

***Deciphering the role of MEF2D splice forms  
during skeletal muscle differentiation.***

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## ABSTRACT

Members of the Mef2 transcription factor family are extensively studied within the muscle field for their ability to cooperate with the myogenic regulatory factors MyoD and myogenin during muscle differentiation. Although it is known that Mef2 pre-mRNAs undergo alternative splicing, the different splice forms have not been functionally annotated. In this thesis, my studies aimed to characterize three Mef2D splice forms: MEF2D $\alpha'\beta$ , MEF2D $\alpha\beta$ , MEF2D $\alpha\emptyset$ . Our results show that MEF2D splice forms can be differentially phosphorylated by p38 MAPK and PKA *in vitro*. Gene expression analysis using cell lines over-expressing each Mef2D splice form suggests that they can differentially activate desmin, myosin heavy chain and myogenin expression. Mass spectrometry analyses from our pull-down assays reveal known and novel MEF2D binding partners. Our work suggests that Mef2D splice forms have overlapping but distinct roles and provides new insight into the importance of Mef2D alternative splicing during skeletal myogenesis.

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## List of abbreviations

|                    |                                                |
|--------------------|------------------------------------------------|
| ATP                | Adenosine triphosphate                         |
| BSA                | Bovine serum albumin                           |
| ChIP               | Chromatin immunoprecipitation                  |
| COS                | CV-1 in origin, SV40 genome                    |
| ddH <sub>2</sub> O | Double-distilled water                         |
| DDX5               | DEAD box protein 5                             |
| Des                | Desmin                                         |
| DMSO               | Dimethyl sulfoxide                             |
| dpc                | Days post coitum                               |
| DMEM               | Dulbecco's modified eagle medium               |
| DTT                | Dithiothreitol                                 |
| EDTA               | Ethylenediaminetetraacetic acid                |
| Ezh2               | Enhancer of zeste 2                            |
| FBS                | Fetal bovine serum                             |
| H3K4ac             | Histone 3, lysine 4, acetylation               |
| H3K27me2           | Histone 3, lysine 27, di-methylation           |
| H3K27me3           | Histone 3, lysine 27, tri-methylation          |
| HDAC               | Histone deacetylase                            |
| HAT                | Histone acetyltransferase                      |
| HMT                | Histone methyltransferase                      |
| HKMT               | Histone lysine methyltransferase               |
| HRP                | Horseradish peroxidase                         |
| Hr(s)              | Hour(s)                                        |
| IP                 | Immunoprecipitation                            |
| IPTG               | Isopropyl $\beta$ -D-1-thiogalactopyranoside   |
| LC-MS/MS           | Liquid chromatography tandem-mass spectrometry |
| LB broth           | Luria-Bertani broth                            |
| MAPK               | Mitogen-activated protein kinase               |
| MB                 | Myoblast                                       |
| MEF2               | Myocyte-specific enhancer factor 2             |
| Min(s)             | Minute(s)                                      |
| MKK6               | MAP kinase kinase 6                            |
| MRF                | Myogenic regulatory factor                     |
| MRF4               | Myogenic regulatory factor 4                   |
| Myh                | Myosin heavy chain                             |
| MT                 | Myotube                                        |
| Myf5               | Myogenic factor 5                              |
| Myog               | Myogenin                                       |
| NE                 | Nuclear extract                                |
| NP-40              | nonyl phenoxypolyethoxylethanol                |
| Pax3               | Paired-box transcription factor 3              |
| Pax7               | Paired-box transcription factor 7              |
| PBS                | Phosphate buffered saline                      |



|          |                                                                 |
|----------|-----------------------------------------------------------------|
| PcG      | Polycomb group                                                  |
| PCR      | Polymerase chain reaction                                       |
| PKA      | Protein kinase A                                                |
| PMSF     | Phenylmethylsulfonyl fluoride                                   |
| RT       | Room temperature                                                |
| RT-PCR   | Reverse transcription polymerase chain reaction                 |
| RT-qPCR  | Quantitative reverse transcription polymerase chain reaction    |
| SD       | Standard deviation                                              |
| SDS      | Sodium dodecyl sulfate                                          |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis                          |
| Sec(s)   | Second(s)                                                       |
| TAD      | Transcriptional activation domain                               |
| TEMED    | Tetramethylethylenediamine                                      |
| TrxG     | Trithorax group                                                 |
| U        | Units                                                           |
| UTX      | Ubiquitously transcribed tetratricopeptide repeat, X chromosome |
| WB       | Western blot                                                    |

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## **Dedication**

This thesis is dedicated to my labmates.

You have been a second family and a home away from home. These last two years have changed me so much and you have been there for me through my transformation. Know that I sincerely and deeply appreciate each of you and will never forget you.

These have been two of the most fulfilling years of my life.

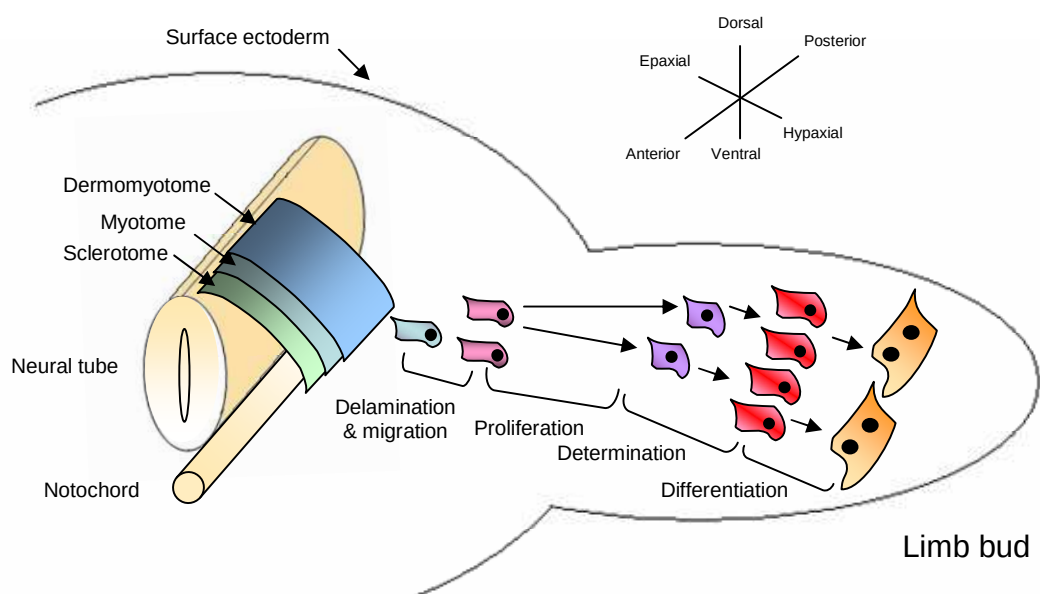
Thank you from the bottom of my heart.

# 1. INTRODUCTION

## ***1.1 Strength in waves: Myogenesis.***

Myogenesis recurs throughout the life of vertebrate organisms and is the process responsible for generating skeletal muscle tissue (reviewed by Sabourin & Rudnicki 2000, Bryson-Richardson & Currie, 2008). Three waves of myogenesis contribute to muscle formation in vertebrates. In mice, the first wave of skeletal myogenesis begins within segments formed along the rostral-caudal axis of the paraxial mesoderm known as somites. A complex orchestration of developmental cues direct dorsal division and differentiation of somites around 9 days post coitum (dpc) to form the dermomyotome which is a transient epithelial-like structure (Fig.1). Cells migrate from the dorsomedial lip of the dermomyotome to form the primary myotome which constitutes the first embryonic muscle mass that later differentiates into deep back muscles. A second wave of myogenesis takes place between 14.5-17.5 dpc and is initiated as centrally located cells of the dermomyotome delaminate and migrate to sites of muscle development like the limb bud where they contribute to secondary muscle formation. At the level of gene expression, paired box domain transcription factors Pax3 and Pax7 are crucial regulators of myogenesis (Fig.2). Pax3 is required for somite segmentation, dermomyotome formation and limb muscle development whereas Pax7 functions primarily to specify and maintain populations of muscle stem cells also known as satellite cells (Seale et al., 2000). Satellite cell activation during postnatal development constitutes the third wave of

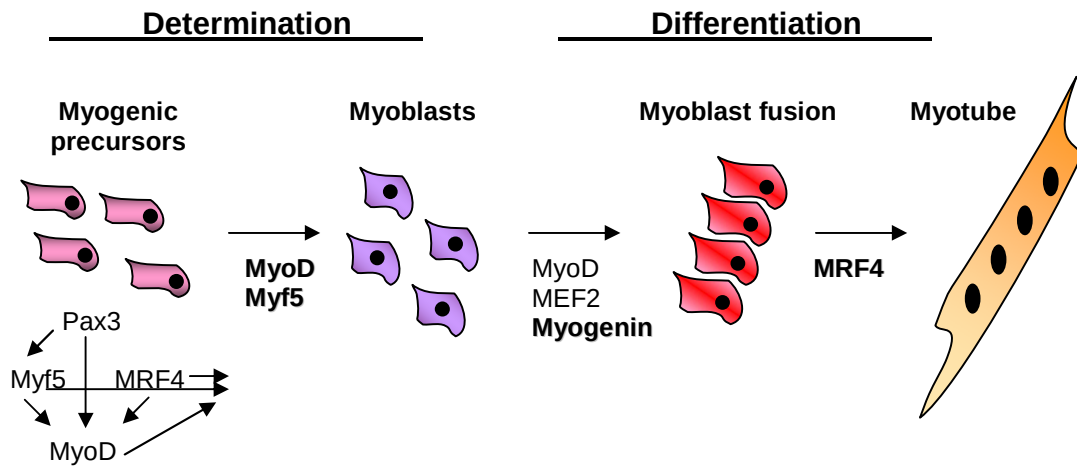
myogenesis also known as adult myogenesis. Another family of transcription factors known as the myogenic regulatory factors (MRFs) robustly initiate the myogenic program downstream of Pax3, leading to the determination and differentiation of dermomyotome-derived myogenic precursors (Fig.2).



**Figure 1. Embryonic myogenesis in the limb bud.** The dermomyotome represents a transient epithelial-like structure which forms as a result of somite differentiation. Delamination and migration of myogenic precursors from the hypaxial lip of the dermomyotome contribute to formation of limb and girdle muscles. Myogenic precursors develop into mature muscle fibres through a progression involving proliferation, determination and differentiation.

### **1.1.1 The Myogenic Regulatory Factors (MRFs).**

Together, Myf5, MyoD, myogenin and MRF4 constitute a subfamily of the basic helix-loop-helix transcription factor superfamily and act by associating with ubiquitous E proteins at the E box consensus motif VCASCTG, which is found in many muscle-specific genes (Cao et al., 2010). The four MRFs were identified between 1987 and 1989 from cDNA library screens based on their ability to convert fibroblasts and other non-muscle cells into myogenic cells when over-expressed (Davis et al., 1987, Braun et al., 1989, Edmondson et al., 1989, Rhodes et al., 1989). The spatio-temporal expression of each MRF during myogenesis has been approximated using *in situ* hybridization in Drosophila and mouse models. In the developing mouse limb bud, Myf5 is the first to be expressed 10-12 dpc followed by MyoD and myogenin which are co-expressed after day 10.5 and lastly by MRF4 which is expressed around 16 dpc. By coupling *in situ* hybridization data with targeted gene knock-out studies in mice, researchers have gained crucial insight into the unique functionality of each MRF. Mice carrying null mutations for MyoD and Myf5 alone display transient skeletal muscle defects, whereas mice null for both alleles exhibit a complete lack of muscle formation (Rudnicki et al., 1993). Myogenin or MRF4 mutant mice, however, primarily display myocyte differentiation defects (Venuti et al., 1995, Yoon et al., 1997). As such, it is thought that MyoD and Myf5 are important early on during the commitment of somitic cells to the myogenic lineage whereas Myogenin and MRF4 are critical during differentiation and late stages of myogenesis (reviewed by Bryson-Richardson and Currie, 2008).



**Figure 2. Schematic illustrating the hierarchical relationship among myogenic regulatory factors.** Early on Pax3, Myf5 and Mrf4 activate MyoD which promotes specification of muscle precursors to myoblasts. Myf5 and MyoD initiate a gene expression profile which commits myogenic precursors along the muscle lineage. Myogenin is subsequently required for early differentiation events and myoblast fusion whereas Mrf4 is required for myotube maturation.

To begin teasing apart the complex gene regulatory networks established and maintained by the MRFs, numerous expression array studies have been conducted with the hope of determining the breadth of genes differentially expressed during myogenesis as well as those differentially targeted by each of the MRFs. One such study conducted a genome-wide analysis of MyoD and myogenin transcription factor binding sites using chromatin immunoprecipitation followed by microarray analysis (ChIP-on-chip), and revealed that the two MRFs regulate a distinct but overlapping set of target genes (Blais et al., 2005). In these studies, many of the genes induced by MyoD during myoblast differentiation were also targeted by myogenin, suggesting that the two participate in a co-operative mechanism of transcriptional activation. A second study corroborated

these results and proposed a mechanism whereby MyoD alone is able to robustly activate early and intermediate differentiation-specific genes, but is insufficient for high-level expression of late genes whose promoter requires both MyoD-dependent histone acetylation and recruitment of myogenin (Cao et al., 2006). In this way, a subset of the overlapping target genes requires sequential activity of the two MRFs and the proper epigenetic environment for full activation. Indeed, it is well established that MRFs play a crucial role in the deposition and maintenance of epigenetic marks at muscle-specific loci via recruitment of histone modifying enzymes (reviewed by Palacios and Puri, 2006). Examples of histone modifying enzymes which have been implicated in the regulation of muscle-specific genes are histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone demethylases.

#### **1.1.2 Epigenetic regulation of skeletal muscle differentiation.**

The concerted effort of MRFs and histone modifying proteins in mediating the transition from proliferating myoblasts to differentiated myocytes has been extensively studied. Key players belong to the polycomb group (PcG) and trithorax group (TrxG) proteins which act antagonistically to regulate numerous developmental processes including skeletal myogenesis (reviewed by Schuettengruber et al., 2007). The PcG protein Enhancer of zeste (Ezh) was first identified based on its role in regulating HOX gene expression during anterior-posterior patterning in *Drosophila* (Simon et al., 1992). The vertebrate homolog Ezh2 has since been implicated in gene repression during the formation of



various tissues including skeletal muscle (Caretti et al., 2004). Ezh2 histone lysine methyltransferase (HKMT) activity is conferred by its SET domain and marks genes for repression through di- and tri-methylation of lysine 27 on histone 3 (H3K27me2 and H3K27me3 respectively) (Sims et al., 2003). Chromatin immunoprecipitation (ChIP) experiments have revealed that Ezh2 and co-repressors YY1 and Histone Deacetylase 1 (HDAC1) are enriched within the regulatory region of some late myogenic genes in proliferating myoblasts, and correlate with increased levels of H3K27me2/3 (Caretti et al., 2004). At the time of transcriptional activation, Ezh2 binding is lost and replaced by MyoD at these loci. Concomitantly, H3K27me2/3 marks are diminished and an increase in acetylation of histone H4 (H4ac) is observed. Increased H4ac has been shown to facilitate mobilization of nucleosomes through recruitment of the TrxG-related SWI/SNF chromatin remodeling complex (de la Serna et al., 2005). SWI/SNF bromodomain-containing ATPase subunits Brg1 and Brm can recognize H4ac marks and stabilize the complex at these loci. This subsequently stabilizes the binding of MyoD to initiate transcriptional activation (de la Serna et al., 2005). Interestingly, enrichment of the H3K27 demethylase UTX can be detected in the myogenin regulatory region after 24hrs of differentiation and coincides with a loss of Ezh2 enrichment as well as loss of the repressive H3K27me3 mark. Subsequently, after 48hrs of differentiation Ash2L1/MLL2(KMT2B) enrichment is maximal and coincides with increased levels of the activating mark H3K4me3 (Seenundun et al., 2010).

## **1.2 The Myocyte Enhancer Factor 2 (Mef2) gene family.**

Numerous activators and repressors have been reported to interact with MRFs, but perhaps the best studied interacting partners are the *Mef2* family of transcription factors. Whereas *S.cerevisiae*, *C.elegans* and *D.melanogaster* contain a single *Mef2* gene, vertebrates have four copies annotated *Mef2A*, *Mef2B*, *Mef2C* and *Mef2D*. MEF2 transcription factors bind the A/T-rich DNA consensus sequence YTA(A/T)<sub>4</sub>TAR as either homo- or heterodimers (reviewed by Potthoff and Olson, 2007). There is a high degree of conservation in the amino (N)-terminal MADS domain (named for the proteins in which the domain was first identified: MCM1, agamous, deficiens, SRF) and in the MEF2 domain which facilitates DNA binding and protein-protein interactions (Potthoff and Olson, 2007). Deletion of this highly conserved N-terminal region in MEF2C has been shown to prevent co-operative binding with either MyoD or myogenin (Molkentin et al., 1995). In contrast, the substantial lack of sequence conservation in the carboxy (C)-terminus transcriptional activation domain (TAD) has been shown to impart distinct functional and regulatory features to vertebrate MEF2 isoforms (reviewed by Potthoff and Olson, 2007).

More than a decade ago, Olson and colleagues demonstrated *in vitro* that MEF2 can bind co-operatively with MyoD or myogenin at muscle-specific loci to enhance gene expression to levels greater than those achieved by MyoD or myogenin alone (Molkentin et al., 1995). Using combinations of MEF2C and MyoD/myogenin mutants lacking DNA-binding, protein dimerization and/or transcriptional activation capabilities, they demonstrated that synergistic gene

activation required only one DNA-binding and transcriptional activation domain from either transcription factor but not from both. They also showed that myogenin must be bound to DNA via an E box motif to initiate myogenesis and that MEF2C interacted specifically with myogenin/E12 heterodimers but not with myogenin or E12 homodimers. Importantly, MEF2 isoforms can also cooperate with MRFs to further regulate MRF expression. Characterization of the minimal myogenin and MRF4 promoters required for tissue-specific expression reveal that both contain MEF2 and E box consensus motifs which are required for robust gene expression (Yee & Rigby, 1993, Naidu et al., 1995). In contrast, MEF2 sites in the proximal MyoD promoter are not required for muscle-specific gene expression (Dechesne et al., 1994). Myf5 regulation is unique in that numerous enhancer modules have been identified that can drive expression in specific anatomical regions of developing vertebrate embryos; likely acquired throughout evolution for fine-tuning expression in response to combinations of developmental cues (Summerbell et al., 2000, Buchberger et al., 2003). Nevertheless, the existence of MEF2 and E-box consensus motifs in most MEF2 and MRF gene regulatory regions suggests that transcriptional regulation and activity of the two protein families is complex and crucial for proper formation of skeletal muscle tissue.

### **1.2.1 Role of MEF2D during skeletal muscle differentiation.**

MEF2D is the focus of studies in skeletal muscle tissue since it is abundant in both myoblasts and myotubes (Breitbart et al., 1993, Martin et al., 1994, Penn et al., 2004). There is also evidence that MEF2D specifically is

involved in MyoD-dependent activation of muscle-genes during differentiation (Penn et al., 2004, Rampalli et al., 2007). Numerous publications underscore the importance of MEF2D as a crucial point of convergence for various signaling cascades during muscle differentiation, including the mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) pathways (reviewed by Pottfoff & Olson 2007).

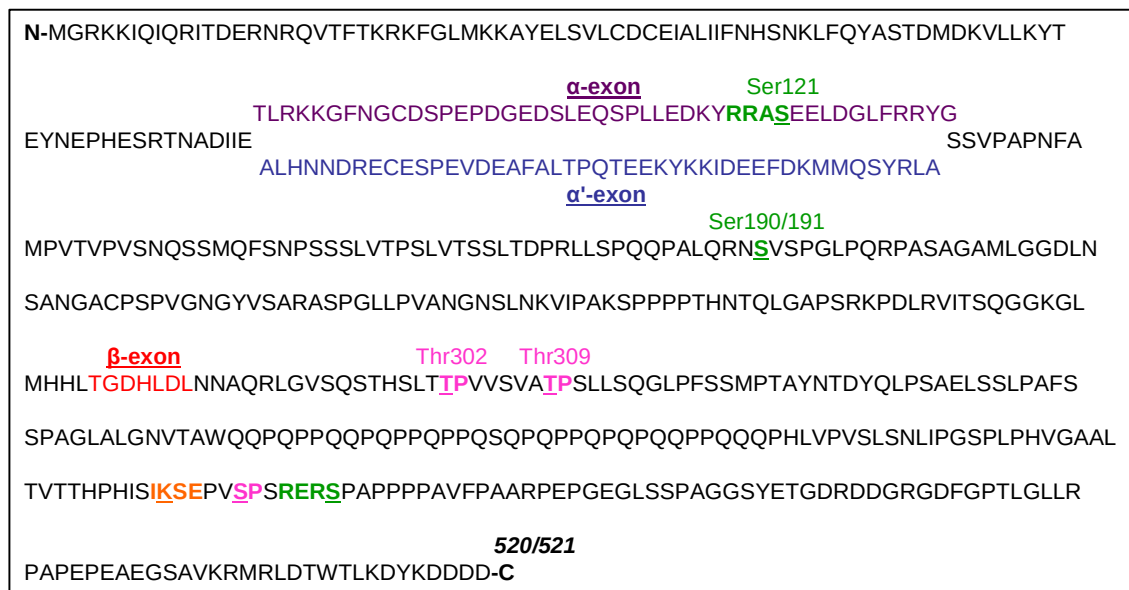
Signaling through the MAPK pathway drives the myogenic program by promoting myoblast differentiation and fusion (Perdiguero et al., 2007). p38 MAPK plays an important role in stabilizing the MyoD-MEF2D complex at the promoter of MyoD target genes during differentiation (Penn et al., 2004). Interestingly, precocious p38 activity results in early expression of late-stage genes, however, MEF2D over-expression is additive such that late-stage gene expression can be detected earlier still (Penn et al., 2004). This is because MEF2D binding recruits active RNA Polymerase II (RNA Pol II) and subsequent phosphorylation of MEF2D by p38 further recruits the TrxG protein complex Ash2L1/MLL2(KMT2B) which deposits H3K4me3 marks that promote active gene transcription (Penn et al., 2004, Rampalli et al., 2007). Several p38 phosphoacceptor sites have been identified within the TAD of MEF2 isoforms that are conserved in MEF2D at sites Thr-302 and Thr-309 (Fig.3; Zhao et al., 1999). Phosphorylation of these threonine residues has been shown to strongly increase MEF2 transcriptional activity (Zhao et al., 1999).

The PKA signaling pathway is another critical regulator of the myogenic program, although unlike the MAPK pathway, it has been shown to inhibit muscle

differentiation (Winter et al., 1993). One mechanism of inhibition occurs through targeted phosphorylation of an E-box consensus site within the N-terminus of MRFs, preventing them from heterodimerizing with E-proteins to transactivate muscle-specific gene promoters (Li et al., 1992, Winter et al., 1993). Another mechanism of PKA-mediated muscle-gene repression described more recently involves nuclear accumulation of HDAC4 and an increased association of HDAC4 with MEF2D (Du et al., 2008). Two PKA phosphoacceptor sites are located in the MADS/MEF2 domain and more recently another two have been identified within the TAD of MEF2D at Ser-121 and Ser-190 (Du et al., 2008). Interestingly, Ser-121 is located within an alternatively spliced region of the protein such that it is present in one splice form but not in another (Fig.3). The functional consequence of Ser-121 loss within the context of muscle differentiation remains unknown.

### ***1.3 Mechanisms of Mef2 regulation in skeletal muscle.***

MEF2 activity is by no means restricted to vertebrate skeletal muscle. It is also required for vertebrate heart development, neuronal differentiation, neural crest development, bone development, T-cell development, and vascular integrity (reviewed by Potthoff and Olson, 2007). With Mef2 expression being quite ubiquitous, the question arises, how is tissue-specific activity achieved? Two mechanisms described to date include the association of Mef2 proteins with HDACs and alternative splicing of Mef2 pre-mRNA (Potthoff and Olson, 2007).



**K/R-X-X-S/T** – PKA consensus sequence (phosphoacceptor site is underlined; McKinsey et al., 2000).

**IKSE** – SUMO1 consensus sequence (sumo-acceptor site is underlined; Grégoire and Yang, 2005).

**S/TP** – p38 phosphoacceptor site is underlined (Cox et al., 2003, Grégoire and Yang, 2005).

**Figure 3. Mouse MEF2D amino acid sequence.** The location of alternatively spliced α-exon, α'-exon and β-exon are indicated. Protein modification sites targeted by PKA, SUMO1 and p38 are indicated in green, orange and pink respectively.

### 1.3.1 HDACs.

In mammals 11 HDACs have been characterized thus far, and are divided into four classes; Class I, Class IIa, Class IIb and Class IV. HDAC loss-of-function studies in mice result in a wide range of phenotypes varying in severity from viable to embryonic lethal (reviewed by Haberland et al., 2009). In skeletal muscle, Class IIa HDACs (HDAC4,-5,-7 and -9) are key mediators of active gene repression at numerous muscle-gene loci often through their association with MEF2 proteins (Lu et al., 2000). There is also evidence suggesting that MEF2C

can activate HDAC9 expression to create a negative-feedback loop for fine-tuning MEF2 activity (Haberland et al., 2007). Still, the complete mechanism of HDAC-mediated MEF2 repression has not been fully elucidated.

Despite constitutive expression of Class IIa HDACs in C2C12 myoblast and myotube cultures *in vitro*, suppression of MEF2 activity is released as a consequence of HDAC dissociation and translocation from the nucleus to the cytoplasm. Interestingly, there are several signaling cascades which affect the subcellular localization of HDACs in skeletal muscle. Calcium/calmodulin-dependent protein kinase (CaMK) signaling results in the direct phosphorylation of HDAC5 which creates docking sites for the protein chaperone 14-3-3, ultimately leading to HDAC5 nuclear export, release of MEF2 suppression and initiation of the terminal differentiation program (McKinsey et al., 2000a, McKinsey et al., 2000b). Conversely, activation of cyclic AMP (cAMP)-dependent PKA has been shown to promote HDAC4 shuttling from the cytoplasm to the nucleus and results in increased nuclear association between HDAC4 and MEF2D (Du et al., 2008).

### **1.3.2 Alternative splicing.**

Although we have known for nearly two decades that the Mef2 family of transcription factors contain alternatively spliced exons, questions of why this is so and how Mef2 splicing is regulated remain largely unanswered. Advances in the field of alternative splicing, however, will undoubtedly shed light on this regulatory process; a process which is responsible for generating incredible protein diversity within a single cell or tissue type from a limited number of genes.

Three of the four vertebrate Mef2 isoforms produce alternatively spliced pre-mRNA. The Mef2C gene contains three alternatively spliced exons annotated alpha ( $\alpha$ ), beta ( $\beta$ ) and gamma ( $\gamma$ ) (Zhu & Gulick, 2004) whereas Mef2A and Mef2D contain only  $\alpha$  and  $\beta$  alternate exons (Breitbart et al., 1993, Martin et al.1994). Interestingly, the  $\gamma$ -exon sequence is conserved in both Mef2A and Mef2D but does not undergo alternative splicing (Zhu & Gulick, 2004).

All characterized transcripts of Mef2 contain either the  $\alpha$ -exon, or the alternative  $\alpha'$ -exon (which represents an alternative Mef2 mRNA product shorter by just one codon). Conversely, the  $\beta$ -exon is included or excluded to generate proteins which vary by 10 amino acids. Intriguingly, the  $\alpha$ ,  $\beta$  and  $\gamma$  exons are all spliced in a tissue-specific manner. Mef2C transcripts containing the  $\gamma$ -exon are found exclusively in the heart, however, skipping of the  $\gamma$ -exon is common in mature skeletal muscle and Mef2C-expressing tissues (Zhu & Gulick, 2004). The  $\beta$ -exon is included in Mef2 transcripts found in mature skeletal muscle, brain and cardiac ventricle while Mef2 transcripts excluding the  $\beta$ -exon are found more ubiquitously (Breitbart et al., 1993). Similarly, the  $\alpha$ -exon can be detected in mature brain, heart and kidney as well as proliferating skeletal myoblasts, whereas the  $\alpha'$ -exon is found exclusively in differentiated skeletal muscle (Martin et al., 1994). Evidence in the literature does not convincingly demonstrate a unique role for muscle-specific MEF2D forms containing the  $\alpha'$ -exon, however, one possibility is that these splice forms are required for proper myoblast fusion and differentiation into mature myotubes. Work by Imbalzano's group supports this possibility, demonstrating that over-expression of a MEF2D  $\alpha'$ -containing splice form can



rescue fusion-defective NIH3T3 fibroblasts undergoing MyoD-induced myogenesis (Ohkawa et al., 2006).

The functional role of MEF2D splice forms has not been carefully studied and often evidence in the literature can be difficult to trace back to one particular splice form or another. It remains largely unknown how the pattern of Mef2D alternative splicing affects responsiveness to various signaling cues or the ability of MEF2D to interact with co-activators and co-repressors. Comparative analysis of MEF2D splice form function will certainly help elucidate the importance of Mef2D alternative splicing during muscle differentiation.

## **1.4 Hypothesis**

Regulated alternative splicing of Mef2D during skeletal muscle development endows MEF2D $\alpha\beta$ , MEF2D $\alpha'\beta$  and MEF2D $\alpha\emptyset$  splice forms with unique biochemical properties that determine their ability to act as activators and/or repressors of gene expression during the muscle differentiation program.

## **1.5 Objectives**

To investigate how Mef2D splicing affects the skeletal muscle differentiation process we have formulated several objectives:

- 1) Determine whether MEF2D splice forms are differentially phosphorylated by p38 $\alpha$  MAPK and PKA.

*- Perform in vitro kinase assays using recombinant Flag-tagged MEF2D splice forms generated with the baculovirus expression system.*

- 2) Determine how the(se) phosphorylation event(s) affect:

- i. p38 $\alpha$ -dependent transcriptional activity of MEF2D splice forms.
- ii. PKA-dependent association of MEF2D splice forms with Class IIa HDACs.

*- Perform RT-qPCR analysis of myogenin, desmin and myosin heavy chain expression in 10T $\frac{1}{2}$ -MyoD-ER fibroblasts stably over-expressing each MEF2D splice form.*

*- Perform Flag pull-down assays using Flag-tagged MEF2D splice forms in vitro phosphorylated by PKA prior to incubation with HeLa cell nuclear extract.*

3) Identify MEF2D splice form interacting partners by mass spectrometry.

- *Perform Flag pull-down assays using Flag-tagged MEF2D splice forms in vitro phosphorylated by p38 $\alpha$  prior to incubation with 48hr differentiated C2C12 cell nuclear extract. Identify associated proteins by LC-MS/MS.*

## 2. MATERIALS

### 2.1 Cell culture

|                       |                                                                                                               |
|-----------------------|---------------------------------------------------------------------------------------------------------------|
| Growth media          | DMEM (Hyclone)<br>10% FBS (Hyclone)<br>5% penicillin/streptomycin (Invitrogen)                                |
| Differentiation media | DMEM (Hyclone)<br>1% horse serum<br>0.1% insulin<br>0.1% transferrin                                          |
| Methylcellulose mix   | 2% Methylcellulose (SIGMA)<br>Dissolved in growth media<br>supplemented with an additional 10%<br>FBS         |
| Suspension media      | 5% FBS (Hyclone)<br>1% penicillin/streptomycin<br>Brought to final volume with<br>methycellulose mix          |
| Trypsin               | 0.5% in PBS (Invitrogen)                                                                                      |
| Complete SFIII media  | Sf-900 III SFM media (Invitrogen)<br>1% penicillin/streptomycin (Invitrogen)                                  |
| PBS, pH 7.4           | 137 mM NaCl<br>2.7 mM KCl<br>10 mM Na <sub>2</sub> HPO <sub>4</sub><br>1.8 mM KH <sub>2</sub> PO <sub>4</sub> |

### 2.3 Western blot

|                 |                              |
|-----------------|------------------------------|
| Blocking buffer | 5% nonfat dry milk in 1X PBS |
|-----------------|------------------------------|

## Primary antibodies

| Antibody (Manufacturer)                      | Species            | Dilution for WB |
|----------------------------------------------|--------------------|-----------------|
| Anti-MEF2D+α'<br>(Genscript)                 | Rabbit, polyclonal | 1:2500          |
| Anti-MEF2D<br>(610774, BD Biosciences)       | Mouse, monoclonal  | 1:2500          |
| Anti-phospho p38α<br>(sc-101759, Santa Cruz) | Rabbit, polyclonal | 1:1000          |
| Anti-HDAC4<br>(2072, Cell Signaling)         | Rabbit, polyclonal | 1:2000          |
| Anti-HDAC5<br>(sc-5252, Santa Cruz)          | Goat, polyclonal   | 1:1000          |
| Anti-Flag M2<br>(F1804, SIGMA)               | Mouse, monoclonal  | 1:2000          |
| Anti-TFIIH (p89)<br>(sc-293, Santa Cruz)     | Rabbit, polyclonal | 1:2000          |

## 5% stacking gel

125 mM Tris-HCl pH 6.8  
5% acrylamide  
0.1% SDS  
0.1% ammonium persulfate  
0.05% TEMED  
Top up with ddH<sub>2</sub>O

## Resolving gel

375 mM Tris-HCl pH 8.8  
10-15% acrylamide  
0.1% SDS  
0.1% ammonium persulfate  
0.05% TEMED  
Top up with ddH<sub>2</sub>O

## 10X Running buffer

Tris base 121 g  
Glycine 577 g  
SDS 40 g  
Top up to 4L with ddH<sub>2</sub>O

|                     |                                                                          |
|---------------------|--------------------------------------------------------------------------|
| 10X Transfer buffer | Tris base 121 g<br>Glycine 577 g<br>Top up to 4L with ddH <sub>2</sub> O |
|---------------------|--------------------------------------------------------------------------|

|                      |                                                                                                                      |
|----------------------|----------------------------------------------------------------------------------------------------------------------|
| 4X SDS sample buffer | 200 mM Tris pH 6.8<br>40% Glycerol<br>8% SDS<br>1 mM EDTA<br>0.1% Bromophenol Blue<br>Top up with ddH <sub>2</sub> O |
|----------------------|----------------------------------------------------------------------------------------------------------------------|

#### ***2.4 RNA extraction and RT-PCR***

|                   |                                                                                                               |
|-------------------|---------------------------------------------------------------------------------------------------------------|
| Random primer mix | 4U RNase OUT (Invitrogen)<br>0.5 µg random primers (Invitrogen)<br>Diluted in DEPC-treated ddH <sub>2</sub> O |
|-------------------|---------------------------------------------------------------------------------------------------------------|

|                                  |                                                                                                                                                       |
|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|
| Reverse Transcriptase master mix | 0.25 X Reverse Transcriptase buffer<br>(from manufacturer)<br>25 mM DTT<br>1.5 mM dNTPs<br>4U RNase OUT<br>Diluted in DEPC-treated ddH <sub>2</sub> O |
|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|

#### ***2.6 Protein expression***

|                           |                                                                                                                                                         |
|---------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|
| Flag equilibration buffer | 20 mM HEPES pH 7.6<br>100 mM KCl<br>1.5 mM MgCl <sub>2</sub><br>5% Glycerol<br>0.2 mM EDTA<br>2 mM DTT<br>0.4 mM PMSF<br>Top up with ddH <sub>2</sub> O |
|---------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|

|                    |                                                                                                                                                          |
|--------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|
| Flag wash buffer 1 | 20 mM HEPES pH 7.6<br>500 mM KCl<br>0.5M Urea<br>15% Glycerol<br>0.2 mM EDTA<br>0.05% NP-40<br>0.2 mM DTT<br>1 mM PMSF<br>Top up with ddH <sub>2</sub> O |
|--------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|

|                     |                                                                                                                                                            |
|---------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Flag wash buffer 2  | 150 mM KCl<br>15% Glycerol<br>0.2 mM EDTA<br>0.01% NP-40<br>0.2 mM DTT<br>1 mM PMSF<br>Top up with ddH <sub>2</sub> O                                      |
| 2X Flag buffer      | 40 mM HEPES pH 7.6<br>300 mM KCl<br>10.4 mM MgCl <sub>2</sub><br>0.4 mM EDTA<br>30% Glycerol<br>20 mM β-Glycerophosphate<br>Top up with ddH <sub>2</sub> O |
| Flag elution buffer | 150 ng/μL 3X Flag peptide<br>1 mg/ml Insulin (Invitrogen)<br>0.7X Flag buffer<br>0.4 mM PMSF<br>0.1 mM DTT                                                 |
| His lysis buffer    | 10 mM Imidazole<br>500 mM KCl<br>25 mM HEPES K <sup>+</sup><br>0.1% NP-40<br>0.5 mM PMSF                                                                   |
| His W10 buffer      | 10 mM Imidazole<br>100 mM KCl<br>25 mM HEPES K <sup>+</sup><br>0.5 mM PMSF                                                                                 |
| His W25 buffer      | 25 mM Imidazole<br>100 mM KCl<br>25 mM HEPES K <sup>+</sup><br>0.5 mM PMSF                                                                                 |
| His elution buffer  | 300 mM Imidazole<br>100 mM KCl<br>25 mM HEPES K <sup>+</sup><br>0.5 mM PMSF                                                                                |

## ***2.7 In vitro kinase assay***

|                       |                                                                                                                                                         |
|-----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|
| 6X p38 Kinase buffer  | 150 mM HEPES pH 7.6<br>150 mM MgCl <sub>2</sub><br>180 mM ATP<br>12 mM DTT<br>60 mM NaF<br>120 mM $\beta$ -Glycerophosphate                             |
| p38 Reaction mixture  | 1 $\mu$ M ATP<br>1X p38 Kinase buffer<br>100-500 $\mu$ Ci/ $\mu$ mol [ $\gamma$ - <sup>32</sup> P] ATP<br>Top up with 1X p38 Kinase buffer              |
| 10X PKA Kinase buffer | 500 mM Tris-HCl pH 7.5<br>10 mM MgCl <sub>2</sub>                                                                                                       |
| PKA Reaction mixture  | 200 $\mu$ M ATP<br>1X PKA Kinase buffer<br>100-500 $\mu$ Ci/ $\mu$ mol [ $\gamma$ - <sup>32</sup> P] ATP<br>10 mM DTT<br>Top up with ddH <sub>2</sub> O |

## ***2.10 Nuclear extract preparation***

|          |                                                                                                                                                                                              |
|----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Buffer A | 10 mM HEPES K <sup>+</sup> , pH 7.6<br>1.5 mM MgCl <sub>2</sub><br>10 mM KCl<br>1 protease inhibitor tablet (Roche)<br>0.5 mM DTT<br>1 mM PMSF                                               |
| Buffer C | 20 mM HEPES K <sup>+</sup> , pH 7.6<br>1.5 mM MgCl <sub>2</sub><br>650 mM KCl<br>0.2 mM EDTA, pH 8.0<br>25% Glycerol (w/v)<br>1 protease inhibitor tablet (Roche)<br>2.0mM DTT<br>0.2mM PMSF |



Buffer D

20 mM HEPES K<sup>+</sup>, pH 7.6  
1.5 mM MgCl<sub>2</sub>  
100 mM KCl  
0.2 mM EDTA, pH 8.0  
25% Glycerol (w/v)  
1 protease inhibitor tablet (Roche)  
2.0 mM DTT  
0.2 mM PMSF

### 3. METHODS

#### **3.1 Cell culture.**

**Adherent cells** - C2C12 mouse myoblast and 10T½ - MyoD-ER mouse fibroblast cells (generous gift from Dr. Stephen Tapscott) were grown in a 37°C incubator and cultured with growth media for up to 8 passages. Cells were allowed to reach 50-60% confluency before switching to differentiation media. Fibroblast cells were converted along the myogenic lineage by switching from growth media to differentiation media containing  $10^{-7}$  M  $\beta$ -estradiol.

**Suspension cultures** - To prepare C2C12 mouse myoblast suspension cultures, adherent cells cultured in growth media supplemented with an additional 10% FBS (20% GM) were first trypsinized and resuspended with 20% GM at a concentration of approximately  $1 \times 10^6$  cells/ml. Resuspended C2C12 cells were transferred to a 50 ml falcon tube (Corning) containing suspension media at a final concentration of  $1 \times 10^6$  cells/10 ml and grown in a 37°C incubator for a maximum of 48 hrs before harvesting for RNA isolation.

**Insect cells** - Non-adherent Sf9 insect cells (Invitrogen) were cultured in sterile spinner flasks containing complete SF9 media and grown in a 27°C incubator. Cell density was maintained between  $0.5-2.0 \times 10^6$  cells/ml.

#### **3.2 DNA cloning and plasmid purification.**

**Cloning** - Inserts corresponding to Mef2D $\alpha\beta$  (Mef2D5), Mef2D $\alpha'\beta$  (Mef2D2) and Mef2D $\alpha\emptyset$  (Mef2D8) were PCR amplified from 10 ng of the corresponding retroviral constructs pcl-Babe-Mef2D5, pcl-Babe-Mef2D2 or pcl-Babe-Mef2D8

(gifts from Dr. Stephen Tapscott) using site-specific primers (see Supplementary Table 1 for primer sequences) and Phusion polymerase according to the manufacturer's protocol (NEB). Inserts and pFBac vector (Invitrogen) were double digested with BclI/NotI and BamHI/NotI, respectively, according to the manufacturer's protocol (NEB). Inserts and vector were then gel purified using the Qiagen Gel Extraction kit and ligated together using T4 Ligase according to the manufacturer's protocol (Invitrogen). Cloning was verified by DNA sequencing using pFB-specific primers (see Supplementary table 1 for primer sequences).

**Transformation** - DNA plasmids were transformed into competent DH5 $\alpha$  bacteria (produced according to the manufacturer's protocol using the Z-compotent E.coli Transformation Kit & Buffer Set; Zymo Research). Briefly, 5  $\mu$ L of each ligation reaction was added to 45  $\mu$ L of competent cells in an eppendorf tube and kept on ice for 30 mins. Approximately 450  $\mu$ L of sterile LB broth (Dulbecco) was added to the competent cell-DNA plasmid mixture and shaken at 225 rpm for 1 hr at 37°C. The bacterial culture was then pelleted by centrifugation and resuspended in only 100  $\mu$ L of supernatant. Resuspended bacteria were spread on a 10 cm LB-agar plate containing the appropriate antibiotics and left in a 37°C incubator overnight.

**Purification** - Single bacterial colonies were used to inoculate 3 ml or 150 ml cultures for mini-prep and maxi-prep plasmid purifications, respectively. Large-scale purifications were performed using the SIGMA Maxi-prep kit according to the manufacturer's protocol. For small-scale purifications, 1.5 ml of bacterial

culture was pelleted at 13,000 rpm at RT for 1 min and resuspended in 200 µL of Solution I (TE Buffer pH 7.5, 100 µg/ml RNase A (SIGMA)). To lyse bacteria, 200 µL of Solution II (0.2N NaOH, 1% SDS) was added to each sample and kept at RT for 5 mins. To neutralize the reaction, 200 µL of Solution III (3M potassium acetate pH 5.5) was added to each sample and kept on ice for 5-10 mins. Cellular debris was pelleted at 13,000 rpm for 10 mins at 4°C. Supernatant was transferred to new eppendorfs containing 500 µL phenol-chloroform, vortexed for 30 secs and centrifuged at 13,000 rpm for 5 mins at 4°C. The aqueous phase was transferred to a new eppendorf tube and DNA was precipitated by the addition of 900 µL of 100% ethanol and recovered by centrifugation at 13,000 rpm for 5 mins at 4°C. DNA pellets were washed with 800 µL of 70% ethanol and spun at 13,000 rpm for 2 mins at RT. DNA pellets were dried upside-down at RT and resuspended in 20-30 µL sterile ddH<sub>2</sub>O. DNA plasmids concentrations were determined using spectrometry (Nanodrop, Thermo Scientific) and stored at -20°C.

### **3.3 Western Blotting.**

Protein samples were resolved by SDS-PAGE on a 10% SDS-polyacrylamide gel (15A for 15 mins; 30A for 45 mins), then transferred to a nitrocellulose membrane (Bio-Rad) for 90 mins (150V) or overnight (20V). After blocking for 1 hr at RT in blocking buffer, membranes were incubated with primary antibody solution overnight at 4°C or for 1 hr at RT. Membranes were then washed with 1X PBS (3X 10 mins) and incubated with the appropriate secondary HRP-conjugated antibody solution. After a final wash with 1X PBS (3X 10mins), membranes were

visualized with Enhanced Chemiluminescence plus HRP substrate (GE Healthcare). The signal was detected on MR film (Ultra Cruz). Before reprobing blots, membranes were incubated in blocking buffer overnight.

### **3.4 RNA extraction and RT-PCR.**

Total RNA was isolated using the RNA STAT60 reagent (AMSBIO) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometry (Nanodrop, Thermo Scientific) with an expected Absorbance<sub>260</sub>/Absorbance<sub>280</sub> ratio of 2.00. Briefly, 3 µL of random primer mix (see Materials) was added to 2 µg total RNA diluted in DEPC water to a final volume of 20 µL and incubated at 70°C for 10 mins; on ice for 2 mins. Then 15 µL of Reverse Transcriptase master mix (see Materials) was added to each reaction and incubated at RT for 10 mins; 42°C for 2 mins. Finally, 50U of M-MuLV Reverse Transcriptase (NEB) was added to each reaction and incubated at 42°C for 50 mins; 70°C for 15 mins; transferred to ice. Total cDNA was diluted to 100 µL with DEPC water and 2 µL was used for PCR analysis with gene-specific primers (see Supplementary Table 1 for primer sequences). PCR reactions were performed using Phusion polymerase (NEB) according to the manufacturer's protocol. PCR conditions were as follows: initial denaturation of 98°C for 2 mins, 35 cycles of denaturation at 98°C for 30 secs, annealing at 55-60°C for 30 secs and extension at 72°C for 30 secs with a final extension for 10 mins at 72°C.

### **3.5 Quantitative RT-PCR (RT-qPCR).**

Quantitative PCR reactions were prepared using 10  $\mu$ L PerfeCTa SYBR Green Supermix (Quanta Biosciences) as per the manufacturer's protocol. Runs were performed using the Corbett Rotor-Gene 6000 (Lifesciences). PCR was performed using gene-specific primers (see Supplementary Table 1 for primer sequences) under the following conditions: first hold at 50°C for 2 mins, second hold at 95°C for 2 mins, 40 cycles of denaturation at 95°C for 15 secs, annealing at 60°C for 20 secs, extension at 72°C for 34 secs with a final extension at 72°C for 10 mins. Levels of the housekeeping gene DDX5 were used to normalize the data.

### **3.6 Protein expression.**

**Baculovirus-mediated expression** - Baculovirus plasmids encoding C-terminally Flag-tagged (FL-) Mef2D $\alpha'$  $\beta$ , Mef2D $\alpha\beta$ , and Mef2D $\alpha\emptyset$  were propagated in competent DH10Bac cells and amplified in Sf9 insect cells according to the Bac-to-Bac® Baculovirus Expression Systems instruction manual (Invitrogen). For protein over-expression, Sf9 cells were seeded at a density of  $20 \times 10^6$  on 15 cm dishes (Corning) and infected with viral supernatant diluted 1:20 with complete SFIII media. Following 72 hrs of infection, cells were collected in 1X PBS (Invitrogen) and pelleted for 5 mins at 1000 rpm at 4°C. The cell pellet was resuspended in 10 ml Flag equilibration buffer and cells were lysed by dounce homogenization with pestle B. Whole Sf9 lysate was clarified by centrifugation at 12,000 rpm for 20 mins at 4°C and purified with anti-Flag M2-agarose beads (SIGMA). Briefly, 200  $\mu$ L Flag resin was washed twice with 4 ml of Flag

equilibration buffer in a 15 ml tube (Stratagene) and recovered via centrifugation at 1000 rpm for 5 mins at 4°C. Flag resin was mixed with approximately 10 ml clarified Sf9 lysate on a rotator for 2 hrs at 4°C. The Flag resin-clarified Sf9 lysate mixture was loaded onto a polypropylene gravity flow column (Biorad) then washed with 4 ml of Flag wash buffer 1 and Flag wash buffer 2 before eluting with Flag elution buffer. Three 300 µL elution fractions were collected following incubation with the resin for 15 mins. All flow-through and elution fractions were analysed via SDS-PAGE using a 12% polyacrylamide gel. Purified proteins were stored at -80°C.

**Bacteria-mediated expression** - A BL-21 bacterial strain stably co-expressing His-p38 $\alpha$ , MEK4 and MEKK-C29 was obtained from Melanie Cobb (University of Texas Southwestern Medical Centre, Dallas Texas) and used to inoculate 2 L of LB broth (Dulbecco) cultured at 37°C until O.D. 0.6 -0.8. Protein expression was induced using 0.4 mM IPTG (SIGMA) for 4 hrs after which the bacterial culture was pelleted at 5000 rpm for 10 mins at 4°C. Cells were resuspended in His lysis buffer and subjected to 4 cycles of sonication (30 secs on, 1 min off; Biorad Sonicator). Whole bacterial lysate was clarified by centrifugation at 20,000 rpm for 30 mins at 4°C. Catalytically active His-p38 $\alpha$  was affinity purified using Ni-NTA resin (Qiagen). Briefly, 2 ml packed Ni-NTA resin was loaded onto a polypropylene gravity flow column (Biorad) and equilibrated with 1 volume His lysis buffer before clarified bacterial lysate was added. The Ni-NTA column was washed with 1 volume His lysis buffer and 2 volumes each His W10 buffer and

His W25 buffer before eluting associated proteins with His elution buffer. All flow-through and elution fractions were analysed by SDS-PAGE using a 15% polyacrylamide gel. Before use in subsequent experiments, activated p38 $\alpha$  was further purified to remove any contaminants by anion exchange chromatography using a MonoQ column (GE Healthcare) on the FPLC AKTA system. Purified proteins were stored at -80°C.

### ***3.7 In vitro phosphorylation.***

Approximately 100 ng-10  $\mu$ g of FL-Mef2D $\alpha\beta$ , FL-Mef2D $\alpha'\beta$  or FL-Mef2D $\alpha\emptyset$  protein was incubated with different dilutions of active His-p38 $\alpha$  (100 or 300 ng) or PKA catalytic subunit (280U, 830U or 2,500U; P6000S, NEB) in the appropriate reaction mixtures (see Materials section 2.7) for 30 mins at 30°C in a thermomixer (Eppendorf). For immediate analysis by SDS-PAGE, kinase reactions were stopped by adding an equal volume of 4X SDS sample buffer and resolved on a 10% polyacrylamide gel by SDS-PAGE. Gels were exposed to film overnight at -80°C.

### ***3.8 Retrovirus transfection and infection.***

**Transfection** - Mef2D $\alpha\beta$ , Mef2D $\alpha'\beta$  and Mef2D $\alpha\emptyset$  in pCL-Babe vector (gift from Dr. Stephen Tapscott, Fred Hutchinson Cancer Research Centre, Seattle Washington) were packaged into retrovirus using HEK293T cells. Approximately 6 million HEK293T cells were used for each sample to be amplified. Cells were trypsinized and resuspended in DMEM containing 10% FBS and 25  $\mu$ M Chloroquin at a concentration of  $1.2 \times 10^6$  cells/ml in a 50 ml tube (Corning) and



kept at 37°C. In a 5 ml tube, 36 µL Lipofectamine 2000 reagent (Invitrogen) was added to 1.5 ml Opti-MEM and incubated at RT for 5 mins. In another 5 ml, tube 6 µg each of Eco envelope protein vector and retroviral plasmid were combined in 1.5 ml Opti-MEM and incubated at RT for 5 mins. Both mixtures were combined for 20 mins at RT, then added to a 10 cm dish containing 5 ml of DMEM + 10% FBS. At this time the cell resuspension was added and cells were mixed manually by rocking and stored overnight in a 37°C incubator. The following morning, transfection media was replaced with fresh DMEM+10% FBS + 1% Penicillin/Streptomycin. Retrovirus supernatant was harvested 72 hrs by scraping, spun at 3000 rpm for 5 mins at 4°C and stored in 1 ml aliquots at -80°C.

**Infection** – One day prior to infection, 10T $\frac{1}{2}$ -MyoDER cells were seeded on a 15 cm dish at 20% confluency. A 1ml aliquot of viral supernatant was thawed and added to the cells along with 4 µg/ml polybrene. Two days following infection, batch cultures were selected using 2 µg/ml puromycin. To convert cells along the myogenic lineage, stable 10T $\frac{1}{2}$ -MyoDER cells were treated with pCL-Babe-MKK6 retrovirus and after 12 hrs of recovery, switched to differentiation media containing 10<sup>-7</sup> M β-estradiol. As a control, 10T $\frac{1}{2}$ -MyoDER cells stably expressing empty pCL-Babe plasmid were also converted. Gene expression was analyzed by RT-qPCR using gene-specific primers (see Supplementary Table 1 for primer sequences).

### **3.9 Flag pull-down assays.**

Sf9 insect cells were infected with baculovirus encoding FL-MEF2D splice forms and lysed as described previously (see Materials & Methods section 3.6 Protein Expression). The concentration of clarified lysate from MEF2D over-expressing and wildtype Sf9 cells was measured using Bradford reagent according to the manufacturer's protocol (Biorad). Approximately 1 mg of clarified lysate was diluted with Flag equilibration buffer in a 4 ml tube (Stratagene) and incubated with 30  $\mu$ L of anti-Flag M2-magnetic beads (SIGMA) on a rotator for 2 hrs at 4°C. The supernatant was removed using a magnetic separator (Active Motif). Flag beads were washed once with Flag wash buffer 1 and twice with Flag wash buffer 2. Flag beads were resuspended with Flag wash buffer 2 and transferred to eppendorf tubes.

To look at the effect of PKA phosphorylation on FL-MEF2D-HDAC association, Flag beads were separated into two aliquots (PKA-treated and un-treated) and underwent *in vitro* phosphorylation using 830U PKA as described previously (see Methods section 3.7 *In vitro* phosphorylation assays). Flag beads were incubated with approximately 400  $\mu$ g of HeLa nuclear extract (see Methods section 3.10) on a rotator overnight at 4°C. Alternatively, to look for putative FL-MEF2D splice form interacting partners, Flag beads were *in vitro* phosphorylated using 300 ng of recombinant p38 $\alpha$  as described previously (see Methods section 3.7 *In vitro* phosphorylation assays). Flag beads were incubated with approximately 400  $\mu$ g of 48 hr differentiated C2C12 nuclear extract (see Methods section 3.10) on a rotator overnight at 4°C.

Following the overnight incubation, the supernatant was removed using a magnetic separator (Active Motif) and Flag beads were washed three times with Flag wash buffer 2. Associated proteins were eluted in three fractions by adding 1 bead volume of 4X SDS sample buffer and boiling the Flag beads for 5 mins. Total flow-through and elution fractions were analysed via SDS-PAGE using a 12% polyacrylamide gel. Polyacrylamide gels were used for western blot analysis to look for specific interacting partners or silver stained in preparation for mass spectrometry analysis (see Methods section 3.3 Western blotting, and 3.11 Mass spectrometry).

### ***3.10 Nuclear extract preparation.***

Adherent HeLa or C2C12 myotubes (30 plates at 70-90% confluency) were harvested by scraping in 5 ml cold PBS, transferred to 50 ml polypropylene tubes (Corning) and pelleted by centrifugation at 3000 rpm for 10 mins at 4°C. To determine the packed cell volume (PCV), cell pellets were resuspended in 5 ml of cold PBS and collected into a single 50 ml tube (Total volume - 5ml (x #aliquots) = PCV). Cells were pelleted as before then resuspended in 5 X PCV of Buffer A and left on ice for 10 mins to swell. To isolate nuclei, the cell resuspension was transferred to an ice-cold Dounce Homogenizer and dounced with 11 strokes of a type B pestle. Nuclei were pelleted by centrifugation at 11,000 rpm for 30 mins at 4°C. The nuclear pellet volume (NPV) was estimated by eye using a comparable volume of water pipetted into a 1.5 ml eppendorf tube. Nuclei were resuspended in 1 NPV of Buffer C, transferred to an ice-cold 2 ml Dounce Homogenizer and dounced with

10 strokes of a type B pestle. The nuclear homogenate was transferred to a 1.5 ml eppendorf tube and rotated overnight at 4°C. Nuclei were isolated by centrifugation at 13,000 rpm for 30 mins at 4°C. The supernatant was injected into a dialysis cassette (3.5K cut-off Slide-A-Lyzer, Pierce) using a 10 ml syringe and needle (BD 23G1) and dialysed in 1L of Buffer D overnight at 4°C. Nuclear extract was removed from the cassette as before, transferred to an eppendorf tube and clarified at 13,000 rpm for 15 mins at 4°C. Nuclear extracts were stored at -80°C.

### ***3.11 Silver staining.***

Protein samples were first resolved by SDS-PAGE. Polyacrylamide gels were then fixed for 30 mins in a solution of 50% ethanol, 10% acetic acid and 40% sterile ddH<sub>2</sub>O. Fixation solution was removed and gels were incubated in 30% ethanol for 15 mins. After washing with sterile ddH<sub>2</sub>O (3x10 mins), gels were sensitized with 0.2 g/L sodium thiosulphate for 1.5 mins. Following a second wash step (3x30 secs), gels were incubated with 2.0 g/L silver nitrate for 25 mins. After washing with sterile ddH<sub>2</sub>O (3x30 secs), gels were incubated in developing solution (60 g/L sodium carbonate, 20 ml/L of the sodium thiosulphate solution used during the sensitization step and 500 µL/L 37% formaldehyde in sterile ddH<sub>2</sub>O). Once the desired intensity of staining was achieved, developing was stopped by discarding the developing solution and adding 6% acetic acid in sterile ddH<sub>2</sub>O for 10mins. Gels were stored in sealed containers for 1-2 weeks in 1% acetic acid at 4°C.

### **3.12 Mass spectrometry.**

Mass spectrometry was performed by Dr. Lawrence Puente at the Ottawa Hospital Research Institute Proteomics Core Facility. Protein samples were resolved by SDS-PAGE and silver stained prior to in-gel digestion (see Methods section 3.10). In-gel digestion was performed using trypsin as previously described (Shevchenko et al., 2007). Overnight digestions were performed using proteomic grade trypsin (Promega). Peptides from gel bands were extracted with 5% formic acid in acetonitrile, dried using the Vacufuge (Eppendorf) and rehydrated in 20  $\mu$ L of 0.1% Trifluoroacetic acid in water. For analysis by LC-MS/MS, peptides were loaded at a rate of 20  $\mu$ L/min onto a Michrom Capillary Peptide Trap using a Finnigan Surveyor HPLC (Thermo Scientific). Following the desalting step, peptides were eluted over 30-60 mins using a 5-40% gradient of acetonitrile with 0.1% formic acid at approximately 300 nL/min. Eluted peptides bound to a second column (75  $\mu$ m x 100 mm) packed with Zorbax SB-C18 5 $\mu$  (Agilent) and were subsequently electrosprayed into an LTQ linear ion-trap mass spectrometer (Thermo Scientific). Mass spectrometry data was acquired in a data-dependant mode which selected the four most intense peaks from each MS spectrum for further fragmentation. To identify unknown proteins, MS/MS data was matched to 148,380 mouse protein sequences from the NCBI database (NCBI nr 20100728) using the MASCOT algorithm version 2.0 (Matrix Science) with the parameters: peptide tolerance  $\pm 1.6$  and mass tolerance  $\pm 0.8$  Da. Protein matches and ion scores were considered significant if they did not exceed the threshold calculated by Mascot based on a p-value of 5%. A secondary criterion

for determining significant protein matches was the identification of at least two unique peptides with significant ion scores calculated by Mascot based on a p-value of 5%. For proteins with Mascot scores below the significance threshold, literature-based evidence supporting an interaction with MEF2D was considered as a tertiary criterion.

### ***13.3 Immunofluorescence.***

One day prior to staining, 10,000 C2C12 cells were seeded on cover slips in 6 well plates containing growth media and stored in a 37°C incubator. Cells were fixed with 1% formaldehyde in 1XPBS for 30 mins at RT. Following three washes with 1XPBS for 5 mins at RT, cells were permeabilized with 0.2% Triton X 100 in 1XPBS for 10 mins at RT. After blocking cells with 10% FBS in 1XPBS containing 0.2% Triton X 100 for 1 hr at RT, primary antibody solution was added (1:200 dilution;  $\alpha$ -Mef2D+ $\alpha'$ ,  $\alpha$ -Mef2D and DAPI dye) and stored overnight at 4°C. Cells were washed as before then incubated with the appropriate secondary antibody solution (1:500 dilution) for 45 mins at RT. Cover slips were mounted on slides and visualized by confocal microscopy (Zeiss).

### ***3.14 Densitometry analysis.***

Western blot films were scanned using an EPSON Expression 1680 scanner and analyzed with ImageJ software (NIH).

### **3.15 Statistical analysis.**

Both student *t*-tests (for pair-wise comparisons) and ANOVA (for multi-way comparisons) statistical analyses were performed using InStat 3 (GraphPad Software).

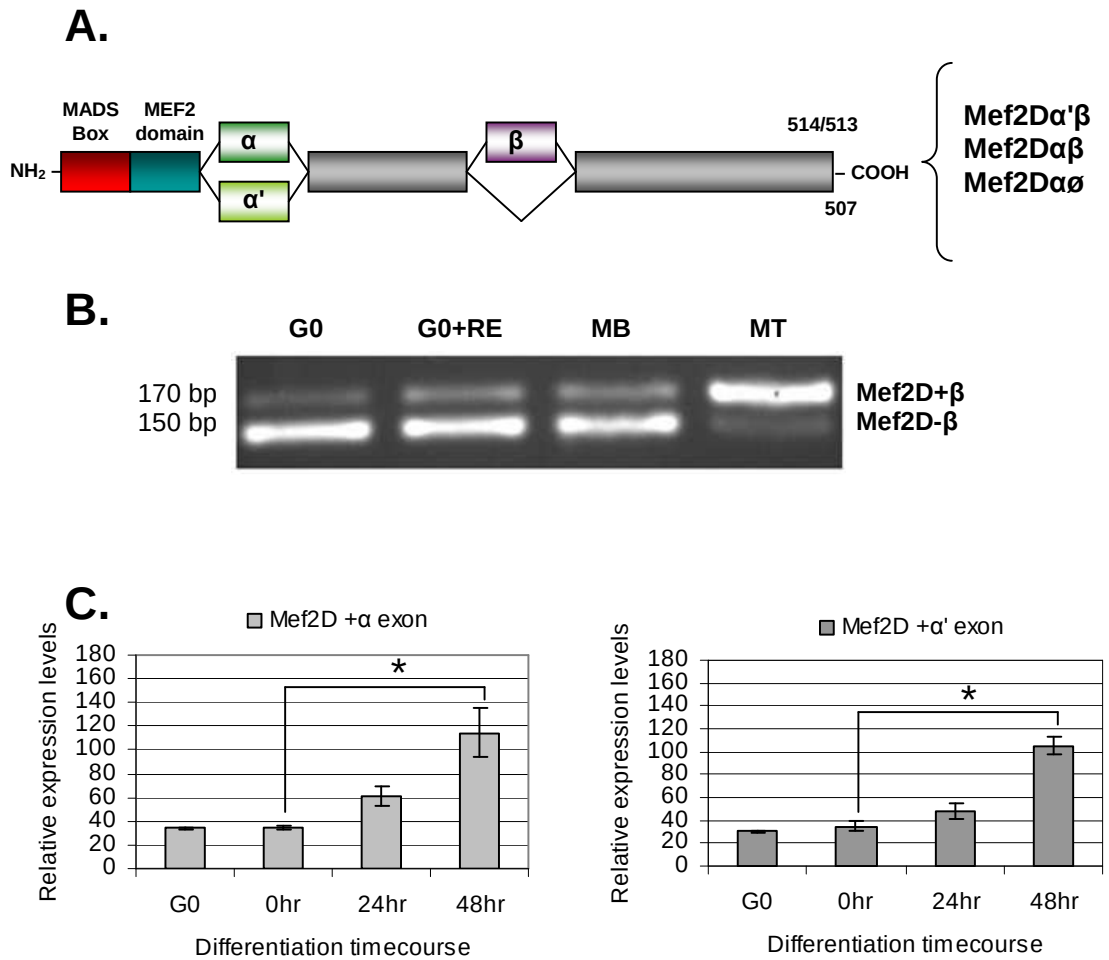
## 4. RESULTS

### ***4.1 Mef2D alternative splicing is regulated during myogenesis.***

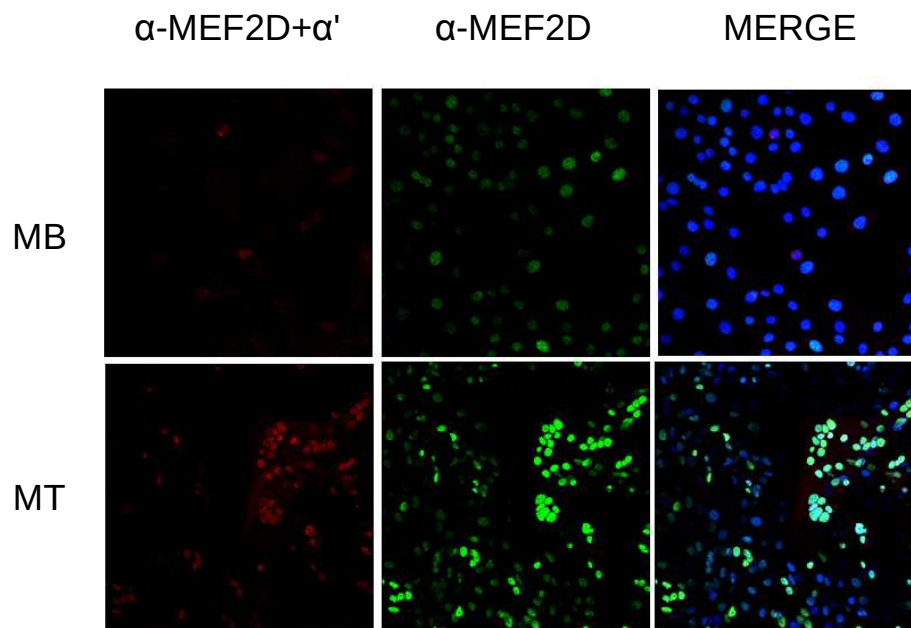
It has been almost two decades since researchers in the muscle field first reported alternative splicing of MEF2D pre-mRNA (Breitbart et al., 1993, Martin et al., 1994, Zhu et al., 2005). Today, the significance of MEF2D splice forms within the context of skeletal muscle differentiation is still a mystery. We began our investigation into the functionality of different MEF2D splice forms (Fig.4A) by first establishing the pattern of Mef2D pre-mRNA splicing in the C2C12 cell culture model of skeletal muscle differentiation. Adherent C2C12 cells were also cultured in non-adherent conditions since this has been shown to change their expression profile such that it mimics quiescent muscle stem cells or satellite cells which are otherwise difficult to isolate (Milasincic et al., 1996). RNA was isolated from non-adherent C2C12 cells (G0), non-adherent cells allowed to re-adhere and enter the cell cycle (G0+RE) and adherent C2C12 cells cultured in growth media (MB) or differentiation media (MT) for 24 or 48 hrs. RT-PCR was performed to detect whether the alternatively spliced  $\beta$ -exon was included or excluded from Mef2D transcripts using primers flanking the alternatively spliced  $\beta$ -exon region (Fig 4A; see Supplementary Table 1 for primer sequences). Interestingly, 170 bp amplicons (Mef2D+ $\beta$ ) were enriched in differentiated myotubes whereas smaller 150 bp amplicons (Mef2D- $\beta$ ) were found primarily in proliferating and non-adherent myoblasts (Fig.4B). Since the  $\alpha$ -exon and  $\alpha'$ -exon differ in size by only 3 nucleotides, the relative abundance of each amplicon was



determined by quantitative reverse transcription PCR (RT-qPCR) using primer sets that specifically recognized the  $\alpha$ -exon or  $\alpha'$ -exon regions (Fig.4A; see Supplementary Table 1 for primer sequences). At the transcript level, both  $\alpha$ -exon-containing transcripts (Mef2D+ $\alpha$  exon) and  $\alpha'$ -exon-containing transcripts (Mef2D+ $\alpha'$  exon) increased during differentiation (Fig.4C). Immunofluorescence analysis, however, reveals that although total MEF2D protein levels increase with differentiation, MEF2D+ $\alpha'$  protein is detected primarily in myotubes (Fig.5; Soji Sebastian, unpublished). Thus, our results corroborate previously published reports that alternative splicing of Mef2D during muscle differentiation favours the inclusion of the  $\beta$ -exon as well as splicing to generate the  $\alpha'$ -exon (Breitbart et al., 1993, Martin et al., 1994, Zhu et al., 2005).



**Figure 4. Mef2D alternative splicing during C2C12 myoblast differentiation.** (A) Schematic representation of alternatively spliced mouse Mef2D proteins: muscle-specific Mef2D $\alpha'\beta$ , Mef2D $\alpha\beta$  and Mef2D $\alpha\emptyset$ . The MADS box (red) and MEF2 domain (blue) are indicated at the amino terminus. Amino acids 87-132 encode the alternatively spliced  $\alpha$ -exon (dark green) and  $\alpha'$ -exon (light green). Amino acids 285-292 encode the alternatively spliced  $\beta$ -exon (purple). (B) RT-PCR analysis of Mef2D transcripts with the  $\beta$ -exon region included (Mef2D+ $\beta$ ) or excluded (Mef2D- $\beta$ ). Total RNA was extracted from C2C12 myoblasts in suspension (G0), proliferation (G0+RE; MB) or 48hr differentiation (MT) conditions. (C) RT-qPCR analysis of Mef2D transcripts with the  $\alpha$ -exon region (Mef2D+ $\alpha$  exon) or the  $\alpha'$ -exon region (Mef2D+ $\alpha'$  exon) included. Total RNA was extracted from C2C12 myoblasts in suspension (G0), proliferation (t=0 hr) or differentiation (t=24 hrs, 48 hrs) conditions. Data is normalized to DDX5. Statistical significance was determined by a student *t*-test;  $p < 0.01$ ;  $N = 3$ . Error bars represent SD.



**Figure 5. Immunofluorescence analysis of MEF2D+ $\alpha'$  protein expression.** C2C12 myoblasts (MB) and 48 hr differentiated myotubes (MT) were stained with antibodies specifically recognizing the  $\alpha'$ -exon region ( $\alpha$ -MEF2D+ $\alpha'$ ; red), total MEF2D ( $\alpha$ -MEF2D; green) or with DAPI (MERGE; blue). This result is representative of multiple independent experiments (Soji Sebastian, unpublished).

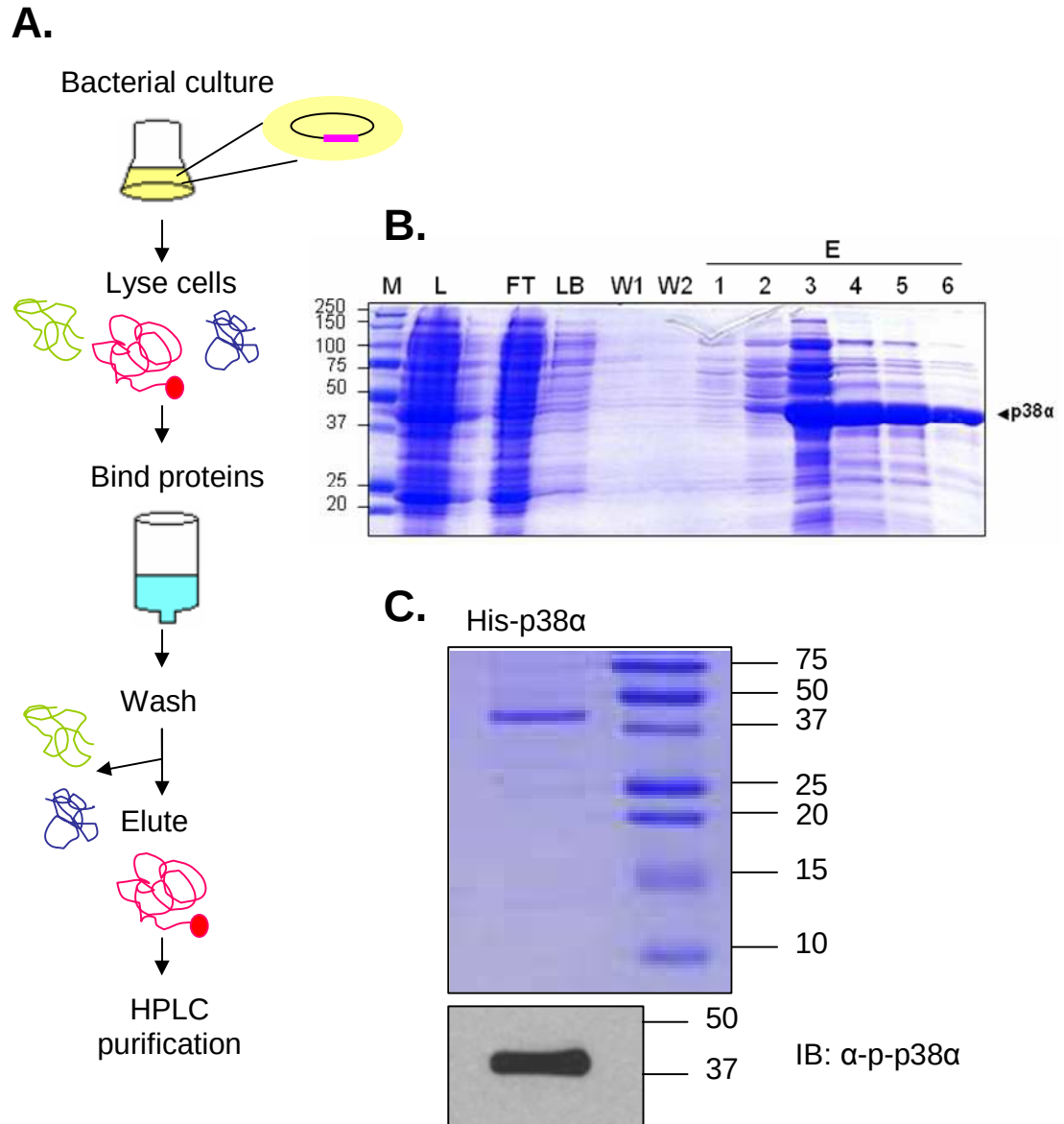
#### **4.2 MEF2D splice forms are differentially phosphorylated by p38 $\alpha$ *in vitro*.**

To functionally annotate MEF2D $\alpha$ , MEF2D $\alpha\beta$  and the muscle-specific MEF2D $\alpha'\beta$  splice form, we first considered how differences in the primary amino acid sequence of each splice form generated via alternative splicing could affect post-translational modifications. Having shown previously that p38 MAPK-dependent phosphorylation of MEF2D is crucial for activation of muscle-specific genes during differentiation (Rampalli et al., 2007); we sought to determine the phosphorylation status of each splice form using *in vitro* p38 $\alpha$  kinase assays.

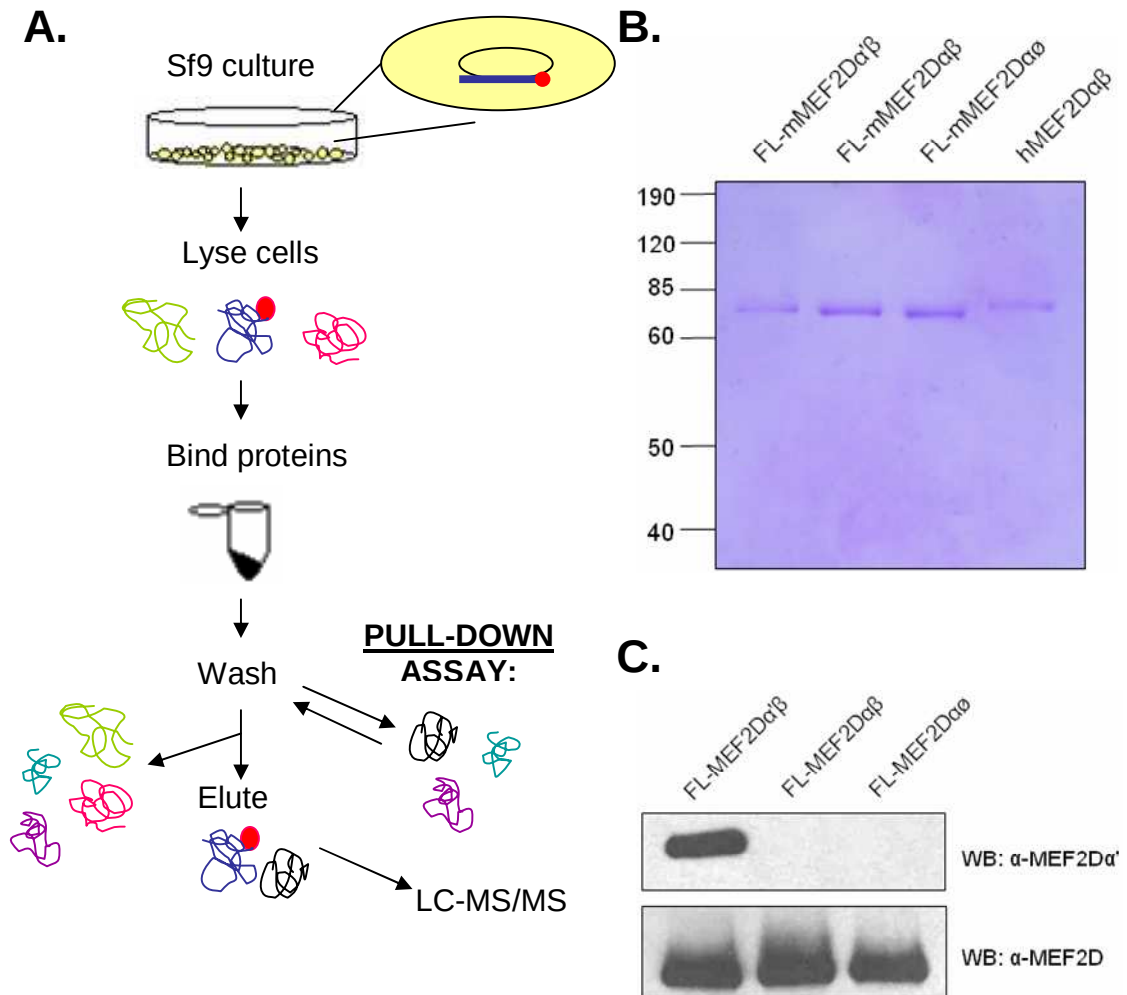
Catalytically active recombinant p38 $\alpha$  was produced using a bacterial strain from a previously published protocol (Khokhlatchev et al., 1997). This particular strain co-expresses a constitutively active form of the upstream kinase in the MAPK signaling cascade MKK6 (annotated MKK6EE) as well as a Histidine-tagged (His-) form of the downstream kinase p38 $\alpha$ ; enabling large-scale purification of catalytically active His-p38 $\alpha$  using nickel affinity chromatography (Fig.6A,B). Several elution fractions (E1-6, Fig.6B) were pooled, dialysed and processed by anion exchange chromatography to increase His-p38 $\alpha$  purity before use in kinase reactions.

Flag-tagged (FL-) MEF2D $\alpha$ , MEF2D $\alpha\beta$ , and MEF2D $\alpha'\beta$  substrates were produced in SF9 insect cells using a baculovirus expression system (Fig. 7A). Constructs encoding each FL-MEF2D splice form were cloned into the pFBac vector and confirmed by DNA sequencing. The identity of purified FL-MEF2D splice forms was confirmed by western blot analysis using antibodies that specifically recognize the  $\alpha$ -exon and/or  $\alpha'$ -exon regions (Fig.7B,C).

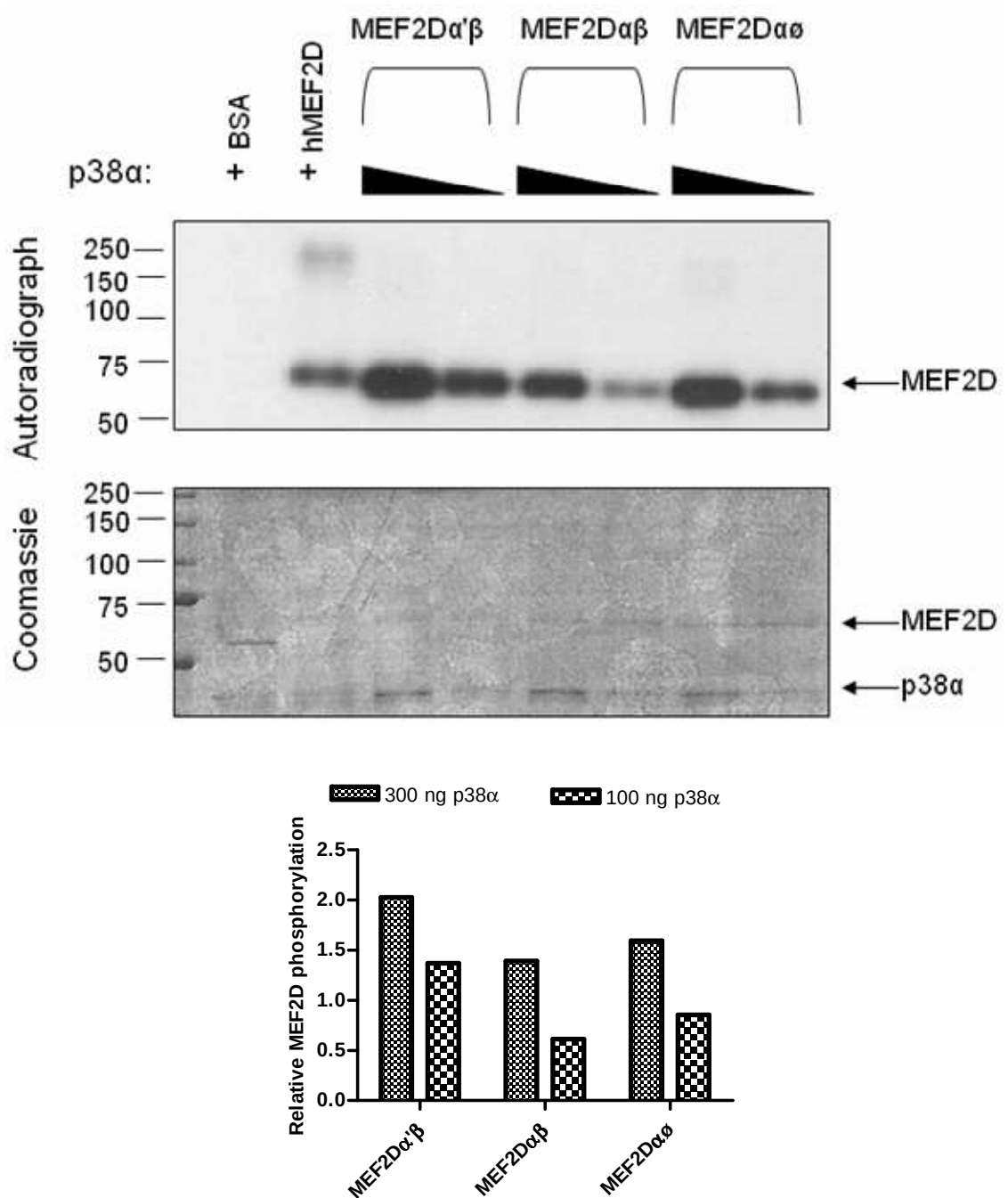
In separate kinase reactions, each FL-MEF2D splice form was incubated with two different concentrations of His-p38 $\alpha$  and [ $\gamma$ -<sup>32</sup>P] ATP. Analysis by autoradiography revealed that FL-MEF2D splice forms were differentially phosphorylated by His-p38 $\alpha$  such that the muscle-specific splice form FL-MEF2D $\alpha'$  $\beta$  was highly phosphorylated relative to FL-MEF2 $\alpha\beta$  which was poorly phosphorylated (Fig.8). Interestingly, FL-MEF2D $\alpha\emptyset$  which lacks the  $\beta$ -exon appeared to be phosphorylated more than FL-MEF2D $\alpha\beta$ , suggesting that in our experimental conditions the  $\beta$ -exon was dispensable for targeting His-p38 $\alpha$  to a subset of phosphoacceptor sites on FL-MEF2D splice forms. To confirm His-p38 $\alpha$  substrate specificity, BSA was used as a negative control and was not phosphorylated by His-p38 $\alpha$  *in vitro* (Fig. 8).



**Figure 6. Purification of activated Histidine-tagged (His-) p38α from bacteria.** (A) Schematic representation of Ni-NTA purification. Bacterial lysate containing over-expressed His-p38α (pink) was applied to Ni-NTA resin. Contaminant proteins (green, blue) were removed in flow-through and wash fractions. His-p38α was eluted with 300 mM Imidazole and further purified by anion exchange chromatography. (B) Coomassie blue-stained polyacrylamide gel of His-p38α following Ni-NTA purification: M=molecular weight marker, L=lysate, FT=flow-through, LB=lysis buffer, W1=wash 1, W2=wash2, E=elution fraction. (C) *Top panel:* Coomassie blue-stained polyacrylamide gel of His-p38α following anion exchange chromatography. *Bottom panel:* Western blot analysis using an anti-phospho-p38α antibody (α-p-p38α) to confirm phosphorylation of His-p38α.



**Figure 7. Purification of Flag-tagged (FL-) MEF2D $\alpha'$  $\beta$ , MEF2D $\alpha\beta$ , and MEF2D $\alpha\emptyset$  splice forms.** (A) Schematic representation of Flag-affinity purification. Sf9 insect cell lysate containing over-expressed FL-MEF2D splice forms (blue) was applied to M2-Flag beads. Contaminant proteins (green, pink, teal, purple) were removed in flow-through and wash fractions. FL-MEF2D splice forms were eluted with 200  $\mu$ g/ml Flag peptide. For Flag pull-down assays, anti-Flag beads were incubated with nuclear extract (NE) prior to the elution step. Associated proteins (black) were identified by LC-MS/MS. (B) Coomassie blue-stained polyacrylamide gel of mouse FL-MEF2D splice forms (FL-mMEF2D $\alpha'$  $\beta$ , FL-mMEF2D $\alpha\beta$ , and FL-mMEF2D $\alpha\emptyset$ ) and human MEF2D (hMEF2D $\alpha\beta$ ) following purification. (C) Western blot analysis of purified FL-MEF2D splice forms using  $\alpha$ -MEF2D $\alpha'$  which recognizes only the muscle-specific splice form or  $\alpha$ -MEF2D which recognizes all splice forms.

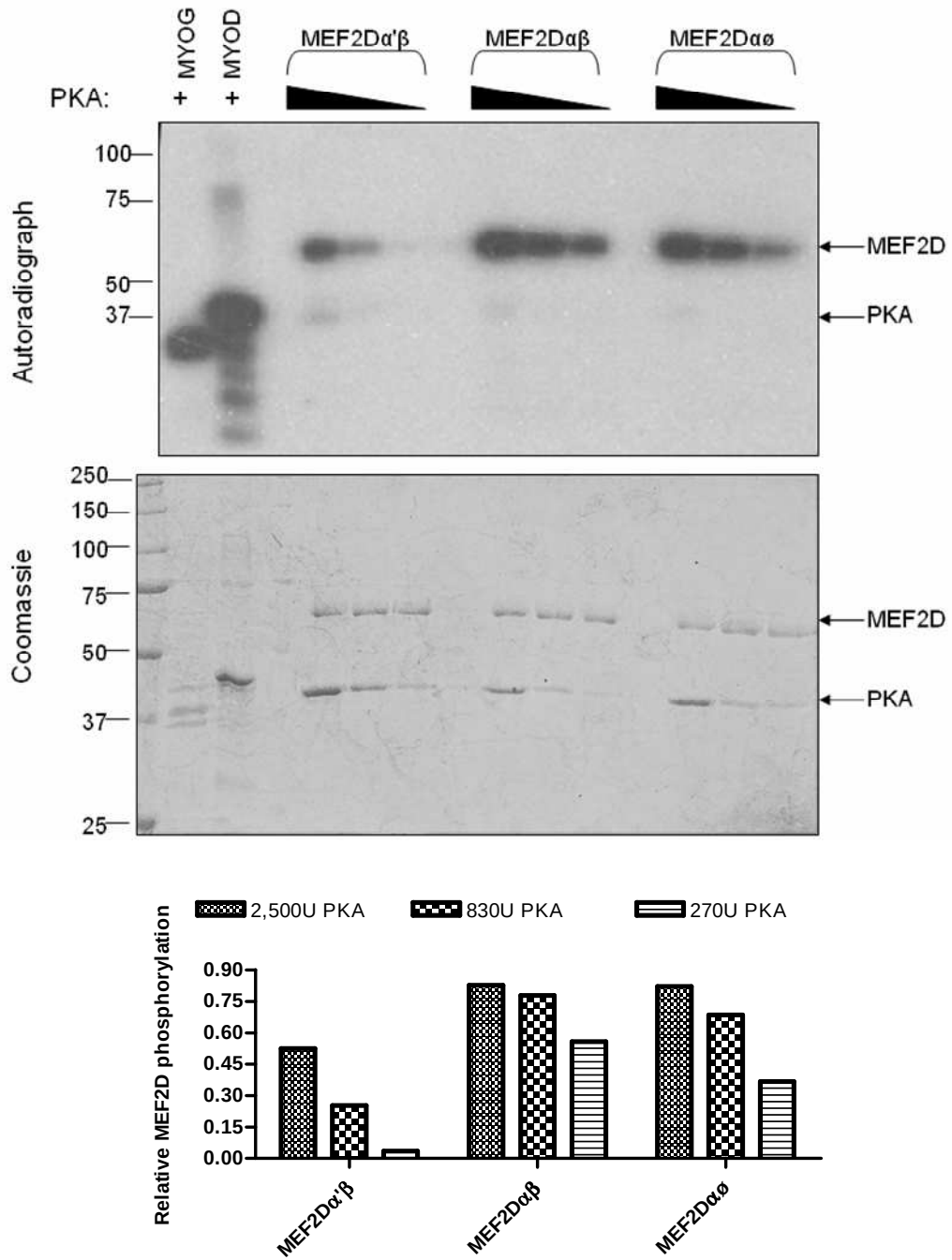


**Figure 8. MEF2D splice forms are differentially phosphorylated by p38α MAP kinase *in vitro*.** Purified FL-MEF2D splice forms were incubated *in vitro* with [ $\gamma$ - $^{32}$ P] ATP and activated p38α (300 ng or 100 ng). Proteins were resolved using SDS-PAGE and visualized by autoradiography or Coomassie blue staining. Bovine serum albumin (BSA) and human MEF2D (hMEF2D) were used as negative and positive controls, respectively. Densitometric analysis of MEF2D phosphorylation was normalized to MEF2D protein levels visualized by Coomassie staining. This result is representative of two independent experiments.



#### **4.3 MEF2D splice forms are differentially phosphorylated by PKA *in vitro*.**

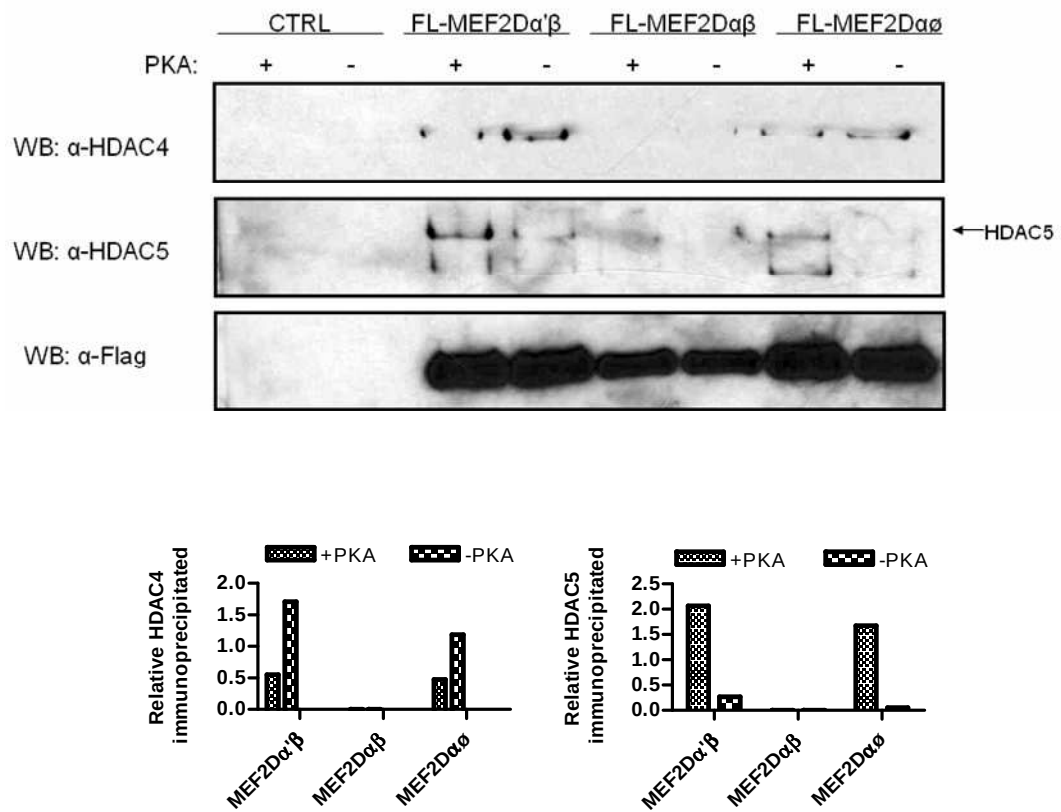
Class IIa HDACs, HDAC4 and HDAC5, are well characterized MEF2 co-factors which negatively affect MEF2 transcriptional activity (Lu et al., 2000). Recent work by John McDermott's group suggests that PKA activity can enhance the association of MEF2D with HDAC4 (Du et al., 2008). This finding prompted us to investigate whether FL-MEF2D splice forms could be differentially phosphorylated by PKA *in vitro*. To this end, commercially available and catalytically active PKA was incubated in separate reactions with FL-MEF2D splice forms in the presence of [ $\gamma$ - $^{32}$ P] ATP. Autoradiography results revealed that the myotube-specific form FL-MEF2D $\alpha'$  $\beta$  was least phosphorylated by PKA *in vitro* compared to FL-MEF2D $\alpha$  $\beta$  and FL-MEF2D $\alpha$  $\delta$  which were phosphorylated to similar extents (Fig.9). The MRFs myogenin and MyoD were used as positive controls since they are known PKA targets (Li et al., 1992, Winter et al., 1993).



**Figure 9. MEF2D splice forms are differentially phosphorylated by PKA *in vitro*.** Purified FL-MEF2D splice forms were incubated *in vitro* with [ $\gamma$ - $^{32}$ P] ATP and PKA (2,500U, 830U or 270U). Proteins were resolved using SDS-PAGE and visualized by autoradiography or Coomassie blue staining. Myogenin (MYOG) and MyoD were used as positive controls. Densitometric analysis of MEF2D phosphorylation was normalized to MEF2D levels visualized by Coomassie staining. This result is representative of three independent experiments.

#### ***4.4 PKA phosphorylation of MEF2D splice forms does not enhance their association with HDAC4.***

Next, we sought to determine whether direct phosphorylation of FL-MEF2D by PKA was responsible for enhancing MEF2D association with HDAC4 as observed by Du and colleagues (Du et al., 2008). Flag pull-down assays followed by western blotting analysis revealed that PKA treatment did not enhance the association of MEF2D splice forms with HDAC4 (Fig.10, Supp. Fig.1). The blot was reprobed with an antibody against HDAC5, another Class IIa HDAC, and surprisingly PKA phosphorylation of FL-MEF2D appeared to enhance its association with HDAC5. Since elution of FL-MEF2D $\alpha\beta$ -associated proteins was inefficient compared to other FL-MEF2D splice forms, it is not conclusive whether different splice forms have different affinities for either of the Class II HDACs.



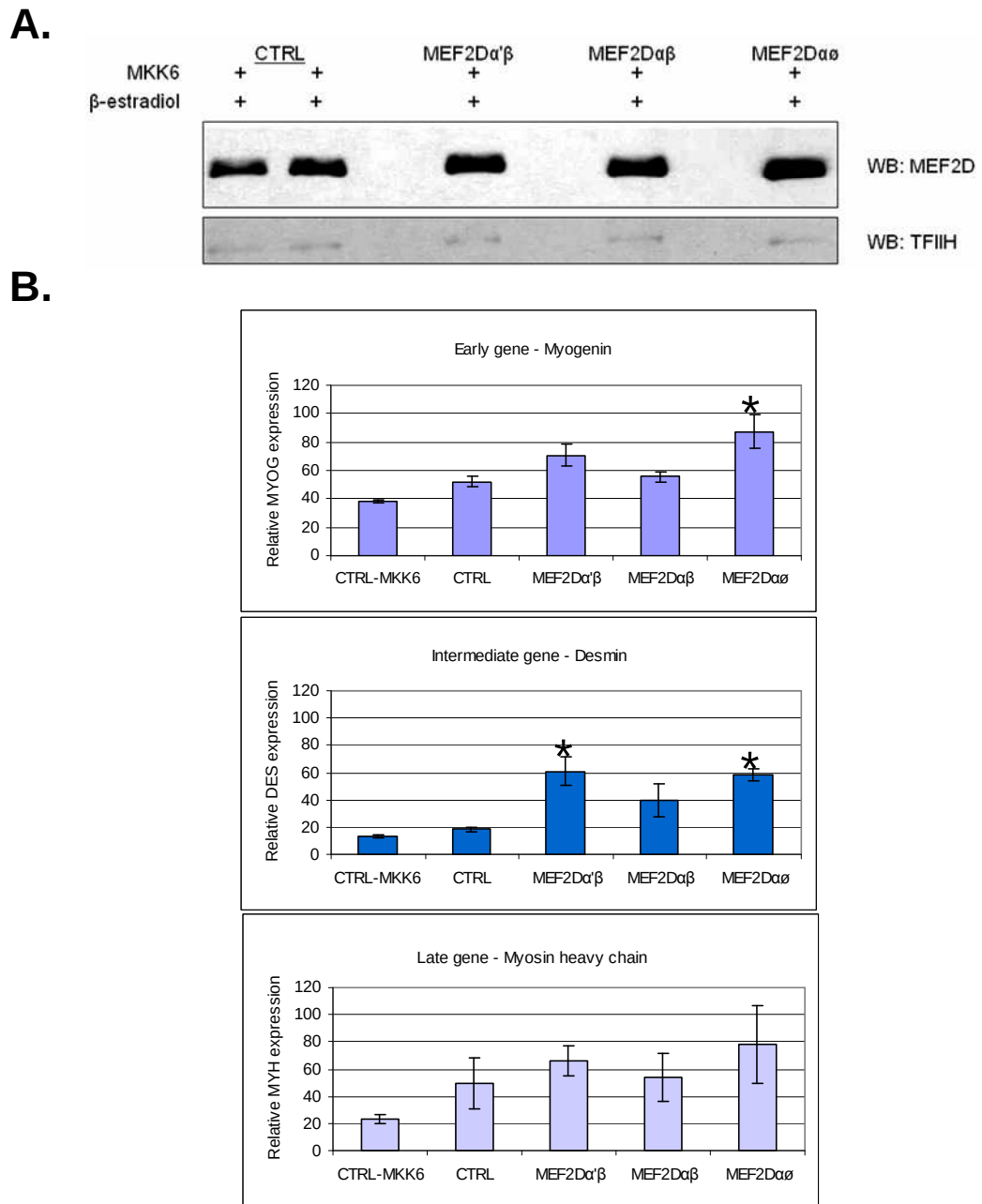
**Figure 10. PKA phosphorylation of MEF2D splice forms increases their association with HDAC5 but not HDAC4.** Flag-tagged MEF2D splice forms (FL-MEF2D $\alpha\beta$ , FL-MEF2D $\alpha\beta$ , and FL-MEF2D $\alpha\emptyset$ ) were immobilized on anti-Flag M2 beads, *in vitro* phosphorylated with or without PKA, then incubated with 48 hr differentiated C2C12 nuclear extract. Associated proteins were eluted using 200  $\mu$ g/ml Flag peptide, resolved by SDS-PAGE and visualized by western blotting with anti-HDAC4, anti-HDAC5 or anti-Flag antibodies. Densitometric analysis of HDAC levels was normalized to FL-MEF2D levels. This result is representative of two independent experiments.

#### **4.5 MEF2D splice forms exhibit different transcriptional activities.**

It is well known that MyoD acts as a crucial regulator of the myogenic program in part by synergizing with MEF2 isoforms to enhance transcription of muscle-specific genes during differentiation (reviewed by Berkes & Tapscott, 2005). To ensure the proper spatio-temporal expression of muscle gene expression during differentiation, MyoD feeds forward to activate MEF2D transcription and the p38 signaling pathway (Penn et al., 2004). Subsequently, MEF2D phosphorylation by p38 $\alpha$  facilitates recruitment of Ash2L/MLL(KMT2B) transcription activating complexes within regulatory regions of muscle-specific genes (Rampalli et al., 2007). Having established that MEF2D splice forms are differentially phosphorylated by p38 $\alpha$  *in vitro*, we next explored the effect of alternative splicing on MEF2D transcriptional activity.

To assess the transcriptional activity of different splice forms, 10T $\frac{1}{2}$ -MyoD-ER fibroblast cell lines stably over-expressing each MEF2D splice form were generated. MEF2D over-expression was confirmed by western blotting analysis (Fig.11A). 10T $\frac{1}{2}$ -MyoD-ER fibroblast cells were used because they stably express a MyoD-estrogen receptor (MyoD-ER) fusion protein which can be induced to translocate into the nucleus and activate muscle genes through the addition of an estrogen analog,  $\beta$ -estradiol. At the same time, retrovirus encoding a constitutively active form of the upstream kinase in the p38 MAPK signaling pathway, MKK6EE, was added to cells to enhance differentiation as reported previously (Penn et al., 2004). RT-qPCR analysis was performed using MEF2D splice form over-expressing cells and revealed that MEF2D splice forms

differentially activate the expression of muscle genes myogenin (Myog), desmin (Des) and myosin heavy chain (Myh). Although MEF2D $\alpha\emptyset$  and the muscle-specific form MEF2D $\alpha'\beta$  could both activate Des expression, MEF2D $\alpha\emptyset$  appeared to be the most potent activator of Myog expression. None of the three Mef2D splice forms appeared to significantly affect Myh expression, however, the large error bars suggest that the quality of this particular data set might be improved upon to yield better results.

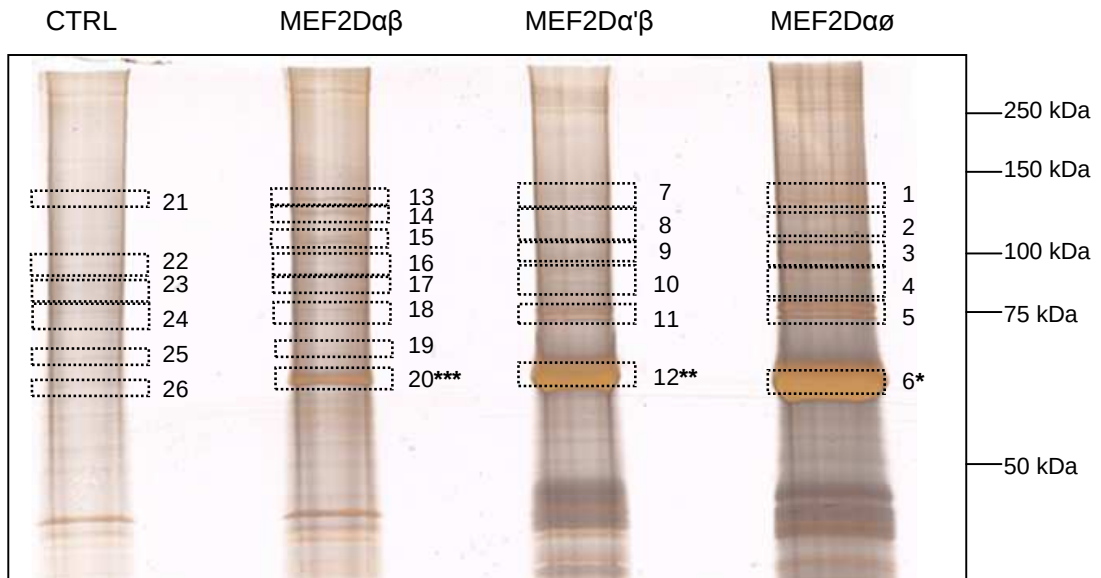


**Figure 11. MEF2D splice forms exhibit different transcriptional activities at MyoD-activated genes in the presence of active p38.** (A) Western blot analysis of 10T $\frac{1}{2}$ -MyoD-ER cells stably expressing a MEF2D splice form (MEF2D $\alpha'\beta$ , MEF2D $\alpha\beta$  or MEF2D $\alpha\emptyset$ ) or an empty vector control (CTRL). (B) MEF2D splice form over-expressing fibroblast cultures were converted using differentiation media supplemented with  $\beta$ -estradiol for 15 hrs. Total RNA was reverse-transcribed and analyzed by qPCR to measure levels of the early myogenic marker myogenin (MYOG), intermediate marker desmin (DES) as well as the late myogenic marker myosin heavy chain (MYH). Data is normalized to DDX5. Statistical significance was determined by mutli-way ANOVA;  $p < 0.01$ ;  $N = 3$ . Error bars represent SD.

#### ***4.6 MEF2D splice forms interact with proteins involved in gene regulation and cell signaling.***

Finally, we chose to investigate the functionality of MEF2D splice forms more broadly by asking whether they associated with similar or different protein complexes during muscle differentiation. Flag pull-down assays were performed using 48 hrs differentiated C2C12 nuclear extract as a source of interacting proteins (Fig.12). The list of associated proteins identified by LC-MS/MS included both putative and previously known interacting partners (Table 1). MEF2C interacted with MEF2D $\alpha\emptyset$ , and MEF2A interacted with both FL-MEF2D $\alpha\emptyset$  and FL-MEF2D $\alpha'\beta$ , although no MEF2 isoforms were identified in the FL-MEF2D $\alpha\beta$  pull-down. Novel Mef2D interacting partners include the cyclic-AMP specific phosphodiesterase PDE4D5 (FL-MEF2D $\alpha'\beta$  band 8, Table 1) and the histone lysine demethylase PHF8 (FL-MEF2D $\alpha\emptyset$  band 1, Table 1).





**Figure 12. Flag pull-down of putative MEF2D splice form interacting partners.** Flag-tagged MEF2D splice forms (\*\*\*=FL-MEF2D $\alpha'$  $\beta$ ; \*\*=FL-MEF2D $\alpha\beta$ ; \*=FL-MEF2D $\alpha\emptyset$ ) were immobilized on anti-Flag M2 beads and incubated with 48 hrs differentiated C2C12 nuclear extract. Associated proteins were eluted using 200  $\mu$ g/ml Flag peptide, resolved by SDS-PAGE and visualized with silver stain. Several protein bands from each lane were excised (dashed boxes), trypsinized and subjected to LC-MS/MS analysis for protein identification. CTRL represents Flag pull-down performed with wildtype Sf9 cell lysate.

**Table 1. Abbreviated list of putative MEF2D splice form interacting partners.**

| Band no.                                                               | Gene symbol | Gene name                                          | MW (kDa) | MASCOT score | Identified peptides |
|------------------------------------------------------------------------|-------------|----------------------------------------------------|----------|--------------|---------------------|
| <b>MEF2D<math>\alpha</math>-associated proteins</b>                    |             |                                                    |          |              |                     |
| 1                                                                      | PHF8        | PHD finger protein 8                               | 102.0    | 32           | 2                   |
| 1                                                                      | SAFB        | Scaffold attachment factor B                       | 105.0    | 35           | 5                   |
| 1                                                                      | CDK13       | Cell division protein kinase 13                    | 164.5    | 26           | 6                   |
| 1                                                                      | HIPK2       | Homeodomain-interacting protein kinase 2           | 127.6    | 25           | 5                   |
| 3                                                                      | MEF2C       | Myocyte-specific enhancer factor 2C                | 54.5     | 60           | 2                   |
| 5                                                                      | HSP701A     | Heat shock protein 701a                            | 69.9     | 73           | 2                   |
| 5                                                                      | MEF2A       | Myocyte-specific enhancer factor 2A                | 53.5     | 118          | 8                   |
| 6                                                                      | MEF2D       | Myocyte-specific enhancer factor 2D                | 55.0     | 503          | 37                  |
| <b>MEF2D<math>\alpha'</math><math>\beta</math>-associated proteins</b> |             |                                                    |          |              |                     |
| 8                                                                      | PDE4D5      | Cyclic AMP specific phosphodiesterase              | 91.0     | 31           | 2                   |
| 12                                                                     | MEF2A       | Myocyte enhancer factor 2A                         | 52.9     | 161          | 7                   |
| 12                                                                     | MEF2D       | Myocyte-enhancer factor 2D                         | 55.0     | 428          | 24                  |
| <b>MEF2D<math>\alpha</math><math>\beta</math>-associated proteins</b>  |             |                                                    |          |              |                     |
| 19                                                                     | EIF4G1      | Eukaryotic translation initiation factor 4 gamma 1 | 54.4     | 44           | 7                   |
| 20                                                                     | MEF2D       | Myocyte-specific enhancer factor 2D                | 55.0     | 217          | 6                   |

## 5. DISCUSSION

Members of the Mef2 transcription factor family, Mef2A-D, are crucial during the differentiation of many cell types in vertebrates. Work by the Dilworth, Imbalzano, and Tapscott groups provide strong evidence suggesting a specific role for MEF2D during skeletal muscle differentiation (Penn et al., 2004, Ohkawa et al., 2006, Rampalli et al., 2007). Interestingly, MEF2D is the only MEF2 isoform present in both immature myoblasts and mature myotubes (Breitbart et al., 1993, Martin et al., 1994, Penn et al., 2004). Evidence that Mef2 proteins are indispensable during myogenesis comes from studies in *Drosophila* which contain a single Mef2 gene (*Dmef2*). One such study demonstrated that loss of *Dmef2* expression blocked formation of all muscle types (Bour et al., 1995).

In this study we aimed to further characterize MEF2D function by distinguishing between three MEF2D splice forms: MEF2D $\alpha\emptyset$ , MEF2D $\alpha\beta$ , and the muscle-specific form MEF2D $\alpha'\beta$ . Since Mef2D alternative splicing was first reported almost two decades ago, we have not gained much insight into how or why it occurs during myogenesis. Most MEF2D studies published to date do not address individual splice forms, likely due in part to the lack of splice-form specific antibodies. To circumvent this problem, we use Flag-tagged MEF2D $\alpha\emptyset$ , MEF2D $\alpha\beta$  and MEF2D $\alpha'\beta$  splice forms in our comparative analysis of their biochemical and/or transcription activator/repressor properties.

### ***5.1 Mef2D alternative splicing is developmentally regulated.***

We began our investigation by confirming the pattern of Mef2D splicing in C2C12 myoblasts. Comparing Mef2D  $\beta$ -exon splicing before (G0, G0+RE, MB)

and after (MT) differentiation, there is a clear shift favouring  $\beta$ -exon inclusion after 48 hrs of differentiation. Although both Mef2D+ $\alpha$  and Mef2D+ $\alpha'$  transcripts increase during the course of differentiation, immunofluorescence data shows that Mef2D+ $\alpha'$  proteins are present in myotubes and not in myoblasts. Taken together, our findings are in agreement with earlier reports describing the existence of a muscle-specific Mef2D splice form: MEF2D $\alpha'\beta$ . Interestingly, the appearance of MEF2D $\alpha'\beta$  after 48 hrs of differentiation correlates with the time-point when myoblasts undergo cell fusion to form multinucleated myotubes (Bijlenga et al., 2000). Although Mef2D mis-splicing is consistently reported as a feature of fusion-defective and partially converted fibroblasts over-expressing MyoD, it is unclear whether exogenous MEF2D $\alpha'\beta$  is sufficient to rescue the fusion defect (Ichida et al., 1998, Russo et al., 1998, Ohkawa et al., 2006).

### **5.2 MEF2D splice forms are differentially phosphorylated by p38 $\alpha$ *in vitro*.**

Numerous groups including our own have demonstrated that MEF2A, MEF2C and MEF2D are all direct targets of the p38 MAP kinase. Moreover, numerous lines of evidence highlight the importance of these phosphorylation events during the differentiation program (Zhu et al., 2004, Penn et al., 2004, Rampalli et al., 2007, Perdiguero et al., 2007, Ramachandran et al., 2008). Our results further demonstrate that MEF2D splice forms can be differentially phosphorylated by p38 $\alpha$  *in vitro*. The muscle-specific form FL-MEF2D $\alpha'\beta$  appears to be highly phosphorylated by activated p38 $\alpha$  relative to the other FL-MEF2D splice forms. Interestingly, the appearance of MEF2D $\alpha'\beta$  protein after 48hrs of differentiation coincides with maximal p38 $\alpha$  signaling in C2C12 cells (Perdiguero

et al., 2007). It is tempting to hypothesize that p38 $\alpha$  signaling may influence Mef2D alternative splicing and promote expression of the MEF2D $\alpha'$  $\beta$  splice form which is most responsive to p38 $\alpha$  activity. In fact, MAPK signaling has been implicated in the regulation of alternative splicing by controlling localization of the splice factor hnRNP A1 (van der Houven van Oordt et al., 2000). Our observation that FL-MEF2D $\alpha\emptyset$  can also be phosphorylated by p38 $\alpha$  supports the conclusion that the  $\beta$ -exon is not absolutely required for p38 docking and phosphorylation of MEF2 (Zhu et al., 2005). However, this finding does not exclude the possibility that  $\beta$ -exon inclusion/exclusion influences which phosphoacceptor sites are targeted by p38 $\alpha$ .

### ***5.3 MEF2D splice forms exhibit different transcriptional activities.***

MyoD is thought to act as a master regulator of differentiation in part by regulating Mef2 expression and p38 signaling to temporally pattern gene expression (Penn et al., 2004). Phosphorylation of MEF2D by p38 $\alpha$  has been shown to activate transcription of muscle-specific genes via recruitment of the Trithorax group Ash2L1/MLL2(KMT2B) methyltransferase complex and subsequent deposition of H3K4me3 marks (Rampalli et al., 2007). In light of our finding that FL-MEF2D splice forms were differentially phosphorylated by p38 $\alpha$ , we next asked whether Mef2D splice forms possessed different transcriptional activities. Although there is disagreement in the literature regarding the transactivation properties of Mef2D splice forms (Breitbart et al., 1993, Martin et al., 1994, Zhu et al., 2005), we found that Mef2D splice forms exhibit different transcriptional activities. MEF2D $\alpha'$  $\beta$  over-expression in converted fibroblasts

appears to activate the intermediate-stage differentiation gene *Des* but is a poor activator of the early-stage gene *Myog*. This finding is consistent with our observation that MEF2D+ $\alpha$ ' protein is abundant in myotubes and suggests a specific role for this splice form in the transcription of some intermediate stage genes. In contrast, MEF2D $\alpha\emptyset$  over-expression significantly increases *Myog* expression in converted fibroblasts relative to the other splice forms. The abundance of Mef2D- $\beta$  transcripts in myoblasts supports the possibility that MEF2D $\alpha\emptyset$  may function predominantly in myoblasts to activate *Myog* expression at the on-set of differentiation. Interestingly, MEF2D $\alpha\beta$  appears to be the least potent activator of both early- and intermediate-stage genes. Since MEF2D+ $\alpha$  proteins are present in myoblasts and myotubes, it is tempting to hypothesize that MEF2D $\alpha\beta$  may be responsible for maintaining basal expression levels of Mef2D target genes in C2C12 myoblasts.

#### ***5.4 MEF2D splice forms are differentially phosphorylated by PKA in vitro.***

In contrast to MEF2D's role during differentiation as a transcriptional activator, recent studies clearly demonstrate that MEF2D can also mediate gene repression prior to the onset of differentiation via a direct interaction with Class IIa histone deacetylases HDAC4 and HDAC5 (Lu et al., 2000, McKinsey et al., 2000, Du et al., 2008, Gordon et al., 2009). One such study by Du and colleagues demonstrated that in COS cells over-expressing constitutively active PKA there is an enhanced association between MEF2D and HDAC4 (Du et al., 2008). This latest finding prompted us to investigate whether FL-MEF2D splice forms could be differentially phosphorylated by PKA. *In vitro* kinase results demonstrate that

relative to FL-MEF2D $\alpha\beta$  and FL-MEF2D $\alpha\emptyset$ , the muscle-specific form FL-MEF2D $\alpha'\beta$  is least phosphorylated by PKA *in vitro*. This finding is consistent with the loss of a known PKA phosphoacceptor site at Ser-121 within the  $\alpha$ -exon (Du et al., 2008). Interestingly, it has been shown that PKA signaling can inhibit muscle differentiation (Lu et al., 1992). Thus, alternative splicing to produce the FL-MEF2D $\alpha'\beta$  splice form which is poorly phosphorylated by PKA would be favourable during the transition from proliferation to differentiation.

#### **5.5 PKA activity can enhance MEF2D association with HDAC5 but not HDAC4.**

Next, we asked how phosphorylation by PKA may be affecting the association between MEF2D and HDAC4. Results from two independent experiments suggest that PKA phosphorylation of FL-MEF2D splice forms is insufficient to enhance their association with HDAC4 (Fig.10, Supp.Fig.1). One explanation for the discrepancy between our results and those previously published (Du et al., 2008) could be that our experiments were carried out *in vitro* rather than *in vivo*. Du and colleagues used COS cells over-expressing constitutively active PKA, MEF2D and HDAC4 which does not exclude the possibility that phosphorylation of HDAC4 or some other Mef2 co-factor enhanced the association with MEF2D. Our *in vitro* experimental conditions, however, ensure that only MEF2D is phosphorylated by PKA. In agreement with our data, Du and colleagues demonstrated that mutating PKA phosphoacceptor sites on MEF2D does not disrupt HDAC4 binding. Having observed no effect of PKA signaling on HDAC4 association with FL-MEF2D splice forms, we asked

whether the same was true for HDAC5, another Class IIa HDAC. Surprisingly, our preliminary result suggests that PKA-dependent phosphorylation of FL-MEF2D $\alpha\beta$  and FL-MEF2D $\alpha\delta$  can enhance their association with HDAC5. FL-MEF2D $\alpha\beta$  did not appear to interact with either HDAC4 or HDAC5, however, it is clear by anti-Flag western blot analysis that less FL-MEF2D $\alpha\beta$  was used in the assay (Fig.10). As such, we cannot state conclusively that FL-MEF2D $\alpha\beta$  does not interact with either Class IIa HDAC.

In proliferating myoblasts, PKA signaling retains HDAC5 in the nucleus (McKinsey et al., 2000) whereas HDAC4 is predominantly cytoplasmic and relocates to the nucleus after myoblasts fusion has occurred (Miska et al., 2001). Within the context of HDAC localization, our data suggests that PKA activity may promote the association of HDAC5 with MEF2D inside the nucleus to inhibit differentiation. This conclusion seems to conflict with the observation that FL-MEF2D $\alpha\beta$  associates with HDAC5 despite being poorly phosphorylated by PKA. One possible explanation is that our *in vitro* conditions do not accurately recapitulate *in vivo* conditions which intricately coordinate Mef2D splicing events with changes in HDAC localization and integration of other signaling pathways including the p38 MAPK pathway.

### **5.6 MEF2D splice forms interact with proteins involved in gene regulation and cell signaling.**

An important determinant of the function carried out by any cellular protein is the protein complex with which it associates. Metaphysically speaking, proteins are who their friends are. MEF2D is no exception; capable of activating or



repressing muscle-gene expression depending on its co-factors and the surrounding cellular environment. We successfully identified putative and known MEF2D binding partners using a Flag pull-down approach followed by protein identification using LC-MS/MS. Although equal volumes of each elution was resolved by SDS-PAGE for mass spectrometry analysis, the data was strongly biased toward FL-MEF2D $\alpha\emptyset$ , likely because more material was recovered from this pull-down than from either of the FL-MEF2D $\alpha'\beta$  or FL-MEF2D $\alpha\beta$  assays (Fig.12). Optimization of our protocol to normalize the protein concentration would correct this disparity and permit more comprehensive analysis of MEF2D splice form-associated proteins. Nevertheless, our analysis reveals both known and novel MEF2D interacting partners. Previously characterized partners include heat shock protein 70 (HSP70) as well as MEF2A and MEF2C which are known to interact with other Mef2 isoforms (Martin et al., 1994, Yang et al., 2009; Table 1). A novel interacting partner identified for FL-MEF2D $\alpha'\beta$  is cAMP-specific phosphodiesterase 4D5 (PDE4D5). The PDE4 enzyme family consist of four isoforms, PDE4A-D, which all hydrolyze cAMP. PDE4D5 plays a crucial role in the formation and maintenance of cAMP gradients within cells, which in turn can regulate PKA activity (Lynch et al., 2007). Perhaps the most interesting protein in our mass spectrometry analysis was the FL-MEF2D $\alpha\emptyset$ -associated protein PHD-finger protein 8 (PHF8). PHF8 acts as a H3K9me1/2 demethylase and has been intensely studied in the last year for its role in X-linked mental retardation (Fortschegger et al., 2010, Kleine-Kohlbrecher et al., 2010), as well as brain and craniofacial development (Qi et al., 2010). In the context of muscle differentiation,

it is tempting to imagine that MEF2D $\alpha$  may associate with PHF8 to facilitate muscle gene activation. PHF8 has been shown to recognize and bind to H3K4me3 marks (Qi et al., 2010) which are known to be deposited at muscle-gene promoters via MEF2D-mediated recruitment of Ash2L1/MLL2(KMT2B) complexes (Rampalli et al., 2007). Perhaps association of PHF8 with the MEF2D-Ash2L1/MLL2(KMT2B) complex promotes spreading of H3K4me3 marks and subsequent gene activation by binding H3K4me3 marks and then removing downstream repressive H3K9me1/2 marks.

Surprisingly, we did not identify Ash2L1 in our mass spectrometry data, despite having previously shown that it interacts with hMEF2D $\alpha\beta$  in a p38 $\alpha$ -dependent manner (Rampalli et al., 2007). One possible explanation is that not enough material was used in the mouse FL-MEF2D $\alpha\beta$  pull-down to provide a comprehensive list of interacting partners. Another possibility is that the strength of the interaction was weak, perhaps because FL-MEF2D $\alpha\beta$  was not adequately *in vitro* phosphorylated by p38 $\alpha$  prior to the pull-down assay.

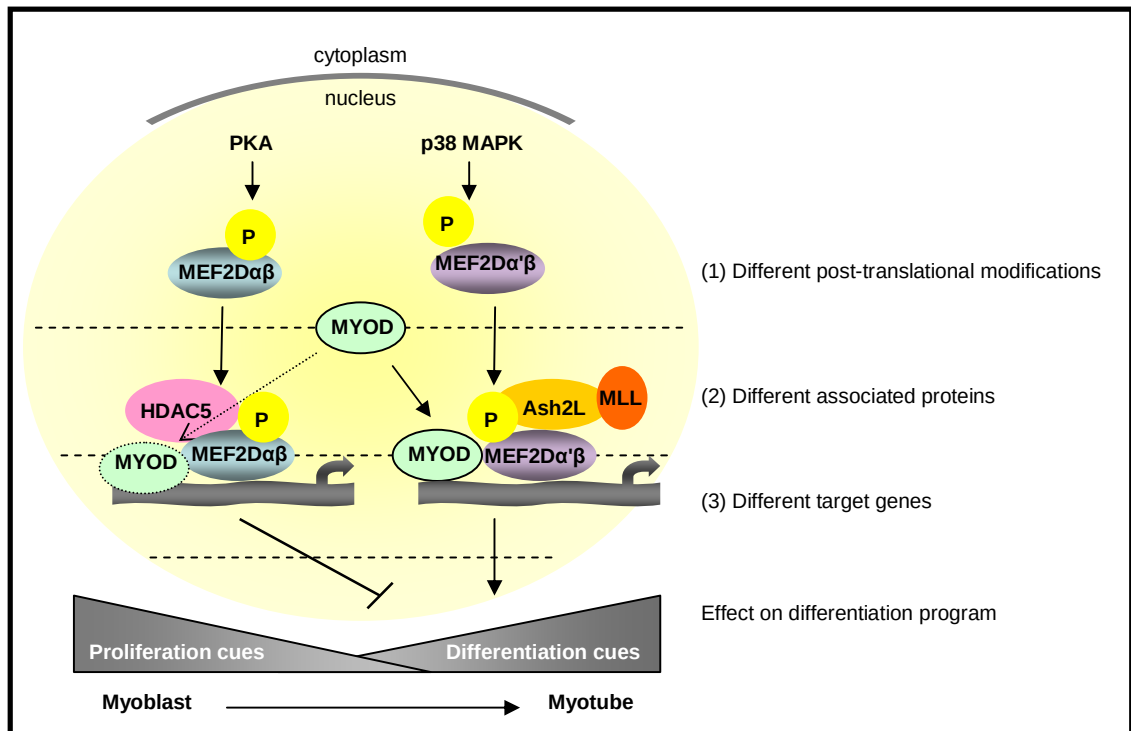
## **5.7 Future Directions**

The field of alternative splicing is evolving faster than ever thanks to innovative new techniques and impressive high-throughput technologies. While molecular biologists continue to elucidate the mechanisms governing alternative splicing and precisely how it is regulated within different cell and tissue types, the importance of this process is undeniable considering estimates that upwards of 70% of human multi-exon genes are alternatively spliced (Johnson et al., 2003). Recently, through the use of custom whole-transcript microarrays, researchers have been able to quantify the enrichment of specific splice factor motifs within tens of thousands of alternatively spliced transcripts across numerous tissue types (Castle et al., 2008). Interestingly, skeletal muscle contains a high proportion of tissue-specific exons compared to other tissues, with motifs for the splice factor proteins PTB (UCUCU) and FOX (UGCAUG) being enriched in the 200 nucleotide region upstream and downstream of muscle-specific exons, respectively (Castle et al., 2008).

Despite being ubiquitously expressed and not muscle-specific, alternative splicing of the transcription factor Mef2D is of interest in our research because of its involvement in the skeletal muscle differentiation program. Also, it is well established that Mef2D alternative splicing produces a muscle-specific form detectable only in differentiated myoblasts (Breitbart et al., 1993, Martin et al., 1994, Zhu et al., 2005), however, questions of how and why remain largely unanswered.

In light of our findings and what remains to be investigated, we propose a hypothetical model which illustrates three explanations for how Mef2D splice forms may differ: (1) they may be subject to different post-translational modifications, (2) they may associate with different protein complexes, and/or (3) they may have different target genes. Undoubtedly these explanations require further testing and are by no means fully proven, however, we will briefly suggest how our own findings support one or more of these explanations and then propose new experiments to test them further. Our model depicts the transition from proliferating myoblasts to differentiated myocytes and the key players involved in promoting or inhibiting differentiation. Vertically, our model is divided into three layers which reflect three mechanisms for how individual MEF2D splice forms might function. Read horizontally, our model illustrates how all three mechanisms could be combined to regulate entry into the myogenic differentiation program.

To consider how post-translational modifications may affect MEF2D splice form function, our model highlights the role of the calcium-sensitive PKA pathway and p38 MAPK pathway. Both kinases have been shown to target MEF2D although with opposing effects such that MEF2D phosphorylation by PKA inhibits differentiation whereas phosphorylation by p38 $\alpha$  promotes differentiation (Du et al., 2008, Rampali et al., 2007).



**Figure 13. Model of Mef2D alternative splicing.** Three layers (1-3) depict how various regulatory mechanisms might contribute to the overall effect MEF2D activity has on the skeletal muscle differentiation program (PKA-mediated repression on the left, p38 MAPK-mediated activation on the right). Layer (1) illustrates differences in post-translational modifications, layer (2) illustrates different associated proteins and layer (3) illustrates how repression/activation of target genes can either block (blunt-end arrow) or promote (arrow) differentiation.

Our findings reveal that recombinant MEF2D splice forms are differentially phosphorylated by PKA and p38 MAPK *in vitro*. We observed that where recombinant MEF2D $\alpha\beta$  is highly phosphorylated by PKA and poorly phosphorylated by p38 $\alpha$ , the exact opposite is true for the muscle-specific form MEF2D $\alpha'\beta$ . Thus, we concluded that MEF2D responsiveness to PKA or p38 signaling can be modulated by alternative splicing. One can imagine that differences in the amino acid sequence of each splice form could lead to changes in phosphoacceptor site preference; ultimately changing the biological outcome. To determine whether phosphoacceptor site selection is different

between MEF2D splice forms it would be crucial to identify the *in vitro* phosphorylated residues using mass spectrometry and then compare the sites across splice forms. One way to validate the importance of these sites *in vivo* would be to employ a  $\beta$ -Galactosidase reporter system. To systematically determine the importance of various phosphoacceptor sites,  $\beta$ -Galactosidase reporter plasmids could be co-transfected into C2C12 cells along with GAL4-MEF2D splice form fusion constructs harbouring point mutations at specific phosphoacceptor sites. In this way, one can draw important connections between alternative splicing, signal responsiveness and resulting changes to the gene expression profile of myoblasts during skeletal muscle differentiation.

We next considered whether association with distinct protein complexes might mediate functional differences between MEF2D splice forms since MEF2D is known to associate with numerous proteins including other transcription factors and histone/chromatin modifiers. For example, the direct interaction of Class II HDACs with MEF2D inhibits its transcriptional activity, whereas Ash2L1/MLL2(KMT2B) binding alongside MEF2D facilitates transcription activation (Lu et al., 2000, Rampalli et al., 2007 respectively). What remains to be seen is whether MEF2D splice forms participate in distinct complexes. One can imagine that these complexes could be temporally and/or spatially distinct such that MEF2D splice forms may associate with the same protein complexes at different times during myogenesis and/or associate with completely different protein complexes at the same time. With these possibilities in mind, one could

perform parallel ChIP experiments for MEF2D, HDAC and Ash2L proteins to compare their enrichment at known MEF2 binding sites before and after C2C12 myoblast differentiation. The  $\alpha$ -exon- and  $\alpha'$ -exon-specific antibodies generated in our laboratory could be used to compare enrichment of the non-muscle MEF2D $\alpha\beta$ /MEF2D $\alpha\emptyset$  forms and the muscle-specific MEF2D $\alpha'\beta$  form. It would also be interesting to see how changes in MEF2D splice form enrichment correlate with changes in HDAC or Ash2L binding at the same genomic loci and ultimately with changes in target gene expression. This kind of ChIP time-course experiment would reveal whether non-muscle and muscle-specific MEF2D splice forms have overlapping or distinct spatiotemporal DNA-binding at MEF2 consensus sites during differentiation.

The third layer of our model illustrates how MEF2D splice forms could possess unique functions via binding to a distinct subset of MEF2 target genes. For high-throughput analysis of genes differentially bound by MEF2D splice forms, ChIP sequencing (ChIP-seq) would be ideal. To this end, one could generate different C2C12 cell lines stably over-expressing a Flag-tagged version of each MEF2D splice form. Anti-Flag antibody could be used to pull-down specific splice forms and the associated chromatin which would then be identified using high-throughput DNA sequencing technology. The disadvantage of using over-expressing cell lines, however, is that because the exogenous protein is exceedingly abundant compared to endogenous levels, one would expect to detect numerous non-specific binding events. Since there are no splice form-

specific antibodies, however, the only alternative would be to use  $\alpha$ -exon-specific antibodies produced in our lab which can recognize the endogenous  $\alpha$ -exon or muscle-specific  $\alpha'$ -exon and not cross-react. Unfortunately these antibodies are not able to distinguish between splice forms with or without the  $\beta$ -exon and as such do not permit assignment of target genes to specific splice forms.

Looking forward, functional annotation of alternatively spliced proteins is crucial to understand how and why cells employ this molecular process to generate such astonishing protein diversity from a limited number of genes. One important application is the design of drugs or compounds capable of targeting disease-specific splice forms. For example, it has recently been shown that hypoxia-inducible factor 1 (HIF-1) subunit  $\alpha$  exists in two splice forms differing by a TAG insertion between exon 1 and 2, and that increased expression of the splice form containing the insertion reflects a breast cancer stage associated with poorer prognosis (Dales et al., 2010). Similarly, researchers have shown that an imbalance in the ratio of receptor for hyaluronan-mediated motility (RHAMM) splice forms correlates with poor survival and increased disease severity (Maxwell et al., 2004). Undoubtedly, the field of alternative splicing will continue to flourish as fresh insights provide new avenues for the development of anti-cancer compounds and a new basis from which to understand cancer progression in humans.



## **5.8 Conclusions**

Muscle differentiation is a crucial stage of skeletal muscle formation within an organism. Furthering our understanding of the molecular events which occur naturally during differentiation can improve our understanding of muscle diseases like Duchenne's muscular dystrophy and myotonic dystrophy; leading to the design of more effective treatments for patients. Our work highlights the importance of Mef2D alternative splicing during muscle differentiation by revealing that splice forms can be differentially targeted by PKA and p38 MAPK, possibly to modulate their association with Class IIa HDACs and/or transcriptional activity. We also observe that splice forms can associate with protein partners involved in gene regulation and cell signaling. Continued investigation into the impact of Mef2D alternative splicing will undoubtedly take our understanding of muscle differentiation to greater depths and significantly change the way we study and treat muscle diseases.

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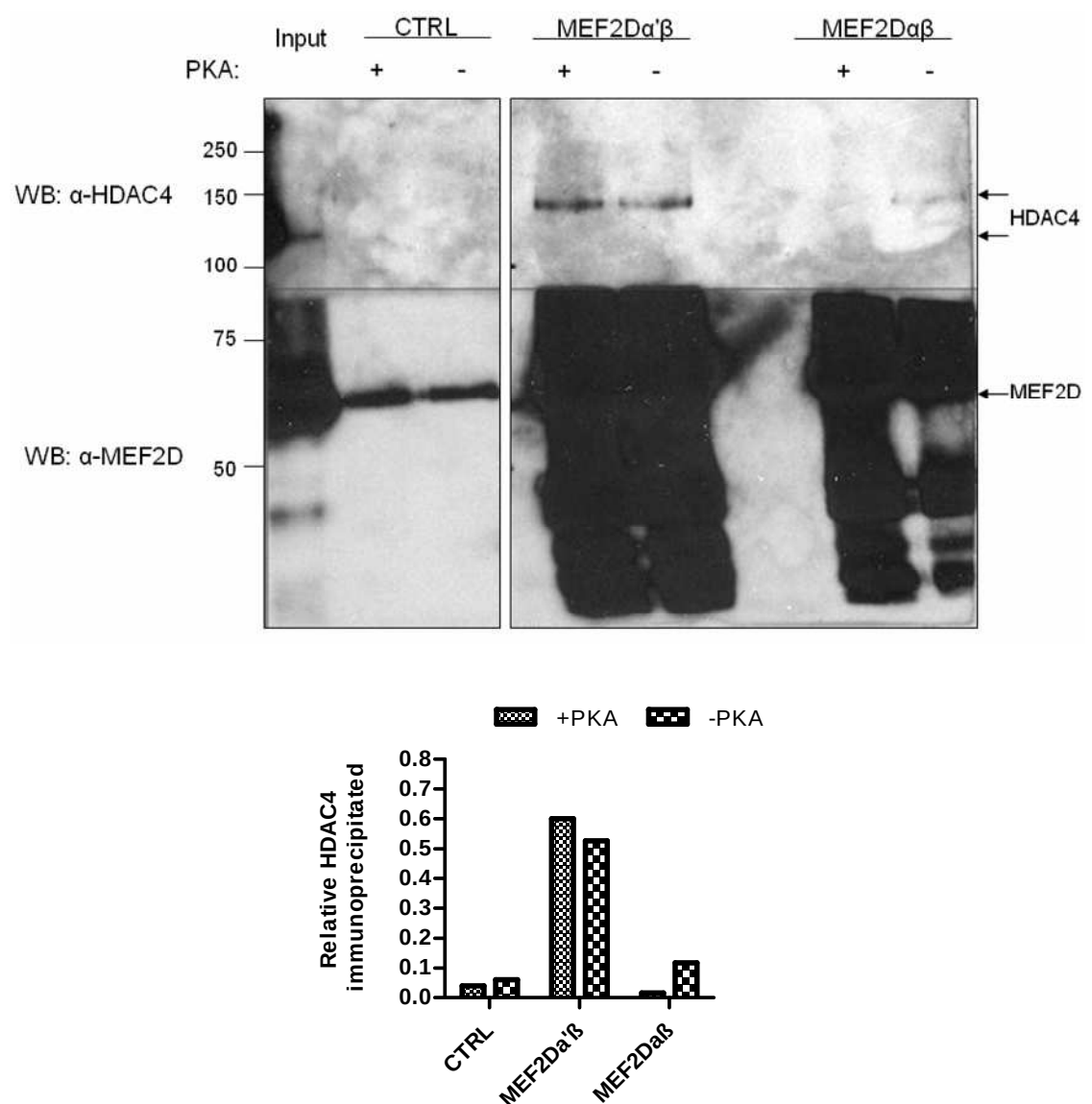
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## APPENDIX I

Supplementary Table 1. Primer sequences.

| Primer name                     | Primer sequence                 |
|---------------------------------|---------------------------------|
| PTB (forward)                   | ACC ACA AAG AGC TCA AGA CCG ACA |
| PTB (reverse)                   | TGA TGA TCT TCA GGA CGG TGC CAA |
|                                 |                                 |
| nPTB (forward)                  | TTT GCT CCA GTA TGG TGA TCC GGT |
| nPTB (reverse)                  | CTG CAG CAG CAT TTG GAA TAG CCA |
|                                 |                                 |
| ETR3 (forward)                  | TAC AAG GGC AAT GGC ACA GAA TGC |
| ETR3 (reverse)                  | CAG CCA TTT GCT GAA TGC CAC TGA |
|                                 |                                 |
| Mef2D $\alpha$ -exon (forward)  | TGA GGA AGA AGG GTT TCA ACG GCT |
| Mef2D $\alpha$ -exon (reverse)  | ACT GCA TGG AGC TCT GAT TGG ACA |
|                                 |                                 |
| Mef2D $\alpha'$ -exon (forward) | TGA GGA AGA AGG GTT TCA ACG GCT |
| Mef2D $\alpha'$ -exon (reverse) | ATG ATG TCA CCA GGG AAG GAG TGA |
|                                 |                                 |
| Mef2D $\beta$ -exon (forward)   | TTC CCA GGG AGG CAA AGG GTT AAT |
| Mef2D $\beta$ -exon (reverse)   | TGG CTG AGT AAA CTT GGT GTT GCC |
|                                 |                                 |
| Myh (forward)                   | ACC TTG CCA AGA AGA AGG ACT CCA |
| Myh (reverse)                   | TGG ATG CGG ATG AAC TTG CCA AAG |
|                                 |                                 |
| Myog (forward)                  | CAT CCA GTA CAT TGA GCG CCT ACA |
| Myog (reverse)                  | AGC AAA TGA TCT CCT GGG TTG GGA |
|                                 |                                 |
| DDX5 (forward)                  | TTC TGA TTG CTA CCG ATC TGG CCT |
| DDX5 (reverse)                  | TGG TAC TGC GAG CAG TTC TTC CAA |
|                                 |                                 |
| Mef2D (forward)                 | TTC AGG CGC TAT GGG TCA TCT GTT |
| Mef2D (reverse)                 | AGG CTC CAT TAG CAC TGT TGA GGT |
|                                 |                                 |
| Mef2A (forward)                 | ATT CTC CAA TTG TGC TTG GCC GAC |
| Mef2A (reverse)                 | AGG AAG TGC CAG ACT GGT CTG     |



**Supplementary Figure 1. PKA phosphorylation of MEF2D splice forms does not enhance their association with HDAC4.** Flag-tagged MEF2D splice forms (FL-MEF2Dα'β, FL-MEF2Dαβ, and FL-MEF2Dαø) were immobilized on anti-Flag M2 beads and incubated with HeLa nuclear extract (Input). Associated proteins were eluted using 200 µg/ml Flag peptide, resolved by SDS-PAGE and visualized by western blotting with anti-HDAC4 or anti-MEF2D antibodies. Densitometric analysis of HDAC4 levels was normalized to FL-MEF2D levels.

## **APPENDIX II**

### **Title: Investigation into the role of Mef2D splicing.**

#### **INTRODUCTION**

Recent estimates predict upwards of 70% of human multi-exon genes are alternatively spliced (Johnson et al., 2003). Through the use of custom whole-transcript microarrays specific for particular tissues and representative of tens of thousands of alternatively spliced transcripts, important insight has been gained into how genes are spliced and which splice factors are at work. Interestingly, skeletal muscle tissue contains a high proportion of tissue-specific exons compared to other tissue types (Castle et al., 2008). Splice factor motifs enriched in skeletal muscle in the 200-nucleotide region upstream of alternatively spliced exons correspond to the splice factor PTB (UCUCU), whereas the motif enriched downstream corresponds to FOX proteins (UGCAUG) (Castle et al., 2008).

Polypyrimidine tract-binding (PTB) proteins are so-called because they bind pyrimidine-rich motifs (Charlet-B et al., 2002). PTB functions largely as a splicing repressor and is known to compete with other splice factors such as ETR-3 for splice sites flanking alternative exons in skeletal muscle (Charlet-B et al., 2002). ETR-3 (also known as CUGBP2) belongs to the CELF (CUGBP1 and ETR-3 like factor) family of proteins and has been shown previously to promote inclusion of cardiac troponin T exon 5 (Goo et al., 2009). Although PTB is ubiquitously expressed, the muscle-restricted microRNA 133 (miR-133) has been shown to down-regulate PTB levels during muscle differentiation (Boutz et al., 2007). Evidence of a MEF2 binding element within an intergenic enhancer in the miRNA

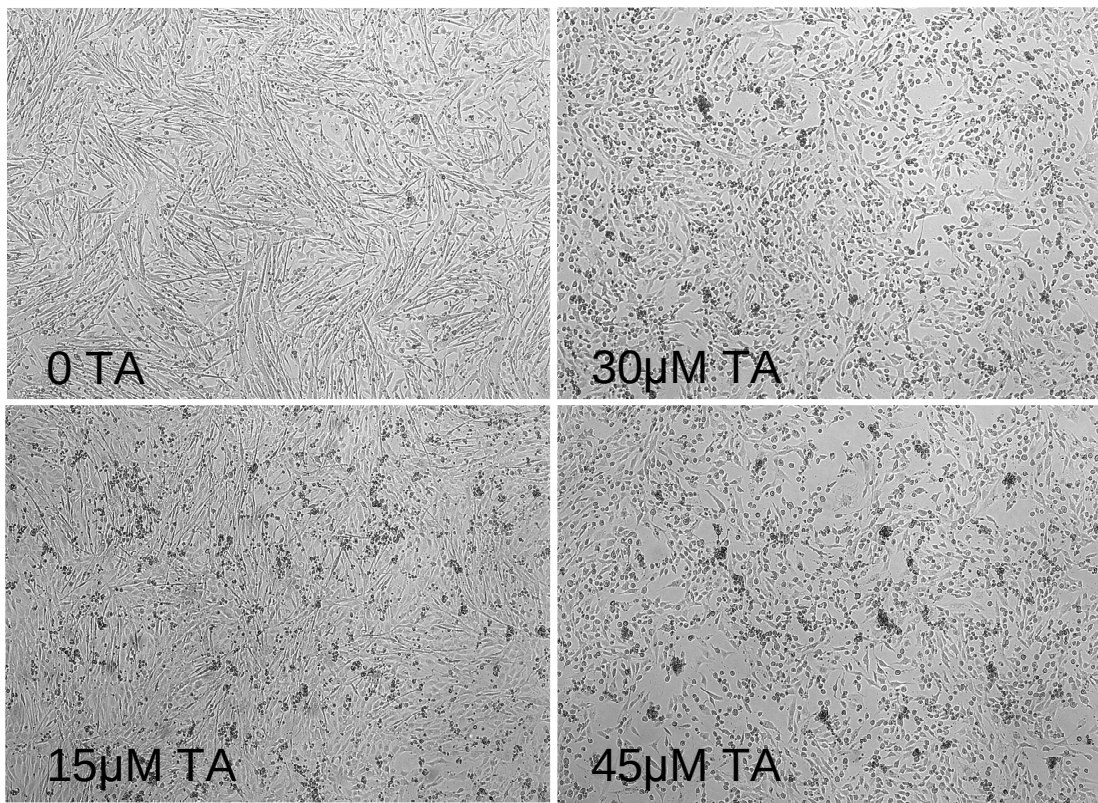
1-133 cluster suggests that Mef2 proteins play a role in fine-tuning PTB expression (Liu et al., 2007).

We decided to test the hypothesis that PTB maybe be involved in skipping of the Mef2D  $\beta$ -exon during differentiation. To this end, we utilized a naturally-occurring polyphenolic compound called tannic acid (TA) which has been studied for its therapeutic effects as a potent anti-mutagenic, anti-oxidant and anti-carcinogenic compound (Khan et al., 2000). Of relevance to our studies, TA-treatment of HEK293T cells was recently shown to increase endogenous PTB expression in a dose-dependent manner through direct activation of the PTB promoter region (Bian et al., 2009). Interestingly, a putative TA negative regulatory sequence (ACTG) and tannic acid-sensitive 13-bp element have been identified using *in vitro* reporter and EMSA assays which suggest that TA may have limited off-target effects (Uchiumi et al., 1998). Our findings reveal that TA-treatment increased PTB expression in differentiating myoblasts but not proliferating myoblasts. Concomitant with this, we observed that TA-treatment resulted in inhibition of myoblast differentiation and mis-splicing of the Mef2D  $\beta$ -exon, providing preliminary evidence correlating PTB expression with Mef2D  $\beta$ -exon splicing during muscle differentiation.

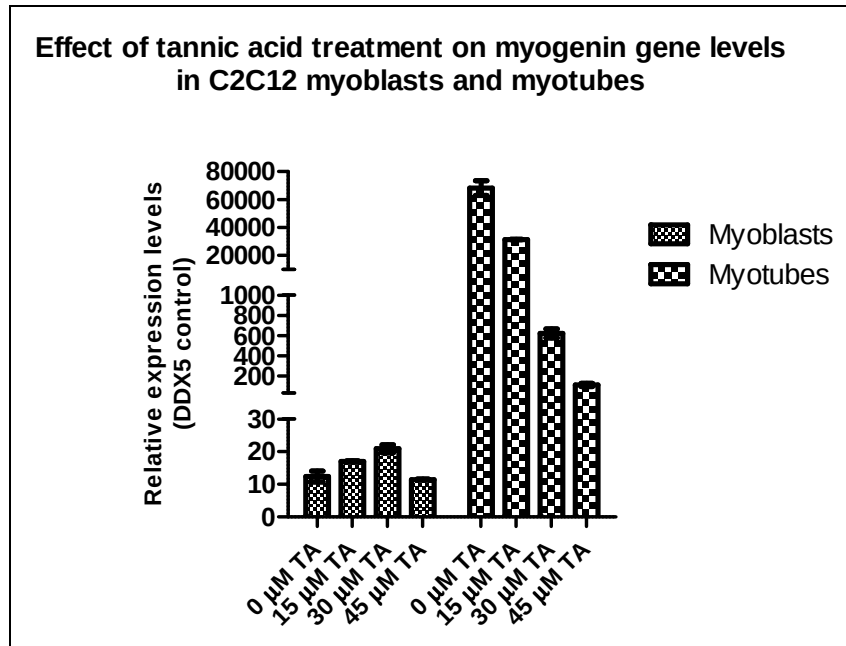
## RESULTS

### Myoblast differentiation is inhibited by TA.

To test the hypothesis that PTB might be responsible for Mef2D  $\beta$ -exon skipping we treated C2C12 myoblasts with varying concentrations of TA ranging from 0-45 $\mu$ M while inducing differentiation for 48 hours (Supp. Fig.2). Surprisingly, TA treatment appeared to inhibit myoblast differentiation. As a measure of the differentiation efficiency we performed RT-qPCR analysis and found that myogenin expression levels decreased upon TA treatment in a dose-dependent manner (Supp. Fig.3).



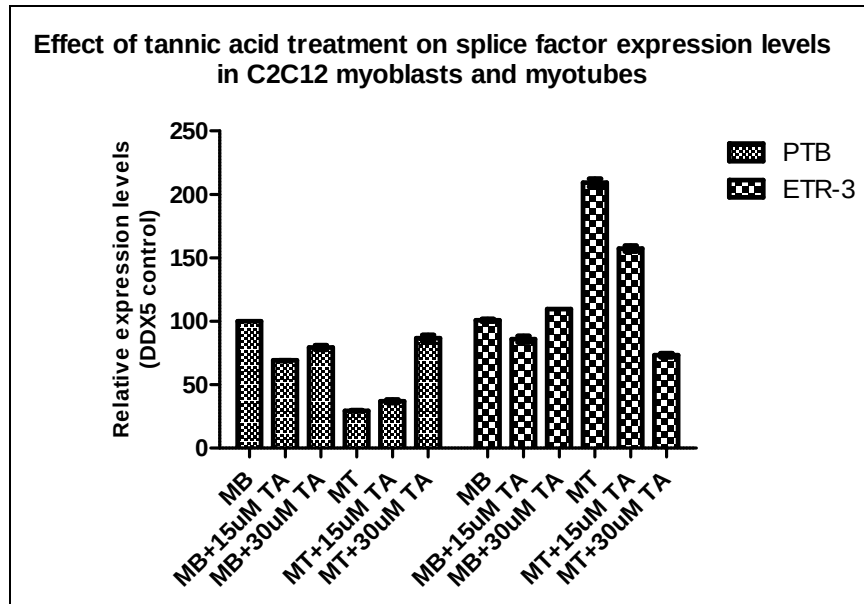
**Supplementary Figure 2. Tannic acid inhibits C2C12 differentiation *in vitro*.** Light microscope images (10X magnification) of C2C12 myoblasts treated with or without tannic acid (0 $\mu$ M, 15 $\mu$ M, 30 $\mu$ M, 45 $\mu$ M) and differentiated for 48hrs in low serum conditions.



**Supplementary Figure 3.** Tannic acid treatment reduces myogenin expression levels in C2C12 myoblasts and myotubes. RT-qPCR analysis of total cDNA reverse transcribed from C2C12 myoblasts (MB) and myotubes (MT, 48hrs differentiation) treated with and without tannic acid (0μM, 15μM, 30μM, 45μM).

#### **TA treatment induces PTB expression in myotubes but not myoblasts.**

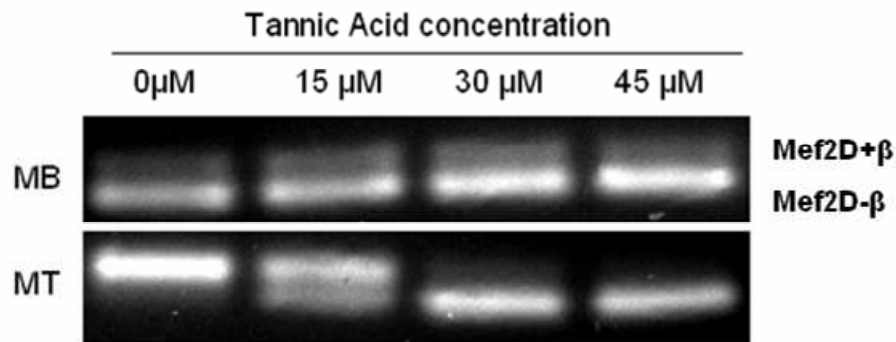
To determine whether this phenotype correlated with PTB expression, we performed RT-qPCR analysis and found that indeed TA treatment induced PTB expression although only in differentiation conditions (Supp.Fig.4). We took this opportunity to look at expression of another splice factor ETR-3 which is known to compete with PTB for splice site binding (Charlet-B et al., 2002). TA treatment appeared to have opposing effects on ETR-3 expression in myotubes, significantly reducing ETR-3 levels in a dose-dependent manner (Supp.Fig.4).



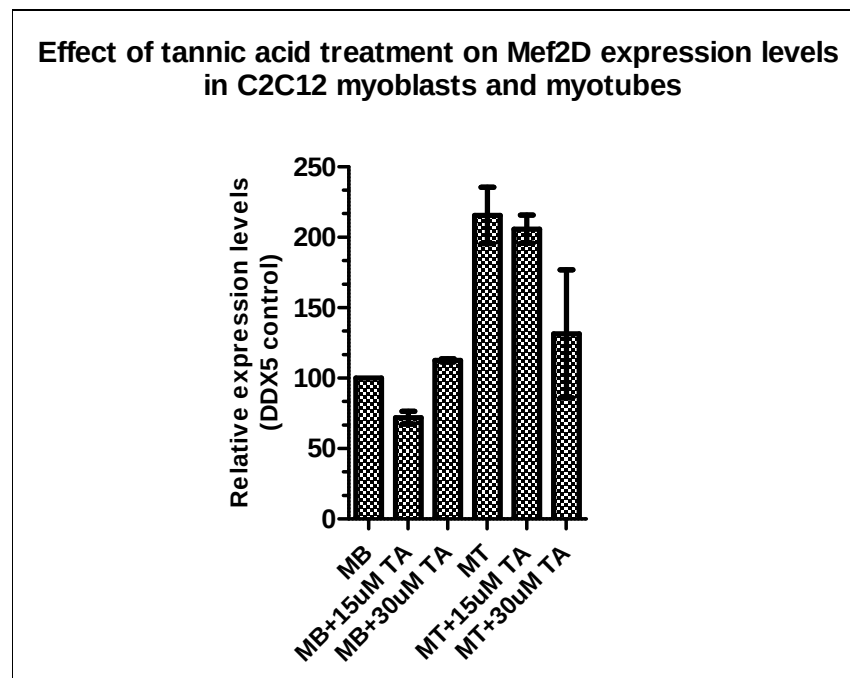
**Supplementary Figure 4. Tannic acid treatment induces PTB expression in differentiating but not proliferating C2C12 myoblasts.** RT-qPCR analysis of total cDNA from C2C12 myoblasts (MB) and myotubes (MT) treated with and without tannic acid (0 $\mu$ M, 15 $\mu$ M, 30 $\mu$ M). Data is normalized to DDX5. Error bars represent SD.

#### The Mef2D $\beta$ -exon is skipped in TA treated myotubes.

To assess the effect of TA-induced PTB expression on splicing of the Mef2D  $\beta$ -exon, we performed regular PCR using  $\beta$ -exon-specific primers and total cDNA reverse transcribed from C2C12 myoblasts and myotubes treated with various concentrations of TA (0-45 $\mu$ M) (Supp.Fig.5). Although we observed no change in the  $\beta$ -exon splice pattern in growing myoblasts, there was a striking shift from  $\beta$ -exon inclusion to skipping when comparing myoblasts differentiated in absence or presence of TA, respectively. Interestingly, the shift in splicing appeared dose-dependent since higher concentrations of TA favoured a higher ratio of Mef2D- $\beta$  relative to Mef2D+ $\beta$  transcripts. Notably, total Mef2D levels were not significantly altered in TA-treated myoblasts and decreased slightly in myotubes (Supp.Fig.6).



**Supplementary Figure 5. Tannic acid treatment results in mis-splicing of the Mef2D β-exon during differentiation.** DNA gel of Mef2D β-exon PCR products amplified from total cDNA reverse-transcribed from myoblasts (MB) or myotubes (MT, 48hrs differentiation) treated with or without tannic acid (0μM, 15μM, 30μM, 45μM).



**Supplementary Figure 6. Effect of tannic acid treatment on MEF2D expression in C2C12 myoblasts and myotubes.** RT-qPCR data of total cDNA from C2C12 myoblasts (MB) and myotubes (MT, 48hrs differentiation) treated with and without tannic acid (0μM, 15μM, 30μM). Data is normalized to DDX5 levels. Error bars represent SD.



## DISCUSSION

In skeletal muscle, motifs for the pre-mRNA splice factor PTB are highly enriched in the region just upstream of alternatively spliced exons (Castle et al., 2008). Research investigating the importance of PTB during myogenesis reveals that the muscle-specific miRNA cluster 1-133 plays a key role in regulating PTB expression (Chen et al., 2006). Interestingly, MEF2 binding to an intergenic enhancer region within this miRNA cluster controls its expression (Liu et al., 2007). For these reasons, we choose to investigate PTB as a potential splice factor involved in Mef2D  $\beta$ -exon splicing.

To test our hypothesis, we utilized a naturally-occurring polyphenolic compound called tannic acid (TA) which has recently been reported to increase PTB expression in HEK293T cells (Bian et al., 2009). Our observations confirm that TA treatment can induce PTB expression, although this was true for differentiating but not proliferating C2C12 myoblasts. One possible explanation for this discrepancy is that endogenous PTB expression is maximal in growth conditions and so any further induction by TA may have been prevented by PTB auto-regulation (Wollerton et al., 2004). Our analysis reveals that not only does TA-treatment increase PTB expression, but it also promotes Mef2D  $\beta$ -exon skipping in differentiating myoblasts. This result is consistent with an increase in PTB; a known splicing repressor that promotes skipping of alternative exons (Xue et al., 2009). Concomitant with this, we observed a decrease in expression of the splice factor ETR-3 which has been shown to antagonize PTB activity and promote inclusion of alternate exons (Charlet-B et al., 2002).

Somewhat surprisingly, our phenotypic and gene expression analysis showed that TA-treatment of differentiating myoblasts inhibited the differentiation program. The decrease in differentiation efficiency appears to be dose-dependent, since myoblasts treated with 15 $\mu$ M TA at the time of differentiation show a modest impairment compared to cells treated with 45  $\mu$ M TA. In agreement with this, myogenin expression levels remained relatively high in differentiating conditions supplemented with lower concentrations of TA. The narrow myotubes and spindle-like morphology of unfused myoblasts suggest that TA may have impaired myoblast fusion, however, a fusion index would be required to determine if this is indeed the case.

Although we were able to implicate PTB in mis-splicing of the  $\beta$ -exon, differentiation inhibition resulting from TA treatment presents an obvious dilemma: was this phenotype the cause or effect of Mef2D mis-splicing? Put another way, was Mef2D mis-splicing a direct result of TA-induced PTB expression or an indirect effect of TA-treatment on progression of differentiation? As with any chemical compound, not all biological effectors and outcomes can be predicted. Likewise, PTB recognizes splice sites in numerous skeletal muscle-specific alternate exons and might therefore indirectly effect Mef2D splicing. More direct approaches like using minigene models would be ideal to elucidate these findings further.

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