SHORT REPORT

Rbfox proteins regulate tissue-specific alternative splicing of Mef2D required for muscle differentiation

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ABSTRACT

Among the Mef2 family of transcription factors, Mef2D is unique in that it undergoes tissue-specific splicing to generate an isoform that is essential for muscle differentiation. However, the mechanisms mediating this muscle-specific processing of Mef2D remain unknown. Using bioinformatic methods, we identified Rbfox proteins as putative modulators of Mef2D muscle-specific splicing. Accordingly, we found direct and specific Rbfox1 and Rbfox2 binding to pre-mRNA in vivo. Gain- and loss-of-function experiments demonstrated that Rbfox1 and Rbfox2 cooperate in promoting Mef2D splicing and subsequent myogenesis. Thus, our findings reveal a new role for Rbfox proteins in regulating myogenesis through activation of essential muscle-specific splicing events.

KEY WORDS: Mef2, Muscle differentiation, Rbfox, Alternative splicing

INTRODUCTION

Together with transcriptional control, alternative splicing is one of the means for organisms to create cell and tissue diversity (Kalsotra and Cooper, 2011). By the alternative usage of exons and splice sites, alternative splicing generates multiple transcript isoforms from the same pre-mRNA. Large-scale studies have revealed that alternative splicing is common in vertebrates (Wang et al., 2008) and affects mostly coding sequences, thus expanding the proteome by generating alternative protein products with distinct functions (Black, 2003; Nilsen and Graveley, 2010). Misregulation of alternative splicing is directly involved in a large variety of genetic disorders and multifactorial diseases as about one third of disease-related human mutations alter splicing (Singh and Cooper, 2012). Muscle was one of the first tissues in which broad use of alternative splicing to increase cellular complexity was documented (Llorian and Smith, 2011). During muscle differentiation, myoblasts give rise to multinucleated myotubes through a temporally ordered series of changes in alternative splicing that generates alternate isoforms of key myogenic factors (Bland et al., 2010; Trapnell et al., 2010). Several candidate cis-elements (Sugnet et al., 2006; Bland et al., 2010) and trans-acting splicing factors involved in regulating muscle-specific alternative splicing have been described. These include Mbnl (Pascual et al., 2006), Rbfox (Kuroyanagi, 2009), Celf (Dasgupta and Ladd, 2012) and Ptb families of proteins (Romanelli et al., 2013). However, how multiple splicing factors coordinate regulation of specific splicing events is poorly understood.

Among the genes that undergo a major isoform switch during muscle differentiation is Mef2D (Trapnell et al., 2010), which encodes a member of the Mef2 family of transcription factors, proteins that are key mediators of signal-dependent transcription controlling differentiation (Pothoff and Olson, 2007). During myogenesis, transcripts of the ubiquitously expressed Mef2D gene undergo alternative splicing to replace exon 1 with the alternate exon 2, which generates a muscle-specific transcriptional activator (Martin et al., 1994; Sebastian et al., 2013). This muscle-specific isoform of Mef2D is able to evade inhibitory PKA signaling, and commits differentiating myoblasts to activate the late-muscle gene expression program (Sebastian et al., 2013). However, the molecular mechanism regulating Mef2D isoform switch ensuring proper temporal control of myogenesis is currently unknown.

Here, we identify the Rbfox1 and Rbfox2 splicing factors as major regulators of Mef2D splicing during muscle differentiation. We provide genetic and biochemical evidence that Rbfox1 and Rbfox2 bind to a specific motif in the intron downstream of the Mef2D exon 2, which leads to its inclusion in myotubes. Consistently, Rbfox1 and Rbfox2 gain- and loss-of-function leads to alterations in Mef2D expression and in muscle differentiation. Our data provide evidence that combinatorial regulation of a single splice site by two splicing regulators determines the fate of an alternative splicing decision essential for myogenesis.

RESULTS AND DISCUSSION

Rbfox proteins are putative positive regulators of Mef2D muscle-specific splicing

In order to identify factors promoting Mef2D exon 2 inclusion in muscle, we used two bioinformatics tools (see Materials and Methods) to conduct an in silico search for putative splicing-factor-binding sites. We focused our analyses on families of splicing factors with a known role in muscle differentiation: Celf, Mbnl, Ptb and Rbfox (Pascual et al., 2006; Kuroyanagi, 2009; Dasgupta and Ladd, 2012; Hall et al., 2013) (supplementary material Fig. S1). Given that the activity (stimulatory or inhibitory) of a splicing factor is often dependent on the position of its target site with respect to the regulated exon (Pascual et al., 2006; Kuroyanagi, 2009; Dasgupta and Ladd, 2012; Hall et al., 2013), we then linked the position of the identified putative binding sites to the predicted output (Mef2D exon 2 inclusion or skipping). Finally, we compared the expression profiles of the different splicing factors to the splicing pattern of Mef2D during a muscle differentiation time course.
A putative binding site for Celf proteins is located downstream of MeF2D α2 exon and, in this position, they would promote exon inclusion (Pascual et al., 2006; Dasgupta and Ladd, 2012). Nevertheless, Celf proteins were not investigated further because their expression levels either do not change or decrease during myogenesis (supplementary material Fig. S2). For similar reasons and because of its broad binding site distribution along MeF2D transcript, we also discounted Mbnl1 and Mbnl2 (supplementary material Fig. S3). By contrast, the biological activity (Grammatikakis et al., 2011; Hall et al., 2013) and the expression pattern of Ptb family, as well as Mbnl3, suggest that they are putative repressors of MeF2D α2 exon inclusion in myoblasts (supplementary material Fig. S4). Finally, a canonical Rbfox-family-binding site (UGCAUG) is located 34 nt downstream of MeF2D α2 exon (Fig. 1A), a position in which it is expected to promote MeF2D α2 exon inclusion (Kuroyanagi, 2009). Intriguingly, Rbfox1 has an expression profile that resembles MeF2D α2 expression during differentiation (Fig. 1B–D). Like
Mef2D a2 (Fig. 1D) (Martin et al., 1994; Sebastian et al., 2013), Rbfox1 is strongly upregulated during muscle differentiation, with a peak of expression at late stages of differentiation, when the bulk of myoblast fusion occurs (Fig. 1B,C). By contrast, Rbfox2 is already present in proliferating myoblasts and its expression does not undergo a dramatic increase during muscle differentiation (Fig. 1B,C). Whereas the possible role of the Ptb family or Mbnl3 as possible negative regulators of Mef2D a2 exon inclusion deserves future work, our results prompted us to further investigate Rbfox proteins as positive regulators of Mef2D muscle-specific splicing.

Rbfox1 and Rbfox2 specifically associate with Mef2D pre-mRNA in vivo
To assess whether Rbfox family members were actually recruited onto the Mef2D primary transcript in muscle cells, we performed endogenous RNA immunoprecipitation following ultraviolet crosslinking (UV-RIP) with antibodies specific for Rbfox1, Rbfox2 or Mef2D a1 and a2 isoforms.

Fig. 2. Alteration of Rbfox1 or Rbfox2 levels affects Mef2D muscle-specific splicing. (A) RT-qPCR of Rbfox1 and Rbfox2 expression in myotubes (MT) transfected with siRNAs targeting selectively Rbfox1 (siRbfox1) or Rbfox2 (siRbfox2), a mixture of both siRNAs (siRbfox1+2) or a non-silencing control (siNSL). (B) Immunoblotting of Rbfox1 and Rbfox2 proteins from cells in A. Tubulin is used as loading control. (C) RT-qPCR of Mef2D isoforms expression in the same cells as in A. (D) RT-qPCR of Mef2D isoforms expression in myoblasts (MB) transfected with either pIRES-Rbfox1 or pIRES-Rbfox2 or both. EV, empty vector control. (E) Immunoblotting of Rbfox1 and Rbfox2 proteins from cells as in D. Tubulin is used as loading control. (F) RT-qPCR of Mef2D splicing pattern in the same cells as in D. Rbfox1 and Rbfox2 expression levels are relative to Gapdh. Mef2D splicing isoforms levels are relative to the total amount of Mef2D transcripts, assayed by a primer set on a constitutive exon (Mef2D all isof.). Data in D and F are represented as fold expression over EV control. Results are mean ± s.e.m., n≥3. *P<0.05; **P<0.01; ***P<0.001 (Student’s t-test).
Rbfox2 or IgG, as control. UV-RIP was performed in multinucleated myotubes, where Mef2D α2 and Rbfox splicing factors are all expressed. UV-RIP was followed by reverse transcription-quantitative PCR (RT-qPCR) to quantify the binding enrichment of Rbfox proteins over the input on specific sites. As shown in Fig. 1E, both Rbfox1 and 2 display a significant enrichment on the Mef2D pre-mRNA region containing the Rbfox-binding site (Mef2D Rbfox BS) when compared to both an upstream region on the same pre-mRNA (Mef2D exon 2) or a region in the non target pre-mRNA Gapdh, used as controls.

As UV light crosslinks proteins that directly interact with RNA, and RT-qPCR allows detection of enrichment on a specific Mef2D

Fig. 3. Rbfox1 and Rbfox2 are both required for muscle differentiation. (A) RT-qPCR of Rbfox1 and Rbfox2 expression in myotubes (MT) infected with lentiviral particles (LV) carrying a shRNA targeting either Rbfox1 (shRbfox1), Rbfox2 (shRbfox2), a mixture of both (shRbfox1+2) or a non-silencing control (shNSL). Data are normalized over Gapdh and shown as fold expression over shNSL control. (B) RT-qPCR of Mef2D isoforms expression in the same cells as in A, normalized to the amount of total Mef2D transcripts (Mef2D all isof.) and shown as fold expression over shNSL control. (C) Immunoblotting analysis of endogenous Mef2D α1 and α2 isoforms in stable Rbfox double-depleted cells. Mef2D pan antibody is used as loading control. (D) Representative immunostaining of myosin heavy chain (Mhc, green) in differentiated cells as described in A. Hoechst 33342 was used to stain nuclei (blue). Scale bars: 200 μm. (E) Quantification of terminally differentiated myotubes in the cell types shown in D. (F) Immunoblotting of Mhc in the same cells as in D. Tubulin is used as loading control. Results are mean±s.e.m., n≥3. *P<0.05; **P<0.01; ***P<0.001 (Student’s t-test).
pre-mRNA region, our data suggest that both Rbfox1 and 2 proteins bind directly and specifically to their target site in Mef2D primary transcript, downstream of the muscle-specific exon α2. Given that Rbfox1 and Rbfox2 can directly interact with each other (Lim et al., 2006), they might work together to promote the inclusion of Mef2D α2 exon in multinucleated myotubes. Intriguingly, a similar cooperation has been already described in zebrafish where the paralogs rbfox11 and rbfox2 regulate partially overlapping networks of alternative splicing events in muscle (Gallagher et al., 2011).

Alterations of Rbfox1 or Rbfox2 levels cause corresponding changes in Mef2D α2 exon splicing

To test our hypothesis, we first transfected proliferating muscle cells with small interfering RNA (siRNA) against Rbfox1, Rbfox2 or both in comparison to a non-silencing siRNA, used as control (Fig. 2A,B). Then, muscle differentiation was induced and the effect of Rbfox silencing on Mef2D α2 exon inclusion was evaluated. Upon transient, selective depletion of either Rbfox1 or Rbfox2 (Fig. 2A,B), we observed a significant reduction of Mef2D α2 isoform that was accompanied by a significant increase of Mef2D α1 splice variant in accordance with the mutually exclusive nature of the two exons (Fig. 2C). The result was also confirmed when both Rbfox1 and Rbfox2 were depleted at the same time (Fig. 2C).

To evaluate the effect of Rbfox1 and Rbfox2 gain-of-function on Mef2D splicing, we transfected proliferating myoblasts with cDNAs encoding either Rbfox1 or Rbfox2 or both (Fig. 2D,E). As shown in Fig. 2F, Rbfox1 and/or Rbfox2 overexpression caused a significant increase in Mef2D α2 exon inclusion when compared to control cells transfected with empty vector (Fig. 2F). Interestingly, Rbfox1 seemed to promote Mef2D α2 exon inclusion with similar strength as Rbfox2, underlying the important contribution of both proteins to Mef2D muscle-specific splicing. Notably, the transient overexpression was performed at myoblast stage, when Mef2D α2 and Rbfox1 are nearly absent, indicating that Rbfox proteins do not require any additional factors to promote Mef2D α2 exon inclusion.

Collectively, our results strongly indicate that Rbfox1 or Rbfox2 is required and sufficient to promote Mef2D α2 isoform production in muscle.

Fig. 4. The differentiation defect of Rbfox1 and Rbfox2 double-knockdown cells is selectively rescued by Mef2D α2. (A) Representative immunofluorescence images for myosin heavy chain (Mhc) or DAPI performed in C2C12 myotubes stably expressing shRNAs targeting both Rbfox1 and Rbfox2 (shRbfox1+2) or a non-silencing control (shNSL) infected with retroviruses expressing either the ubiquitous (Mef2D α1) or muscle-specific (Mef2D α2) isoforms of Mef2D. Scale bars: 100 μm. (B) Quantification of terminally differentiated myotubes in the cell types shown in A. Values are presented as mean±s.e.m., n≥3. **P<0.01; ns, not significant (Student’s t-test). (C) Mef2D splicing isoforms overexpression visualized using Mef2D pan antibody.
**Rbfox1 and Rbfox2 are both required for muscle differentiation**

Given that Mef2D α2 is essential for myogenesis (Sebastian et al., 2013), we investigated the relative contribution of Rbfox1 and 2 to muscle differentiation. For this purpose, we generated stable single and double Rbfox-knockout down cell lines. Cells were collected 3 days after the induction of muscle differentiation, and Mef2D splicing was evaluated by RT-qPCR. In line with the transient Rbfox depletion results, single and double Rbfox stable depletion caused a significant reduction of Mef2D α2 exon inclusion and a significant increase of the mutually exclusive Mef2D α1 splice variant, when compared to non-silencing control cells (Fig. 3A,B). Results were confirmed at protein levels (Fig. 3C). To assess the phenotypic effect on muscle differentiation, we performed immunofluorescence staining and western blot analyses of myosin heavy chain (Mhc), a marker of terminal muscle differentiation. Despite the fact that Rbfox proteins are regulated differently during muscle differentiation, stable knockdown of either Rbfox1 or Rbfox2 caused a significant impairment of muscle differentiation compared to control cells (Fig. 3D–F). In particular, depleted cells either failed to fuse or they formed very thin myotubes compared to the control differentiated cells (Fig. 3D). This failure to form multinucleated myotubes in the absence of Rbfox1 or Rbfox2 was accompanied by a decreased expression of muscle differentiation genes such as Myh3 (Fig. 3F), a result similarly observed previously (Singh et al., 2014).

**Mef2D α2, but not α1, specifically rescues myogenic defects caused by Rbfox depletion**

We have previously shown that Mef2D α2 is required for muscle differentiation (Sebastian et al., 2013). Given that Rbfox1 and Rbfox2 depletion inhibits Mef2D α2 production and muscle differentiation, we examined whether the ectopic Mef2D α2 expression could rescue the muscle impairment in cells lacking Rbfox. To test this, we infected cells depleted for both Rbfox1 and Rbfox2 with retroviral vectors carrying either Mef2D α1 or α2 splice variant and quantified muscle differentiation. Although the Rbfox-depleted cells ectopically expressing Mef2D α1 still failed to differentiate, the expression of Mef2D α2 isoform significantly rescued the myogenic defects due to Rbfox loss (Fig. 4). Taken together, our results provide a strong link between Rbfox expression, Mef2D α2 production and muscle differentiation.

**Conclusions**

Alternative splicing is a major contributor to the increased proteome complexity of higher eukaryotes. Alternative splicing widely occurs in muscle and aberrant alternative splicing contributes to the major forms of muscular dystrophy. However, the molecular mechanisms regulating alternative splicing and ensuring proper temporal control of myogenesis are poorly understood. In this study, we focused our attention on the post-transcriptional regulation of Mef2D and, in particular, to a peculiar alternative splicing event that gives rise to Mef2D α2, a muscle-specific isoform needed to promote transcription of late differentiation genes. Based on our results, we propose that the main players of this regulatory network are Rbfox1 and Rbfox2 that together promote Mef2D α2 exon inclusion. Rbfox2 seems to be the major regulator as, when depleted in muscle cells, it strongly disrupts Mef2D splicing pattern. However, Rbfox1 appears to be required as well, because its overexpression strongly stimulates Mef2D α2 exon inclusion. Accordingly, Rbfox1 and Rbfox2 are both required for myogenesis as muscle differentiation impairment occurs when both proteins are depleted.

In conclusion, we have characterized a new role for Rbfox1 and Rbfox2 as key mediators of Mef2D splicing that generate a form of the transcription factor that is essential for terminal muscle differentiation.

**MATERIALS AND METHODS**

**In silico analysis**

The region of Mef2D primary mRNA including α2 exon and 200 nt upstream and downstream of it was analyzed for the presence of putative splicing factors binding sites with the following software: http://sfnmap.technion.ac.il/index.html; http://www.intromi.it/splicing.html. Output candidates with a known role in muscle were further investigated as described in the text.

**RNA extraction, reverse transcription and RT-qPCR**

RNA purification from myoblasts and myotubes, DNaseI treatment, cDNA synthesis and qPCR analysis were performed as previously described (Neguembor et al., 2013). PCR primers are listed in supplementary material Table S1. Normalization details are provided in the figure legends for each data set.

**Cell culture, transfection, generation of stable lines and fusion index analysis**

C2C12 cells culture, differentiation and fusion index analyses were performed as previously described (Neguembor et al., 2013). For transient depletion experiments, 24 h after induction of differentiation cells were transfected with siRNA against Rbfox1 and/or Rbfox2 or non-silencing control (SMARTpool: ON-TARGETplus Rbfox1 L-041929-01, Rbfox2 L-051552-01, non-silencing control D-001810-10; Thermo Scientific) following the manufacturer’s instructions. Cells were collected 3 days after transfection. For overexpression experiments, cells were transfected with pIREs:Rbfox1, pIREs:Rbfox2 or the empty control using Lipofectamine-LTX (Invitrogen) and collected 24 h after transfection. Stable knockdown cells were generated by lentiviral transduction with pLKO.1 constructs carrying short hairpin RNA (shRNA) for Rbfox1 (5′-CCCAACTAGACACAACTTCTGAAA-3′) and/or Rbfox2 (5′-GCCATTTAAGAGTTTAGTAA-3′) or non-silencing control (5′-TTCATCTTGTCGACGAGGAT-3′), according to the manufacturer’s instructions (Dharmacon), and maintained as polyclonal populations under 0.5 μg/ml puromycin selection.

**UV-crosslinked RNA immunoprecipitation**

The UV-RIP assay was carried out essentially as described previously (Cabanian et al., 2012). Wild-type C2C12 cells were seeded in 15-cm dishes for each RNA immunoprecipitation. 72 h after differentiation cells were UV crosslinked (two irradiations of 100,000 μJ/cm²), lysed and treated with 30 U of Turbo DNase (Ambion). 10% of supernatant was saved as input and the rest used in RNA immunoprecipitation. 10 μg of the following antibodies were used for the immunoprecipitation: anti-Rbfox1 (LS-C181983, LSBio), anti-Rbfox2 (A300-864A, Bethyl) or anti-lgG (Jackson Laboratory) antibodies. RNA was extracted with Trizol and analyzed by RT-qPCR. All experiments were repeated at least three times.

**Immunoblotting**

Immunoblots were performed using the following antibodies: anti-Rbfox1 [1D10, as described previously (Lee et al., 2009)], anti-Rbfox2 (#A300-864A, Bethyl Laboratories), anti-Mef2D (pan-Mef2D; #610774, BD Biosciences), anti-Mef2D α1 and α2 (as previously described in Sebastian et al., 2013), anti-Mhc (MF20 from the Developmental Studies Hybridoma Bank) and anti-tubulin (#049K4767, Sigma) as loading control.

**Rescue of Rbfox differentiation defect with Mef2D isoforms**

Rescues of Rbfox knockdown by Mef2D overexpression were performed as described in Sebastian et al., 2013. Subconfluent control shNSL cells
and cells depleted of both Rbfox1 and Rbfox2 (ahRbfox1+2) were infected with retrovirus expressing either Mef2D α1 or Mef2D α2 for 2 days. Infected cells were plated and differentiated for 3 days with 2% horse serum and insulin. Fusion indexes were calculated as previously described (Neguembor et al., 2013). The overexpression of Mef2D was visualized using a Mef2D pan antibody (#610774, BD Biosciences).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0a (GraphPad Software, San Diego). Statistical significance was calculated by Student’s t-test on at least three independent experiments. P-values are denoted as *P<0.05; **P<0.01; ***P<0.001. Details of each dataset are provided in the corresponding figure legends.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

V.R. designed and performed experiments, analyzed results, and wrote the manuscript. S.S. performed Mef2D rescue and immunoblot. F.J.D. and D.G. designed experiments, analyzed results and wrote the manuscript.

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Supplementary material

Supplementary material available online at http://jcs.biologists.org lookup/supp doi:10.1242/jcs.161059/DC1

References


Figure S1. Position of putative binding sites for splicing factors with a role in muscle in Mef2D pre-mRNA. Sequence of the region of Mef2D transcript including α2 exon and 200 nt upstream (on the top) and downstream of it (at the bottom). Each square identifies the target sequence of a specific splicing factor, following the color code present in the scheme: yellow for Mbnl, blue for Ptb, black for Rbfox and pink for Celf.
Figure S2. Celf expression levels either do not change or decrease during myogenesis. A RT-qPCR of the members of Celf proteins. Celf4 is not shown as it is not expressed in muscle. B Scheme of the position-dependent function of Celf.
Figure S3. Mbnl1/2 expression levels during myogenesis.
A RT-qPCR of Mbnl1 and 2. B Scheme of the position-dependent function of Mbnl1 and 2.
A

**Mbnl3**

![Graph](image1)

**Ptb**

![Graph](image2)

B

![Diagram](image3)

**Figure S4.** Mbnl3 and Ptb are putative repressors of Mef2D α2 exon inclusion.

Table S1. List of primers

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