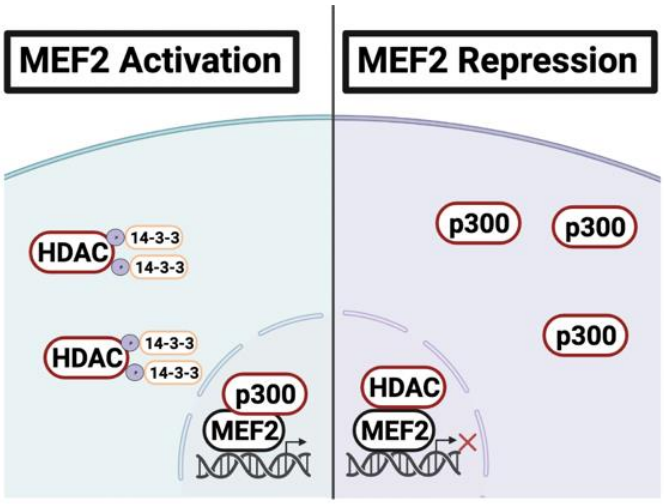

Figure 7: Schematic of the transcriptional switch function of MEF2 proteins. MEF2 proteins can activate the transcription of muscle-specific genes when bound to the DNA or inhibit transcription depending on cofactor interactions.

While direct protein-protein interactions control MEF2 functionality, signaling pathways, kinases, and post-translational medications will influence MEF2 function. Class 2a HDAC binding is facilitated by the concentration of cytoplasmic Ca^{2+} ions, which in turn activate calcium/calmodulin-dependent kinase (CaMK). When CaMK phosphorylates the HDAC protein, it is released from MEF2 and binds to a 14-3-3 protein (Jayathilaka et al., 2012; Lu, McKinsey, Nicol, et al., 2000).

Many kinases are known to interact directly with MEF2 at specific resides. Kinases such as p38 mitogen-activated protein kinases (MAPK), ERK5, PKA, PKC, and GSK3β have all been shown to phosphorylate different MEF2 isoforms at specific amino acid residues in all 3 domains.

The p38/MAPK-MEF2 relationship has been well studied in skeletal muscle as it is required for proper myogenesis to occur (Cox et al., 2003). Phosphorylation of MEF2 by p38/MAPK activates the protein to bind to target genes for gene expression but has also been shown to be over-activated in cardiac hypertrophy. The TAD of MEF2A contains a p38 docking site required for proper MAPK phosphorylation. p38 has been shown to phosphorylate three different amino acid residues on MEF2A in the TAD, Threonine-312, Threonine-319, and Serine-452. In addition, in MEF2C residues Threonine-293, Threonine-300, and Serine-387 are phosphorylated by p38/MAPK. MEF2D influence via phosphorylation activity of p38/MAPK has been recorded but not concluded. ERK5 phosphorylated MEF2C and MEF2A at the conserved residue Serine-387 (Cox et al., 2003; Zhao et al., 1999).

Finally, in addition to these kinase influences, MEF2 is involved in numerous signaling pathways such as Ca^{2+} signaling, Wnt, Notch, PI3K/AKT, and TGFβ (Chen et al., 2017; Wiedmann et al., 2005). The PI3K/AKT pathway is involved in metabolism, cell growth, and angiogenesis. In skeletal muscle, this pathway has been shown to induce muscle hypertrophy, an abnormal increase in skeletal muscle mass. AKT, a serine-threonine protein kinase, induces skeletal muscle growth by activating certain muscle-specific genes. AKT phosphorylates FOXO transcriptional repressors, which in turn affect factors associated with muscle atrophy. The PI3k/AKT pathway can also stimulate MEF2 activity to increase the expression of certain MEF2 target genes, including *Krüppel-like Factor 2* (*KLF2),* an anti-inflammatory transcription factor (Wiedmann et al., 2005).

3. Overview of Protein-Protein interactions

Proteins are small molecules often referred to as the basic building blocks of life. They are composed of amino acids encoded by genes that form peptide structures. While many people may

associate proteins primarily with muscle, they are involved in a multitude of enzymatic and DNA-related processes. Proteins can interact with a variety of signaling molecules such as ligands in phosphorylation cascades, bind to DNA to promote or repress gene expression, and bind to other proteins through protein-protein interactions (PPIs) (Gillies & Lieber, 2011). These physiological roles make proteins a good target for drug therapies. Disruptions in PPIs are found in many diseases, such as DMD, and can have catastrophic effects on the development of a healthy embryo and adult (Wong et al., 2020). In the body, protein-protein interactions are complex structures that govern many cellular, metabolic, and physiological processes. Some examples of protein-protein interactions in the cell include the maintenance of the cytoskeleton, protein synthesis, cell cycle control, and transcription. Tight control of these interactions is necessary to prevent the progression of cancer and potential degenerative diseases. Current drug therapies have shifted focus to targeting the interactions between proteins to disrupt complexes that may form in certain diseases. In addition, emerging cancer therapy research has targeted proteins such as Tumour protein P53 (p53), a protein involved in cell cycle control that, when mutated, is a hallmark of cancer cells (Yang et al., 2015). Therefore, understanding how p53 interacts with its surrounding environment, including other proteins, can help us understand how to stop the mechanisms that lead to cancer. Due to the multitude of post-translational modifications and the complexity of PPIs, understanding how individual proteins can play multiple roles is critical to our understanding of development and disease.

3.1 Protein interactome screen with mass spectrometry

Many new technologies have aided in the investigation of protein networks. While many assays can be used to test binary interactions, understanding a broader network can provide more data on potential novel pathways and interactions. In addition, whole protein screening of more

than binary interactions can be performed at different stages of the cell cycle or even differentiation, providing valuable information about cellular development.

Mass spectrometry is a common high-throughput used to study protein interactions (Moustafa et al., 2023). To perform MS experiments, samples of interest are digested and purified, fragmented, and captured by mass spectrometers. The products of these samples are identified using computational methods that measure the peaks of each peptide. Since the inception of MS, many variations and improvements have been made to increase the rigour and accuracy of the system (Domon & Aebersold, 2006; Mehmood et al., 2015).

Protein MS takes advantage of MS technology and applies it to the study of protein mapping, structure, and quantification. Three different approaches can be used to perform proteomic studies with MS. A top-down approach uses whole proteins in a sample with a matrix-associated laser desorption ionization (MADI) MS and is lower throughput (Domon & Aebersold, 2006). The second is middle-down proteomics using large peptide fragments around 20kDa and finally bottom-up proteomics digests peptide fragments even further and is a high throughput method for peptide identification via peptide sequence. Protein MS is a powerful tool for constructing protein interactome networks (Domon & Aebersold, 2006; Mehmood et al., 2015). An interactome is a map of all the interactions of a given protein. This list of proteins can be categorized by function, cellular location, and enriched for gene ontology analysis. While this approach to studying protein interactions cannot demonstrate how the interactions are occurring or their underlying mechanisms, it gives a strong foundation for studying novel protein interactions (Richards et al., 2021).

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is a powerful analytical tool for protein identification to generate protein networks. This technique involves a

liquid chromatography (LC) separation step and a mass spectrometry detection step as outlined in Figure 8 (Pitt, 2009). The sample is first separated by an LC column based on molecular weight and chemical composition, such as polarity. After ionization of the sample to select the mass-to-charge ratio, the fragments are analyzed in a mass spectrometer. Depending on the experimental design, proteins can be digested into large peptide fragments, short fragments, or left as whole proteins prior to sample addition to the column. In tandem with MS, the initial separation that LC has given a level of strength to increase the capability of the technique (Pitt, 2009).

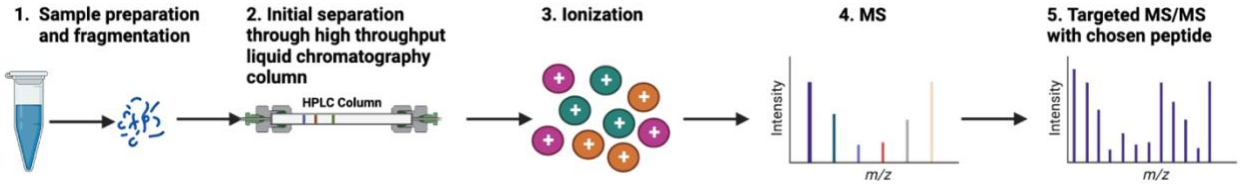

Figure 8. Schematic overview of the LC-MS/MS technique. The LC-MS/MS technique adds an additional separation stage using liquid chromatography prior to the ionization and read-out stages.

3.2 GFP-nanobody protein screening

Another way to generate these complex protein interactome maps is to use green fluorescent protein (GFP) tags and nanobodies to purify GFP-conjugated proteins and their binding partners. Found in jellyfish, GFP has been used in many biochemical applications such as fluorescence imaging and affinity purification (Svendsen et al., 2017). GFP is also extremely versatile, and new technology has allowed the molecule to be conjugated to nanobodies. Nanobodies are like antibodies in their ability to recognize and bind to specific epitopes. However, they are dissimilar in size due to nanobodies being composed of a single antigen-binding variable domain without a light chain domain (de Beer & Giepmans, 2020). Nanobodies were discovered in camelid animals and are approximately 12-15kDA in size compared to larger 150kDa IgG

antibodies. Companies such as Chromotek® have developed GFP-binding nanobodies that can be conjugated to bait proteins to create fusion proteins. These fusion proteins can be incubated with prey protein samples for affinity purification. Nanobodies are an emerging alternative to antibodies for various experiments including affinity purification due to their stability at high temperatures, varying pH, and lower cost (de Beer & Giepmans, 2020). Together, this construct of GFP-bound nanobodies has emerged in recent studies to isolate GFP-tagged proteins and the tagged proteins' binding partners (Zhang et al., 2020).

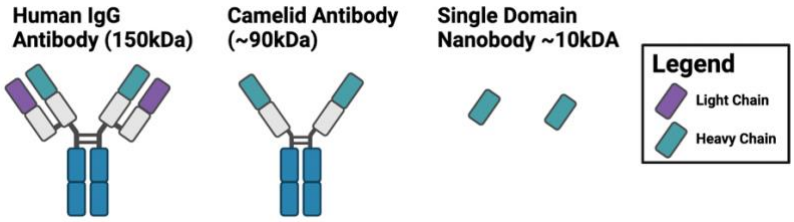

Figure 9: Schematic of a nanobody in comparison to a conventional IgG antibody. The nanobody structure maintains the heavy chain seen in a conventional IgG antibody but lacks the light chain.

Statement of purpose

Proper development of both skeletal and cardiac muscle is necessary for survival, therefore understanding the mechanisms and interactions that drive these processes is both crucial to both our understanding of human development and how to combat striated muscle diseases. Myocyte Enhancer Factor 2, MEF2, is a transcriptional switch that plays a pivotal role in both the maintenance and the function of a variety of tissues, including striated muscle. Previous literature has well characterized many protein-protein interactions with MEF2A during myogenesis and cardiomyocyte survival and cardiogenesis. In addition, MEF2A null mice have neonatal lethality and impaired myoblast differentiation. However, there are still many areas surrounding the role of MEF2 in satellite stem cells, muscle progenitors, and the control of MEF2 during the differentiation of myoblasts to myotubes. Recent literature has linked MEF2 to novel protein interactions that are present in cachexia, muscle wasting due to underlying disease, and cardiac hypertrophy, the abnormal enlargement of the heart. The **purpose of this study was to investigate and characterize novel protein-protein interactions with MEF2** during the different stages of skeletal myogenesis, myoblast proliferation and differentiation, and cardiomyocytes. The objective of the study was to use a proteomic approach using liquid chromatography-mass spectrometry (LC-MS/MS) and nanobodies for affinity purification. Through the implementation of a variety of biochemical approaches, the results indicate a novel repressive protein-protein interaction between MEF2A and Forkhead box protein 1 (FOXP1) through the antagonization of the mitogen-activated kinase (MAPK) p38 phosphorylation of MEF2A via FOXP1. Thus, characterization and investigation of this novel interaction will increase our understanding of the regulation of MEF2A during both developmental processes such as myogenesis and disease phenotypes such as cardiac hypertrophy.

Chapter 2: FoxP1 Represses MEF2A in Striated Muscle

Sydney Steiman[1,2,3], Tetsuaki Miyake[1,2,3] and John C. McDermott[1,2,3*]

[1]Department of Biology, York University, Toronto, ON, M3J 1P3, Canada

[2]Muscle Health Research Centre (MHRC), York University, Toronto, ON, M3J 1P3, Canada

[3]Centre for Research in Biomolecular Interactions (CRBI), York University, Toronto, ON, M3J 1P3, Canada

* To whom correspondence should be addressed.

Tel: +1-416-736-2100x30344; Fax: +1-416-736-5698; email: jmcderm@yorku.ca.

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Author Contributions

Experimental Design
Sydney Steiman, Tetsuaki Miyake, and John C. McDermott

Mass Spectrometry
Tetsuaki Miyake

Drafting Manuscript
Sydney Steiman

Editing Manuscript
Sydney Steiman, Tetsuaki Miyake, and John C. McDermott

Conducting Experiments

Tetsuaki Miyake:	Figure 10A
	Figure 12D
Sydney Steiman:	Figure 10B-E
	Figure 11A-D
	Figure 12A-C
	Figure 13A-F
	Figure 14A-E
	Figure 15A-E
	Figure 16A-C

Abstract

Myocyte Enhancer Factor 2 (MEF2) is a transcription factor complex encoded by genes *mef2a-d*. MEF2 proteins belong to the MADS-box DNA binding protein superfamily involved in many developmental pathways including myogenesis and the survival of cardiomyocytes. MEF2 proteins interact with myogenic regulatory factors and modulate the expression of muscle-specific genes. Thus, unbiased characterization of the MEF2A interactome would expand our understanding of MEF2 functions. A GFP-nanotrap purification followed by LC-MS/MS proteomic analysis was employed to identify MEF2A interacting proteins. Following gene ontology analysis, we focused on an interaction between MEF2A and its novel interacting protein, FOXP1, in striated muscle. FOXP1 was found in the MEF2A-immunocomplex in muscle cells, and ectopic expression of FOXP1 delays myogenic differentiation. FOXP1 inhibited MEF2A activation on myogenic reporter genes driven by the regulatory regions of *creatine kinase muscle* and *myogenin* genes. Additionally, siRNA-mediated deletion of FOXP1 in myoblasts and cardiomyocytes enhances MEF2A *trans*activation properties. Lastly, various biochemical experiments indicate that FOXP1 antagonizes p38 MAPK activation of MEF2A through Threonine-312 phosphorylation. Collectively, we documented a novel repressive interaction of FOXP1 and MEF2A in proliferating striated muscle cells.

Introduction

Striated muscle fulfills a vital function for all metazoans, facilitating movement, blood circulation, and digestion (Frontera & Ochala, 2015). The complex development of striated muscle requires strict control and balance of transcriptional regulators and gene expression. During embryogenesis, both myogenesis, the development of skeletal muscle, and cardiogenesis, the progression of cardiomyocytes into a functional heart muscle, is controlled by basic helix-loop-helix myogenic regulatory factors (MRFs) and a multitude of cofactors (Hernandez-Hernandez et al., 2017) (Taylor & Hughes, 2017). Diseases such as muscular dystrophies, sarcopenias, and cardiac hypertrophy arise when the natural progression of muscle maintenance and formation is disrupted (Medrano & Naya, 2017; Wong et al., 2020). One protein that is critical for striated muscle formation during embryogenesis and the regeneration of skeletal muscle post-natally is Myocyte Enhancer Factor 2 (MEF2). MEF2 protein complexes, encoded by the *mef2a-d* genes, are MADS-box (MCM1, Agamous, Deficiens and SRF) domain-containing transcription factors with the ability to homo- and heterodimerize through a unique conserved MEF2 domain. MEF2 complexes bind to a conserved DNA sequence [(T/C)TA(A/T)$_4$TA(G/A)] and cooperate with MRFs for the expression of a plethora of muscle-specific genes (Cornwell & McDermott, 2023; Wu et al., 2011). Each MEF2 isoform consists of 3 functional domains, a conserved N-terminal MADS-box of 57 amino acids followed by a 29 amino acid MEF2 domain as well as a highly variable transcriptional activation domain at the C-terminus that confers individual function to both the isoform and the post-translationally modified MEF2 protein (Potthoff & Olson, 2007). MEF2 protein activity is expressed as early as day 7.5 post coitum with persistent expression of MEF2A and MEF2D in adult cardiac and skeletal muscle. MEF2 function is influenced by both direct protein binding as well as by cofactors such as class IIa histone deacetylases (HDACs),

MAPK/p38 phosphorylation cascades, and Ca^{2+} signaling pathways. In addition, MEF2 proteins can modulate several developmental signaling pathways including Wingless/Integrated (Wnt), Notch, and the PI3K/AKT inflammatory pathway (Frey & Olson, 2003; Wiedmann et al., 2005).

The role of MEF2 during myogenesis has been well characterized as a factor required for the differentiation program of proliferative myoblasts into mature multinucleated myofibers (Liu et al., 2014). The activation of MEF2 activity is particularly robust following the activation of quiescent muscle stem cells called satellite cells upon stimulation of muscle growth or replacement of damaged muscle. In quiescent satellite cells, MEF2s and certain MRFs are expressed but muscle-specific genes are not expressed, suggesting that MEF2 activity is repressed (Dumont et al., 2015; Taylor & Hughes, 2017). In addition to facilitating skeletal myogenesis, MEF2 activity functions as a pro-survival factor in cardiomyocytes (Hashemi et al., 2015). However, MEF2 has also been implicated in cardiac hypertrophy, a condition in which abnormal thickening of the heart muscle mediates the progression of heart failure. Mouse models have shown that alterations in the expression of MEF2A in the heart can be lethal and disrupt myoblast differentiation (Akazawa & Komuro, 2003; Cornwell & McDermott, 2023; Kolodziejczyk et al., 1999). MEF2A has also been implicated in cancer cachexia, a continuous decline in skeletal muscle mass and is the cause of up to 30% of cancer-related deaths due to either heart or respiratory failure. Recent literature has suggested that there is a dysregulation in MEF2 protein activity through the upregulation of transcriptional repressors and other unknown cofactors (Neyroud et al., 2021).

To fully understand the regulation of MEF2A in skeletal muscle we undertook a systematic unbiased proteomic screen approach to document a MEF2A interactome in striated muscle. This was carried out using a GFP-nanotrap affinity purification followed by LC-MS/MS analysis. This approach provided an interactome map of both known and potentially novel protein-protein

interactions with MEF2A. This interactome map will provide further opportunities to dissect the roles of MEF2A in striated muscle regulation. Categorization of the interactome list and GO analysis revealed a number of novel candidate proteins to investigate including proteins involved in muscle hypertrophy and muscle adaptation. We focused on the identification of Forkhead box protein 1 (FOXP1), a transcriptional repressor recently implicated in sarcopenia and cardiac hypertrophy, as a candidate for further study (Neyroud et al., 2021; Wang et al., 2004). Further experimentation supports a model in which MEF2A repression mediated by FOXP1 is vital to maintain myoblasts in an undifferentiated state in the proliferative or quiescent states. These observations contribute to our ongoing understanding of muscle development and the precise balance between transcription factor regulation and muscle differentiation.

Results

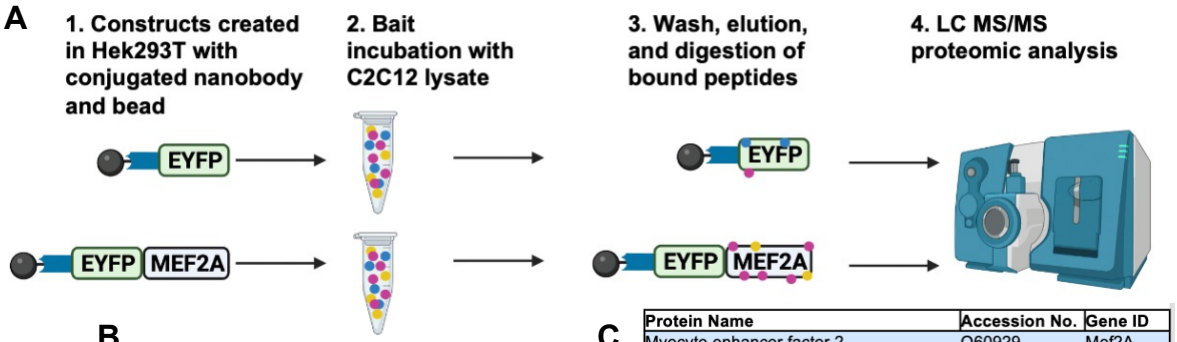
MEF2A interactome analysis in skeletal muscle.

While the role of MEF2 proteins as transcriptional switches and their numerous cofactors have been well documented in various tissues (Moustafa et al., 2023; Mughal et al., 2015), a comprehensive proteomic analysis to establish a network of binding partners in skeletal muscle remains outstanding. Here, we aimed to construct an interactome network using a GFP-nanobody trap affinity purification in tandem with LC-MS/MS to identify proteins interacting with MEF2A in skeletal muscle. To construct this network, EYFP-tagged MEF2A proteins were conjugated to GFP-nanobody magnetic beads for affinity purification without overexpressing MEF2A proteins. Fig. 10A displays the workflow depicting our approach to generate an interactome network. Expression of EYFP and EYFP-MEF2A was confirmed by Western blotting analysis (Fig. 10B).

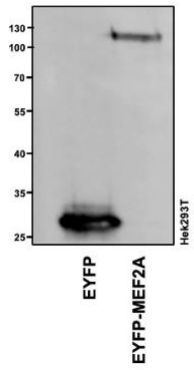
The criteria for including proteins as potential interactors were dependent on the spectral count, the total number of spectra identified for a protein, and the presence of the protein in both replicates. Proteins found in both replicates and not in the GFP conditional control were considered unique proteins. In addition, proteins found in both EYFP-MEF2A replicates with more than 3-fold enrichment over the GFP control were also included. It resulted in a list of 36 proteins (28 unique proteins, (8 > than 3-fold) enriched proteins) (Fig. 10C).

To further characterize this protein interactome network, Gene Ontology (GO) analysis was performed to identify enriched biological processes and molecular functions. Among the processes and functions shown in Fig. 10D, we document several GO terms that have been associated with known MEF2A function, including cardiac muscle hypertrophy and histone deacetylase binding. Interestingly, several categories not previously associated with MEF2A were also identified, such as disordered domain-specific binding. Due to a combination of our interest in myogenesis and the

importance of the muscle-related categories, we decided to focus on interactions within these GO terms (Fig. 10E). Of particular interest from this interactome network was the putative association with Forkhead box protein 1 (FOXP1), which has previously been documented to play a critical role in cardiomyocyte maintenance and sarcopenia.



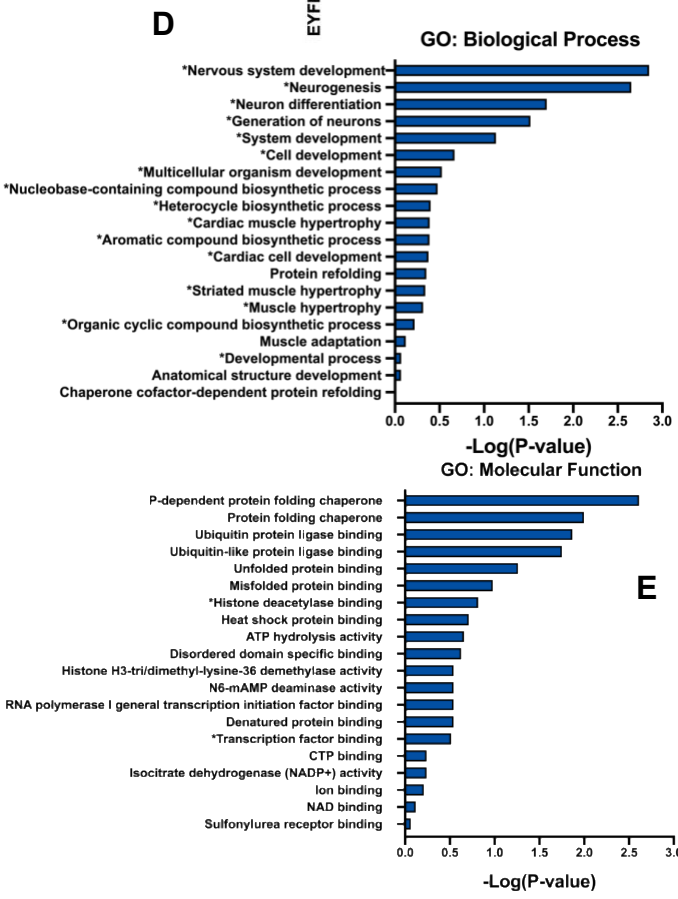
B



C

Protein Name	Accession No.	Gene ID
Myocyte enhancer factor 2	Q60929	Mef2A
Heat shock 70 kDa protein 1-like	P16627	Hspa1l
Ran-binding protein 9	Q8BIV3	Ranbp6
Forkhead box protein P1	P58462	Foxp1
Golgi resident protein GCP60	Q8BMP6	Acbd3
Low-density lipoprotein receptor-related protein 1B	Q9JI18	Lrp1b
DPY30 domain-containing protein 1	Q9D9T0	Dydc1
26S proteasome non-ATPase regulatory subunit 3	P14685	Psmd3
Insulin receptor substrate 4	Q9Z0Y7	Irs4
Cxxc-type zinc finger protein 5	Q91WA4	Cxxc5
Protein jagged-1	Q9QX0	Jag1
Neuritin-like protein	Q8C4W3	Nrn1l
Transmembrane protein 87B	Q8BKU8	Tmem87b
Ras association domain-containing protein 10	Q8BL43	Rassf10
DNA-directed RNA polymerase 1 subunit RPA49	Q8K202	Polr1e
Ovochymase-2	Q7M761	Ovch2
Isocitrate dehydrogenase (NADP) mitochondrial	P54071	Idh2
VSP10 domain-containing receptor	Q9EPR5	Sorcs2
Homeobox protein Hox-C10	P31257	Hoxc10
L-lactate dehydrogenase A chain	P06151	Ldha
Protein c10	O35127	Grcc10
Lysine-specific demethylase 4A	Q8BW72	Kdm4a
Solute carrier family 2, facilitated glucose transporter membrane 7	P0C6A1	Slc2a7
Luc7-like protein 3	Q5SUF2	Luc7l3
Retinoic acid-induced protein 1	Q61818	Rai1
Zinc finger protein 518A	B2RRF6	Znf518a
ATP-dependent DNA helicase Q1	Q9Z129	Recql
Adenosine deaminase-like protein	Q80SY6	Adal
Heat shock 70 kDa protein 1A*	Q61696	Hspa1a
Laminin subunit gamma-1*	P02468	Lamc1
Protein bassoon*	O88737	Bsn
Transmembrane protein 52*	Q9D702	Tmem52
Adhesion G-protein coupled receptor F1*	Q8VEC3	Adgrf1
Immunoglobulin-like and fibronectin type 3 domain-containing protein*	Q3KNY0	Igfn1
Heat shock protein HSP 90-beta*	P11499	Hsp90ab1
PDZ and LIM domain protein 2*	Q8R1G6	Pdlim2

D



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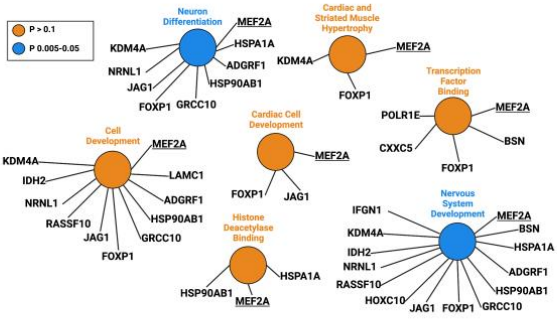


Figure 10. The MEF2A interactome in skeletal muscle. A. Schematic overview of the GFP-nanobody trap affinity purification experiment using a skeletal muscle lysate followed by LC-MS/MS. **B.** EYFP conjugation to a MEF2A construct confirmed by Western blot analysis. **C.** Table showing the uniquely enriched peptides in blue and the >3-fold enriched peptides identified by mass spectrometry analysis. Proteins in blue are unique proteins not found in the GFP control samples while the proteins in orange with an * were found in both the MEF2A and GFP control with a ≥ 3 fold enrichment **D.** Gene Ontology (GO) analysis of the identified interactome peptides for both the biological processes and molecular functions, known MEF2A pathways are indicated with an *. **E.** Schematic representation of the GO terms of interest along with P-values.

FOXP1 interacts with MEF2A in myogenic cells and decreases in expression during muscle differentiation.

First, we wanted to verify the interaction between MEF2A with FOXP1. FOXP1 is a well-characterized transcriptional repressor (Lozano et al., 2021; Rocca et al., 2017). Gene Ontology analysis categorized FOXP1 together with many of the established MEF2A-related categories, including cardiac and striated muscle hypertrophy. By immunofluorescence analysis, we detected both MEF2A and FOXP1 in the nuclei of proliferating myoblasts (Fig. 11A). A protein-protein interaction of ectopically expressed FLAG-MEF2A and HA-FOXP1 was confirmed by FLAG immunoprecipitation followed by Western blotting analysis of the precipitated protein complex (Fig. 11B). Furthermore, endogenous FOXP1 was found in the immunocomplex precipitated with MEF2A antibody (Fig. 11C) suggesting the formation of a FOXP1/MEF2A protein complex in myoblasts.

We further sought to determine whether there is a functional relationship between MEF2A and FOXP1. To this end, we investigated the potential functional impact of ectopic FOXP1 expression on the *trans*activation properties of MEF2A. 4XMEF2 luciferase reporter gene assay was used to quantify the transcriptional effect of the presence or absence of ectopically expressed FOXP1. Fig. 11D indicates that the ectopic expression of MEF2A activated the reporter gene and that the addition of ectopic expression of FOXP1 significantly reporter gene activity by MEF2A expression. Since MEF2A activation is essential for myogenesis with MRFs (Taylor & Hughes,

2017; Wu et al., 2011) and FOXP1 inhibits MEF2A activity, we hypothesized that FOXP1 might play an important role in restricting MEF2A activity in proliferating myoblasts.

Following the initial characterization experiments of the MEF2A/FOXP1 interaction, the pattern of FOXP1 expression during myogenic differentiation was examined. Western blotting analysis documents that FOXP1 protein expression is substantial in myoblasts in the proliferative phase but decreases upon initiation of the differentiation and progression of myoblasts into mature multi-nucleated fused myotubes (Fig. 12A). Upregulation of Myogenin and MCK proteins and their accumulation indicated the progression of myotube formation. Immunofluorescence analysis of differentiated C2C12 cultures at different stages of differentiation depicts that FOXP1 expression was sequestered in the nucleus relative to the cytoplasm (Fig. 12B). Since it is well documented that MEF2A protein expression is present at both the proliferative myoblast and myotube stages of myogenesis, our observations support the idea that FOXP1 is required for the maintenance of the undifferentiated state by suppressing MEF2A activity in proliferating myoblasts.

We further extended this idea to a heterogenous myotube culture conditions to determine differences in the pattern of FOXP1 expression between differentiated myotubes and cells that do not form multinucleated myotubes but are quiescent reserved cells (RCs). We reasoned that if FOXP1 is required for the maintenance of the undifferentiated state, FOXP1 might be differentially expressed in myotubes and RCs. To test this idea, we fractionated a heterogenous C2C12 population under DM conditions into myotubes and quiescent reserved cells. In Fig. 12C Western blot analysis of each fraction indicates that FOXP1 protein is undetectable in the myotube fraction but clearly detected in the RC fraction suggesting that FOXP1 expression is preserved in undifferentiated cells whether they are proliferating or quiescent. A differentiated primary mouse

satellite cell primary culture exhibited similar expression patterns of FOXP1 in undifferentiated cells by immunofluorescence analysis (Fig. 12D). Cumulatively, these data highlight FOXP1 protein expression in undifferentiated cells in both the proliferative and quiescent myoblasts states and its subsequent decline in the differentiation stages of myogenesis.

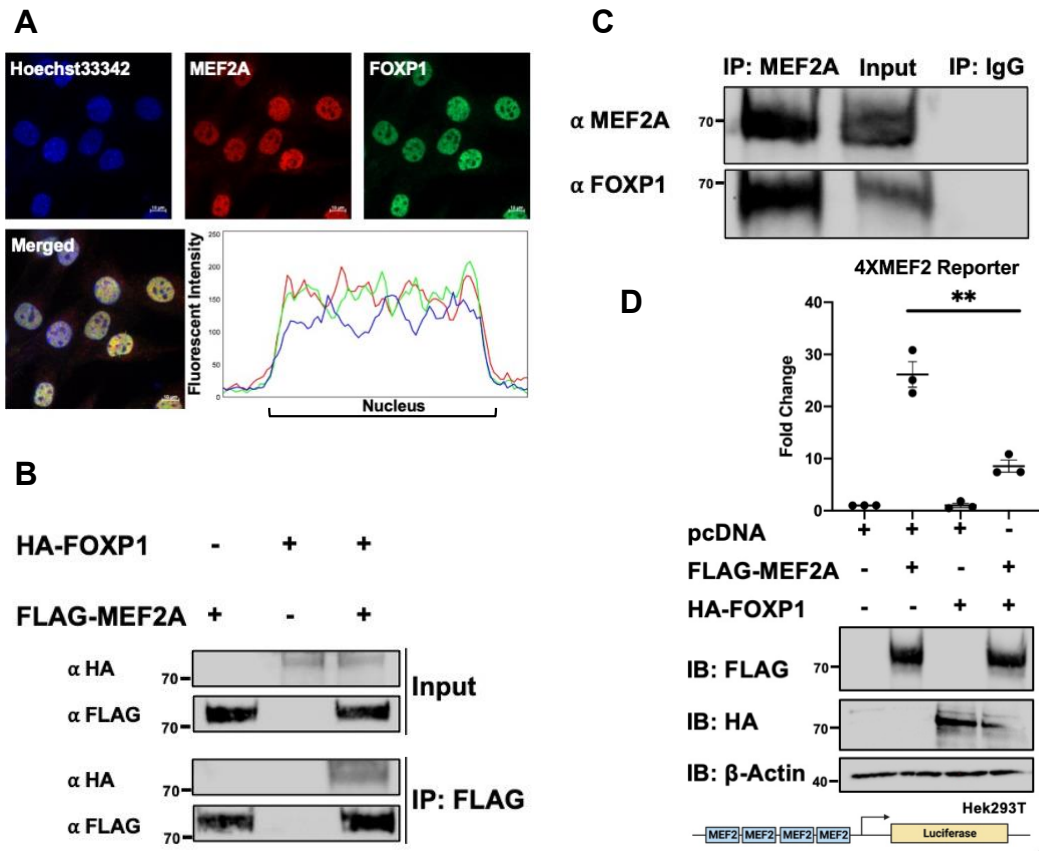


Figure 11. FOXP1 interacts and colocalizes with MEF2A in myoblasts. A. Immunofluorescence analysis of a fixed myoblast culture shows endogenous MEF2A expression in red and FOXP1 protein expression in green localized to the in the nuclei of myoblasts. **B.** Hek293T cells with ectopically expressed HA-FOXP1 and FLAG-MEF2A were subjected to a FLAG immunoprecipitation. Lysates with non-transfected FLAG-MEF2A and HA-FOXP1 were used as controls. **C.** A C2C12 lysate was subjected to a MEF2A immunoprecipitation with an IgG control. Co-immunoprecipitated FOXP1 was visualized by Western blotting analysis. **D.** Hek293T cells were transfected with a 4XMEF2-luc construct in tandem with FLAG-MEF2A and HA-FOXP1. A schematic of the 4XMEF2-luc construct is shown below. *Renilla* luciferase was used to normalize transfection efficiency and pcDNA was used as a control for endogenous protein activity. Each dot represents one biological replicate.

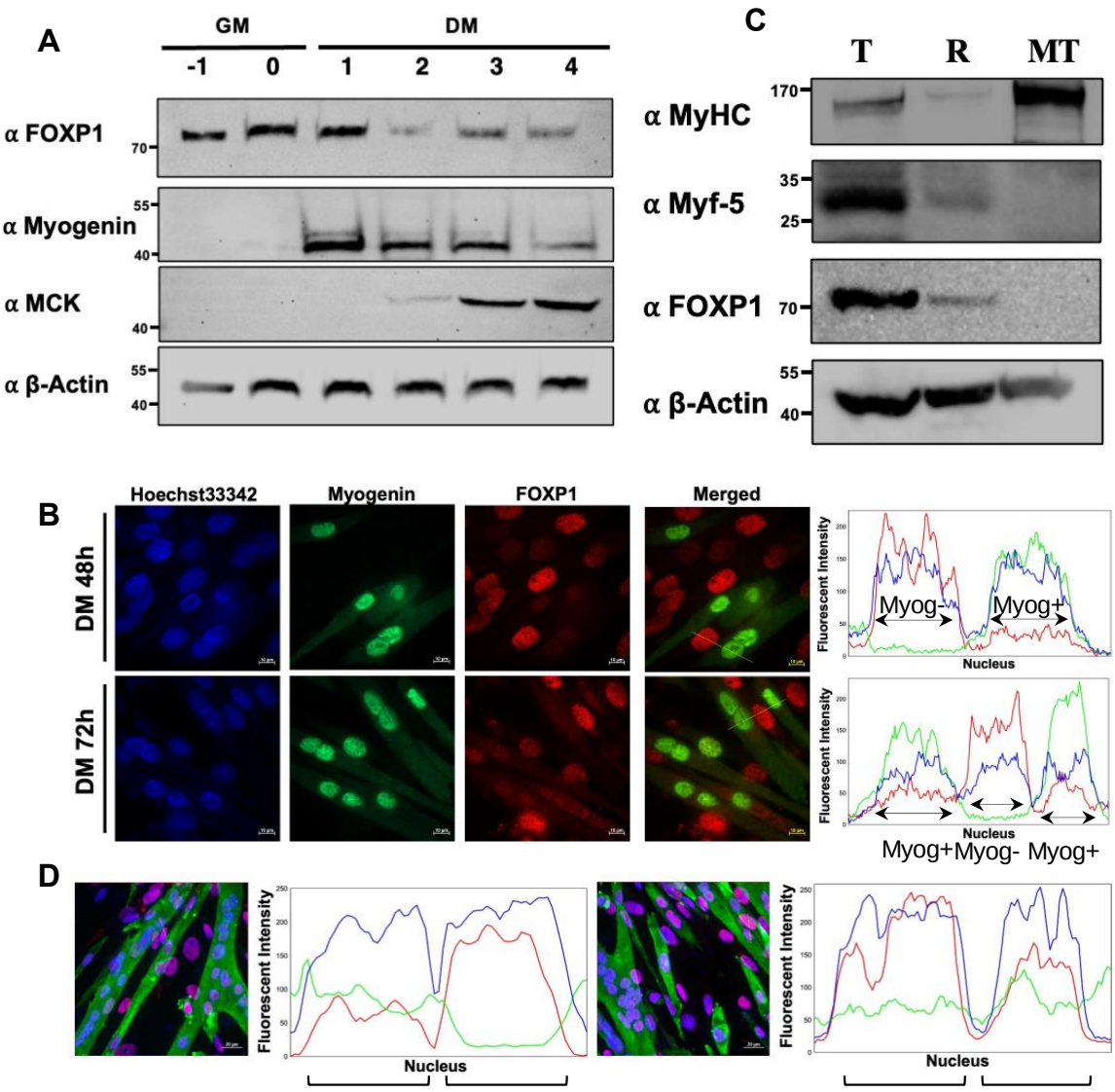


Figure 12. FOXP1 protein decreases during myoblast differentiation. **A.** C2C12 myoblast lysates were collected from cultures incubated in GM and DM for Western blot analysis. Myogenic differentiation markers Myogenin and MCK were used as controls for differentiation, while β-actin was used as a loading control. **B.** Immunofluorescence analysis of a fixed differentiated C2C12 culture showing the endogenous levels of Myogenin in green and FOXP1 in red in the Hoeschst33342 (blue) stained nuclei of myotubes. **C.** A C2C12 cell culture grown in DM was fractionated into a myotube, reserve cell, and total protein lysate for 48h before Western blotting analysis. **D.** Immunofluorescence analysis of a fixed primary mouse satellite cell culture to visualize the endogenous expression of myosin heavy chain in green and FOXP1 in red. The intensity blot of FOXP1, MyHC and Hoechst33342 is shown with the yellow line.

FOXP1 gain and loss of function during myoblast differentiation.

Based on our observations of FOXP1 expression in myoblasts and knowledge of the importance of MEF2A in myogenic differentiation, we determined if FOXP1 could regulate the differentiation process. To investigate this, both a gain of function by ectopic expression of FOXP1 and a loss of function by siRNA targeting FOXP1 were employed.

Initially, we tested Myogenin induction as a MEF2 target gene and marker for the initiation of myotube formation, with ectopic expression of FOXP1. As is well documented in the literature, Western blotting analysis (Fig. 13A) confirmed the upregulation of Myogenin protein level at 48h in DM (Tripathi et al., 2019). However, when HA-FOXP1 was ectopically expressed, Myogenin protein levels failed to be induced. Ectopic expression of HA-FOXP1 reduced the number of Myogenin-positive cells in the population in comparison to a control condition (Fig. 13B). Further immunofluorescence analysis showed that there was a decrease in the number of Myogenin positive cells under conditions of high FOXP1 protein expression.

Taken together, an increase in FOXP1 protein expression results in a delay in the normal progression of myoblast differentiation. To further our understanding of the potential importance of FOXP1 during myogenesis, we employed a loss-of-function approach with siRNA targeting FOXP1. For a loss-of-function analysis, 3 independent siRNAs targeting different regions of FOXP1 mRNA were employed along with a scrambled control. The siRNAs and control were transfected and confirmed efficient reduction of FOXP1 expression (Supp. 1). Western blotting analysis was done during both growth and during the course of differentiation. Compared to scrambled conditions, siRNA-treated cultures exhibited precocious expression of Myogenin (Fig. 13C). Due to FOXP1 depletion, both Myogenin and Muscle Creatine Kinase protein expression were expressed one day earlier than controls upon induction of differentiation. This was further

observed by immunofluorescence analysis quantifying the number of Myogenin-expressing nuclei, which was significantly higher in the FOXP1 depleted condition by the siRNA compared to the scrambled control (Fig. 13D). Finally, to investigate how the loss of FOXP1 function may affect MEF2A activity, 4XMEF2 reporter gene activity assay with 3 independent siRNAs targeting FOXP1 was performed. The reporter gene analysis in Fig. 13E shows that the depletion of FOXP1 resulted in an increase in reporter gene activity. Taken collectively, these data support a model in which FOXP1 suppresses MEF2A function to prevent premature muscle differentiation in proliferating or quiescent myoblasts.

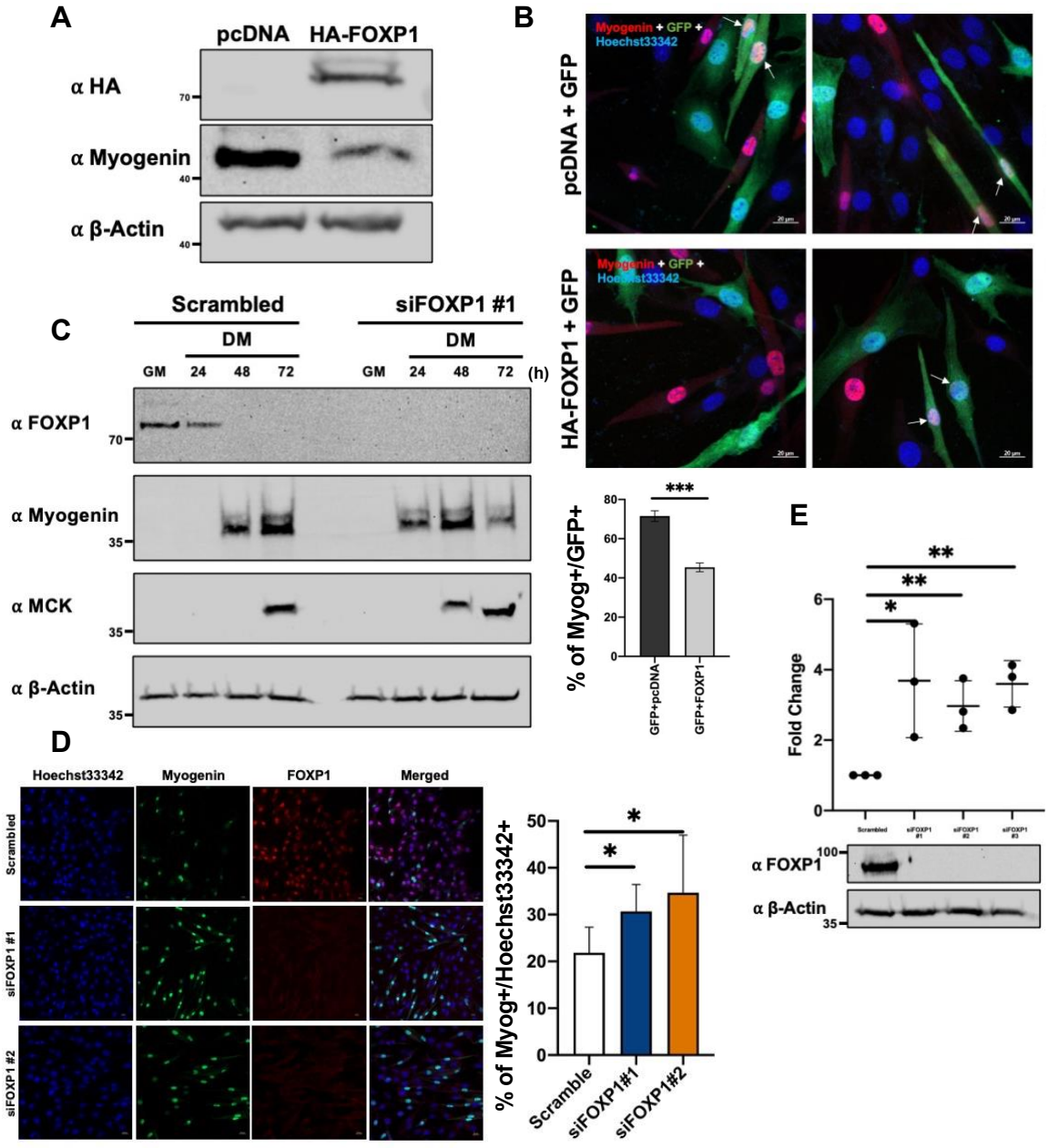


Figure 13. Gain and loss of function of FOXP1 during myoblast differentiation. A. Western blot analysis of a 48h DM C2C12 lysate with ectopic expression of HA-FOXP1 and pcDNA control. **B.** Immunofluorescence of fixed transfected 48h DM C2C12 plates, GFP positive cells were counted over 3 biological replicates. The graph shows the percentage of Myogenin expressing GFP transfected cells. **C.** C2C12 lysates were collected during GM and DM conditions with post-transfection of a scrambled control or siRNA targeting FOXP1 and visualized by Western blotting analysis. **D.** Immunofluorescence visualization followed by the quantification of Myogenin expressing nuclei with an associated bar graph for 3 biological replicates. **E.** C2C12 cells were transiently transfected with a 4XMEF2-luc, a *Renilla* luciferase transfection control, and either a

scrambled or siRNA targeting FOXP1. 3 biological replicates were analyzed and the statistical significance was calculated using GraphPad Prism 8.0 using an independent t-test.

FOXP1 antagonizes MEF2A transcriptional activation activity.

While the data on synthetic reporter genes indicate that FOXP1 antagonizes MEF2A *trans*activation, we next employed the use of well-characterized natural myogenic gene reporters that are known to be regulated by MEF2. A *creatine muscle kinase* (*ckm*) and a *myogenin* promoter driving a luciferase reporter gene were used for this analysis. Fig. 14A-B shows that ectopic expression of FLAG-MEF2A activates both the *ckm* and *myogenin* reporter; however, when cells were co-transfected with HA-FOXP1, there was a decrease in reporter gene activation by MEF2A. Collectively, this reporter gene analysis demonstrates the repressive nature of FOXP1 on MEF2A-driven transcriptional activity which results in the reduction of not only synthetic MEF2 reporter gene activation but also natural myogenic promoter activity.

MEF2A activity is regulated by a variety of co-factors. Among them, class II HDACs and P300 have been reported to regulate MEF2A activity associated with MEF2's MADS/MEF2 domain. To determine which MEF2A domain is required to facilitate this FOXP1 interaction, several GAL4-DNA binding domain (DBD) fusion constructs were used on a 5XUpstream Activator Sequence (UAS) luciferase reporter gene system. A map of the different constructs with the GAL4-DBD at the N-terminus is shown in Fig. 14C. Fig. 14D reporter gene analysis with ectopic expression of all GAL4-DBD-MEF2A fusion proteins activated the reporter. However, the addition of HA-FOXP1 reduced GAL4-DBD-MEF2A driven reporter gene activity. Of specific note was the conserved FOXP1 repression of the GAL4-DBD-MEF2A fusion proteins containing only a few amino acids within the *trans*activation domain. In addition, if the TAD of MEF2A was replaced with the VP16 *trans*activation domain (MEF2A 1-91a.a.(MADS/MEF2)-VP-16), this MEF2A 1-91-VP16 was not repressed in the presence of HA-FOXP1 (Fig. 14E). This revealed

that the MEF2A's MADS/MEF2 domain is not sufficient but the *trans*activation domain, in particular amino acids 273-373, are necessary for FOXP1-mediated repression.

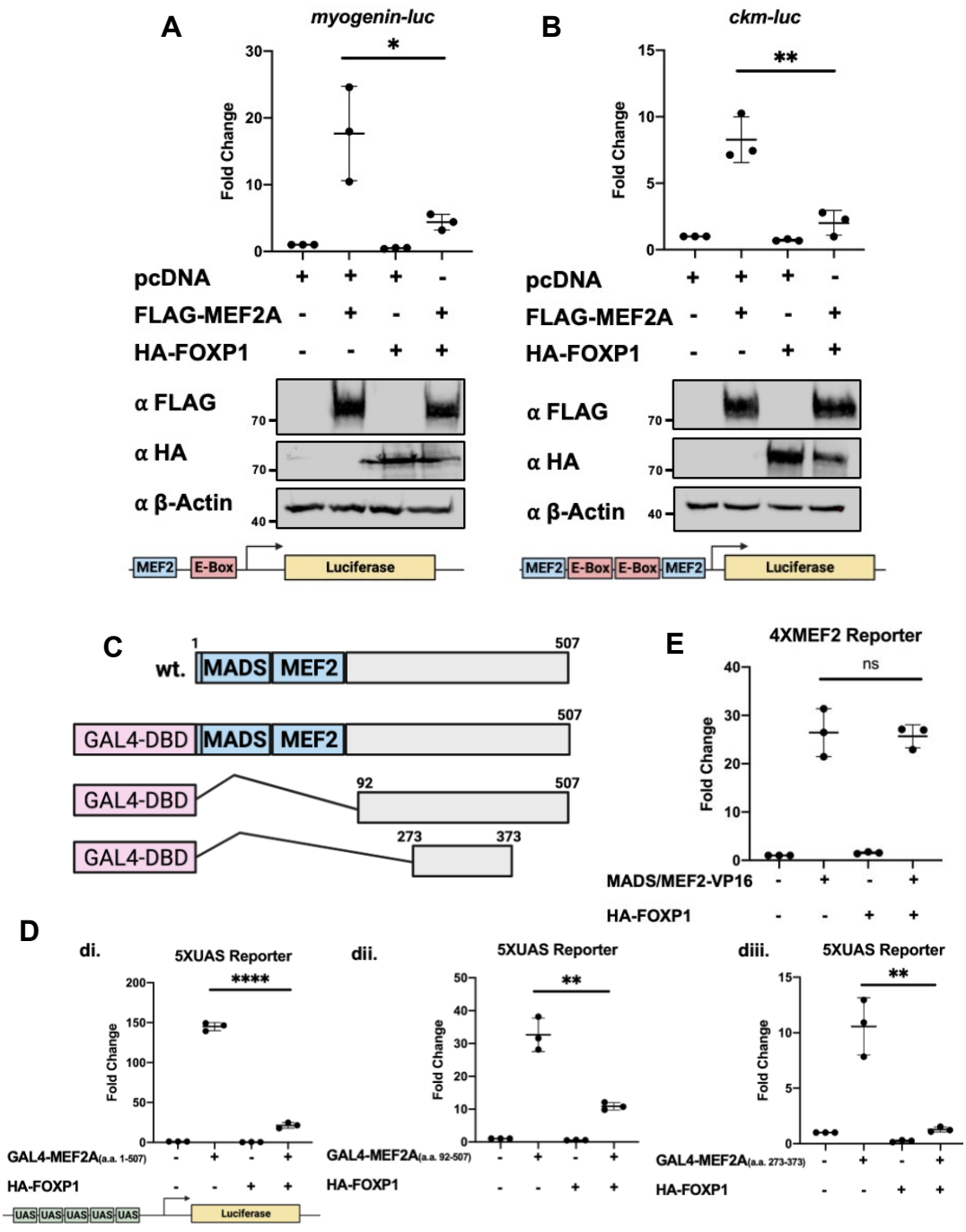


Figure 14. FOXP1 antagonizes MEF2A *trans*activation. A. FLAG-MEF2A in combination with and without HA-FOXP1 was ectopically expressed in C2C12 with a *myogenin*-luc reporter gene, Western blot analysis confirming the transfection of the constructs is shown below. **B.** FLAG-MEF2A in combination with and without HA-FOXP1 was ectopically expressed in C2C12 using a *ckm*-luc reporter gene, Western blot analysis confirming the transfection of the constructs is shown below. **C.** Schematic of the 3 GAL4-DBD-MEF2A fusion proteins, the numbers indicate the amino acid residues within the MEF2A peptide. **D.** Hek293T transfected with the GAL4-DBD

-MEF2A fusion constructs in combination with HA-FOXP1 and a 5XUAS-luc reporter for GAL4-DBD binding. **E.** Hek293T culture was transfected with a MADS/MEF2 domain fused to a VP16 *trans*activation domain in combination with HA-FOXP1 and a 5XUAS-luc reporter. *Renilla* luciferase was used as a transfection control in all experiments and pcDNA was used to control for endogenous protein activity. The error bars represent s.e.m. compared to the relevant control conditions *P<0.01, **P<0.001, ****P<0.0001. For each reporter experiment 3 biological replicates were conducted.

FOXP1 repression of MEF2A is associated with de-phosphorylation of the MEF2A transcriptional activation domain.

Since the experimental data with GAL4-DBD-MEF2A fusions pointed to an interaction of FOXP1 with the C-terminal TAD of MEF2A, we sought to determine how FOXP1 might repress MEF2A transcriptional activity through its TAD. Previously, we have documented that the TAD of MEF2A is highly regulated by p38MAPK phosphorylation (Cox et al., 2003). In addition, this region of the TAD overlaps with a p38MAPK docking site (Barsyte-Lovejoy et al., 2002). We, therefore, hypothesized that FOXP1 repression might be facilitated by disruptions in TAD phosphorylation by p38 MAPK. In Fig. 15A, reporter gene analysis with a p38MAPK inhibitor SB203580 but not its inactive analog SB202474 was employed. As expected, a decrease in MEF2A transcriptional activity on the 4XMEF2 reporter gene was observed with p38MAPK inhibition. Conversely, FOXP1 repressive activity on MEF2A was not evident under conditions when p38 MAPK was inhibited. To further test this idea, the level of MEF2A phosphorylation at Thr-312 (one of the core p38MAPK target sites on MEF2A) (Cox et al., 2003) was assessed by Western blotting analysis. As previously documented, activation of the p38 MAPK by a constitutively active upstream kinase, MKK6(EE), was confirmed by phosphor-Thr-312 analysis of MEF2A (Fig. 15B). As predicted, the level of phosphorylation of MEF2A at Thr-312 was markedly decreased with ectopic HA-FOXP1 expression even in the presence of robust MKK6/p38MAPK activation. Immunofluorescence analysis confirmed that the level of Thr-312 phosphorylation was decreased in C2C12 cells expressing ectopic HA-FOXP1 protein (Fig. 15C).

One of the possibilities by which FOXP1 prevents phosphorylation of MEF2A at Thr-312 by p38MAPK was FOXP1 directing phosphatase activity toward MEF2A (Cox et al., 2003; Rashid et al., 2014). To investigate this possibility, we used a pan-phosphatase inhibitor, Okadaic acids. Fig. 15D Western blot analysis confirmed that ectopic expression of HA-FOXP1 reduced the phosphorylation of Thr-312 of MEF2A, but in the presence of the phosphatase inhibitor, ectopically expressed HA-FOXP1 did not reduce Thr-312 phosphorylation levels. Furthermore, in the presence of a phosphatase inhibitor, FOXP1 failed to repress MEF2A transcriptional activity measured by MEF2 luciferase reporter gene activity (Fig. 15E). Since we have previously documented repression of MEF2A function by protein phosphatase 1 alpha (PP1α) (Perry et al., 2009), we tested if PP1α is in a MEF2A/FOXP1 protein complex. Western blotting analysis of a FLAG-immunoprecipitation revealed the FLAG-FOXP1 indeed co-immunoprecipitated with HA-PP1α (Fig. 15F). This analysis suggests that FOXP1 maintains MEF2A repression by preventing MEF2A from phosphorylation mediated activation by p38MAPK and that this repression is mediated by the formation of a repressive immunocomplex including a phosphatase, for example, PP1α in undifferentiating cells.

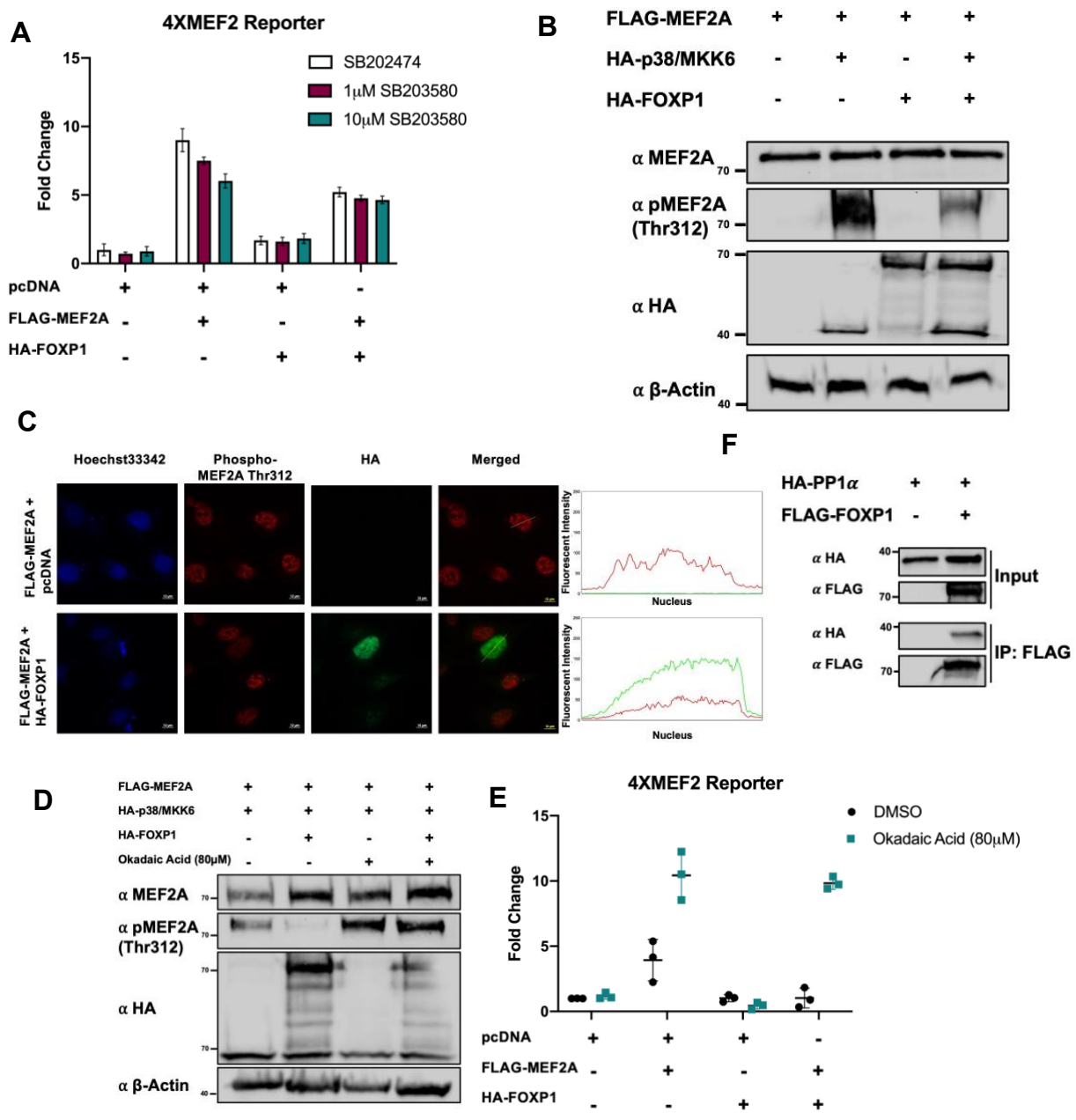


Figure 15. MEF2A phosphorylation by p38 MAPK is disrupted by FOXP1. A. A C2C12 culture was transiently transfected with 4XMEF2-luc for 16h and then treated with a p38 inhibitor (SB203580) and its negative control (SB202474) for 6h. **B.** Western blot analysis of a Hek293T lysates with ectopically expressed FLAG-MEF2A in combination with p38/MKK6 and HA-FOXP1 blotting for phosphorylated MEF2A Thr-312 and HA and β-actin as a loading control. **C.** Immunofluorescence analysis of fixed C2C12 cells transfected with FLAG-MEF2A and pcDNA or HA-FOXP1 probed for phosphorylated MEF2A Thr-312. **D.** Western blot analysis of C2C12 lysates with FLAG-MEF2A, p38/MKK6 and with and without HA-FOXP1 for 16h followed by incubation with Okadaic acid for 6h. **E.** A C2C12 culture was transiently transfected with

4XMEF2-luc reporter gene for 16h followed by Okadaic acid for 6h. The control cells were treated with the solvent (DMSO), *Renilla* luciferase was used as a transfection control and pcDNA was used to control for endogenous protein activity. **F.** Hek293T cells were transfected with HA-PP1α in combination with FLAG-FOXP1 for a FLAG immunoprecipitation using FLAG beads followed by Western blot analysis.

FOXP1 depletion enhances MEF2A activity and hypertrophy in primary cardiomyocytes.

Since MEF2A is implicated in cardiac hypertrophy as well as myoblast differentiation (Cornwell & McDermott, 2023), we turned our attention to cardiomyocytes to further document the potential role of FOXP1 as a MEF2A repressor. As observed in the skeletal muscle, in isolated rat primary cardiomyocyte lysates, FOXP1 immunoprecipitated with MEF2A (Fig. 16A). In addition, siRNA-mediated silencing of FOXP1 expression enhanced MEF2 reporter gene activity in cardiomyocytes (Fig. 16B). Therefore, we postulate that FOXP1 may also form a repressive complex with MEF2A protein in cardiomyocytes.

Finally, it has been previously documented that there is an upregulation of MEF2A activity in cardiac hypertrophic conditions (Moustafa et al., 2023). Therefore, we hypothesized that the depletion of FOXP1 might enhance the hypertrophic cardiomyocyte phenotype due to de-repression of MEF2A activity. First, we confirmed that treatment with one of the well-characterized cardiac hypertrophy-inducing reagents, phenylephrine (PE), caused a significant increase in cell size by the assessment of the size change of rat PCM cells by immunofluorescence analysis with Wheat Germ Agglutinin (WGA) staining (Fig 16C). Furthermore, we document that silencing FOXP1 expression by siRNA resulted in a significant increase in cell area compared to the scrambled control in response to PE suggesting that FOXP1 depletion exacerbated the PE-induced hypertrophy by de-repressing MEF2 activity. While this idea needs to be further addressed *in vivo*, the possible role of FOXP1 as a regulator of cardiac hypertrophy warrants further investigation.

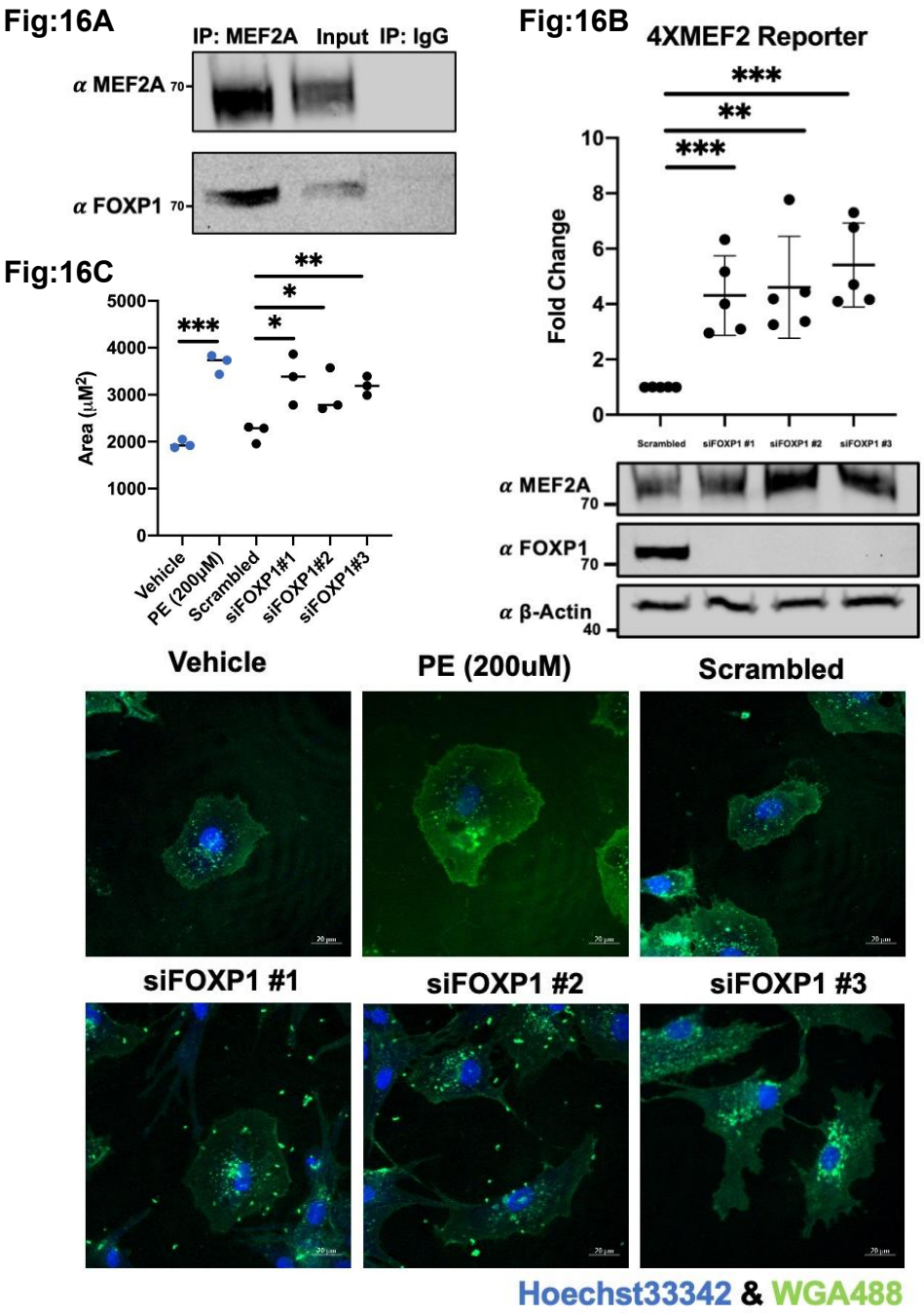


Figure 16. FOXP1 depletion in rat primary cardiomyocytes. A. Primary cardiomyocyte lysates were subjected to MEF2A immunoprecipitation with an IgG control. **B.** Primary cardiomyocytes were transfected with a 4XMEF2-luc reporter gene with a scrambled control and siRNA targeting FOXP1, Western blot analysis is shown below. **C.** Immunofluorescence and cell area quantification of primary cardiomyocytes treated with phenylephrine or siRNA targeting FOXP1 in serum-free media for 48h. A scrambled negative control and DMSO solvent were used as controls for 3 biological replicates. Cell area was measured using WGA488 staining and ImageJ analysis. The error bars represent the s.e.m. compared to the relevant control conditions, *P<0.01, **P<0.002, ***P<0.0001.

Discussion

In this MEF2A interactome study, we have identified a novel MEF2A binding protein, FOXP1, that negatively regulates MEF2A transcriptional activity during myogenesis and striated muscle maintenance. Previous research has begun to characterize the role of MEF2A in activating transcription for muscle-specific genes and cardiomyocyte survival (Moustafa et al., 2023). In addition, while MEF2 isoforms share a conserved MADS-box domain and MEF2 domain, the *trans*activation domain confers a wide range of specificity and function through post-translational modifications. The *trans*activation domain of MEF2A facilitates interactions with MAPK, CaMK, and protein phosphatases (Moustafa et al., 2023; Perry et al., 2009). The function of MEF2 proteins is primarily determined by the interacting various protein cofactors with which it interacts. Studies have shown that HDACs are important repressors of MEF2 proteins through interactions within the MADS/MEF2 domain (Jayathilaka et al., 2012; Zhao et al., 2005). Recent literature has also suggested a potential role for MEF2 in skeletal muscle wasting that is not yet fully understood and may depend on previously unrecognized protein interactions. We aimed to address these potential avenues using an unbiased high-throughput proteomic approach to identify novel protein-protein interactions using MEF2A as a bait protein in a skeletal muscle context. The interactome generated using this approach revealed several novel candidate MEF2A protein interactions without modification of protein-expression profile by the MEF2A overexpression. GO analysis of this interactome data set suggested the involvement in processes such as cardiac hypertrophy, disordered domain-specific binding, and nervous system development. Here, we present these interactome data along with an in-depth analysis of the interaction of MEF2A with FOXP1, a transcriptional co-repressor. The data suggest that this interaction may have important implications for our understanding of MEF2A regulation in striated muscle.

An important indicator of the progression from myocyte proliferation to myotube differentiation during myogenesis is the induction of myogenic regulatory factors by MEF2A (Hernandez-Hernandez et al., 2017). Activation of MEF2A, which results in the upregulation of factors such as Myogenin, promotes the differentiation program that leads to the formation of multinucleated myotubes. Strategic activation of MEF2A is required for proper myogenesis to occur at the correct stages of skeletal muscle development. Thus, dysregulation of MEF2A activation during the proliferative stages may affect the later stages of myogenesis. We propose that FOXP1, an enriched protein identified in the MEF2A interactome screen, is a novel regulator of MEF2A function during proliferation in myoblasts as well as potentially modulating cardiomyocyte gene expression. Briefly, we propose that in the presence of FOXP1, MEF2A activation is suppressed, thereby maintaining cells in an undifferentiated state. In addition, we present evidence that this antagonistic protein interaction targets the *trans*activation domain of MEF2A by inhibiting the phosphorylation of MEF2A by the p38MAPK pathway.

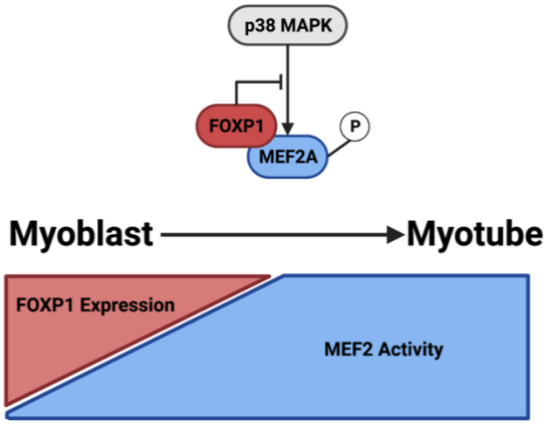


Figure 17: Proposed model of the MEF2A:FOXP1 interaction. Models of FOXP1 expression during the myogenesis and the differentiation of proliferative myoblast to multinucleated myotubes and a model of MEF2A regulation by FOXP1. The schematic on top shows that FOXP1 expression and repressive activity decreases during differentiation where MEF2A activity increases. The bottom schematic shows the MEF2A transcriptional regulation by FOXP1.

Although the function of MEF2 proteins through repressive HDAC interactions occurring in the MADS/MEF2 domain has been very well documented (Jayathilaka et al., 2012; Lu, McKinsey, Zhang, et al., 2000), our research has revealed an additional novel repression mechanism of MEF2A in which FOXP1 targets the C-terminal transactivation domain of MEF2A. This repression is likely mediated by antagonizing the ability of p38MAPK, a well-characterized activator of MEF2A, to phosphorylate and activate MEF2A. Further studies mapping this interaction will further our understanding of this protein interaction and the respective domains required in each protein.

The characterization of impaired MEF2A function by FOXP1 repression during cardiac hypertrophy may reveal novel pathways of MEF2A regulation. In view of this, we speculated that the interaction with FOXP1 might influence cardiomyocyte gene expression, which led us to further investigate this interaction in cardiac muscle. We document that the interaction between MEF2A and FOXP1 is conserved in cardiomyocytes and may modulate the pro-hypertrophic role of MEF2 in response to phenylephrine treatment of primary cardiomyocytes. In addition to primary cardiomyocyte cultures, the implementation of satellite cell cultures gives great insight into the function and maintenance of MEF2A transcriptional regulation.

Activation of MEF2A through the p38 MAPK signaling pathway is a key regulatory pathway that confers robust activation of MEF2 *trans*activation properties. Previous literature has suggested that MEF2A phosphorylation at conserved C-terminal residues enhances the transcriptional activation of *ckm* and *myogenin* genes. Given our current observations, we propose that this activation pathway is antagonized by FOXP1 complexing with MEF2A to inhibit phosphorylation. In addition, in light of these data, we propose that recruitment of PP1α into a large immunocomplex with FOXP1:MEF2A may contribute to the de-phosphorylation and

maintaining MEF2A in a repressive state. Further studies can investigate if FOXP1 repression of MEF2A is facilitated through binding to AT-rich sequences on muscle-specific genes.

Collectively, the results presented in this study suggest that FOXP1 may play a critical role in muscle gene regulation by targeting the MEF2A transcription factor. In addition, the MEF2A protein interactome network provides a valuable resource for future studies to investigate novel protein interactions with MEF2A. These observations contribute to a better understanding of the molecular mechanisms underlying muscle development and maintenance and may have implications for the development of therapies for diseases such as cardiac hypertrophy and sarcopenias.

Future directions, implications, and limitations

In this study, we have further characterized the role that MEF2A plays during myogenesis by identifying a novel protein-protein interaction with FOXP1 using an LC-MS/MS proteomic approach. Through our interactome network, we have identified new potential pathways in which MEF2A is involved, such as domain-disordered specific binding, as well as expanding the known categories in which MEF2A plays a critical role. Through proteomic and biochemical analysis, we were able to provide evidence that a FOXP1:MEF2A interaction plays a role in the function of MEF2A in striated muscle and that the presence and absence of FOXP1 can in turn affect the progression of myogenesis. However, while these findings are significant in broadening our understanding of myogenesis, cardiomyocyte maintenance, and myocyte differentiation, future work is necessary to focus on further characterizing this interaction *in vivo*. As well, additional novel candidate proteins that interact with MEF2A should be investigated to expand the already vast array of signaling pathways and functions that converge on MEF2A in regulating transcription. While the data presented in this study were obtained in cell culture only, similar methods and experimental approaches can be used to validate MEF2A protein interactions can be used in other model systems.

In addition to *in vivo* muscle investigation, shifting to other tissue models such as the brain where Gene Ontology identified MEF2A and FOXP1 as factors in neuronal maintenance is also of considerable interest. FOXP1 transcriptional repression is maintained in brain tissue and disruptions in FOXP1 expression can lead to deleterious phenotypes (Ferland et al., 2003). Additionally, MEF2A transcriptional activity is important for proper brain development and neuronal cell maintenance. Disruptions in MEF2A have been associated with the upregulation of autophagy-related genes, which have been implicated in the progression of Alzheimer's disease

(Li et al., 2021). Therefore, further investigation of our identified MEF2A and FOXP1 interaction in neuronal cells may determine the significance and conservation of this protein-protein interaction. Due to our investigation of this protein interaction focusing on striated muscle tissue, the formation of a larger immunocomplex with other cofactors are signaling pathways that could affect this interaction in other tissue types.

Lastly, our unbiased proteomic LC-MS/MS approach to creating an interactome network for MEF2A revealed a large number of novel binding candidates that could be of importance to investigate further. While the focus for this project was characterizing only one interaction discovered from the MEF2A interactome list, FOXP1, other factors should be further investigated. One protein to investigate in a myogenic context is KDM4A (lysine-specific demethylase 4A) which is a known player in myogenesis through demethylating different myogenic regulatory factors such as MyoD (Zhu et al., 2021). Recent literature has reported that KDM4A demethylates the chromatin of muscle-specific genes and that KDM4A knockout models appear to have impaired myogenesis as well as satellite cell differentiation (Zhu et al., 2021). Therefore, this protein would be a good putative candidate to investigate in order to model how satellite cell activation and differentiation are affected by a possible KDM4A:MEF2A interaction.

Materials and Methods

Cell culture

C2C12 myoblasts and Hek293T cells were obtained from American Type Culture Collection (ATCC, CRL-1772, and CRL-3216, respectively). Cells were cultured in growth medium (GM) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco), and L-Glutamine (HyClone), supplemented with 1% penicillin-streptomycin (Invitrogen, ThermoFisher) and 10% fetal bovine serum (FBS). Cells were maintained in a humidified incubator at 5% CO_2 and 37 ˚C. Myotube formation was induced by replacing GM with a differentiation medium (Wiedmann et al.), consisting of DMEM supplemented with 2% FBS and 1% penicillin-streptomycin.

Primary cardiomyocyte preparation

Hearts were isolated from 1-3 days old Sprague Dawley rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp). Whole hearts were isolated from sacrificed pups and digested with trypsin (Promega) and collagenase (Worthington Biochemical Corp) and re-suspended in DMEM/F12 (Gibco) supplemented with 1% penicillin-streptomycin, 50mg/L gentamycin (Invitrogen, ThermoFisher), and 10% FBS. Cells were pre-plated for removal of non-cardiomyocytes at 37 ˚C for 1 hour and counted using a Hemocytometer. Cardiomyocytes were seeded on gelatin-coated plates overnight in F12/DMEM growth medium. Cells were replenished with fresh media the following day for subsequent experimentation.

Transfections

For ectopic protein expression in Hek293T and C2C12 myoblasts, cells were transfected using polyethyleneimine (PEI) at a ratio of 1:3 for DNA to PEI (mass/mass) respectively.

Cardiomyocytes were transfected using Lipofectamine 2000 (Invitrogen) using instructions provided by the manufacturer. Cell medium was replenished 16h post-transfection and harvested 8 hours later. For small interfering RNA (siRNA) experiments, cells were transfected with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions at 200μM of siRNA. All transfections were prepared in Opti-MEM (Gibco) medium in separate tubes, then mixed and incubated at room temperature for 15min before being added to the media.

Plasmids

FLAG-FOXP1 was a gift from Stefan Koch (Addgene plasmid # 153145 ; http://n2t.net/addgene:153145 ; RRID:Addgene_153145). psDNA3.1 Foxp1A was a gift from Anjana Rao (Addgene plasmid # 16362 ; https://n2t.net/addgene:16362 ; RRID:Addgene_16362). EYFP ORF was amplified with the primers including HinDIII and EcoRI site and inserted into pcDNA3 (Invitrogen) for pcDNA3-EYFP. MEF2A was constructed by insertion of mouse MEF2A ORF amplified by PCR primers with EcoRI and XhoI sites from mouse cDNA generated from C2C12 total RNA using SuperscriptIII (Invitrogen) with oligo-dT primer into pcDNA3-EYFP. GAL4-MEF2A, VP16, and HA-PP1 α have been described previously (Perry et al., 2009). 4XMEF2-Luc and 3X-FLAG-MEF2Aconstrcuts have been described previously (Moustafa et al., 2023). *Ckm*-luc, *MyoG*-luc, and *Renilla* plasmid (pRL-*Renilla,* Promega) have all been described previously (Tripathi et al., 2022).

Gene silencing

Depletion of FOXP1 using small interfering RNA (siRNA) was done using siRNA (Sigma-Aldrich). For C2C12, cells were transfected with siFOXP1 #1 (SASI_Mm01_00141050),

siFOXP1 #2 (SASI_Mm01_00141051), and siFOXP1 #3 (SASI_Mm-1_00141052), and universal scrambled siRNA (SIC001) were used at 75nM concentrations. For cardiomyocytes, siFOXP1 #1 (SASI_Rn01_00044538), siFOXP1 #2 (SASI_Rn01_00044539), and siFOXP1 #3 (SASI_Rn01_00044540), and universal scrambled siRNA (SIC001) were used at 200nM concentrations.

Antibodies

Antibodies for GFP (rat, monoclonal, #3H9) was from ChromoTek, FOXP1 (rabbit, polyclonal, #2005S) and HA (rabbit, polyclonal, #C29F4) was purchased from Cell Signaling. β-Acting (mouse, monoclonal, #sc-47778), Myf-5 (C-20) (rabbit, polyclonal, #sc-302), and MCK (mouse, monoclonal, #sc-365046) was purchased from Santa Cruz Biotechnology. FLAG (mouse, monoclonal, #F3165) and Phospho-MEF2A (Thr312) (mouse, polyclonal, #PA5-36666) was purchased from Sigma-Aldrich. MyoG (mouse, monoclonal, #F5D) and MyHC (mouse, monoclonal, MF20) were purchased from Developmental Studies Hybridoma Bank (DSHB). Rabbit MEF2A polyclonal antibodies were produced in-house with the assistance of the Animal Care Facility at York University (Toronto, ON, Canada).

Pharmacological treatments

To induce hypertrophy in the cardiomyocytes, phenylephrine (Sigma-Aldrich, 200μM) and vehicle control were added to the media and cardiomyocytes were cultured for 48 before harvesting for subsequent experimentation. C2C12 myoblasts were treated with a p38/MAPK inhibitor (SB203580; 1μM and 10μM) or its inactive analogue (SB202474; 1μM and 10μM) for 24h then harvested.

Protein extraction and Western blot

Cells were harvested using NP-40 lysis buffer containing 0.5% (V/V) NP-40, 50nM Tris-HCl, 150nM NaCl, 10mM sodium pyrophosphate, 2mM EDTA, 0.1M NaF, protease inhibitor cocktail (Sigma-Aldrich), 1mM phenyl methyl sulfonyl fluoride (Sigma-Aldrich), and 1mM sodium orthovanadate (Bioshop). For C2C12 differentiated cultures, plates were fractionated adding 1% trypsin for 2min at room temperature before collecting detached myotubes, and the remaining myotubes were further removed by repeating incubation with 1% trypsin. After brief washing with PBS, the remaining reserve cells were harvested using a cell scrapper. Extracted proteins were denatured in an SDS loading buffer at 100 ˚C for 10min and loaded on an 8 or 10% SDS-polyacrylamide gel for size separation by electroporation at 100V for 1 hour. The proteins in the gel were transferred onto an Immobilon-FL polyvinylidene difluoride membrane (Millipore) with cold transfer buffer (gly+Tris). Blots were blocked with 5% skim milk in Tris-buffered saline (TBS)-T (10mM Tris-HCl, 150mM NACl with 10% 0.1% Tween 20) for 1 hour. Membranes were incubated overnight at 4 ˚C with primary antibody in 1% skim milk in TBS-T. Primary antibodies included MEF2A (1:500), FOXP1 (1:1000), FLAG (1:1000), HA (1:1000), and beta-actin (1:1000). The following day the blots were washed with TBST and incubated at room temperature with secondary antibody conjugated to horseradish peroxide (HRP). Protein was detected by the HRP substrate (Bio-Rad) and visualized and recorded using an iBright CL1500 Imaging system (ThermoFisher).

GFP-Nanobody trap sample preparation for LC-MS/MS

EYFP/EYFP-MEF2A proteins were produced in HEK293T cells transfected with corresponding expression vectors. EYFP/EYFP-MEF2A proteins were immobilized onto the GFP binding peptide (GBP) nanobody conjugated magnetic beads (ChromoTech #gtma) and other cellular proteins were removed by twice times washes with NP40 lysis buffer and then 1X RIPA buffer. The resultant protein GBP-beads complex was further incubated with the proteins extracted from C2C12 cells at 4 ˚C with agitation in NP40 lysis buffer. After 2X washing with NP40 lysis buffer and 1X washing with PBS, the remaining proteins were subjected to on-bead trypsin digestion and analyzed by mass-spec analysis (SPARC, University of Toronto).

Immunoprecipitation

Non-transfected cardiomyocytes and C2C12 myoblasts were harvested using the procedure described above. Immunoprecipitation was performed using Dynabeads Protein G (Invitrogen, #10003D) washed with PBS and then incubated with Rabbit IgG 1:1000 (Cell Signaling; #2729) or MEF2A antibody 1:500 in 1mL PBS overnight. The following day the beads were washed with PBS to remove unbound antibodies and then incubated with 1.2mg of protein lysate overnight. Beads were washed once more with PBS and eluted with SDS loading buffer at 100 ˚C for 10min. For FLAG immunoprecipitations, anti-FLAG M2 magnetic beads (Sigma, #M8823) were washed with PBS prior to incubation with 1mg of lysate overnight. Beads were washed with PBS and the protein complex on the beads was eluted using a 3X FLAG peptide solution for 1 hour. Eluted proteins were analyzed by Western blotting.

Immunofluorescence

C2C12 myoblasts and cardiomyocytes were seeded onto glass-bottom plates (MatTek Corp) and washed with cold PBS prior to fixation using 4% paraformaldehyde solution at room temperature for 10min. Cells were permeabilized using 90% ice-cold methanol for 10min then washed with PBS and incubated with blocking buffer (5% FBS in PBS) at room temperature for an hour. Plates were then incubated overnight at 4 ˚C with the primary antibody in blocking buffer. Plates were washed with PBS and incubated at room temperature with an Alexa fluorophore-conjugated secondary antibody (Life Technologies) in a blocking buffer. To mark nuclei, Hoechst33342 (Sigma-Aldrich) was added to 1μM in PBS. To mark cell membrane, WGA488 (Biotium #29022-1) was added to 1μM in PBS. Cells were visualized using a Zeiss Observer Z1 confocal fluorescent microscope with a Yokogawa CSU-XI spinning disk and processed using ZEN 2.5 (blue) software (Zeiss).

Reporter gene assays

C2C12 and cardiomyocytes were washed with PBS and harvested in Luciferase lysis buffer (Promega) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Luciferase enzymatic activity was measured using a luminometer (Berthold) using a Firefly luciferase substrate (Promega) and Renilla luciferase substrate (Promega). Luciferase values were normalized to transfection efficiency corresponding to Renilla luciferase activity. The average of the triplicates was used for the calculation of fold activation relative to the control. The error bar represents the standard deviation of the three biological replicates in the experiment.

References

Akazawa, H., & Komuro, I. (2003). Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res*, *92*(10), 1079-1088. https://doi.org/10.1161/01.RES.0000072977.86706.23

Amadei, G., Handford, C. E., Qiu, C., De Jonghe, J., Greenfeld, H., Tran, M., Martin, B. K., Chen, D. Y., Aguilera-Castrejon, A., Hanna, J. H., Elowitz, M. B., Hollfelder, F., Shendure, J., Glover, D. M., & Zernicka-Goetz, M. (2022). Embryo model completes gastrulation to neurulation and organogenesis. *Nature*, *610*(7930), 143-153. https://doi.org/10.1038/s41586-022-05246-3

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B., & Herrmann, B. G. (2003). Wnt3a plays a major role in the segmentation clock controlling somitogenesis. *Dev Cell*, *4*(3), 395-406. https://doi.org/10.1016/s1534-5807(03)00055-8

Barsyte-Lovejoy, D., Galanis, A., & Sharrocks, A. D. (2002). Specificity determinants in MAPK signaling to transcription factors. *J Biol Chem*, *277*(12), 9896-9903. https://doi.org/10.1074/jbc.M108145200

Black, B. L. (2007). Transcriptional pathways in second heart field development. *Semin Cell Dev Biol*, *18*(1), 67-76. https://doi.org/10.1016/j.semcdb.2007.01.001

Brade, T., Pane, L. S., Moretti, A., Chien, K. R., & Laugwitz, K. L. (2013). Embryonic heart progenitors and cardiogenesis. *Cold Spring Harb Perspect Med*, *3*(10), a013847. https://doi.org/10.1101/cshperspect.a013847

Buckingham, M., & Relaix, F. (2015). PAX3 and PAX7 as upstream regulators of myogenesis. *Semin Cell Dev Biol*, *44*, 115-125. https://doi.org/10.1016/j.semcdb.2015.09.017

Chen, L., Wang, D., Wu, Z., Ma, L., & Daley, G. Q. (2010). Molecular basis of the first cell fate determination in mouse embryogenesis. *Cell Res*, *20*(9), 982-993. https://doi.org/10.1038/cr.2010.106

Chen, X., Gao, B., Ponnusamy, M., Lin, Z., & Liu, J. (2017). MEF2 signaling and human diseases. *Oncotarget*, *8*(67), 112152-112165. https://doi.org/10.18632/oncotarget.22899

Conway, S. J., Henderson, D. J., Kirby, M. L., Anderson, R. H., & Copp, A. J. (1997). Development of a lethal congenital heart defect in the splotch (Pax3) mutant mouse. *Cardiovasc Res*, *36*(2), 163-173. https://doi.org/10.1016/s0008-6363(97)00172-7

Cornwell, J. D., & McDermott, J. C. (2023). MEF2 in cardiac hypertrophy in response to hypertension. *Trends Cardiovasc Med*, *33*(4), 204-212. https://doi.org/10.1016/j.tcm.2022.01.002

Cox, D. M., Du, M., Marback, M., Yang, E. C., Chan, J., Siu, K. W., & McDermott, J. C. (2003). Phosphorylation motifs regulating the stability and function of myocyte enhancer factor 2A. *J Biol Chem*, *278*(17), 15297-15303. https://doi.org/10.1074/jbc.M211312200

de Beer, M. A., & Giepmans, B. N. G. (2020). Nanobody-Based Probes for Subcellular Protein Identification and Visualization. *Front Cell Neurosci*, *14*, 573278. https://doi.org/10.3389/fncel.2020.573278

Domon, B., & Aebersold, R. (2006). Mass spectrometry and protein analysis. *Science*, *312*(5771), 212-217. https://doi.org/10.1126/science.1124619

Dumont, N. A., Bentzinger, C. F., Sincennes, M. C., & Rudnicki, M. A. (2015). Satellite Cells and Skeletal Muscle Regeneration. *Compr Physiol*, *5*(3), 1027-1059. https://doi.org/10.1002/cphy.c140068

Estrella, N. L., Desjardins, C. A., Nocco, S. E., Clark, A. L., Maksimenko, Y., & Naya, F. J. (2015). MEF2 transcription factors regulate distinct gene programs in mammalian

skeletal muscle differentiation. *J Biol Chem*, *290*(2), 1256-1268. https://doi.org/10.1074/jbc.M114.589838

Ferland, R. J., Cherry, T. J., Preware, P. O., Morrisey, E. E., & Walsh, C. A. (2003). Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. *J Comp Neurol*, *460*(2), 266-279. https://doi.org/10.1002/cne.10654

Frey, N., & Olson, E. N. (2003). Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*, *65*, 45-79. https://doi.org/10.1146/annurev.physiol.65.092101.142243

Frontera, W. R., & Ochala, J. (2015). Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int*, *96*(3), 183-195. https://doi.org/10.1007/s00223-014-9915-y

Gerri, C., Menchero, S., Mahadevaiah, S. K., Turner, J. M. A., & Niakan, K. K. (2020). Human Embryogenesis: A Comparative Perspective. *Annu Rev Cell Dev Biol*, *36*, 411-440. https://doi.org/10.1146/annurev-cellbio-022020-024900

Gillies, A. R., & Lieber, R. L. (2011). Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve*, *44*(3), 318-331. https://doi.org/10.1002/mus.22094

Gossett, L. A., Kelvin, D. J., Sternberg, E. A., & Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol Cell Biol*, *9*(11), 5022-5033. https://doi.org/10.1128/mcb.9.11.5022-5033.1989

Gritz, E., & Hirschi, K. K. (2016). Specification and function of hemogenic endothelium during embryogenesis. *Cell Mol Life Sci*, *73*(8), 1547-1567. https://doi.org/10.1007/s00018-016-2134-0

Hashemi, S., Salma, J., Wales, S., & McDermott, J. C. (2015). Pro-survival function of MEF2 in cardiomyocytes is enhanced by beta-blockers. *Cell Death Discov*, *1*, 15019. https://doi.org/10.1038/cddiscovery.2015.19

He, J., Ye, J., Cai, Y., Riquelme, C., Liu, J. O., Liu, X., Han, A., & Chen, L. (2011). Structure of p300 bound to MEF2 on DNA reveals a mechanism of enhanceosome assembly. *Nucleic Acids Res*, *39*(10), 4464-4474. https://doi.org/10.1093/nar/gkr030

Hernandez-Hernandez, J. M., Garcia-Gonzalez, E. G., Brun, C. E., & Rudnicki, M. A. (2017). The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. *Semin Cell Dev Biol*, *72*, 10-18. https://doi.org/10.1016/j.semcdb.2017.11.010

Horst, D., Ustanina, S., Sergi, C., Mikuz, G., Juergens, H., Braun, T., & Vorobyov, E. (2006). Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis. *Int J Dev Biol*, *50*(1), 47-54. https://doi.org/10.1387/ijdb.052111dh

Jayathilaka, N., Han, A., Gaffney, K. J., Dey, R., Jarusiewicz, J. A., Noridomi, K., Philips, M. A., Lei, X., He, J., Ye, J., Gao, T., Petasis, N. A., & Chen, L. (2012). Inhibition of the function of class IIa HDACs by blocking their interaction with MEF2. *Nucleic Acids Res*, *40*(12), 5378-5388. https://doi.org/10.1093/nar/gks189

Kolodziejczyk, S. M., Wang, L., Balazsi, K., DeRepentigny, Y., Kothary, R., & Megeney, L. A. (1999). MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Curr Biol*, *9*(20), 1203-1206. https://doi.org/10.1016/S0960-9822(00)80027-5

Kubalak, S. W., Miller-Hance, W. C., O'Brien, T. X., Dyson, E., & Chien, K. R. (1994). Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *J Biol Chem*, *269*(24), 16961-16970. https://www.ncbi.nlm.nih.gov/pubmed/8207020

Lassar, A. B. (2009). The p38 MAPK family, a pushmi-pullyu of skeletal muscle differentiation. *J Cell Biol*, *187*(7), 941-943. https://doi.org/10.1083/jcb.200911123
Le Grand, F., & Rudnicki, M. A. (2007). Skeletal muscle satellite cells and adult myogenesis. *Curr Opin Cell Biol*, *19*(6), 628-633. https://doi.org/10.1016/j.ceb.2007.09.012
Li, H., Wang, F., Guo, X., & Jiang, Y. (2021). Decreased MEF2A Expression Regulated by Its Enhancer Methylation Inhibits Autophagy and May Play an Important Role in the Progression of Alzheimer's Disease. *Front Neurosci*, *15*, 682247. https://doi.org/10.3389/fnins.2021.682247
Lindskog, C., Linne, J., Fagerberg, L., Hallstrom, B. M., Sundberg, C. J., Lindholm, M., Huss, M., Kampf, C., Choi, H., Liem, D. A., Ping, P., Varemo, L., Mardinoglu, A., Nielsen, J., Larsson, E., Ponten, F., & Uhlen, M. (2015). The human cardiac and skeletal muscle proteomes defined by transcriptomics and antibody-based profiling. *BMC Genomics*, *16*(1), 475. https://doi.org/10.1186/s12864-015-1686-y
Liu, N., Nelson, B. R., Bezprozvannaya, S., Shelton, J. M., Richardson, J. A., Bassel-Duby, R., & Olson, E. N. (2014). Requirement of MEF2A, C, and D for skeletal muscle regeneration. *Proc Natl Acad Sci U S A*, *111*(11), 4109-4114. https://doi.org/10.1073/pnas.1401732111
Lozano, R., Gbekie, C., Siper, P. M., Srivastava, S., Saland, J. M., Sethuram, S., Tang, L., Drapeau, E., Frank, Y., Buxbaum, J. D., & Kolevzon, A. (2021). FOXP1 syndrome: a review of the literature and practice parameters for medical assessment and monitoring. *J Neurodev Disord*, *13*(1), 18. https://doi.org/10.1186/s11689-021-09358-1
Lu, J., McKinsey, T. A., Nicol, R. L., & Olson, E. N. (2000). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. *Proc Natl Acad Sci U S A*, *97*(8), 4070-4075. https://doi.org/10.1073/pnas.080064097
Lu, J., McKinsey, T. A., Zhang, C. L., & Olson, E. N. (2000). Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell*, *6*(2), 233-244. https://doi.org/10.1016/s1097-2765(00)00025-3
Majidi, S. P., Reddy, N. C., Moore, M. J., Chen, H., Yamada, T., Andzelm, M. M., Cherry, T. J., Hu, L. S., Greenberg, M. E., & Bonni, A. (2019). Chromatin Environment and Cellular Context Specify Compensatory Activity of Paralogous MEF2 Transcription Factors. *Cell Rep*, *29*(7), 2001-2015 e2005. https://doi.org/10.1016/j.celrep.2019.10.033
Maroto, M., Bone, R. A., & Dale, J. K. (2012). Somitogenesis. *Development*, *139*(14), 2453-2456. https://doi.org/10.1242/dev.069310
Massenet, J., Gardner, E., Chazaud, B., & Dilworth, F. J. (2021). Epigenetic regulation of satellite cell fate during skeletal muscle regeneration. *Skelet Muscle*, *11*(1), 4. https://doi.org/10.1186/s13395-020-00259-w
Medrano, J. L., & Naya, F. J. (2017). The transcription factor MEF2A fine-tunes gene expression in the atrial and ventricular chambers of the adult heart. *J Biol Chem*, *292*(51), 20975-20988. https://doi.org/10.1074/jbc.M117.806422
Mehmood, S., Allison, T. M., & Robinson, C. V. (2015). Mass spectrometry of protein complexes: from origins to applications. *Annu Rev Phys Chem*, *66*, 453-474. https://doi.org/10.1146/annurev-physchem-040214-121732
Molkentin, J. D., & Dorn, G. W., 2nd. (2001). Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev Physiol*, *63*, 391-426. https://doi.org/10.1146/annurev.physiol.63.1.391

Mora, S., Yang, C., Ryder, J. W., Boeglin, D., & Pessin, J. E. (2001). The MEF2A and MEF2D isoforms are differentially regulated in muscle and adipose tissue during states of insulin deficiency. *Endocrinology*, *142*(5), 1999-2004. https://doi.org/10.1210/endo.142.5.8160

Moustafa, A., Hashemi, S., Brar, G., Grigull, J., Ng, S. H. S., Williams, D., Schmitt-Ulms, G., & McDermott, J. C. (2023). The MEF2A transcription factor interactome in cardiomyocytes. *Cell Death Dis*, *14*(4), 240. https://doi.org/10.1038/s41419-023-05665-8

Mughal, W., Nguyen, L., Pustylnik, S., da Silva Rosa, S. C., Piotrowski, S., Chapman, D., Du, M., Alli, N. S., Grigull, J., Halayko, A. J., Aliani, M., Topham, M. K., Epand, R. M., Hatch, G. M., Pereira, T. J., Kereliuk, S., McDermott, J. C., Rampitsch, C., Dolinsky, V. W., & Gordon, J. W. (2015). A conserved MADS-box phosphorylation motif regulates differentiation and mitochondrial function in skeletal, cardiac, and smooth muscle cells. *Cell Death Dis*, *6*(10), e1944. https://doi.org/10.1038/cddis.2015.306

Nebbioso, A., Manzo, F., Miceli, M., Conte, M., Manente, L., Baldi, A., De Luca, A., Rotili, D., Valente, S., Mai, A., Usiello, A., Gronemeyer, H., & Altucci, L. (2009). Selective class II HDAC inhibitors impair myogenesis by modulating the stability and activity of HDAC-MEF2 complexes. *EMBO Rep*, *10*(7), 776-782. https://doi.org/10.1038/embor.2009.88

Neyroud, D., Nosacka, R. L., Callaway, C. S., Trevino, J. G., Hu, H., Judge, S. M., & Judge, A. R. (2021). FoxP1 is a transcriptional repressor associated with cancer cachexia that induces skeletal muscle wasting and weakness. *J Cachexia Sarcopenia Muscle*, *12*(2), 421-442. https://doi.org/10.1002/jcsm.12666

Olson, E. N., Arnold, H. H., Rigby, P. W., & Wold, B. J. (1996). Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell*, *85*(1), 1-4. https://doi.org/10.1016/s0092-8674(00)81073-9

Perry, R. L. S., Yang, C., Soora, N., Salma, J., Marback, M., Naghibi, L., Ilyas, H., Chan, J., Gordon, J. W., & McDermott, J. C. (2009). Direct Interaction between Myocyte Enhancer Factor 2 (MEF2) and Protein Phosphatase 1α Represses MEF2-Dependent Gene Expression. *Molecular and Cellular Biology*, *29*(12), 3355-3366. https://doi.org/10.1128/mcb.00227-08

Pitt, J. J. (2009). Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *Clin Biochem Rev*, *30*(1), 19-34. https://www.ncbi.nlm.nih.gov/pubmed/19224008

Pon, J. R., & Marra, M. A. (2016). MEF2 transcription factors: developmental regulators and emerging cancer genes. *Oncotarget*, *7*(3), 2297-2312. https://doi.org/10.18632/oncotarget.6223

Potthoff, M. J., & Olson, E. N. (2007). MEF2: a central regulator of diverse developmental programs. *Development*, *134*(23), 4131-4140. https://doi.org/10.1242/dev.008367

Pourquie, O. (2003). Vertebrate somitogenesis: a novel paradigm for animal segmentation? *Int J Dev Biol*, *47*(7-8), 597-603. https://www.ncbi.nlm.nih.gov/pubmed/14756335

Rashid, A. J., Cole, C. J., & Josselyn, S. A. (2014). Emerging roles for MEF2 transcription factors in memory. *Genes Brain Behav*, *13*(1), 118-125. https://doi.org/10.1111/gbb.12058

Richards, A. L., Eckhardt, M., & Krogan, N. J. (2021). Mass spectrometry-based protein-protein interaction networks for the study of human diseases. *Mol Syst Biol*, *17*(1), e8792. https://doi.org/10.15252/msb.20188792

Rocca, D. L., Wilkinson, K. A., & Henley, J. M. (2017). SUMOylation of FOXP1 regulates transcriptional repression via CtBP1 to drive dendritic morphogenesis. *Sci Rep*, *7*(1), 877. https://doi.org/10.1038/s41598-017-00707-6

Rose, B. A., Force, T., & Wang, Y. (2010). Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. *Physiol Rev*, *90*(4), 1507-1546. https://doi.org/10.1152/physrev.00054.2009

Schultz, E., & Jaryszak, D. L. (1985). Effects of skeletal muscle regeneration on the proliferation potential of satellite cells. *Mech Ageing Dev*, *30*(1), 63-72. https://doi.org/10.1016/0047-6374(85)90059-4

Segales, J., Perdiguero, E., & Munoz-Canoves, P. (2016). Regulation of Muscle Stem Cell Functions: A Focus on the p38 MAPK Signaling Pathway. *Front Cell Dev Biol*, *4*, 91. https://doi.org/10.3389/fcell.2016.00091

Seth, M., Sumbilla, C., Mullen, S. P., Lewis, D., Klein, M. G., Hussain, A., Soboloff, J., Gill, D. L., & Inesi, G. (2004). Sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA) gene silencing and remodeling of the Ca2+ signaling mechanism in cardiac myocytes. *Proc Natl Acad Sci U S A*, *101*(47), 16683-16688. https://doi.org/10.1073/pnas.0407537101

Severs, N. J. (2000). The cardiac muscle cell. *Bioessays*, *22*(2), 188-199. https://doi.org/10.1002/(SICI)1521-1878(200002)22:2<188::AID-BIES10>3.0.CO;2-T

Shadrin, I. Y., Khodabukus, A., & Bursac, N. (2016). Striated muscle function, regeneration, and repair. *Cell Mol Life Sci*, *73*(22), 4175-4202. https://doi.org/10.1007/s00018-016-2285-z

Steventon, B., Busby, L., & Arias, A. M. (2021). Establishment of the vertebrate body plan: Rethinking gastrulation through stem cell models of early embryogenesis. *Dev Cell*, *56*(17), 2405-2418. https://doi.org/10.1016/j.devcel.2021.08.012

Svendsen, A., Kiefer, H. V., Pedersen, H. B., Bochenkova, A. V., & Andersen, L. H. (2017). Origin of the Intrinsic Fluorescence of the Green Fluorescent Protein. *J Am Chem Soc*, *139*(25), 8766-8771. https://doi.org/10.1021/jacs.7b04987

Tam, S. K., Gu, W., Mahdavi, V., & Nadal-Ginard, B. (1995). Cardiac myocyte terminal differentiation. Potential for cardiac regeneration. *Ann N Y Acad Sci*, *752*, 72-79. https://doi.org/10.1111/j.1749-6632.1995.tb17407.x

Taylor, M. V., & Hughes, S. M. (2017). Mef2 and the skeletal muscle differentiation program. *Semin Cell Dev Biol*, *72*, 33-44. https://doi.org/10.1016/j.semcdb.2017.11.020

Tripathi, S., Miyake, T., & McDermott, J. C. (2019). Smad7:beta-catenin complex regulates myogenic gene transcription. *Cell Death Dis*, *10*(6), 387. https://doi.org/10.1038/s41419-019-1615-0

von Maltzahn, J., Jones, A. E., Parks, R. J., & Rudnicki, M. A. (2013). Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc Natl Acad Sci U S A*, *110*(41), 16474-16479. https://doi.org/10.1073/pnas.1307680110

Wang, B., Weidenfeld, J., Lu, M. M., Maika, S., Kuziel, W. A., Morrisey, E. E., & Tucker, P. W. (2004). Foxp1 regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation. *Development*, *131*(18), 4477-4487. https://doi.org/10.1242/dev.01287

Watson, A. J., & Barcroft, L. C. (2001). Regulation of blastocyst formation. *Front Biosci*, *6*, D708-730. https://doi.org/10.2741/watson

Watson, A. J., Natale, D. R., & Barcroft, L. C. (2004). Molecular regulation of blastocyst formation. *Anim Reprod Sci*, *82-83*, 583-592. https://doi.org/10.1016/j.anireprosci.2004.04.004

Watt, A. J., Battle, M. A., Li, J., & Duncan, S. A. (2004). GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc Natl Acad Sci U S A*, *101*(34), 12573-12578. https://doi.org/10.1073/pnas.0400752101

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *Adv Physiol Educ*, *27*(1-4), 201-206. https://doi.org/10.1152/advan.00025.2003

Wen, Y., Bi, P., Liu, W., Asakura, A., Keller, C., & Kuang, S. (2012). Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. *Mol Cell Biol*, *32*(12), 2300-2311. https://doi.org/10.1128/MCB.06753-11

Wiedmann, M., Wang, X., Tang, X., Han, M., Li, M., & Mao, Z. (2005). PI3K/Akt-dependent regulation of the transcription factor myocyte enhancer factor-2 in insulin-like growth factor-1- and membrane depolarization-mediated survival of cerebellar granule neurons. *J Neurosci Res*, *81*(2), 226-234. https://doi.org/10.1002/jnr.20556

Wong, T. W. Y., Ahmed, A., Yang, G., Maino, E., Steiman, S., Hyatt, E., Chan, P., Lindsay, K., Wong, N., Golebiowski, D., Schneider, J., Delgado-Olguin, P., Ivakine, E. A., & Cohn, R. D. (2020). A novel mouse model of Duchenne muscular dystrophy carrying a multi-exonic Dmd deletion exhibits progressive muscular dystrophy and early-onset cardiomyopathy. *Dis Model Mech*, *13*(9). https://doi.org/10.1242/dmm.045369

Wu, W., Huang, X., Cheng, J., Li, Z., de Folter, S., Huang, Z., Jiang, X., Pang, H., & Tao, S. (2011). Conservation and evolution in and among SRF- and MEF2-type MADS domains and their binding sites. *Mol Biol Evol*, *28*(1), 501-511. https://doi.org/10.1093/molbev/msq214

Xia, X., Yu, C. Y., Bian, M., Sun, C. B., Tanasa, B., Chang, K. C., Bruffett, D. M., Thakur, H., Shah, S. H., Knasel, C., Cameron, E. G., Kapiloff, M. S., & Goldberg, J. L. (2020). MEF2 transcription factors differentially contribute to retinal ganglion cell loss after optic nerve injury. *PLoS One*, *15*(12), e0242884. https://doi.org/10.1371/journal.pone.0242884

Yamanaka, Y., Hamidi, S., Yoshioka-Kobayashi, K., Munira, S., Sunadome, K., Zhang, Y., Kurokawa, Y., Ericsson, R., Mieda, A., Thompson, J. L., Kerwin, J., Lisgo, S., Yamamoto, T., Moris, N., Martinez-Arias, A., Tsujimura, T., & Alev, C. (2023). Reconstituting human somitogenesis in vitro. *Nature*, *614*(7948), 509-520. https://doi.org/10.1038/s41586-022-05649-2

Yang, Z. J., Broz, D. K., Noderer, W. L., Ferreira, J. P., Overton, K. W., Spencer, S. L., Meyer, T., Tapscott, S. J., Attardi, L. D., & Wang, C. L. (2015). p53 suppresses muscle differentiation at the myogenin step in response to genotoxic stress. *Cell Death Differ*, *22*(4), 560-573. https://doi.org/10.1038/cdd.2014.189

Yoon, C., Song, H., Yin, T., Bausch-Fluck, D., Frei, A. P., Kattman, S., Dubois, N., Witty, A. D., Hewel, J. A., Guo, H., Emili, A., Wollscheid, B., Keller, G., & Zandstra, P. W. (2018). FZD4 Marks Lateral Plate Mesoderm and Signals with NORRIN to Increase Cardiomyocyte Induction from Pluripotent Stem Cell-Derived Cardiac Progenitors. *Stem Cell Reports*, *10*(1), 87-100. https://doi.org/10.1016/j.stemcr.2017.11.008

Zammit, P. S. (2017). Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin Cell Dev Biol*, *72*, 19-32. https://doi.org/10.1016/j.semcdb.2017.11.011

Zhang, Z., Wang, Y., Ding, Y., & Hattori, M. (2020). Structure-based engineering of anti-GFP nanobody tandems as ultra-high-affinity reagents for purification. *Sci Rep*, *10*(1), 6239. https://doi.org/10.1038/s41598-020-62606-7

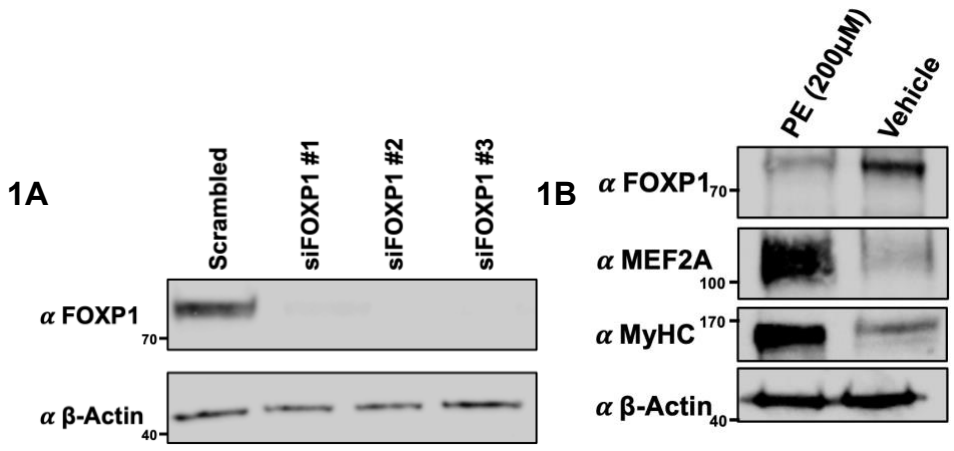
Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., di Padova, F., Olson, E. N., Ulevitch, R. J., & Han, J. (1999). Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol*, *19*(1), 21-30. https://doi.org/10.1128/MCB.19.1.21

Zhao, X., Sternsdorf, T., Bolger, T. A., Evans, R. M., & Yao, T. P. (2005). Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. *Mol Cell Biol*, *25*(19), 8456-8464. https://doi.org/10.1128/MCB.25.19.8456-8464.2005

Zhu, Q., Liang, F., Cai, S., Luo, X., Duo, T., Liang, Z., He, Z., Chen, Y., & Mo, D. (2021). KDM4A regulates myogenesis by demethylating H3K9me3 of myogenic regulatory factors. *Cell Death Dis*, *12*(6), 514. https://doi.org/10.1038/s41419-021-03799-1

Zlabinger, K., Spannbauer, A., Traxler, D., Gugerell, A., Lukovic, D., Winkler, J., Mester-Tonczar, J., Podesser, B., & Gyongyosi, M. (2019). MiR-21, MiR-29a, GATA4, and MEF2c Expression Changes in Endothelin-1 and Angiotensin II Cardiac Hypertrophy Stimulated Isl-1(+)Sca-1(+)c-kit(+) Porcine Cardiac Progenitor Cells In Vitro. *Cells*, *8*(11). https://doi.org/10.3390/cells8111416

Supplementary Data



Supplementary Figure 1. A. Western blot analysis of C2C12 lysates following siRNA targeting FOXP1 testing. **B.** Western blot analysis of primary cardiomyocytes treated with phenylephrine showing decreased expression of FOXP1 compared to vehicle.