Tissue-specific splicing of a ubiquitously expressed transcription factor is essential for muscle differentiation

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Alternate splicing contributes extensively to cellular complexity by generating protein isoforms with divergent functions. However, the role of alternate isoforms in development remains poorly understood. Mef2 transcription factors are essential transducers of cell signaling that modulate differentiation of many cell types. Among Mef2 family members, Mef2D is unique, as it undergoes tissue-specific splicing to generate a muscle-specific isoform. Since the ubiquitously expressed (Mef2Da1) and muscle-specific (Mef2Da2) isoforms of Mef2D are both expressed in muscle, we examined the relative contribution of each Mef2D isoform to differentiation. Using both in vitro and in vivo models, we demonstrate that Mef2D isoforms act antagonistically to modulate differentiation. While chromatin immunoprecipitation (ChIP) sequencing analysis shows that the Mef2D isoforms bind an overlapping set of genes, only Mef2Da2 activates late muscle transcription. Mechanistically, the differential ability of Mef2D isoforms to activate transcription depends on their susceptibility to phosphorylation by protein kinase A (PKA). Phosphorylation of Mef2Da1 by PKA provokes its association with corepressors. Conversely, exon switching allows Mef2Da2 to escape this inhibitory phosphorylation, permitting recruitment of Ash2L for transactivation of muscle genes. Thus, our results reveal a novel mechanism in which a tissue-specific alternate splicing event has evolved that permits a ubiquitously expressed transcription factor to escape inhibitory signaling for temporal regulation of gene expression.

[Keywords: alternative splicing; Mef2; PKA signaling; gene expression; HDAC; Ash2L]

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Genome-wide studies have highlighted the capacity of cells to exploit the 20,000 genes of the human genome to produce a far more complex proteome through the generation of alternate transcripts [The ENCODE Project Consortium et al. 2012]. A major contributor to this cellular complexity is alternative splicing of exons, which has been proposed to occur universally in multixenon genes [Wang et al. 2008]. The generation of these alternatively spliced transcripts can occur through several mechanisms, including [1] the inclusion/exclusion of an exon, [2] alternate 3’ or 5’ splice donor/acceptor sites, or [3] the alternate use of mutually exclusive exons [Kalsotra and Cooper 2011]. In the case of mutually exclusive exons, the controlled use of alternate exons allows for the expression of functionally distinct protein isoforms that have the potential to perform specific tasks in a temporally or spatially restricted manner. Furthermore, ontology analysis suggests that transcripts incorporating mutually exclusive exons are likely to play a role in tissue-specific functions such as differentiation [Wang et al. 2008]. However, our knowledge concerning the role of specific alternative splicing events in regulating differentiation remains limited.

During myogenesis, myoblasts undergo a temporally ordered series of changes in gene expression, giving rise to multinucleated myotubes [Bergstrom et al. 2002]. These alterations are extensive, as RNA sequencing (RNA-seq) analysis has shown that 7770 genes change their expression during myotube formation [Trapnell et al. 2010]. In addition to these changes in transcript abundance, alternate mRNA processing leads to a switch in the major isoform of 7% of alternative spliced genes [Trapnell et al. 2010].
This drastic change in the myogenic transcriptome suggests an important role for alternative splicing in establishing the myotube fate. Among the genes that undergo a major isoform switch during myogenesis is the transcription factor *Mef2D* (Trapnell et al. 2010). *Mef2D* is a member of the Mef2 family of transcription factors, which act as key mediators of signal-dependent transcription in many different cell types to control developmental processes such as differentiation (Potthoff and Olson 2007). Interestingly, the switch in *Mef2D* isoforms observed during muscle differentiation is a result of an alternate use of mutually exclusive exons [see Fig. 1] that generates an isoform that is expressed exclusively in skeletal and cardiac muscle (Martin et al. 1994). However, the functional relevance of this muscle-specific isoform of *Mef2D* to muscle development remains unknown. Here we sought to elucidate the differential roles of the ubiquitously expressed (*Mef2Da1*) and muscle-specific (*Mef2Da2*) isoforms of *Mef2D* in skeletal myogenesis.

**Results**

*The muscle-specific isoform of Mef2D drives the late stages of myogenesis*

Advances in our understanding of the relative roles of *Mef2D* isoforms in myogenesis have been hampered by the lack of reagents that distinguish between the endogenous proteins. Consequently, we generated antibodies that discriminate between the muscle-specific *Mef2Da2* isoform and its ubiquitously expressed counterpart, *Mef2Da1* [Fig. 1A]. Western blot of recombinant *Mef2D* proteins demonstrate the specificity of our antibodies [Supplemental Fig. 1]. Furthermore, these antibodies recognize the endogenous *Mef2D* isoforms in differentiating myoblasts using Western blot, immunofluorescence, and immunoprecipitation [Supplemental Fig. 1; data not shown]. Importantly, a similar efficiency of immunoprecipitation is observed with both isoform-specific antibodies.

Initially, we characterized the expression of *Mef2D* isoforms during murine myogenesis. Temporal analysis demonstrates a clearly distinct pattern of expression for the two *Mef2D* isoforms [Fig. 1B,C]. In the case of the ubiquitously expressed *Mef2Da1*, the protein is present in proliferating myoblasts and continues to accumulate throughout differentiation. In contrast, the muscle-specific *Mef2Da2* is absent from proliferating myoblasts and is strongly induced between 48 and 72 h of differentiation [Fig. 1B,C]. This pattern of expression suggests a role for *Mef2Da2* in late myogenesis.

To further explore the role of *Mef2D* isoforms in myogenesis, we created isogenic C2 myoblast lines where the exogenous expression of either *Mef2Da1* or *Mef2Da2* can be induced upon doxycycline (Dox) treatment [Supplemental Fig. 2A]. Interestingly, myoblasts induced to express *Mef2Da2* formed multinucleated, myosin heavy chain (MHC)-positive cells more quickly than the control cells [Fig. 2A; Supplemental Fig. 2B], suggesting an accelerated differentiation. In contrast, expression of *Mef2Da1* appeared to block myogenesis, as fewer MHC$^+$ cells were observed in this population at late stages (120 h) of differentiation. Having observed opposing effects of *Mef2Da1* and *Mef2Da2* on differentiation in cultured myoblasts, we next examined whether these disparate roles of *Mef2D* isoforms are also observed in vivo using a model of mouse muscle regeneration. Indeed, injection of...
retrovirus expressing Mef2Dα1 into cardiotoxin-injured tibialis anterior (TA) muscle resulted in severely impaired regeneration where myofiber repair was delayed (Fig. 2B; Supplemental Fig. 3A), resulting in an accumulation of undifferentiated MyoD+ muscle cells in the interstitial space (Supplemental Fig. 4). In contrast, injection of retrovirus expressing Mef2Dα2 resulted in improved muscle regeneration with an increased muscle fiber caliber compared with the contralateral control TA muscle (Fig. 2B; Supplemental Fig. 3B). Furthermore, only the injection of Mef2Dα2 could mediate the previously described Mef2D-dependent fiber type switching in the TA muscle (Potthoff et al. 2007) from a predominantly fast type MHC-IIb myofiber toward a slower muscle isotype that contained a mixture of MHC-IIb and MHC-IIa myofibers (Supplemental Fig. 5). Thus, Mef2Dα1 and Mef2Dα2 have antagonistic effects on myogenesis and muscle regeneration.

Examination of Mef2Dα2-expressing myoblasts showed that MHC proteins were expressed at the earliest stages of differentiation (0 h) (Fig. 2A). This temporal shift in MHC expression led us to examine whether Mef2Dα2 expression would allow for the expression of muscle genes in proliferating myoblasts. To our surprise, RT-qPCR analysis demonstrates that expression of Mef2Dα2, but not Mef2Dα1, can activate Myh3 along with other late myogenic genes [Mylpf and Tnnt1] in proliferating myoblasts [Supplemental Fig. 6]. To determine whether the expression of these late markers of myogenesis was due to a precocious differentiation, we examined the ability of Mef2D isoforms to activate expression of Myog and Ckm—two muscle-specific genes normally up-regulated at intermediate stages of myogenesis. In contrast to Myh3, which is strongly up-regulated by ectopic expression of Mef2Dα2 in proliferating myoblasts, we observed no change in transcription of Myog or Ckm upon expression of the muscle-specific isoform (Supplemental Fig. 6). Taken together, our findings suggest that alternate splicing generates the muscle-specific Mef2Dα2 isoform to temporally regulate activation of late muscle genes.

We next sought to understand the mechanism underlying the differential ability of Mef2D isoforms to activate late muscle gene expression. Two potential models were envisaged: (1) Mef2Dα1 and Mef2Dα2 recruit similar coregulatory proteins to an alternate subset of muscle genes or (2) Mef2Dα1 and Mef2Dα2 bind to a similar set of genomic loci, leading to the recruitment of alternate coregulatory proteins. We therefore explored the contribution of these two models to the functional differences observed between Mef2D isoforms.

**Figure 2.** Mef2Dα1 and Mef2Dα2 have opposing effects on muscle differentiation and regeneration. (A) Stable isogenic C2iFRT myoblast lines were induced to express Mef2Dα1, Mef2Dα2, or control (parental cells). After 24 h of Dox treatment, cells were incubated in low-serum medium supplemented with Dox to permit differentiation in the presence of exogenous Mef2D isoforms [or control]. Immunofluorescence was performed using antibodies recognizing MHC or DAPI as indicated. (B) Cardiotoxin-injured mouse TA muscles were infected with retrovirus expressing Mef2Dα1, Mef2Dα2, or vector control. After 15 d of regeneration, cross-sections were stained using hematoxylin and eosin. The cross-sectional area of individual fibers was calculated and plotted. n = 3.
Genome-wide binding shows that Mef2D isoforms bind to a largely overlapping set of genes

We first explored the contribution of altered genomic targeting to the differential ability of Mef2D isoforms to activate transcription. For this, we performed chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq) to examine the genomic distribution of Mef2Dα1 and Mef2Dα2 in myotubes. Fragmented chromatin was immunoprecipitated using our isoform-specific antibodies to isolate DNA associated with the endogenous Mef2Dα1 and Mef2Dα2 proteins [Supplemental Figs. 7, 8]. Bioinformatic analysis of ChIP-seq data identified 7910 binding sites for Mef2α1, while the muscle-specific isoform Mef2Dα2 bound to 9103 sites that are not present in the control [Fig. 3A; Supplemental Table 1]. Comparison of the Mef2D-binding sites using a highly stringent cutoff of \( P < 10^{-6} \) revealed that 66% of Mef2Dα1-bound peaks are also bound by Mef2Dα2 [Fig. 3A]. However, when the stringency is modestly reduced to \( P < 10^{-4} \), the overlap in binding of Mef2Dα1 at Mef2Dα2-bound peaks increases to 85% (data not shown). Consistent with the fact that Mef2Dα1 and Mef2Dα2 bind to a largely overlapping set of genomic loci, position weight matrices for Mef2D-binding elements observed under these peaks show that both isoforms of the protein recognize a nearly identical consensus sequence of CTAAAAATAG [Fig. 3B]. A de novo search for DNA sequences enriched under peaks identified Mef2 and E-box sequences [i.e., MyoD/Myog/Mrf5/Mrf4-binding sites] as the two most highly represented DNA elements at both Mef2Dα1 and Mef2Dα2 genomic targets [data not shown]. Interestingly, examination of genomic loci bound preferentially by either Mef2Dα1 or Mef2Dα2 did not identify Mef2-binding sites as the most highly represented DNA sequences found under these peaks [Supplemental Fig. 9]. This finding suggests that the differential binding observed between Mef2Dα1 and Mef2Dα2 at a small subset of genes is likely due to altered targeting of the transcription factor to DNA through an indirect tethering mechanism.

To assess the importance of overlapping Mef2Dα1 and Mef2Dα2 genomic binding to myogenesis, we performed gene ontology (GO) analysis of peaks bound by both isoforms. Genes targeted by both Mef2Dα1 and Mef2Dα2 are strongly associated with GO annotations defining a role in muscle development [Fig. 3C; Supplemental Table 1]. Among these genes, we examined Mef2D binding at the HDAC9 gene, which is known to participate in a negative feedback loop with Mef2 proteins [Haberland et al. 2007]. At this locus, we observed peaks within the HDAC9 promoter for both Mef2Dα1 and Mef2Dα2 that overlap with MyoD binding [Fig. 3D]. Similarly, we observed that the promoters of Myh3, Titin, Myog, Tnt1, Ckm, and Mylpf are bound equally well by both Mef2Dα1 and Mef2Dα2 in differentiating myotubes [Supplemental Fig. 8]. RNA-seq analysis of transcripts that are up-regulated at 24 h of differentiation when expression of Mef2D isoforms is induced in C2 cells identified 227 genes activated by Mef2Dα2 [Supplemental Table 1]. Combining the RNA-seq data set with Mef2D peaks within 50 kb of the transcription start site identified 107 genes whose expression may be directly modulated by Mef2Dα2 [Supplemental Table 1; Supplemental Fig. 10]. Consistent with Mef2Dα2 modulating the late muscle gene expression program, GO analysis of putative Mef2Dα2 direct target genes showed a highly significant enrichment for genes involved in muscle development and muscle contraction [Supplemental Fig. 10A]. Importantly, although Mef2Dα1 bound to most of the direct transcriptional targets of Mef2Dα2, exogenous expression of the ubiquitous Mef2D isoform did not lead to a significant increase in expression at any of these genes [Supplemental Table 1; Supplemental Fig. 10B]. Similarly, ChIP-qPCR analysis of Mef2D isoform binding in proliferating myoblasts demonstrates that both Mef2Dα1 and Mef2Dα2 bind to the Myh3 and Mylpf promoters [Supplemental Fig. 6B], although only Mef2Dα2 binding gives rise to transcriptional activation. Taken together, these results demonstrate that altered DNA binding is not the major determinant of the differential activation of transcription by Mef2D isoforms.

The muscle-specific Mef2Dα2 isoform preferentially interacts with coactivator complexes

To assess the possibility that Mef2Dα1 and Mef2Dα2 associate with alternate coregulatory proteins to regulate muscle gene expression, we performed a proteomic analysis of proteins that interact with each Mef2D isoform. Initially, we examined proteins that interact with recombinant Mef2Dα1 and Mef2Dα2. In a pull-down assay, Flag-tagged Mef2Dα1 or Mef2Dα2 proteins were used as bait to isolate interacting proteins from nuclear extracts prepared from myotubes [Supplemental Fig. 11]. Mass spectrometry analysis of Mef2Dα1- or Mef2Dα2-interacting proteins [Table 1] identified components of the coactivator Ash2L methyltransferase complexes, co-repressor HDAC9 deacetylase complexes, and the SWI/SNF ATP-dependent chromatin remodelling complexes that have previously been reported to associate with Mef2D [Ohkawa et al. 2006, 2007; Rampalli et al. 2007; Du et al. 2008]. Qualitative analysis of the identified peptides showed that recombinant Mef2Dα1 and Mef2Dα2 interact with the same sets of proteins [Table 1]. This finding suggests that the exchange of mutually exclusive exons in Mef2D does not generate proteins with distinct protein–protein interaction domains. However, the experiment was performed with recombinant Mef2D isoforms. To determine whether Mef2Dα1 and Mef2Dα2 isoforms also interact with the same sets of proteins in their native cellular context, we immunoprecipitated endogenous Mef2Dα1 and Mef2Dα2 proteins from myotube extracts and tested the interaction with the previously identified cofactors by Western blot [Fig. 4A]. Strikingly, in these conditions, we observed differential interactions for Mef2Dα1 versus Mef2Dα2. Precisely, the muscle-specific Mef2Dα2 interacts with the Ash2L coactivator complex but not the repressive HDAC4 and HDAC9 proteins. Conversely, the ubiquitously expressed Mef2Dα1 does not associate with...
Ash2L but interacts strongly with HDAC4 and HDAC9. Therefore, in contrast to their recombinant counterparts, endogenous Mef2D isoforms interact with distinct protein complexes in differentiating myotubes—Mef2Dα1 interacts with corepressors, while Mef2Dα2 interacts with coactivators.

**Figure 3.** Mef2D isoforms bind a largely overlapping set of genes involved in muscle development. ChIP-seq analysis was performed on C2 myotubes that had been differentiated for 120 h. Chromatin was immunoprecipitated with antibodies recognizing either Mef2Dα1 or Mef2Dα2. To control for nonspecific binding to the antibodies, control ChIP-seq experiments were performed with antibodies that were preincubated with peptides corresponding to the epitope and labeled as Mef2Dα1 (blocked) or Mef2Dα2 (blocked). (A) Venn diagram showing the overlap between the 9103 Mef2Dα2 peaks and the 7910 Mef2Dα1 peaks identified by ChIP-seq. (B) Logo diagrams of the position weight matrix of the sequence generated based on the enrichment of Mef2-binding sites observed in peaks bound by either Mef2Dα1 or Mef2Dα2. (C) The top GO terms for biological processes (BP) are shown for the group of 5204 genes that are bound equally well by both Mef2Dα1 and Mef2Dα2. (D) The sequencing reads are mapped across the HDAC9 locus for ChIP experiments performed using Mef2Dα1, Mef2Dα2, Mef2Dα1 (blocked), Mef2Dα2 (blocked), and MyoD. Peaks for both Mef2Dα1 and Mef2Dα2 that overlap with MyoD binding are observed in the proximal promoter region (boxed in red).
phosphorylated by p38 on the activating phosphorylation of Mef2D by p38 demonstrates that both Mef2D isoforms were efficiently phosphorylated by p38α. Having observed that the activating phosphorylation of Mef2D by p38α occurs in both Mef2Dα1 and Mef2Dα2, we next explored the possibility that the isoforms may be differentially affected by inhibitory PTMs. Transactivation by Mef2D is known to be inhibited through phosphorylation of Ser119 [S119] and S190 by PKA, which enables an association with class II HDACs [Du et al. 2008]. Importantly, alternative exon usage in Mef2Dα2 removes the S119 residue targeted by PKA in the Mef2Dα1 isoform [see Fig. 4B]. Thus, we examined whether the Mef2Dα1 and Mef2Dα2 isoforms are differentially phosphorylated by PKA. Consistent with previous observations [Du et al. 2008], Mef2Dα1 is strongly phosphorylated by PKA [Fig. 4C]. In contrast, Mef2Dα2 is resistant to phosphorylation by PKA even though the phosphorylation target site S189[190] remains intact [Fig. 4C]. These findings confirm the hypothesis that Mef2D isoforms are differentially modified by PTMs. Thus, overall, our results show that alternative exon usage regulates the ability of Mef2D protein to be phosphorylated by PKA.

### Table 1. Proteomics analysis of Mef2Dα1- and Mef2Dα2-interacting proteins

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Recombinant Flag-tagged Mef2Dα1 and Mef2Dα2 proteins were used as bait to pull down interacting proteins from nuclear extracts prepared from C2 myotubes. Proteins interacting with the Mef2D isoforms were separated by SDS-PAGE and analyzed by mass spectrometry as described in the Supplemental Material. Peptides: The number of unique peptides identified for each protein; Mascot score: the confidence in the proper identification of the protein; —: no peptides were identified for that particular protein.

**Mef2Dα2 protein evades inhibitory protein kinase A (PKA) signaling**

Based on the difference in protein-binding specificity observed between recombinant and endogenous proteins, we reasoned that the altered interactions could be modulated by post-translational modifications [PTMs]. Indeed, several PTMs have been shown to regulate Mef2D function in vivo [Potthoff and Olson 2007]. Important to transcriptional activation, phosphorylation of Mef2D by p38α MAPK [p38α] at positions Thr308 [T308] and T315 [see Fig. 4B] is required for recruitment of the Ash2L coactivator complexes to muscle genes [Rampalli et al. 2007]. We therefore examined the propensity of Mef2Dα1 and Mef2Dα2 to be phosphorylated by p38α. Using recombinant proteins, in vitro kinase assays demonstrate that both Mef2Dα1 and Mef2Dα2 are efficiently phosphorylated by p38α [Fig. 4C]. Having observed that the activating phosphorylation of Mef2D by p38α occurs

**Mutation of PKA phosphorylation sites allows Mef2Dα1 to activate myogenic transcription**

To better understand the importance of the differential phosphorylation of Mef2D isoforms by PKA, we examined how PKA activity changes during differentiation. Previous studies have shown that PKA signaling is required at various stages of myogenesis to mediate CREB-dependent gene expression [Berdeaux and Stewart 2012]. Using antibodies that recognize CREB phosphorylated at S133 to measure PKA activity, we observed that the PKA phosphorylates CREB in proliferating myoblasts, and the kinase remains active as myoblasts transition to myotubes [Fig. 4D]. Thus, consistent with the requirement for activated CREB in myogenesis [Berdeaux and Stewart 2012], PKA activity is maintained throughout myotube formation.

The fact that PKA activity is maintained throughout differentiation suggests that the differential propensity to be phosphorylated by PKA could be responsible for the differential interaction of Mef2D isoforms with corepressor versus coactivators. To test this possibility, we generated the mutant Mef2Dα1[S119A/S190A], which is resistant to phosphorylation by PKA [Du et al. 2008]. Similar to results observed with the endogenous protein, overexpressed Mef2Dα1 did not interact with Ash2L but showed a strong interaction with HDAC9 [Fig. 5A]. However, inhibition of phosphorylation by PKA using Mef2Dα1[S119A/S190A] led to a marked reduction in the interaction between the mutant protein and the repressive HDAC9 protein. Surprisingly, the loss in interaction with HDAC9 is concurrent with the appearance of an association with the coactivator Ash2L that normally interacts with Mef2Dα2. Thus, phosphorylation of Mef2D proteins by PKA is a primary determinant of the interaction with transcriptional coactivators or corepressors.

Finally, we examined whether the switch in Mef2D isoforms could represent a requirement for the transcription factor to evade inhibitory PKA signaling to mediate
Alternative splicing evades inhibitory PKA signaling

Figure 4. The muscle-specific isoform of Mef2Dα1 is resistant to phosphorylation by PKA. (A) The Ash2L complex preferentially interacts with the Mef2Dα2 isoform. Nuclear extracts were prepared from C2 cells that had been induced to differentiate for 120 h. Endogenous Mef2D proteins were immunoprecipitated with antibodies recognizing Mef2Dα1 or Mef2Dα2 or with nonspecific IgG. Western blots were performed using the antibodies indicated. (B) Schematic representation of the positioning of serine residues phosphorylated by PKA and threonine residues phosphorylated by p38α MAPK in Mef2Dα1 (and the equivalent residues in Mef2Dα2). (C) In vitro kinase assays were performed using either purified p38α MAPK (top panels) or PKA (bottom panels) in the presence of either Mef2Dα1 or Mef2Dα2. Mef2D proteins (100 ng) were incubated with varying concentrations of recombinant p38α MAPK or PKA for 30 min in the presence of 4 μCi of [γ-32P]-ATP. Proteins were then separated by SDS-PAGE and exposed to autoradiographic [Autorad] film. Parallel reactions lacking [γ-32P]-ATP were separated by SDS-PAGE and stained with Coomassie blue. (D) PKA activity in C2 cells was determined at different stages of differentiation by examining the amount of phosphorylated CREB present in whole-cell extracts. Western blots were performed on whole-cell extracts using the antibodies indicated.

Discussion

Alternative splicing of transcription factors has been shown to direct tissue-specific gene expression by modifying the specificity of DNA-binding element recognition of the protein, resulting in the activation of an alternate set of genes (Reed et al. 2010; Gabut et al. 2011). Performing the first comparative genome-wide analysis of alternate transcription factor isoforms, we determined that the generation of a muscle-specific Mef2Dα2 isoform does not alter the DNA-binding element recognition of the transcription factor compared with the more ubiquitous Mef2Dα1 isoform. Instead, we found that alternate usage of mutually exclusive exons plays a crucial role in controlling the transcriptional activity of Mef2D during differentiation by altering its susceptibility to phosphorylation by PKA. Specifically, the Mef2Dα1 isoform that is phosphorylated by PKA acts as a transcriptional repressor, while the PKA-resistant Mef2Dα2 isoform activates late muscle gene expression. Thus, we identified a previously unappreciated mechanism by which a ubiquitously expressed transcription factor undergoes tissue-specific alternative exon usage to permit temporal activation of gene expression during differentiation.

A role for the Mef2 family of proteins in modulating gene expression in response to cell signaling pathways is well established (Potthoff and Olson 2007). Among the signaling pathways known to regulate Mef2 activity in development, PKA and p38 MAPK signaling are known to converge on Mef2D function during myogenesis. Studies using reagents that did not distinguish between...
Mef2Dα1 and Mef2Dα2 have shown that PKA represses Mef2D function (Du et al. 2008), while p38α stimulated its transactivation (Penn et al. 2004; Rampalli et al. 2007). In the case of p38α, phosphorylation of Mef2D allows the recruitment of Ash2L/MLL2-containing methyltransferase complexes to establish trimethylation of histone H3 (H3K4me3) within promoters of MyoD target genes to facilitate transcription (Rampalli et al. 2007; Aziz et al. 2010). In contrast, PKA-mediated phosphorylation of Mef2D engages an interaction between the transcription factor and class II HDACs to mediate transcriptional repression (Du et al. 2008). The use of antibodies that allow us to distinguish between Mef2Dα1 and Mef2Dα2 confirm these original findings, as Mef2D proteins can interact with both corepressors and coactivators during myogenesis. However, our analysis of specific isoforms demonstrates that it is not the same Mef2D molecules that interact with these antagonizing coregulatory molecules. Instead, we found that the ubiquitous Mef2Dα1 interacts with the corepressors HDAC4 and HDAC9 through an interaction enabled by PKA-mediated phosphorylation. Splicing of the Mef2D transcripts during myogenesis then generates a muscle-specific protein that is resistant to PKA phosphorylation and associates with the Ash2L coactivator complex. This represents a significant advance in our understanding of myogenesis, as previous studies showed that PKA phosphorylates Mef2D to block its ability to drive myogenesis (Du et al. 2008). This inhibitory effect of PKA on Mef2D function was perplexing due to the fact that PKA signaling is required in myogenesis to facilitate CREB-dependent transactivation and myoblast fusion (Chen et al. 2005; Mukai and Hashimoto 2008; Stewart et al. 2011; Berdeaux and Stewart 2012). Our finding that Mef2D undergoes a splicing event during differentiation that renders the transcription factor resistant to PKA inhibition resolves this paradox and highlights the need to study isoform-specific functions of proteins during development.

Resolving the paradox of continued PKA signaling during myogenesis is an important finding that identifies an alternate mechanism that evolved for cells to overcome inhibitory signaling pathways during differentiation. Indeed, common models for development describe the down-regulation of signaling pathways that are inhibitory to differentiation concomitant with the up-regulation of prodifferentiation signals to permit progression toward a
new cell fate [Iglesias-Bartolome and Gutkind 2011]. However, PKA signaling is both promyogenic through phosphorylation of CREB (Chen et al. 2005; Mukai and Hashimoto 2008; Stewart et al. 2011; Berdeaux and Stewart 2012) and inhibitory to myogenesis through phosphorylation of Mef2D [Du et al. 2008]. Thus, the muscle-specific switch in exon usage for Mef2D evolved to evade the inhibitory effects of PKA signaling, allowing transcriptional activation of Mef2D target genes in the presence of promyogenic PKA signaling. While we showed that the exon switching is used by Mef2D to evade phosphorylation by PKA, it remains to be determined how extensively splicing is used to evade signaling pathways in development. However, we propose that isoform switching may be a widely used mechanism to modify susceptibility of a protein to cell signaling, as recent studies suggest that tissue-specific exons often encode disordered peptides that are enriched for sites of phosphorylation [Buljan et al. 2012].

Profiling of Mef2Dα1 and Mef2Dα2 protein levels during myogenesis demonstrated a temporal control of isoform abundance where a clear switch in exon usage allows accumulation of the muscle-specific Mef2Dα2 starting at an intermediate stage of differentiation. The timing of this splicing event suggests a major role for Mef2Dα2 in ensuring the proper temporal activation of late gene expression during myogenesis. Previous studies of myogenesis have identified a role for Me2D in establishing the proper temporal activation of a subset of muscle genes [Penn et al. 2004]. Furthermore, studies in zebrafish have demonstrated that Me2D proteins are important to the activation of the late stages of the myogenic program [Hinits and Hughes 2007]. Based on these observations, we propose a model for the temporal regulation of late muscle gene expression through Mef2D isoform switching (Fig. 6).

In this model, Mef2Dα1 is the predominant isoform of the transcription factor expressed in the early myogenesis. Active PKA signaling in the cell catalyzes phosphorylation of Mef2Dα1 to establish a HDAC-dependent transcriptional repression at late muscle genes. As differentiation progresses, Mef2D transcripts undergo alternative splicing, leading to the establishment of two distinct isoforms: the ubiquitous Mef2Dα1 isoform that continues to act as a transcriptional repressor and a muscle-specific Mef2Dα2 isoform that is resistant to PKA signaling and permits the transcriptional activation of the late muscle gene expression program. As our ChIP-seq studies have shown that Mef2Dα1 and Mef2Dα2 bind to overlapping subsets of target genes in the late stages of myogenesis, we propose that an ongoing competition between the two isoforms

**Figure 6.** Model for the temporal regulation of late muscle gene expression through the use of alternate exons. During the early stages of differentiation, the ubiquitous isoform of Mef2D (Mef2Dα1) is expressed almost exclusively. The majority of the Mef2Dα1 are phosphorylated by active PKA signaling, leading to a recruitment of class II HDAC4/9 onto muscle promoters to maintain a transcriptionally repressed state. At later stages of differentiation, alternative splicing of Mef2D transcripts gives rise to a second population of Mef2D proteins (Mef2Dα2). The Mef2Dα2 isoform, which cannot be phosphorylated by PKA, competes with Mef2Dα1 for binding to target genes. While binding of PKA-phosphorylated Mef2Dα1 leads to recruitment of class II HDACs to repress the promoter, Mef2Dα2 (and unphosphorylated Mef2Dα1) leads to recruitment of the Ash2L complexes to muscle promoters to activate gene expression. This competition would result in an ongoing dynamic exchange of repressive and activating Mef2D transcription factors that is proposed to act as a rheostat to ensure an optimal level of transcription to mediate formation of function myotubes.
exists for binding to Mef2 elements within the promoter, leading to the recruitment of both coactivator and corepressor proteins to muscle genes. Such a competition has previously been demonstrated for glucocorticoid receptors, where continuous cycling on and off the MMTV promoter (McNally et al. 2000) allows for dynamic exchange of transcription factor isoform binding at a single genomic site (Voss et al. 2011). Similarly, a dynamic exchange of Mef2Dax1 and Mef2Dax2 binding in differentiating myotubes would permit the alternate association of coactivators and corepressors at the promoter region of late muscle genes to achieve optimal expression levels required for healthy muscle development and function. This model is completely consistent with previous genome-wide studies that demonstrated that HDAC and histone acetyltransferase proteins are both highly enriched at actively transcribed genes to ensure efficient gene expression (Wang et al. 2009).

In conclusion, we showed that the muscle-specific isoform of Mef2D plays a key role in establishing the late muscle gene expression program. This role is enabled through a temporally regulated switch in exon usage that allows Mef2D to evade inhibitory phosphorylation by PKA. Thus, we identified a novel role for the alternate use of mutually exclusive exons to modulate the transition in function of a transcription factor from repressor to activator through altered susceptibility to an inhibitory PTM.

Materials and methods

Antibodies

Rabbit polyclonal antibodies that specifically recognize the alternate isoforms of Mef2D were generated by Genscript using keyhole limpet hemocyanin-coupled peptides—CELDGLFRYGST [for Mef2Dax1] or CPQTEEEKKIDEEF [for Mef2Dax2]. The pan-Mef2D mouse monoclonal antibody was purchased from BD Biosciences (catalog no. 610774). Ash2L antibody has been previously described (Demers et al. 2007). The DPF-30 antibody was generated in a rabbit using a His-tagged full-length mouse DPF-30 protein as antigen. The following antibodies recognizing different forms of MHC were obtained from the Developmental Studies Hybridoma Bank: embryonic MHC (MF20), MHC-I (A4.840), MHC-IIa (SC-71), and MHC-IIb (BF-F3). CREB (Santa Cruz Biotechnology, catalog no. SC-186), phospho-CREB (Ser133) [Cell Signaling, catalog no. 9191], HDAC4 [Cell Signaling, catalog no. 2072], HDAC9 [Abcam, catalog no. 109446], tubulin [Developmental Studies Hybridoma Bank, catalog no. 6G7], and myogenin (Santa Cruz Biotechnology, catalog no. sc12732) antibodies are commercially available.

Cell culture

C2 myoblasts (Yaffe and Saxel 1977) were maintained in Dulbecco’s modified medium (4.5 g/L glucose) supplemented with 10% fetal calf serum, 4 mM L-glutamine, and 1.5 g/L sodium bicarbonate while not allowing cell density to achieve >80% confluent. To differentiate myoblasts, cells were allowed to achieve 90% confluence prior to a shift in culture medium to Dulbecco’s modified medium containing 2% horse serum with insulin (10 μg/mL) and transferrin (10 μg/mL). Differentiation medium was changed every 24 h for up to 5 d.

Generation of isogenic C2 lines expressing Mef2D isoforms

A C2 line containing a single insertion of an FRT recombination element was generated using the Flp-In system (Invitrogen). The C2iFRT cell line was then generated by constitutively express the Tet repressor by selecting for cells transfected with the pPyCAGIP-TetR plasmid. Clones were then screened by immunofluorescence to ensure that they retained the potential to differentiate (express Myog and Myh3 while forming multinucleated myotubes) upon serum withdrawal. The cDNAs encoding Mef2Dax1, Mef2Dax2, Mef2Dax2(S189A), Mef2Dax1(S190A), or Mef2Dax1(S119/190A) were cloned into the multiple cloning site of a modified pCDNA5/FRT plasmid, where the CMV promoter was replaced by a CMV containing a Tet operator. Plasmids were transfected separately into C2iFRT cells and selected for hygromycin expression as outlined in the Flp-In system protocol. Batch cultures were then screened for their ability to differentiate [as described above] and Mef2D expression. Batch cell lines were termed C2iFRT-Mef2Dax1, C2iFRT-Mef2Dax2, C2iFRT-Mef2Dax2(S189A), C2iFRT-Mef2Dax1(S190A), or C2iFRT-Mef2Dax1(S119/190A) based on the form of Mef2D that was exogenously expressed when induced with Dox at a concentration of 0.5 μM.

ChIPs

C2 myoblasts [7 × 10⁷ cells] were differentiated for 5 d. Proteins were cross-linked to DNA for 30 min using 1% formaldehyde in Dulbecco’s modified Eagle medium (DMEM) at 22°C. After 30 min, the formaldehyde-containing medium was removed, and cells were washed with 125 mM glycine in PBS for 10 min at 22°C. Recovered cells were resuspended in a hypotonic lysis buffer (25 mM Hapes at pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, 0.5 mM DTT, 0.3 mM PMSF). After 45 min on ice, myotubes were lysed using a 2-mL dounce homogenizer [β-pestle]. Pellets recovered from homogenate were resuspended in nuclease buffer [50 mM Tris HCl at pH 7.5, 140 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂]. MNase (Sigma) was added to the chromatin at a concentration of 0.12 U per 100 μg of ribonucleic acids [measured as in Brand et al. 2008] and incubated for 5 min at 37°C. Fragmentation of the DNA was stopped by the addition of one-tenth volume of MNase stop buffer (11 mM EDTA, 11 mM EGTA). The chromatin was then pelleted at 4000 rpm for 10 min at 4°C. The pellet was resuspended in shearing buffer (50 mM Tris HCl at pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5% SDS, 1% Triton X-100) and sonicated using a Bioruptor for 80 cycles of 30 sec on and 60 sec off to further fragment the chromatin to obtain fragments of 150–300 base pairs [bp]. After sonication, the chromatin was then centrifuged to pellet debris, and the supernatant was transferred to fresh Eppendorf tubes for immunoprecipitation with antibodies of interest as previously described (Seenundun et al. 2010). Libraries were generated from immunoprecipitated DNA as outlined in the Illumina DNA Library Construction kit protocol, except that 21 rounds of PCR were performed for amplification, and DNA fragments ranging from 200 to 350 bp were isolated during size selection on agarose gel. DNA libraries were sequenced on an Illumina Hi-Seq 2000 at the McGill University and Genome Quebec Innovation Center. The DNA sequences of primer sets used for ChiP-qPCR analysis are available on request.

High-throughput DNA sequencing analysis

Sequences were extracted using GApipeline. Reads were aligned by Burrows-Wheeler alignment [BWA] to the mouse genome (mm9). Duplicate sequences were discarded to minimize the effects of PCR amplification. Each read was extended in the sequencing orientation to a total of 200 bases to infer the coverage at...
Alternative splicing evades inhibitory PKA signaling

Regeneration in mice

Mouse studies were performed in accordance with the University of Ottawa regulations for animal care and handling. The left and right TA muscles of wild-type CD-1 mice (obtained from Charles River) were injured by injection of cardiotoxin as previously described (Liu et al. 2012). After 48 h of recovery, retrovirus expressing either Mef2D or Mef2DΔ2 (multiplicity of infection [MOI] 1 × 10⁶) was injected into one of the regenerating TA muscles, while the contralateral regenerating muscle was injected with a control retrovirus. After 3, 7, or 15 d of recovery, TA muscles were isolated and sectioned (12 μm thickness). For TA samples taken at 15 d of regeneration, the muscles were prepared for paraffin inclusion as described in Liu et al. (2012). For TA samples prepared at 3 and 7 d of regeneration, unfixed muscles were embedded in OCT compound, frozen in isopentane cooled with liquid nitrogen, and then sectioned (12 μm thickness). To assess regeneration, cross-sections were prepared for histological examination on a ScanScope CS microscope [Aperio] after staining with hematoxylin and eosin. Changes in fiber size distribution between muscles injected with Mef2D-expressing virus and the control were determined using a nonparametric [Wilcoxon] test. Fiber type switching was assessed by immunofluorescence using antibodies recognizing different isoforms of MHC. After immunostaining, coverslips were mounted in mounting medium with DAPI [Vector Laboratories, Inc., H-1500] and imaged using a Zeiss LSM 510 Meta confocal microscope.

Protein–protein interaction analysis

Nuclear extracts were prepared from differentiated C2 myotubes [5 d] as previously described [Weil et al. 1979] with the following modifications. Nuclear pellets were resuspended in one pellet volume of modified buffer C (20 mM Hepes at pH 7.6, 1.5 mM MgCl₂, 650 mM KCl, benzamid [2.5 U per 10⁶ cells], 0.2 mM PMSE, 0.5 mM DTT, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate) and rotated for 30 min at 4°C. Nuclear homogenates were then diluted with one pellet volume of buffer E (20 mM Hepes at pH 7.6, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM PMSE, 0.5 mM DTT, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate). Extracted proteins were recovered from the nuclei by centrifugation at 15,000g for 30 min at 4°C. Nuclear extracts were then quantitated for protein content prior to interaction studies.

For immunoprecipitation studies of endogenous proteins, 10 μg of specific antibody or rabbit IgG were prebound to 20 μL of protein A Dynabeads. Flag immunoprecipitations were performed using M2-agarose (Sigma). Mef2D-associated proteins were immunoprecipitated as previously described (Chaturvedi et al. 2012) and eluted by incubating with 5% acetic acid for 5 min at 22°C. The acetic acid was removed from the recovered proteins by evaporation using a SpeedVac [Eppendorf] before proceeding with Western blot analysis.

In the pull-down studies, baculovirus-expressed Flag-Mef2DΔ2 or Flag-Mef2DΔ2 proteins were purified from Sf9 insect cells using M2-agarose (Sigma) as previously described [Rampalli et al. 2007]. Briefly, M2-agarose-bound proteins were washed in stringent conditions (500 mM KCl) and then equilibrated in immunoprecipitation buffer (20 mM Hepes at pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSE, 0.5 mM DTT). Flag-Mef2D-bound M2 agarose beads were then incubated with nuclear extracts for 4 h at 4°C. After washing, proteins were eluted with 3X-Flag peptide (Sigma) and separated by SDS-PAGE. After visualization with Coomassie blue, protein bands were excised for mass spectrometry analysis.

Mass spectrometry

Protein identification was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada). Gel bands were in-gel-digested according to the method of Shevchenko et al. (2006). Peptide extracts were concentrated by SpeedVac [Eppendorf] and resuspended in 0.1% trifluoroacetic acid. Peptides were loaded onto a peptide trap (Zorbax SB300-C18, Agilent) for 5 min at 15 μL/min using a Dionex UltiMate 3000 RSLC nano high-pressure liquid chromatography [HPLC]. Peptides were eluted over a 60-min gradient of 3%–45% acetonitrile with 0.1% formic acid at 0.3 μL/min onto a 10-cm analytical column [New Objective PicoFrit self-packed with Zorbax C18] and sprayed directly into a LTQ Orbitrap XL hybrid mass spectrometer using a nanospray source [Thermo Scientific]. Mass spectra were acquired in a data-dependent fashion, with MS scans acquired in the Fourier Transform cell, while MS² scans were acquired in the ion trap module.

Tandem mass spectrometry [MS/MS] spectra were matched against a custom database comprised of mouse sequences in SwissProt [2011_07 version of uniprot_sprot.fasta.gz from ftp://

RNA-seq

C2iFRT-Mef2DΔ2, C2iFRT-Mef2DΔ2, or control [C2iFRT] cells were grown in proliferative conditions for 24 h in the presence of Dox. Near confluence, cells were then differentiated for a further 24 h in the presence of Dox. Total RNA was isolated from cells using the RNeasy minikit [Qiagen] using standard conditions. Library preparation and 100-bp paired-end RNA-seq were performed using standard Illumina procedures for the HiSeq 2000 platform.

The RNA-seq data were analyzed using the Bowtie, TopHat, CuffLinks, and CummeRbund software suite [Trapnell et al. 2012]. Briefly, paired-end 100-bp reads were mapped to the mm9 genome [University of California at Santa Cruz] and known mm9 transcripts (RefSeq mm9 build37.2) using Bowtie version 2.0.2 (Langmead and Salzberg 2012) with default parameters. Briefly, M2-agarose-bound proteins were washed in stringent conditions (500 mM KCl) and then equilibrated in immunoprecipitation buffer (20 mM Hepes at pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSE, 0.5 mM DTT). Flag-Mef2D-bound M2 agarose beads were then incubated with nuclear extracts for 4 h at 4°C. After washing, proteins were eluted with 3X-Flag peptide (Sigma) and separated by SDS-PAGE. After visualization with Coomassie blue, protein bands were excised for mass spectrometry analysis.

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Tandem mass spectrometry [MS/MS] spectra were matched against a custom database comprised of mouse sequences in SwissProt [2011_07 version of uniprot_sprot.fasta.gz from ftp://
Immunofluorescence

Cells were fixed using 4% paraformaldehyde, permeabilized with 0.5% Triton X-100/PBS, and then blocked with 5% BSA in PBS. Primary antibodies were diluted with PBS/5% BSA solution and incubated overnight at 4°C. After washing, cells were incubated with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, A11001) and/or Alexa Fluor 546 (Invitrogen, A11010). Prior to visualization, coverslips were mounted in Fluor 488 (Invitrogen, A11010) and/or Alexa Fluor 546 (Invitrogen, A11010). Images were taken using an Olympus FluoView FV1000 confocal microscope.

Kinase assay

Baculovirus-expressed Flag-Mef2Dα1 or Flag-Mef2Dα2 proteins were purified from Sf9 insect cells using M2-agarose (Sigma). His-Mef2D proteins (100 ng) were incubated with either His-p38 kinase or PKA and [γ-32P]ATP in a buffer containing 10 mM Hepes (pH 7.4), 50 mM KCl, 1.5 mM MgCl2, 10% glycerol, 0.5 mM EGTA, and 10 mM β-glycerophosphate for 30 min at 37°C. Reactions were then separated on 10% SDS-PAGE gels, dried, and exposed to autoradiographic film. Parallel reactions lacking [32P]ATP were run on 10% SDS-PAGE gels and stained for protein content with Coomassie blue.

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References


Martin JF, Miano JM, Hustad CM, Copeland NG, Jenkins NA, Olson EN. 1994. A MEF2 gene that generates a muscle-specific
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Tissue-specific splicing of a ubiquitously expressed transcription factor is essential for muscle differentiation

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References
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