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FoxP1 Represses MEF2A in Striated Muscle

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ABSTRACT

Myocyte enhancer factor 2 (MEF2) proteins are involved in multiple developmental, physiological, and pathological processes in vertebrates. Protein-protein interactions underlie the plethora of biological processes impacted by MEF2A, necessitating a detailed characterization of the MEF2A interactome. A nanobody based affinity-purification/mass spectrometry strategy was employed to achieve this goal. Specifically, the MEF2A protein complexes were captured from myogenic lysates using a GFP-tagged MEF2A protein immobilized with a GBP-nanobody followed by LC-MS/MS proteomic analysis to identify MEF2A interactors. After bioinformatic analysis, we further characterized the interaction of MEF2A with a transcriptional repressor, FOXP1. FOXP1 coprecipitated with MEF2A in proliferating myogenic cells which diminished upon differentiation (myotube formation). Ectopic expression of FOXP1 inhibited MEF2A driven myogenic reporter genes (derived from the *creatine kinase muscle* and *myogenin* genes) and delayed induction of endogenous myogenin during differentiation. Conversely, FOXP1 depletion enhanced MEF2A *trans*activation properties and myogenin expression. The FoxP1:MEF2A interaction is also preserved in cardiomyocytes and FoxP1 depletion enhanced cardiomyocyte hypertrophy. FOXP1 prevented MEF2A phosphorylation and activation by the p38MAPK pathway. Overall, these data implicate FOXP1 in restricting MEF2A function in order to avoid premature differentiation in myogenic progenitors and also to possibly prevent re-activation of embryonic gene expression in cardiomyocyte hypertrophy.

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Introduction

Striated muscle fulfills a vital function for all metazoans, facilitating movement, blood circulation, and digestion.[1] 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 transcription factors and a multitude of cofactors.[2,3] Diseases such as muscular dystrophies, sarcopenias, and cardiac hypertrophy arise due to aberrant patterns of gene expression.[4,5] One protein that is critical for muscle formation during embryogenesis and the regeneration of skeletal muscle postnatally 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. Dimeric 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.[6,7] Each MEF2 isoform consists of three 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.[8] MEF2 protein activity is expressed as early as day 7.5 postcoitum 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.[9–14] In addition, MEF2 proteins can modulate several developmental signaling pathways including Wingless/Integrated (Wnt), Notch, and the PI3K/AKT inflammatory pathway.[15,16]

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.[17] 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.[3,18] In addition to facilitating skeletal myogenesis, MEF2 activity functions as a prosurvival factor in cardiomyocytes.[19] However, MEF2 has also been implicated in cardiac hypertrophy, a

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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.[7,12,20] MEF2A has also been implicated in cancer cachexia, a condition in which skeletal muscle mass progressively declines and leads 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.[21]

To further understand the regulation of MEF2A in skeletal muscle we undertook a systematic unbiased proteomic screen approach to document the MEF2A interactome in myogenic cells. This was carried out using a GFP-nanotrap affinity purification followed by LC-MS/MS analysis.[22] 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,[21,23] as a candidate for further study. Further experimentation supports a model in which MEF2A repression by FOXP1 is vital to maintain myoblasts in an undifferentiated state in the proliferative or quiescent states. Moreover, hyperactivation of MEF2A by FoxP1 depletion leads to enhanced cardiomyocyte hypertrophy. These observations contribute to our ongoing understanding of MEF2 function and the necessity for precise balance of transcription factor activity for biological processes such as muscle differentiation and cardiomyocyte hypertrophy.

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,[24,25] 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[22] to identify proteins interacting with MEF2A in skeletal muscle. To characterize the interactome, EYFP-tagged MEF2A proteins (bait protein) were immobilized on GFP binding protein (GBP)-nanobody magnetic beads for affinity purification. To enhance the detection of nuclear localized MEF2A interacting (prey) proteins, nuclear enriched protein fractions were prepared from proliferating C2C12 cells which were then incubated with the bait to capture MEF2A interacting proteins (see Methods for further details). Figure 1A displays the workflow depicting the approach to generate an interactome network. Expression of EYFP and EYFP-MEF2A were confirmed by Western blot analysis (Figure 1B).

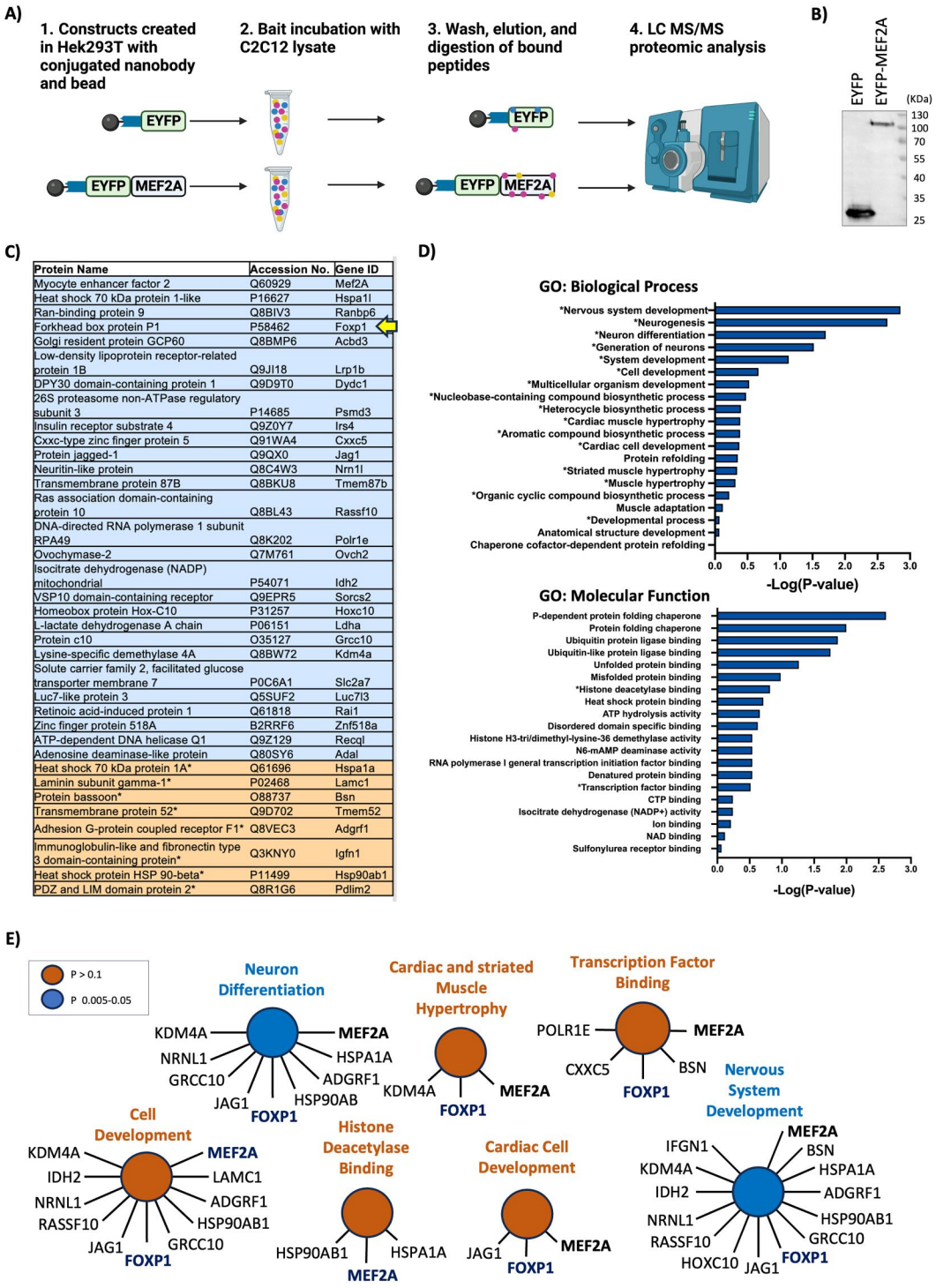
The criteria for protein identification as potential interactors was dependent on the total number of unique peptides ($\geq$2), and the presence of the protein in both biological replicates. Proteins found in both replicates and not in the GFP conditional control were considered as unique candidate interacting proteins. In addition, proteins found in both EYFP-MEF2A replicates with more than 3-fold enrichment over the GFP control were also included in the initial informatic analysis. Application of these criteria resulted in a list of 36 proteins (28 unique proteins, 8 enriched proteins) (Figure 1C).

To further characterize this protein interactome network, Gene Ontology (GO) analysis was performed to identify enriched biological processes and molecular functions. Among the Biological Processes and Molecular Functions shown in Figure 1D, several GO terms are documented that have been previously associated with known MEF2A function, including cardiac muscle hypertrophy and histone deacetylase binding. Interestingly, several categories not previously associated with MEF2A were also identified, for example disordered domain-specific binding. Due to a combination of our interest in myogenesis and the importance of the muscle-related categories, we focused on interactors within these GO terms (Figure 1E). 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.[26–28]

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

First, the biochemical interaction between MEF2A and FOXP1 identified in the interactome screen was confirmed. FOXP1 is a well characterized transcriptional repressor.[29,30] Gene Ontology analysis categorized FOXP1 together with many of the established MEF2A related categories, including cardiac and striated muscle hypertrophy (Figure 1E). By immunofluorescence analysis, we detected both MEF2A and FOXP1 in the nuclei of proliferating myoblasts (Figure 2A). The protein-protein interaction of ectopically expressed FLAG-MEF2A and HA-FOXP1 was also confirmed by FLAG immunoprecipitation followed by Western blotting analysis of the precipitated protein complex (Figure 2B). Furthermore, endogenous FOXP1 was found in the immunocomplex precipitated with MEF2A antibody (Figure 2C) suggesting the formation of an endogenous FOXP1/MEF2A protein complex in myoblasts.

Next, we 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. A 4XMEF2 luciferase reporter gene assay[31] was used to quantify the transcriptional effect of the presence or absence of ectopically expressed FOXP1 with MEF2A. Figure 2D indicates that ectopic expression of MEF2A activated the reporter gene as expected, and that the addition of ectopically expressed FOXP1 suppressed reporter gene activation by MEF2A. Since MEF2A activation is essential for myogenesis along with



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

Figure 1. 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 fusion to a MEF2A protein confirmed by Western blot analysis. (C) Table showing the putatively interacting proteins uniquely identified in EYFP-MEF2A in blue and the >3-fold enriched peptides (EYFP-MEF2A/EYFP) by the mass spectrometry analysis. (D) Gene ontology (GO) analysis of the identified proteins for both the biological processes and molecular functions, known MEF2A pathways are indicated with an asterisk. (E) Schematic representation of identified putative interacting proteins in the GO terms of interest along with *P*-values.

MRFs[3,6] and FOXP1 inhibits MEF2A activity, we hypothesized that FOXP1 might play an important role in restricting MEF2A activity in proliferating myoblasts when the cells have to be maintained in an undifferentiated state to avoid premature differentiation.

Following the initial characterization of the MEF2A/FOXP1 interaction, the pattern of FOXP1 expression during myogenic differentiation was examined. Western blotting analysis indicated that FOXP1 protein expression is substantial in myoblasts in the proliferative phase and decreases upon initiation of the differentiation and progression of myoblasts into mature multinucleated fused myotubes (Figure 3A). 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 was

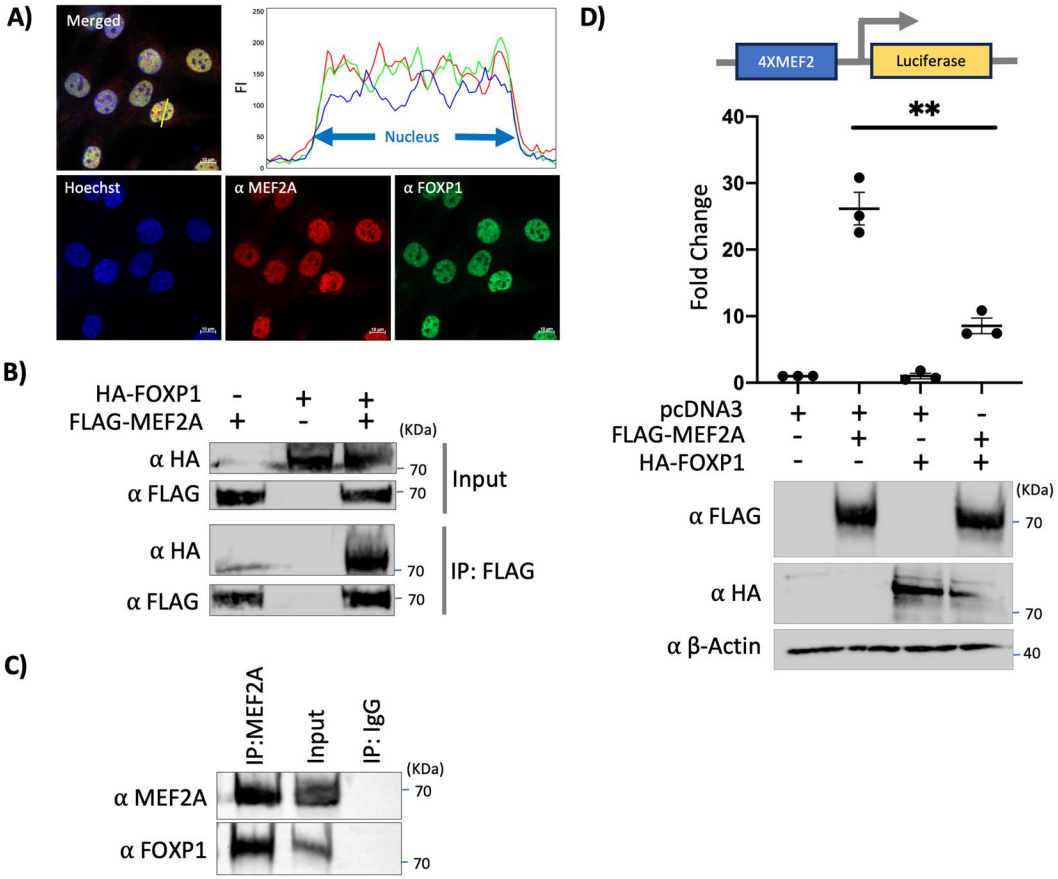


Figure 2. FOXP1 interacts and colocalizes with MEF2A in myoblasts. (A) Immunofluorescence analysis of a fixed myoblasts shows endogenous MEF2A in red and FOXP1 in green localized at the nuclei marked by Hoechst33342 in blue. The intensity blot of the MEF2A and FOXP1 signals is shown over the yellow line in the merged image. (B) Hek293T cells with ectopically expressed HA-FOXP1 and FLAG-MEF2A were subjected to a FLAG immunoprecipitation. Lysates with either FLAG-MEF2A or HA-FOXP1 transfected were used as controls. (C) A C2C12 myoblasts lysate was subjected to a MEF2A immunoprecipitation with MEF2A antibody or 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 reporter is shown. 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.

predominantly localized in the nucleus (Figure 3B). Since it is well documented that MEF2A protein expression is present at both the proliferative myoblast and myotube stages of myogenesis,[33] our observations support the idea that FOXP1 is required for the maintenance of the undifferentiated state by suppressing nuclear MEF2A activity in proliferating myoblasts.

We further extended this idea to 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 in differentiation conditions (termed reserve cells, RCs).[34,35] We reasoned that if FOXP1 is required for the maintenance of the undifferentiated state, FOXP1 might be differentially expressed in myotubes compared to RCs. To test this idea, we fractionated a heterogenous C2C12 population under DM conditions into myotubes and quiescent reserve cells. In Figure 3C Western blot analysis of each fraction indicates that FOXP1 protein was undetectable in the differentiated myotube fraction but clearly detected in the RC fraction, suggesting that FOXP1 expression is preserved in undifferentiated reserve cells but not in myotubes. Of note, a differentiated primary mouse satellite cell primary culture exhibited similar expression patterns of FOXP1 in undifferentiated cells by immunofluorescence analysis (Figure 3D). To further document the impact of FoxP1 expression on the myogenic differentiation program, we ectopically expressed mCherry-FoxP1 or mCherry alone in myogenic cells and assessed the incorporation of mCherry signals into myotubes under DM conditions. As documented in Figure 3E, there is substantial incorporation of mCherry signal into myotube nuclei in the mCherry alone condition. Conversely, we could not identify signal from mCherry-FoxP1 in any myotube nuclei, thus indicating that expression of FoxP1 is incompatible with myogenic differentiation.

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 further, both a gain of function by ectopic expression of FOXP1 and loss of function approach by siRNA targeting FOXP1 were employed.

Initially, we tested myogenin induction as a MEF2 target gene and marker for the initiation of differentiation, with ectopic expression of FOXP1. As is well documented in the

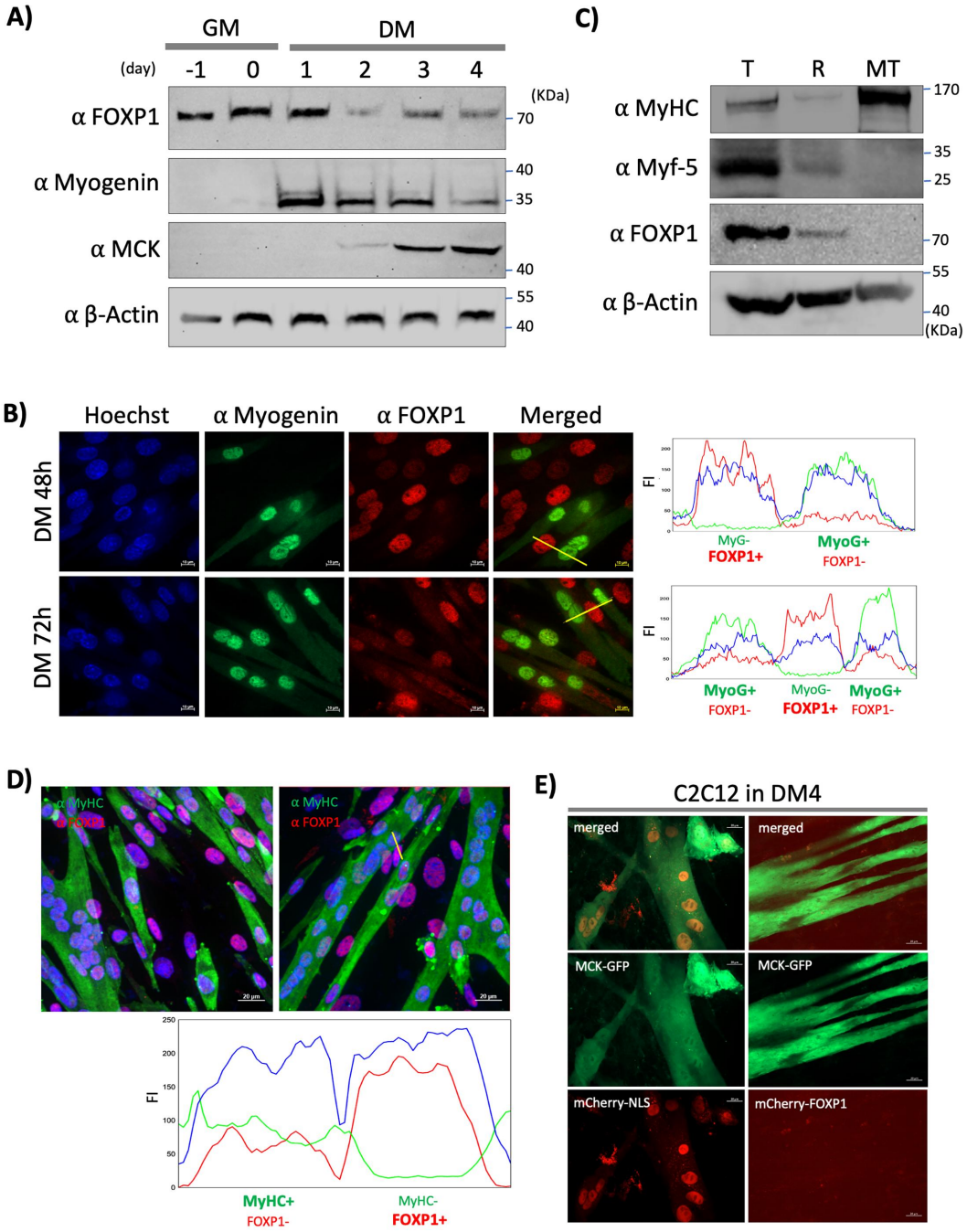


Figure 3. FOXP1 protein decreases during myoblast differentiation. (A) C2C12 lysates were collected from cultures incubated in GM and DM for Western blot analysis. Myogenic differentiation markers myogenin and MCK were used as controls for differentiation, while β-actin was used as a loading control. The numbers indicating the molecular weight (KDa). (B) Immunofluorescence analysis of a fixed differentiated C2C12 culture showing endogenous levels of myogenin in green and FOXP1 in red in the Hoeschst33342 (blue) stained nuclei. The intensity blot of the myogenin (green) and FOXP1[32] signals is shown over the yellow line in the merged image. (C) A C2C12 cell culture grown in DM for 48 h was fractionated into a myotube, reserve cell, and total protein lysate were subjected to the Western blotting analysis (T = total, R = reserve cell, MT = myotubes). (D) Immunofluorescence analysis of a fixed primary mouse satellite cell culture to visualize endogenous expression of myosin heavy chain (MyHC) in green and FOXP1 in red. The intensity blot of FOXP1, MyHC and Hoechst33342 is shown with the yellow line. (E) C2C12 cells were transfected with MCK-GFP reporter gene construct and either mCherry-NLS or mCherry-FOXP1 expression construct. Myotube formation was induced by reduction of mitogenic stimulus (GM to DM). The cells were maintained for 4 days in DM and then GFP and mCherry signals were visualized by confocal fluorescence microscopy.

literature,[36] Western blotting analysis (Figure 4A) confirmed that upregulation of myogenin protein level occurs at approximately 48 h in DM.[37,38] However, when HA-FOXP1 was ectopically expressed, myogenin protein levels failed to be induced at the normal rate. Ectopic expression of HA-FOXP1 reduced the number of myogenin-positive cells in the population in comparison to a control condition (Figure 4B). Further immunofluorescence analysis showed that there was a decrease in the number of Myogenin-positive cells under conditions of 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 next employed

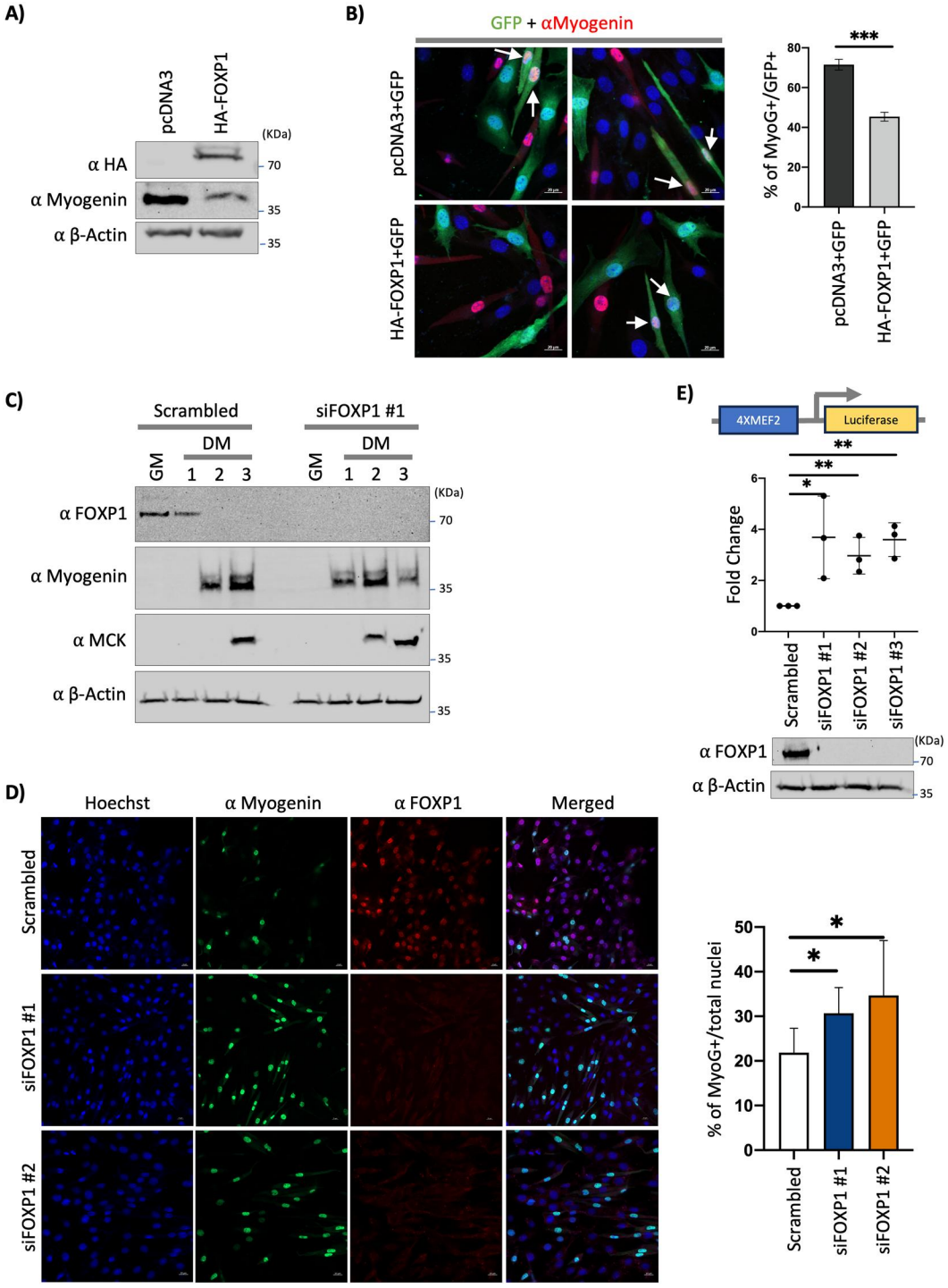


Figure 4. Gain and loss of function of FOXP1 during myoblast differentiation. (A) Western blot analysis of a 48 h DM C2C12 lysate with ectopic expression of HA-FOXP1 and pcDNA3 control. (B) Immunofluorescence images of fixed C2C12 cells transfected with indicated constructs kept in DM for 48 h. GFP positive and GFP/myogenin double positive cells were counted over three 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 expression levels of indicated proteins were analyzed by Western blotting. (D) Immunofluorescence analysis of a DM 48 h C2C12 culture transfected with a scrambled and siRNA targeting FOXP1 with myogenin in green and FOXP1 in red. The quantification of myogenin expressing nuclei of each condition is shown in an associated bar graph for three 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. Three biological replicates were analyzed, and the statistical significance was calculated using GraphPad Prism 8.0 with error bars representing SEM. $^{*}P < 0.001$ and $^{**}P < 0.0001$.

a loss-of-function approach with siRNA silencing of FOXP1. For this loss-of-function analysis, three independent siRNAs targeting different regions of FOXP1 mRNA were employed along with a scrambled control. The siRNAs and control were transfected and efficient reduction of FOXP1 expression was confirmed (Supplementary Figure 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 (Figure 4C). Due to FOXP1 depletion, both Myogenin and

MCK protein expression were expressed earlier than controls upon induction of differentiation. This was further supported by immunofluorescence analysis quantifying the number of Myogenin-positive nuclei, which was significantly higher in the FOXP1 depleted condition compared to the scrambled control (Figure 4D). Finally, to investigate how the loss of FOXP1 function may affect MEF2A activity, 4XMEF2 reporter gene activity assay with three independent siRNAs targeting FOXP1 was performed. The reporter gene analysis in Figure 4E shows that the depletion of FOXP1 resulted in an increase in MEF2 dependent reporter gene activity.

Cumulatively, these data (Figure 3 and Figure 4) highlight robust FOXP1 protein expression in undifferentiated proliferative and quiescent myoblasts and its subsequent decline during the differentiation stages of myogenesis. Importantly, the same trend is observed in primary mouse satellite cell cultures, in which FoxP1 expression is high in MyHC negative cells and low in differentiated MyHC positive cells (Figure 3D). The pattern of expression of FoxP1 in primary satellite cells and our documentation of its repressive function on MEF2A suggests a potentially important role of FoxP1 in adult muscle regeneration. Taken collectively, these data support a model in which FOXP1 suppresses MEF2A function to prevent premature muscle differentiation in proliferating or quiescent myoblasts.

FOXP1 antagonizes MEF2A transactivation properties

While the data on the synthetic reporter gene assay system indicates that FOXP1 antagonizes MEF2A *trans*activation, we next employed the use of well characterized natural myogenic gene promoter/enhancer-based reporter genes that are known to be regulated by MEF2. A creatine kinase muscle (*ckm*)[39,40] and also a *myogenin* promoter[41,42] based luciferase gene reporters were used for this analysis. Figure 5A and B indicates that ectopic expression of FLAG-MEF2A activates both the *ckm* and myogenin reporter genes as expected; however, when cells were cotransfected 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 (4xMEF2-Luc), but also natural myogenic promoter activity (*ckm* and myogenin).

MEF2A activity is regulated by a variety of cofactors. Among them, class II HDACs and P300 have been reported to regulate MEF2A activity primarily through MEF2's MADS/MEF2 domain.[43,44] To delineate which region of MEF2A is required to facilitate the FOXP1 interaction, several GAL4-DNA binding domain (DBD) fusion constructs were used on a 5X upstream activator sequence (UAS) luciferase reporter gene system.[45] A map of the different constructs fused with the GAL4-DBD at the N-terminus is shown in Figure 5C.[46] Figure 5D documents reporter gene analysis with ectopic expression of GAL4-DBD-MEF2A fusion proteins. These studies revealed that the region between aa 273 and 373 within the MEF2A *trans*activation domain were sufficient for repression by FOXP1. In addition, if the TAD of MEF2A was replaced with the VP16 *trans*activation domain fused to the MADS-MEF2 domain (MADS/MEF2-VP-16), this fusion was not repressed in the presence of HA-FOXP1 (Figure 5E) providing further evidence that the MADS-MEF2 domain is not required for FOXP1 repression. Immunoprecipitation experiments presented in Figure 5F, we detected a very weak interaction of FoxP1 with MEF2A 1–91 in this assay compared to the robust interaction observed with the full length protein. We attribute this marginal interaction with the capability of MEF2A 1–91 to dimerize with the endogenous MEF2A which would then bridge the FoxP1 interaction. In summation, these data indicate that FoxP1 mediated MEF2A repression occurs through the C terminal *trans*activation region of MEF2A.

FOXP1 repression of MEF2A is associated with hypophosphorylation 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.[47] In addition, this region of the TAD overlaps with a p38MAPK docking site.[48] We, therefore, hypothesized that FOXP1 repression might be facilitated by disruptions in TAD phosphorylation by p38MAPK. In Figure 5A, reporter gene analysis with a specific p38MAPK inhibitor (SB203580) and its inactive analogue (SB202474) was employed.[49,50] As previously observed, a decrease in MEF2A transcriptional activation on the 4XMEF2 reporter gene was observed with p38MAPK inhibition. Conversely, FOXP1's repressive activity on MEF2A was not evident under conditions when p38MAPK was inhibited. This led us to postulate that FoxP1 might antagonize MEF2A phosphorylation by p38 MAPK. To further test this idea, the level of MEF2A phosphorylation at Thr-312 (one of the core p38MAPK target sites on MEF2A)[47,51] was assessed by Western blotting analysis. As previously documented, activation of the p38MAPK by a constitutively active form of the upstream kinase, MKK6(EE),[52] was confirmed by phospho-Thr-312 analysis of MEF2A (Figure 6B). 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 MKK6EE/p38MAPK activation. Immunofluorescence analysis also confirmed that the level of Thr-312 phosphorylation was decreased in C2C12 cells expressing ectopic HA-FOXP1 protein (Figure 6C). It is important to note that Thr 312 phosphorylation detection by phosphoantibodies is used as a proxy for p38 MAPK activation of MEF2A but there are multiple additional p38 MAPK phosphoacceptor sites on MEF2A that cumulatively determine its response to p38 MAPK activation.[47]

One possibility by which FOXP1 antagonizes phosphorylation of MEF2A at Thr-312 was by directing phosphatase activity toward MEF2A, since we previously characterized an interaction between MEF2 and protein phosphatase 1 α (PP1α). To investigate this possibility, we initially used a pan-phosphatase inhibitor, okadaic acid (OA). Western blot analysis (Figure 6D) confirmed that ectopic expression of HA-FOXP1

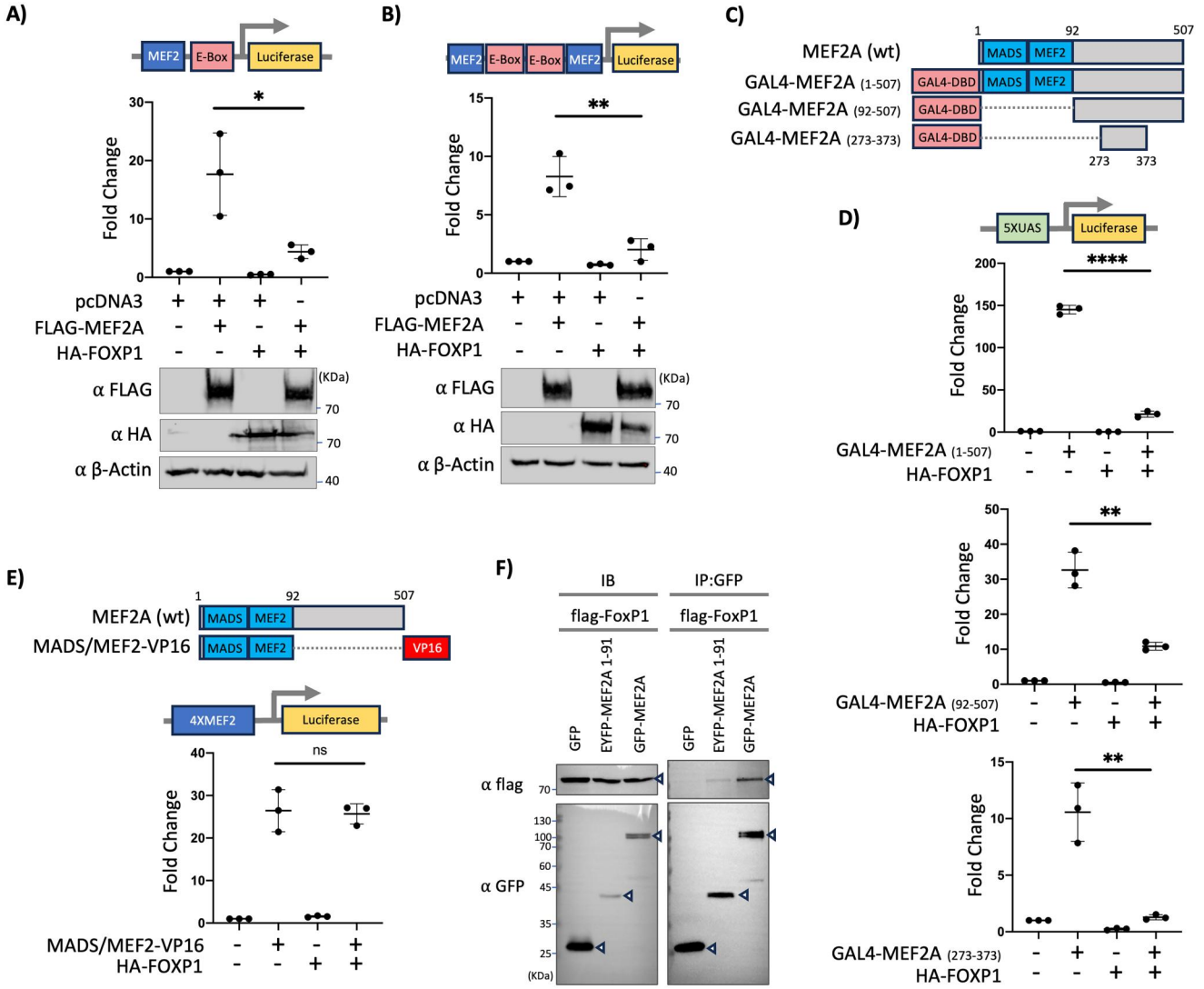


Figure 5. FOXP1 antagonizes MEF2A *trans*activation. (A) FLAG-MEF2A in combination with HA-FOXP1 or pcDNA3 was ectopically expressed in C2C12 with a myogenin-luc reporter gene, Western blot analysis confirming the transfection of the constructs is shown below. (B) FLAG-MEF2A in combination with HA-FOXP1 or pcDNA3 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 three 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. (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 pcDNA3 (not shown) was used to equal total amount of DNA transfected in each condition. The error bars represent SEM compared to the relevant control conditions $^{*}P < 0.01$, $^{**}P < 0.001$, $^{****}P < 0.0001$. (F) C2C12 cells were transfected with indicated expression constructs and harvested the next day. Total cell lysates (10 μg) were subjected to immunoblotting (IB) analysis with α flag (for flag-FOXP1) or α GFP (for GFP/EYFP tagged MEF2A) antibodies. The same lysate (150 μg) was immunoprecipitated with GFP-nanotrap beads. After elution from the beads, 120 μg and 30 μg equivalent volume of eluant was used for the detection of flag (flag-FOXP1) and GFP (EYFP-MEF2A (1-91), GFP-MEF2A) tagged proteins respectively. White triangles indicate corresponding peptides.

reduced the phosphorylation of Thr-312 of MEF2A as we previously observed, but in the presence of the OA phosphatase inhibitor, ectopically expressed HA-FOXP1 did not reduce Thr-312 phosphorylation levels. We did notice a possible reduction in HA FoxP1 expression with OA in this experiment (Figure 6D) but further experiments indicated no appreciable effect of OA on endogenous or ectopic FoxP1 expression (Supplementary Figure 3A and B). Furthermore, in the presence of the OA phosphatase inhibitor, FOXP1 failed to repress MEF2A transcriptional activity measured by 4XMEF2 luciferase reporter gene activity (Figure 6E). Since we have previously documented repression of MEF2A function by PP1α,[46] we tested the possibility that PP1α might complex with MEF2A/FOXP1. Western blotting analysis of a FLAG-immunoprecipitation revealed that FLAG-FOXP1 does indeed co-immunoprecipitate with HA-PP1α (Figure 6F). However, it proved difficult to discern between separate FOXP1/PP1α and FoxP1/MEF2A complexes versus a three-way FoxP1/MEF2A/PP1α complex using this approach. Overall, these data suggest that FOXP1 represses phosphorylation mediated MEF2A activation by p38MAPK, and that this repression could be mediated by the formation of a repressive immunocomplex including a phosphatase, for example, PP1α, in undifferentiated cells.

FOXP1 depletion enhances MEF2A activity and hypertrophy in primary cardiomyocytes

Since MEF2A is implicated in cardiac hypertrophy,[7,53] we turned our attention to the other striated muscle counterpart,

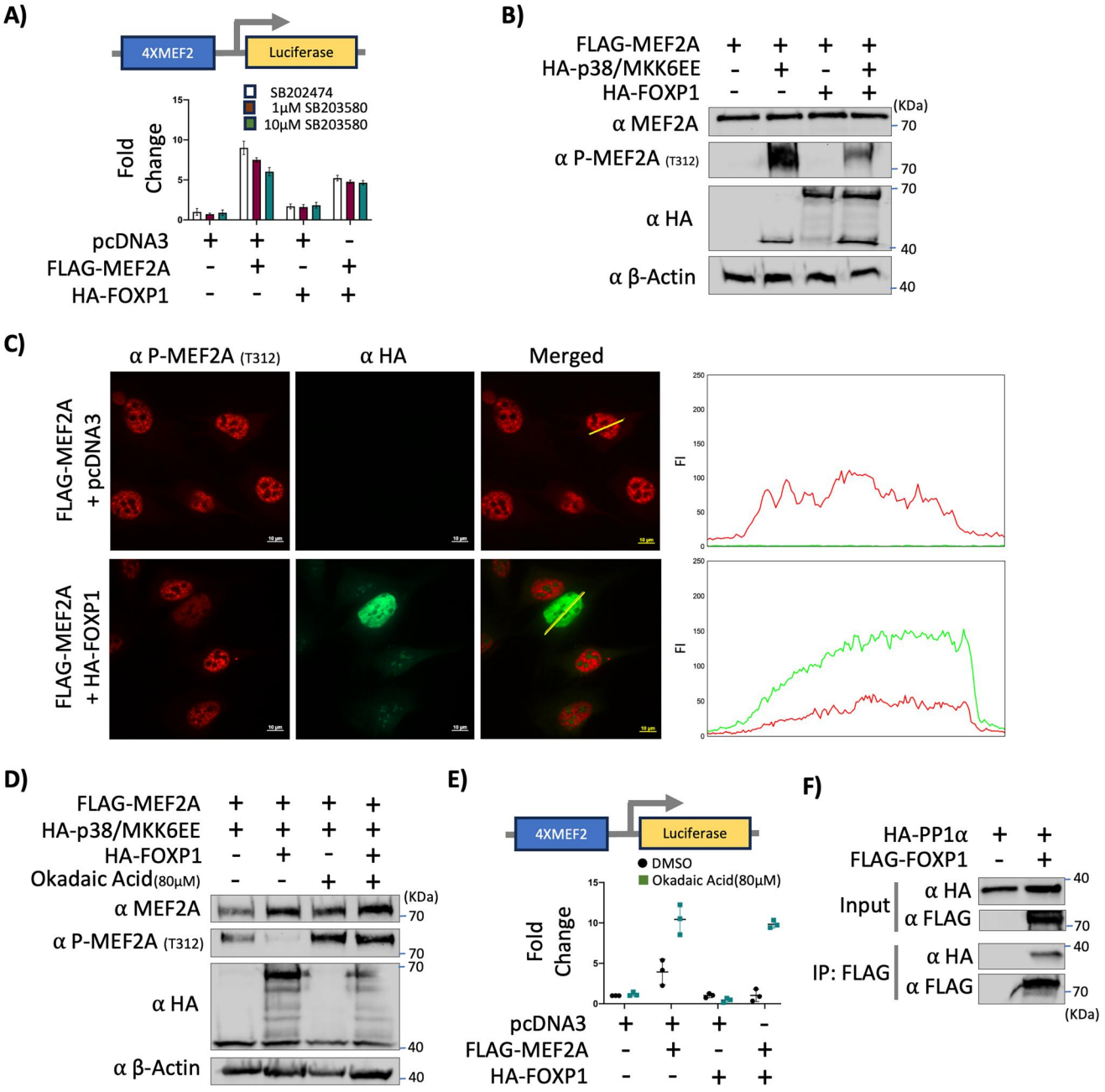


Figure 6. MEF2A phosphorylation by p38 MAPK is disrupted by FOXP1. (A) A C2C12 culture was transiently transfected with 4XMEF2-luc for 16 h and then treated with a p38 inhibitor (SB203580) and its negative control (SB202474) for 6 h. (B) Western blot analysis of a Hek293T lysates with ectopically expressed FLAG-MEF2A in combination with p38/MKK6 and HA-FOXP1 blotting for phosphorylated MEF2A (P-MEF2A) at Thr-312 and HA and β-actin as a loading control. (C) Immunofluorescence analysis of fixed C2C12 cells transfected with FLAG-MEF2A and pcDNA3 or HA-FOXP1 probed for phosphorylated MEF2A at Thr-312[32] and HA (green). Signal intensity of P-MEF2A (T-312) (green) was graphed with/without HA-FOXP1 expression (green). (D) Western blot analysis of C2C12 lysates with FLAG-MEF2A, p38/MKK6 and with HA-FOXP1 or pcDNA3 for 16 h followed by incubation with okadaic acid for 6 h. (E) A C2C12 culture was transiently transfected with 4XMEF2-luc reporter gene for 16 h followed by okadaic acid for 6 h. The control cells were treated with the solvent (DMSO), Renilla luciferase was used as a transfection control. (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.

cardiomyocytes. To document the potential role of FOXP1 as a more general repressor of MEF2A function in striated muscle. As observed in the skeletal muscle, in isolated rat primary cardiomyocyte lysates, FOXP1 immunoprecipitated with MEF2A (Figure 7A). In addition, siRNA-mediated silencing of FOXP1 expression enhanced MEF2 reporter gene activity in cardiomyocytes (Figure 7B). Therefore, we postulate that FOXP1 can form a repressive complex with MEF2A protein in cardiomyocytes.

Finally, it has been well documented that there is a critical upregulation of MEF2A activity under cardiac hypertrophic conditions.[24] Therefore, we hypothesized that depletion of FOXP1 might promote cardiomyocyte hypertrophy due to de-repression of MEF2A activity. First, we confirmed that treatment with one of the well characterized cardiac hypertrophy inducing reagents, phenylephrine (PE),[54] provoked a significant increase in cell surface area of neonatal rat ventricular cardiomyocytes by staining with wheat germ

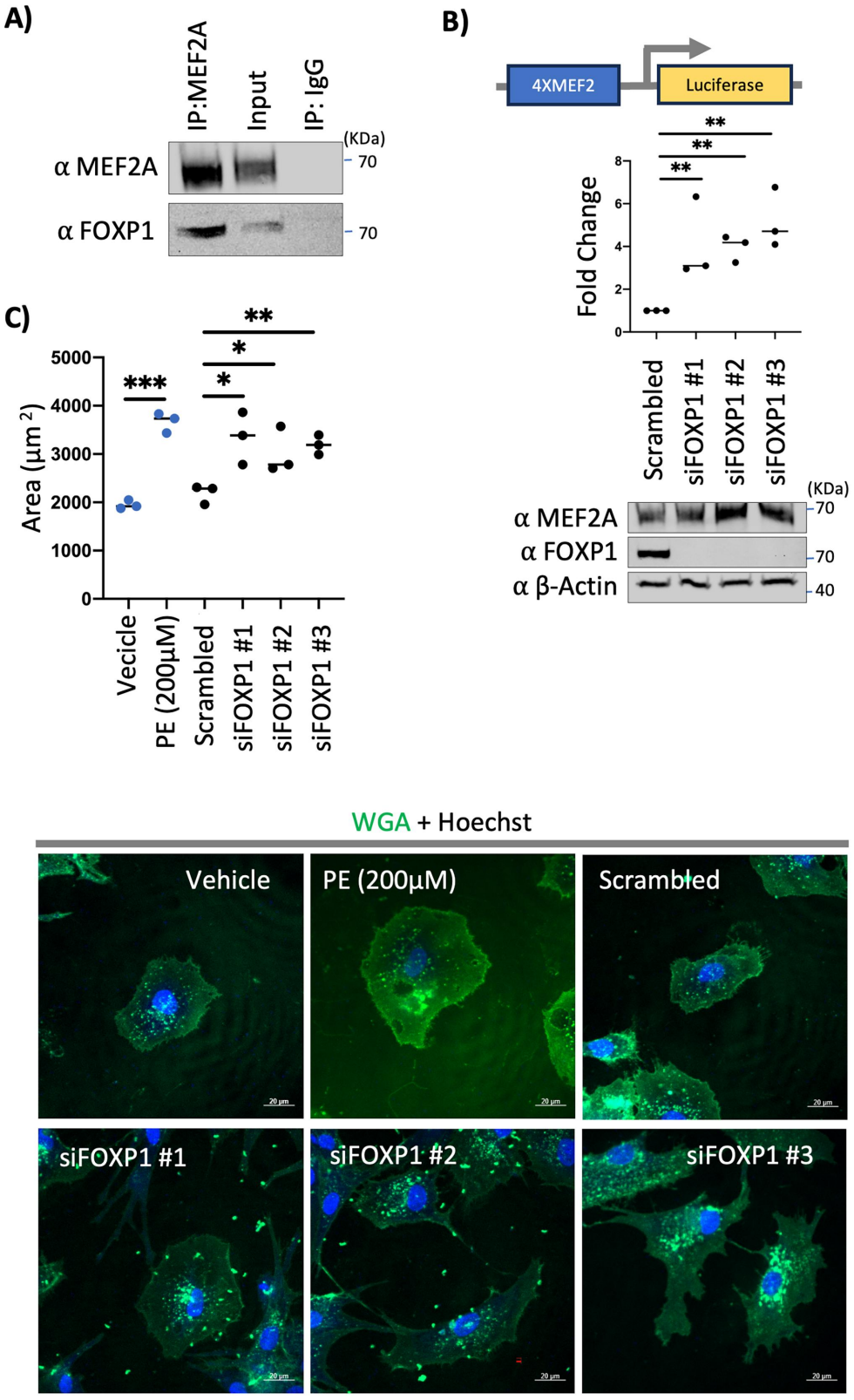


Figure 7. FOXP1 depletion in rat primary cardiomyocytes. (A) Primary cardiomyocyte lysates were subjected to immunoprecipitation with MEF2A antibody or an IgG control. Presence of indicated proteins were analyzed by Western blotting. (B) Primary cardiomyocytes were transfected with a 4XMEF2-luc reporter gene with a scrambled control or 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 48 h. A scrambled control and DMSO solvent were used as controls for three biological replicates. Cell area was measured using WGA488 staining and ImageJ. The error bars represent the SEM. compared to the relevant control conditions, $^{*}P < 0.01$, $^{**}P < 0.002$, $^{***}P < 0.0001$.

agglutinin (WGA) (Figure 7C). This treatment also resulted in an accumulation of MEF2A, MyHC and downregulation of FOXP1 protein (Supplementary Figure 2). Moreover, we document that silencing FOXP1 expression by siRNA resulted in an enhanced cell area compared to the scrambled control in PE treated cardiomyocytes, suggesting that FOXP1 depletion enhanced cardiomyocyte hypertrophy, possibly by further derepressing MEF2 activity. While this idea needs to be further addressed in vivo, the possible role of FOXP1 as a negative regulator of cardiac hypertrophy has important clinical implications and warrants further investigation.

Discussion

In this MEF2A interactome study, we have identified a novel MEF2A binding protein, FOXP1, that negatively regulates MEF2A *trans*activation properties in myogenic cells. Previous research has begun to characterize the role of MEF2A in activating transcription for muscle-specific genes and also cardiomyocyte survival.[19,24] 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.[24,46] The function of MEF2 proteins is primarily determined by interacting with various protein cofactors. Studies have shown that class II HDACs are important repressors of MEF2 proteins through interactions within the MADS/MEF2 domain.[43,44] Recent literature has also suggested a potential role for MEF2 in skeletal muscle wasting[55] that is not yet fully understood and may depend on previously unrecognized protein interactions. In this study, we aimed to address these potential avenues using an unbiased high-throughput proteomic approach to identify novel protein-protein interactions with MEF2A. The interactome generated using this approach revealed several novel candidate MEF2A protein interactions. GO analysis of this interactome data set suggested the involvement in processes such as transcription factor binding, cardiac cell development, striated muscle hypertrophy, cardiac hypertrophy, disordered domain-specific binding, and neuron differentiation. Here, we present these interactome data along with an in-depth analysis of the interaction of MEF2A with FOXP1, a transcriptional corepressor. These 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 the MRF, myogenin, by MEF2A.[2] Upregulation of factors such as myogenin, promote the differentiation program that leads to the formation of multinucleated myotubes.[56,57] 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 impact the later stages of myogenesis. Here, we propose that FOXP1, an enriched protein identified in the MEF2A interactome screen, is a novel regulator of MEF2A function in proliferating myoblasts. In addition, our data indicate that the MEF2A:FOXP1 interaction may be operative in modulating cardiomyocyte gene expression.

Although the function of MEF2 proteins through repressive HDAC interactions occurring in the MADS/MEF2 domain has been very well documented,[9,43] our research has revealed an additional novel repressive mechanism on MEF2A in which FOXP1 targets the C-terminal *trans*activation 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.[45,58] Future studies mapping this interaction in detail 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 have relevance for heart disease since hypertrophy is a feature of the progression to heart failure. We document that the interaction between MEF2A and FOXP1 is conserved in cardiomyocytes and may modulate the prohypertrophic role of MEF2 in response to phenylephrine treatment of primary cardiomyocytes.

Activation of MEF2A through the p38MAPK signaling pathway is a key regulatory pathway that confers robust activation of MEF2 *trans*activation properties.[45,58] Previous literature has suggested that MEF2A phosphorylation at conserved C-terminal residues enhances the transcriptional activation of *ckm* and myogenin genes.[41,59] Given our current observations, we propose that this activation pathway is antagonized by FOXP1 leading to inhibition of MEF2A phosphorylation and activation. A schematic depicting a distillation of these data is provided in Figure 8. Our data is consistent with the possible recruitment of PP1α into a large immunocomplex with FOXP1:MEF2A which may contribute to the dephosphorylation and maintenance of MEF2A in a repressive state.

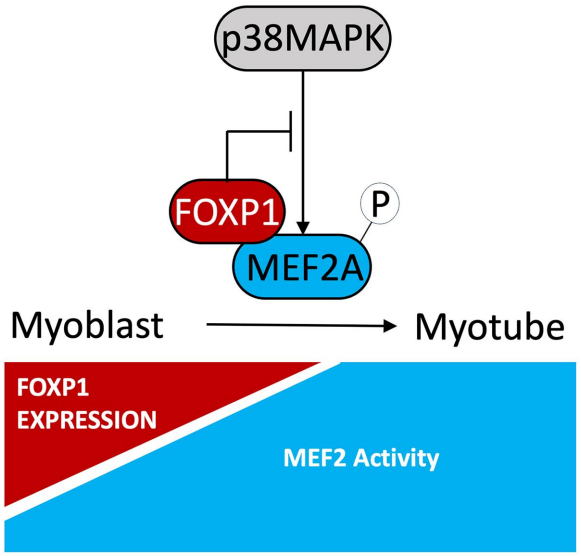


Figure 8. Proposed model of the MEF2A:FOXP1 interaction during myogenesis. The schematic indicates that FOXP1 represses p38 MAPK dependent MEF2A activity under proliferative conditions in myoblasts. This repressive mechanism is reduced as the differentiation program proceeds and FOXP1 expression is downregulated.

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 additional novel protein interactions with MEF2A. These observations contribute to our knowledge of the molecular mechanisms underlying MEF2A regulation and may also have implications for our understanding of cardiac hypertrophy and skeletal muscle differentiation.

Materials and methods

Cell culture

C2C12 myoblasts and Hek293T cells were obtained from American Type Culture Collection (ATCC, CRL-1772, and CRL-3216, respectively). Primary mouse skeletal muscle satellite cells were purchased from iXCells Biotechnologies (10MU-033). 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) or 20% FBS for the primary cells. Cells were maintained in a humidified incubator at 5% CO_2 and 37 °C. Myotube formation was induced by replacing GM with differentiation medium,[16] consisting of DMEM supplemented with 2% FBS and 1% penicillin-streptomycin.

Primary cardiomyocyte preparation

Hearts were isolated from 1 to 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 resuspended in DMEM/F12 (Gibco) supplemented with 1% penicillin-streptomycin, 50 mg/L gentamycin (Invitrogen, ThermoFisher), and 10% FBS. Cells were pre-plated for removal of noncardiomyocytes at 37 °C for 1 h 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).[60] Cardiomyocytes were transfected using Lipofectamine 2000 (Invitrogen) using instructions provided by the manufacturer. Cell medium was replenished 16 h post-transfection and harvested 8 h later. For small interfering RNA (siRNA) experiments, cells were transfected with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions at 200 μM of siRNA. All transfections were prepared in Opti-MEM (Gibco) medium in separate tubes, then mixed and incubated at room temperature for 15 min 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 with into pcDNA3-EYFP. mCherry-FOXP1 was constructed by insertion of mCherry ORF at HindIII/EcoRI and PCR amplified FOXP1 was further inserted at EcoRI/XhoI site to maintain reading frame of mCherry. GAL4-MEF2A, VP16, and HA-PP1 α have been described previously.[46] 4XMEF2-Luc and 3X-FLAG-MEF2Aconstrcuts have been described previously.[24] *ckm*-luc, *myog*-luc, and *Renilla* plasmid (pRL-*Renilla*, Promega) have all been described previously.[61] The reporter construct pMCK-EGFP was a kind gift from A. Ferrer-Martinez (Universitat de Barcelona, Spain).

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 75 nM 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 200 nM 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-Actin (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) was purchased from Developmental Studies Hybridoma Bank (DSHB). Rabbit MEF2A polyclonal antibody 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, 200 μM) and vehicle control were added to

the media and cardiomyocytes were cultured for 48 h before harvesting for subsequent experimentation. C2C12 myoblasts were treated with a p38/MAPK inhibitor (SB203580; 1 μM and 10 μM) or its inactive analogue (SB202474; 1 μM and 10 μM) (Sigma-Aldrich, #444190) for 24 h then harvested.

Protein extraction and western blot

Cells were harvested using NP-40 lysis buffer containing 0.5% (V/V) NP-40, 50 nM Tris-HCl, 150 nM NaCl, 10 mM sodium pyrophosphate, 2 mM EDTA, 0.1 M NaF, protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenyl methyl sulfonyl fluoride (Sigma-Aldrich), and 1 mM sodium orthovanadate (Bioshop). For C2C12 differentiated cultures, plates were fractionated adding 1% trypsin for 2 min 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, remaining reserve cells were harvested using a cell scrapper. Extracted proteins were denatured in SDS loading buffer at 100 °C for 10 min and loaded on an 8 or 10% SDS-polyacrylamide gel for size separation by electroporation at 100 V for 1 h. The proteins in the gel were transferred onto an Immobilon-FL polyvinylidene difluoride membrane (Millipore) with cold transfer buffer (25 mM Tris-HCl, 192 mM glycine at pH8.3 with 10% methanol (vol/vol)). Blots were blocked with 5% skim milk in Tris-buffered saline (TBS)-T (10 mM Tris-HCl, 150 mM NACl with 10% 0.1% Tween 20) for 1 h. 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), β-actin (1:1000), GFP (1:1000). The following day the blots were washed with TBST and incubates 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 vector. 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 two washes with NP40 lysis buffer and then 1X RIPA buffer. The resultant protein GBP-beads complex were then incubated with nuclear protein enriched extract from C2C12 cells. In brief, C2C12 cells were harvested and centrifuged at 500 G at 4 °C for 5 min, and cells were resuspended in hypotonic buffer (20 mM Tris-HCl pH7.0, 10 mM NaCl, 3 mM $MgCl_2$) and NP40 was added to 0.05% and vortexed at 4 °C for 5 min. Centrifugation was then performed at 500 G at 4 °C for 10 min to pellet the nuclei. To extract nuclear proteins, nuclei were resuspended in NP40 lysis buffer and vortexed for 30 min at 4 °C. Following this, cellular debris was pelleted by centrifugation at 10,000 G at 4 °C for 15 min. The nuclear protein enriched soluble fraction was then incubated with the EYFP/EYFP-MEF2A bait proteins immobilized on GFP binding peptide (GBP) nanobody conjugated magnetic beads at room temperature for 30 min. Unbound proteins were depleted by 3× washes with NP40 lysis buffer and 1× wash with PBS, remaining proteins were subjected to on-bead trypsin digestion. Mass spectrometry was performed at the SPARC BioCentre, Hospital for Sick Children, Toronto, Canada.

Immunoprecipitation

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

Immunofluorescence

C2C12 myoblasts and cardiomyocytes were seeded onto glass-bottom plates (MatTek Corp, P35GCOL-1.5-10-C) and washed with cold PBS prior to fixation using 4% paraformaldehyde solution at room temperature for 10 min. Cells were permeabilized using 90% ice cold methanol for 10 min 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 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 blocking buffer. To mark nuclei, Hoechst33342 (ThermoFisher, H3570) was added to 1 μM in PBS. To mark cell membrane, WGA488 (Biotium #29022-1) was added to 1 μM in PBS. To mark cell 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, #78440). Luciferase enzymatic activity was measured using a luminometer (Berthold) using a Firefly luciferase substrate (Promega, E1501) and Renilla luciferase substrate (Promega, E2820). Luciferase values were normalized to transfection efficiency corresponding 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.

Acknowledgments

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The materials and reagents described herein are freely available to the research community to use for noncommercial purpose and will be provided through contact with the corresponding author. The mass spectrometry data have been deposited with the ProteomeXchange Consortium via the PRIDE[62] partner repository with the dataset identifier PXD050042.

References

1. Frontera WR, Ochala J. Skeletal muscle: a brief review of structure and function. Calcif Tissue Int. 2015;96:183–195. doi:10.1007/s00223-014-9915-y.
2. Hernández-Hernández JM, García-González EG, Brun CE, Rudnicki MA. The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. Semin Cell Dev Biol. 2017;72:10–18. doi:10.1016/j.semcdb.2017.11.010.
3. Taylor MV, Hughes SM. Mef2 and the skeletal muscle differentiation program. Semin Cell Dev Biol. 2017;72:33–44. doi:10.1016/j.semcdb.2017.11.020.
4. Medrano JL, Naya FJ. The transcription factor MEF2A fine-tunes gene expression in the atrial and ventricular chambers of the adult heart. J Biol Chem. 2017;292:20975–20988. doi:10.1074/jbc.M117.806422.
5. Wong TWY, Ahmed A, Yang G, Maino E, Steiman S, Hyatt E, Chan P, Lindsay K, Wong N, Others GD. 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. 2020;13(9):dmm045369.
6. Wu W, Huang X, Cheng J, Li Z, de Folter S, Huang Z, Jiang X, Pang H, Tao S. Conservation and evolution in and among SRF- and MEF2-type MADS domains and their binding sites. Mol Biol Evol. 2011;28:501–511. doi:10.1093/molbev/msq214.
7. Cornwell JD, McDermott JC. MEF2 in cardiac hypertrophy in response to hypertension. Trends Cardiovasc Med. 2023;33:204–212. doi:10.1016/j.tcm.2022.01.002.
8. Potthoff MJ, Olson EN. MEF2: a central regulator of diverse developmental programs. Development. 2007;134:4131–4140. doi:10.1242/dev.008367.
9. Lu J, McKinsey TA, Zhang CL, Olson EN. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Mol Cell. 2000;6:233–244. doi:10.1016/s1097-2765(00)00025-3.
10. Quinn ZA, Yang CC, Wrana JL, McDermott JC. Smad proteins function as co-modulators for MEF2 transcriptional regulatory proteins. Nucleic Acids Res. 2001;29:732–742. doi:10.1093/nar/29.3.732.
11. Mao Z, Bonni A, Xia F, Nadal-Vicens M, Greenberg ME. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science. 1999;286:785–790. doi:10.1126/science.286.5440.785.
12. Kolodziejczyk SM, Wang L, Balazsi K, DeRepentigny Y, Kothary R, Megeney LA. MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. Curr Biol. 1999;9:1203–1206. doi:10.1016/S0960-9822(00)80027-5.
13. Youn HD, Sun L, Prywes R, Liu JO. Apoptosis of T cells mediated by Ca^{2+}-induced release of the transcription factor MEF2. Science. 1999;286:790–793. doi:10.1126/science.286.5440.790.
14. Blaeser F, Ho N, Prywes R, Chatila TA. Ca(2+)-dependent gene expression mediated by MEF2 transcription factors. J Biol Chem. 2000;275:197–209. doi:10.1074/jbc.275.1.197.
15. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol. 2003;65:45–79. doi:10.1146/annurev.physiol.65.092101.142243.
16. Wiedmann M, Wang X, Tang X, Han M, Li M, Mao Z. 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. 2005;81:226–234. doi:10.1002/jnr.20556.
17. Liu N, Nelson BR, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN. Requirement of MEF2A, C, and D for skeletal muscle regeneration. Proc Natl Acad Sci USA. 2014;111:4109–4114. doi:10.1073/pnas.1401732111.
18. Dumont NA, Bentzinger CF, Sincennes MC, Rudnicki MA. Satellite cells and skeletal muscle regeneration. Compr Physiol. 2015;5:1027–1059. doi:10.1002/cphy.c140068.
19. Hashemi S, Salma J, Wales S, McDermott JC. Pro-survival function of MEF2 in cardiomyocytes is enhanced by beta-blockers. Cell Death Discov. 2015;1:15019. doi:10.1038/cddiscovery.2015.19.
20. Akazawa H, Komuro I. Roles of cardiac transcription factors in cardiac hypertrophy. Circ Res. 2003;92:1079–1088. doi:10.1161/01.RES.0000072977.86706.23.
21. Neyroud D, Nosacka RL, Callaway CS, Trevino JG, Hu H, Judge SM, Judge AR. FoxP1 is a transcriptional repressor associated with cancer cachexia that induces skeletal muscle wasting and weakness. J Cachexia Sarcopenia Muscle. 2021;12:421–442. doi:10.1002/jcsm.12666.
22. Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC, Leonhardt H. A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. Mol Cell Proteomics. 2008;7:282–289. doi:10.1074/mcp.M700342-MCP200.
23. Wang B, Weidenfeld J, Lu MM, Maika S, Kuziel WA, Morrisey EE, Tucker PW. Foxp1 regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation. Development. 2004;131:4477–4487. doi:10.1242/dev.01287.
24. Moustafa A, Hashemi S, Brar G, Grigull J, Ng SHS, Williams D, Schmitt-Ulms G, McDermott JC. The MEF2A transcription factor interactome in cardiomyocytes. Cell Death Dis. 2023;14:240. doi:10.1038/s41419-023-05665-8.
25. Mughal W, Nguyen L, Pustylnik S, da Silva Rosa SC, Piotrowski S, Chapman D, Du M, Alli NS, Grigull J, Halayko AJ, et al. A conserved MADS-box phosphorylation motif regulates differentiation and mitochondrial function in skeletal, cardiac, and smooth muscle cells. Cell Death Dis. 2015;6:e1944. doi:10.1038/cddis.2015.306.
26. Bai S, Kerppola TK. Opposing roles of FoxP1 and Nfat3 in transcriptional control of cardiomyocyte hypertrophy. Mol Cell Biol. 2011;31:3068–3080. doi:10.1128/MCB.00925-10.
27. Zhang Y, Li S, Yuan L, Tian Y, Weidenfeld J, Yang J, Liu F, Chokas AL, Morrisey EE. Foxp1 coordinates cardiomyocyte proliferation through both cell-autonomous and nonautonomous mechanisms. Genes Dev. 2010;24:1746–1757. doi:10.1101/gad.1929210.
28. Jiang X, Ji S, Yuan F, Li T, Cui S, Wang W, Ye X, Wang R, Chen Y, Zhu S. Pyruvate dehydrogenase B regulates myogenic differentiation via the FoxP1-Arih2 axis. J Cachexia Sarcopenia Muscle. 2023;14:606–621. doi:10.1002/jcsm.13166.

29. Lozano R, Gbekie C, Siper PM, Srivastava S, Saland JM, Sethuram S, Tang L, Drapeau E, Frank Y, Buxbaum JD. FOXP1 syndrome: a review of the literature and practice parameters for medical assessment and monitoring. J Neurodev Disord. 2021;13:18.
30. Rocca DL, Wilkinson KA, Henley JM. SUMOylation of FOXP1 regulates transcriptional repression via CtBP1 to drive dendritic morphogenesis. Sci Rep. 2017;7:877. doi:10.1038/s41598-017-00707-6.
31. Gordon JW, Pagiatakis C, Salma J, Du M, Andreucci JJ, Zhao J, Hou G, Perry RL, Dan Q, Courtman D, and others., et al. Protein kinase A-regulated assembly of a MEF2{middle dot}HDAC4 repressor complex controls c-Jun expression in vascular smooth muscle cells. J Biol Chem. 2009;284:19027–19042. doi:10.1074/jbc.M109.000539.
32. Majidi SP, Reddy NC, Moore MJ, Chen H, Yamada T, Andzelm MM, Cherry TJ, Hu LS, Greenberg ME, Bonni A. Chromatin environment and cellular context specify compensatory activity of paralogous MEF2 transcription factors. Cell Rep. 2019;29:2001–2015.e5. doi:10.1016/j.celrep.2019.10.033.
33. Naya FJ, Olson E. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Curr Opin Cell Biol. 1999;11:683–688. doi:10.1016/s0955-0674(99)00036-8.
34. Jang M, Scheffold J, Røst LM, Cheon H, Bruheim P. Serum-free cultures of C2C12 cells show different muscle phenotypes which can be estimated by metabolic profiling. Sci Rep. 2022;12:827. doi:10.1038/s41598-022-04804-z.
35. Schöneich C, Dremina E, Galeva N, Sharov V. Apoptosis in differentiating C2C12 muscle cells selectively targets Bcl-2-deficient myotubes. Apoptosis. 2014;19:42–57. doi:10.1007/s10495-013-0922-7.
36. Myer A, Olson EN, Klein WH. MyoD cannot compensate for the absence of myogenin during skeletal muscle differentiation in murine embryonic stem cells. Dev Biol. 2001;229:340–350. doi:10.1006/dbio.2000.9985.
37. Tripathi S, Miyake T, McDermott JC. Smad7:beta-catenin complex regulates myogenic gene transcription. Cell Death Dis. 2019;10:387. doi:10.1038/s41419-019-1615-0.
38. Andrés V, Walsh K. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol. 1996;132:657–666. doi:10.1083/jcb.132.4.657.
39. Cserjesi P, Olson EN. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. Mol Cell Biol. 1991;11:4854–4862. doi:10.1128/mcb.11.10.4854-4862.1991.
40. Nakatsuji Y, Hidaka K, Tsujino S, Yamamoto Y, Mukai T, Yanagihara T, Kishimoto T, Sakoda S. A single MEF-2 site is a major positive regulatory element required for transcription of the muscle-specific subunit of the human phosphoglycerate mutase gene in skeletal and cardiac muscle cells. Mol Cell Biol. 1992;12:4384–4390.
41. Edmondson DG, Cheng TC, Cserjesi P, Chakraborty T, Olson EN. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol Cell Biol. 1992;12:3665–3677. doi:10.1128/mcb.12.9.3665-3677.1992.
42. Buchberger A, Ragge K, Arnold HH. The myogenin gene is activated during myocyte differentiation by pre-existing, not newly synthesized transcription factor MEF-2. J Biol Chem. 1994;269:17289–17296.
43. Jayathilaka N, Han A, Gaffney KJ, Dey R, Jarusiewicz JA, Noridomi K, Philips MA, Lei X, He J, Ye J, and others., et al. Inhibition of the function of class IIa HDACs by blocking their interaction with MEF2. Nucleic Acids Res. 2012;40:5378–5388. doi:10.1093/nar/gks189.
44. Zhao X, Sternsdorf T, Bolger TA, Evans RM, Yao TP. Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. Mol Cell Biol. 2005;25:8456–8464. doi:10.1128/MCB.25.19.8456-8464.2005.
45. Ornatsky OI, Cox DM, Tangirala P, Andreucci JJ, Quinn ZA, Wrana JL, Prywes R, Yu YT, McDermott JC. Post-translational control of the MEF2A transcriptional regulatory protein. Nucleic Acids Res. 1999;27:2646–2654. doi:10.1093/nar/27.13.2646.
46. Perry RLS, Yang C, Soora N, Salma J, Marback M, Naghibi L, Ilyas H, Chan J, Gordon JW, McDermott JC. Direct interaction between myocyte enhancer factor 2 (MEF2) and protein phosphatase 1α represses MEF2-dependent gene expression. Mol Cell Biol. 2009;29:3355–3366. doi:10.1128/MCB.00227-08.
47. Cox DM, Du M, Marback M, Yang EC, Chan J, Siu KW, McDermott JC. Phosphorylation motifs regulating the stability and function of myocyte enhancer factor 2A. J Biol Chem. 2003;278:15297–15303. doi:10.1074/jbc.M211312200.
48. Barsyte-Lovejoy D, Galanis A, Sharrocks AD. Specificity determinants in MAPK signaling to transcription factors. J Biol Chem. 2002;277:9896–9903. doi:10.1074/jbc.M108145200.
49. de Angelis L, Zhao J, Andreucci JJ, Olson EN, Cossu G, McDermott JC. Regulation of vertebrate myotome development by the p38 MAP kinase-MEF2 signaling pathway. Dev Biol. 2005;283:171–179. doi:10.1016/j.ydbio.2005.04.009.
50. Dionyssiou MG, Nowacki NB, Hashemi S, Zhao J, Kerr A, Tsushima RG, McDermott JC. Cross-talk between glycogen synthase kinase 3beta (GSK3beta) and p38MAPK regulates myocyte enhancer factor 2 (MEF2) activity in skeletal and cardiac muscle. J Mol Cell Cardiol. 2013;54:35–44. doi:10.1016/j.yjmcc.2012.10.013.
51. Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, Han J. Regulation of the MEF2 family of transcription factors by p38. Mol Cell Biol. 1999;19:21–30. doi:10.1128/MCB.19.1.21.
52. Puri PL, Wu Z, Zhang P, Wood LD, Bhakta KS, Han J, Feramisco JR, Karin M, Wang JY. Induction of terminal differentiation by constitutive activation of p38 MAP kinase in human rhabdomyosarcoma cells. Genes Dev. 2000;14:574–584. doi:10.1101/gad.14.5.574.
53. Ikeda S, He A, Kong SW, Lu J, Bejar R, Bodyak N, Lee K-H, Ma Q, Kang PM, Golub TR, et al. MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. Mol Cell Biol. 2009;29:2193–2204. doi:10.1128/MCB.01222-08.
54. Clerk A, Michael A, Sugden PH. Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine: a role in cardiac myocyte hypertrophy? J Cell Biol. 1998;142:523–535. doi:10.1083/jcb.142.2.523.
55. Moretti I, Ciciliot S, Dyar KA, Abraham R, Murgia M, Agatea L, Akimoto T, Bicciato S, Forcato M, Pierre P, et al. MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity. Nat Commun. 2016;7:12397. doi:10.1038/ncomms12397.
56. Sassoon D, Lyons G, Wright WE, Lin V, Lassar A, Weintraub H, Buckingham M. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature. 1989;341:303–307. doi:10.1038/341303a0.
57. Wright WE, Sassoon DA, Lin VK. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell. 1989;56:607–617. doi:10.1016/0092-8674(89)90583-7.
58. Chang CI, Xu BE, Akella R, Cobb MH, Goldsmith EJ. Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. Mol Cell. 2002;9:1241–1249. doi:10.1016/s1097-2765(02)00525-7.
59. Liu ML, Olson AL, Edgington NP, Moye-Rowley WS, Pessin JE. Myocyte enhancer factor 2 (MEF2) binding site is essential for C2C12 myotube-specific expression of the rat GLUT4/muscle-adipose facilitative glucose transporter gene. J Biol Chem. 1994;269:28514–28521. doi:10.1016/S0021-9258(18)46957-8.
60. Longo PA, Kavran JM, Kim MS, Leahy DJ. Transient mammalian cell transfection with polyethylenimine (PEI). Methods Enzymol. 2013;529:227–240. doi:10.1016/B978-0-12-418687-3.00018-5.
61. Miyake T, Aziz A, McDermott JC. Maintenance of the undifferentiated state in myogenic progenitor cells by TGFbeta signaling is Smad independent and requires MEK activation. Int J Mol Sci. 2020;21(3):1057. doi: 10.3390/ijms21031057.
62. Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, Kundu DJ, Prakash A, Frericks-Zipper A, Eisenacher M, et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. Nucleic Acids Res. 2022;50:D543–D552. doi:10.1093/nar/gkab1038.