Rbfox2-Coordinated Alternative Splicing of Mef2d and Rock2 Controls Myoblast Fusion during Myogenesis

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SUMMARY

Alternative splicing plays important regulatory roles during periods of physiological change. During development, a large number of genes coordinately express protein isoform transitions regulated by alternative splicing; however, the mechanisms that coordinate splicing and the functional integration of the resultant tissue-specific protein isoforms are typically unknown. Here we show that the conserved Rbfox2 RNA binding protein regulates 30% of the splicing transitions observed during myogenesis and is required for the specific step of myoblast fusion. Integration of Rbfox2-dependent splicing outcomes from RNA-seq with Rbfox2 iCLIP data identified Mef2d and Rock2 as Rbfox2 splicing targets. Restored activities of Mef2d and Rock2 rescued myoblast fusion in Rbfox2-depleted cultures, demonstrating functional cooperation of protein isoforms generated by coordinated alternative splicing. The results demonstrate that coordinated alternative splicing by a single RNA binding protein modulates transcription (Mef2d) and cell signaling (Rock2) programs to drive tissue-specific functions (cell fusion) to promote a developmental transition.

INTRODUCTION

The basic transcription and cell signaling events required for myoblast differentiation are well characterized (Braun and Gautel, 2011; Hindi et al., 2013). Previous results from our lab and others (Bland et al., 2010; Hall et al., 2013; Trapnell et al., 2010) have demonstrated extensive alternative splicing transitions during myoblast differentiation. However, splicing factors responsible for generation of muscle-specific isoforms of genes during myogenesis are largely unknown. In addition, we have previously shown collective alternative splicing of several transcripts in distinct temporal clusters during myoblast differentiation; however, it is not well understood if these muscle-specific protein isoforms cooperate to control specific aspects of the myogenic program (Bland et al., 2010). Misregulated splicing contributes to disease phenotypes in skeletal muscle in diseases such as myotonic dystrophy (DM) and facioscapulohumeral muscular dystrophy (FSHD), demonstrating an essential role of regulated splicing for proper skeletal muscle function (Gabellini et al., 2006; Pistoni et al., 2013; Singh and Cooper, 2012).

The Rbfox family of RNA-binding proteins is highly conserved from C. elegans to mammals and has been shown to regulate alternative splicing (Gallagher et al., 2011; Kuroyanagi et al., 2007; Zhang et al., 2008). Mammals contain three Rbfox paralogs, Rbfox1 (A2BP1), Rbfox2 (RBM9), and Rbfox3 (NeuN or HRNBP3). Rbfox1 and Rbfox2 are expressed in brain, heart, and skeletal muscle; Rbfox2 is also expressed in ovary, hematopoietic cells, and embryonic stem cells, while Rbfox3 expression is restricted to neurons (Jin et al., 2003; Kim et al., 2009; Underwood et al., 2005). Nestin-cre mediated knockout of Rbfox1 or Rbfox2 in brain produced increased susceptibility to seizures and compromised cerebellum development, respectively (Gehman et al., 2011, 2012). The finding of differing phenotypes for Rbfox1 or Rbfox2 loss of function despite largely overlapping expression suggests that the paralogs perform separable biological functions. Consistent with this, splicing analysis showed that some exons are more responsive to either Rbfox1 or Rbfox2 (Gehman et al., 2011, 2012).

Rbfox homologs have been shown to regulate muscle-specific splicing in C. elegans (Fukumura et al., 2007; Kuroyanagi et al., 2007). Similarly, a role for Rbfox homologs in zebrafish heart...
and skeletal muscle development was demonstrated by morpholino-mediated knockdown of both Rbfox1 and Rbfox2 that showed reduced heart rate, abnormal morphology of skeletal muscle myofibril arrangement, and complete muscle paralysis (Gallagher et al., 2011). While Rbfox1 and Rbfox2 are highly expressed in adult mammalian skeletal muscle (Jin et al., 2003; Underwood et al., 2005), few Rbfox muscle-specific splicing targets have been identified, and a role for Rbfox proteins in myogenesis has not been defined.

Our previous computational analysis of alternative exons regulated during myoblast differentiation identified the Rbfox binding motif as highly enriched and conserved within the introns flanking regulated exons (Bland et al., 2010). Here we show that Rbfox2, but not Rbfox1, is required for myoblast differentiation. Rbfox2 depletion does not disrupt the core myogenic program but rather prevents myoblast fusion, a late and essential step of muscle differentiation. To identify Rbfox2 targets required for fusion, we first defined the Rbfox regulatory network using RNA-seq of Rbfox2-depleted cultures. The results indicate that Rbfox2 regulates different gene sets with regard to both gene expression and alternative splicing in undifferentiated and differentiated cells. Rbfox2 knockdown expression of >6,000 genes, but the effects are relatively small, with less than 2-fold change for 75% of the genes. However, Rbfox2 depletion has a strong effect on alternative splicing. Rbfox2 represses the differentiated patterns of both splicing and gene expression in undifferentiated cells and activates myogenic patterns upon differentiation. To identify differentiation-associated splicing transitions that are directly associated with Rbfox2, we performed individual-nucleotide resolution UV-crosslinking and immunoprecipitation (iCLIP). Among these events, we identified performed individual-nucleotide resolution UV-crosslinking and complete muscle paralysis (Gallagher et al., 2011). While Rbfox1 and Rbfox2 are highly expressed in adult mammalian skeletal muscle (Jin et al., 2003; Underwood et al., 2005), few Rbfox muscle-specific splicing targets have been identified, and a role for Rbfox proteins in myogenesis has not been defined.

The fusion defect in Rbfox2-depleted cultures was rescued by expression of a siRNA-resistant Rbfox2 cDNA using a doxy-cycline inducible lentiviral construct (Figures S1B and S1C) (Meerbrey et al., 2011). The knockdown of endogenous Rbfox2 and expression of exogenous myc-tagged Rbfox2 in these cultures was confirmed by immunostaining (Figure S1B) and western blotting (Figure S1D). Additionally, expression of myc-Rbfox2 restored all tested Rbfox2-dependent splicing changes (Figure S1E). These results indicate that inhibition of myoblast fusion by Rbfox2 knockdown was a direct effect of Rbfox2 loss of function.

Given that Rbfox1 is induced during differentiation, we were surprised to find that Rbfox1 depletion only moderately affected C2C12 differentiation (Figures 1B and 1C). In addition, the fusion defect resulting from Rbfox2 knockdown was not rescued by lentiviral-mediated expression of Rbfox1 (data not shown) indicating that Rbfox1 is not sufficient to rescue the defects caused by loss of Rbfox2. Consistent with minor effects on differentiation and in contrast to the effects of Rbfox2 knockdown, RNA-seq analysis indicated that knockdown of Rbfox1 in differentiated cultures showed little effect on the transcriptome (see below). Interestingly, knockdown of Rbfox2 resulted in downregulation of Rbfox1, while knockdown of Rbfox1 did not produce changes in Rbfox2 expression (Figures 1D and S2A).

To characterize the molecular step of myoblast differentiation that is inhibited by Rbfox2 depletion, we performed western blotting for known markers of differentiation (Figure 1A). The results indicated that expression of myogenin, Mef2a, cyclinD1 as well as late (Mef2c, Myh3, fast skeletal muscle troponin T, phospho-Erk) markers of differentiation was largely unaffected in Rbfox2 knockdown cultures. These results indicated that loss of Rbfox2 did not disrupt the core of the myogenic program but rather blocked a specific and critical step required for fusion of myoblasts to form multinucleated myotubes.

**RESULTS**

**Rbfox2 Is Required for Myoblast Fusion**

To analyze Rbfox function in skeletal muscle, we used the C2C12 mouse myoblast cell line which is derived from adult muscle stem (satellite) cells (Blau et al., 1985; Yaffe and Saxel, 1977). When cultured in high-serum medium, these cells rapidly proliferate as mononucleated myoblasts with myogenic potential. Withdrawal of serum when cells become confluent leads to up-regulation of myogenic transcription factors, exit from the cell cycle, cell alignment, and fusion into multinucleated myotubes that resemble immature myofibers. Our western blot and RNA-seq data indicated that Rbfox1 RNA and protein are undetectable in undifferentiated cultures and are expressed upon differentiation (Figures S1A and S2A available online). Rbfox2 is expressed in both cell states, increasing slightly upon differentiation (Figure 1A, top panel; Figure S1A). To investigate the role of Rbfox proteins in muscle differentiation, we performed siRNA-mediated knockdown of Rbfox2 or Rbfox1 in undifferentiated cultures and maintained the knockdown throughout differentiation (Figures 1A and 1D). Knockdown of Rbfox2 inhibited fusion of myoblasts during differentiation as indicated by significantly reduced fusion index (Figures 1B and 1C). Similar results were observed for five siRNAs targeting different constitutively spliced regions of the Rbfox2 mRNA, two of which are shown in Figures 1B–1D. As described previously for human embryonic stem cells (Yeo et al., 2009), Rbfox2 knockdown resulted in cell loss; however, Rbfox2 and control cells were standardized to >90% confluent upon differentiation induction. Importantly, while Rbfox2-depleted myoblasts do not fuse in differentiation conditions, the cells express Myh3, a late stage marker of differentiation (Figures 1A and 1B). This result indicated that a specific and late stage of myoblast differentiation was affected by loss of Rbfox2.

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**Rbfox-Mediated Transcriptome Changes during Muscle Differentiation**

To identify transcriptome changes that were responsive to Rbfox2 and/or Rbfox1, we performed RNA-seq using poly(A)+ RNA from undifferentiated and differentiated (day 4) cultures treated with Rbfox2 or scrambled siRNAs and differentiated cultures treated with Rbfox1 siRNAs. Knockdown of Rbfox1 and
Rbfox2 was confirmed by western blot analysis in cultures treated in parallel to those used for RNA-seq (Figure S2A). For most cultures, RNA-seq was performed in at least two biological replicates. Each RNA sample produced 132–169 million paired-end, 100 nt reads, of which 58%–88% mapped uniquely to the mouse genome (Supplemental Experimental Procedures). Technical and biological replicates demonstrated a high level of reproducibility both for differential gene expression (R² > 0.98) and alternative splicing (R² > 0.98) (Figures S2B and S2C).

Gene expression was determined based on calculated fragments per kilobase of transcript per million mapped reads (FPKM) and differentially expressed genes were identified by edgeR with a false discovery rate (FDR) less than 1% in each experimental condition (Robinson et al., 2010). Comparative analysis of gene expression in undifferentiated and differentiated cultures treated with scrambled siRNAs identified 6,412 differentially expressed genes (Figure 2A, top panel; Table S1 and Table S4). This result was comparable to previous RNA-seq results from a C2C12 differentiation time course (Trapnell et al., 2010). We also identified alternate promoter changes in 129 genes and 3’ UTR changes in 52 genes (Table S4). We identified 609 splicing events in 500 genes that underwent

Figure 1. Rbfox2, but Not Rbfox1, Is Required for Terminal Skeletal Muscle Differentiation

(A) Neither early nor late myogenic markers are affected by Rbfox2 depletion during differentiation. Western blots of myogenic markers from C2C12 cultures treated with scrambled or Rbfox2 siRNAs and harvested at the indicated days following addition of differentiation medium (U is undifferentiated), Ponceau S (Pon S) staining of western blot membrane.

(B) Depletion of Rbfox2 but not Rbfox1 prevents myoblast fusion. Cultures were immunostained (day 4) for myosin heavy chain (Myh3) and DAPI counterstained after treatment with the indicated siRNAs, scrambled (Scr), Rbfox2 siRNA #1 (F2s1), Rbfox2 siRNA #2 (F2s2), Rbfox1 siRNA #1 (F1s1).

(C) Quantified fusion indices from (B). Standard error of the mean from three independent cultures is shown. *** indicates p < 0.001 as determined by one-way ANOVA using Newman-Keuls posttest.

(D) Western blot of parallel cultures from (B) for indicated proteins. See also Figure S1.
differentiation-dependent splicing transitions with a change in percent spliced in (PSI) values of ≥20% (Wang et al., 2008). These were classified according to splicing pattern with cassette exons predominating (84%) (Table S2). We identified 268 genes that underwent both alternative splicing (ΔPSI ≥ 20%) and gene expression (FDR < 1%) transitions during differentiation and 232 genes that are exclusively regulated at the level of alternative splicing (Figure 2A, top panel), highlighting a role for regulated splicing during myogenesis.

RNA-seq showed relatively few changes in gene expression (154 genes with FDR < 1%) or alternative splicing (25 events with ΔPSI ≥ 20%) in response to Rbfox1 knockdown in differentiated cultures (Table S4; data not shown). In contrast, knockdown of Rbfox2 resulted in substantial effects on gene expression and alternative splicing in both undifferentiated and differentiated cultures (Table S4). Interestingly, the set of genes with altered expression versus the set with alternative splicing exhibited very little overlap, suggesting regulation of gene expression by two separate mechanisms in cells lacking Rbfox2 (Figure 2A, middle and bottom panels). With regard to differentially expressed genes, the effects of Rbfox2 depletion were relatively modest in that approximately 75% showed less than a 2-fold effect (FPKM > 5). Knockdown of Rbfox2 in undifferentiated or differentiated cultures resulted in differential expression of 945 genes, only 85 of which (9%) were affected in both cell states (Figure S2D; Table S1 and Table S4). Of the 502 genes whose expression was affected by Rbfox2 depletion in undifferentiated cells, most (84%) are regulated during differentiation, and 93% of these express the differentiated pattern upon Rbfox2 depletion, strongly suggesting a repressive role for Rbfox2 in the undifferentiated state (Figure S2D; data not shown). However, 364 of 528 (69%) genes affected by Rbfox2 knockdown in differentiated cultures are normally regulated during differentiation, and 50% (183) of these retain the undifferentiated pattern,
suggesting complex Rbfox2-mediated regulation of gene expression in the differentiated state (Figure S2D; data not shown). Altered gene expression in a Rbfox-dependent manner has been reported to occur indirectly in neurons by alternative splicing of transcription factors and/or signaling molecules that modulate the activity of transcription regulators (Fogel et al., 2012).

We validated the alternative splicing changes identified by RNA-seq by performing RT-PCR for more than 60 splicing events with ΔPSIs ranging from 20% to 90% based on RNA-seq data. Our overall validation rate was ~83%, demonstrating robust detection of alternative splicing by our computational analysis of RNA-seq data (Figure 2B; data not shown). Of the 609 splicing events that were regulated during differentiation, 182 (30%) were affected by Rbfox2 knockdown in either undifferentiated and/or differentiated cultures (ΔPSI ≥ 20%; Figure 2D; Table S2). As observed for the gene expression changes associated with Rbfox2 depletion, different splicing events were affected in undifferentiated or differentiated cultures, and loss of Rbfox2 produced the pattern of the other cell state (Figures 2D and 2E). These results indicate that Rbfox2 represses the differentially splicing pattern for one set of events in undifferentiated cells and activates splicing of other events in differentiated cultures. Some genes are expressed in both undifferentiated and differentiated states but are spliced preferentially in undifferentiated or differentiated cultures (Figures S2E and S2F; Table S2), suggesting differential activity of Rbfox2 in two states.

Because Rbfox2 is primarily known to be a splicing regulator and the gene expression changes associated with Rbfox2 depletion were modest relative to the splicing changes, we focused on alternatively spliced genes for follow up experiments. We performed ingenuity pathway analysis (IPA) to identify functional categories of genes that undergo splicing transitions during differentiation that are Rbfox2 dependent (182 events, Figure 2C). IPA identified significant enrichment of genes that control aspects of cell-signaling pathways such as RhoA, calcium signaling, and chemokine signaling that have been shown to regulate fusion of mammalian myoblasts (Figure 2F) (Hindi et al., 2013). This analysis provided a data set of splicing events that we used to investigate the mechanism of the fusion defect observed in the absence of Rbfox2.

**Identification of Direct Rbfox2 Splicing Targets**

We performed an unbiased de novo motif analysis using multiple em for motif elicitation (MEME) to identify enriched hexamers within 150 nt flanking cassette exons altered in undifferentiated or differentiated cultures by Rbfox2 knockdown (Bailey et al., 2006). The most enriched hexamer both upstream and downstream was the Rbfox binding motif, UGCAUG. Combining motif analysis with RNA-seq data demonstrated that this motif strongly correlated with increased inclusion or skipping in Rbfox2 knockdown when located upstream or downstream of the exon, respectively (Figure 3A). This is also consistent with previous observations that Rbfox binding downstream of the regulated exon promotes inclusion of the exon, whereas binding upstream inhibits inclusion of the downstream exon (Yeo et al., 2009; Zhang et al., 2008). Moreover, the motif was found particularly enriched within 200 nt of the regulated exon (Figure 3A).

To separate splicing events affected by Rbfox2 depletion due to secondary effects from those most likely to be direct Rbfox2 splicing targets, we performed iCLIP analysis in undifferentiated and 4-day differentiated C2C12 cultures as described (König et al., 2010). Cell extracts from UV-exposed cultures were used to isolate Rbfox2-specific protein-RNA complexes for library preparation. The specificity of the library for RNA associated with Rbfox2 was demonstrated upon isolation of the Rbfox2-RNA complexes and cDNA library preparation (Figures 3B and 3C). The reads from four replicates performed in both undifferentiated and differentiated cultures were merged for a total of 10,399,805 reads from undifferentiated cultures, and 6,216,495 reads for differentiated cultures, 58% and 61% of which mapped to the genome, respectively. These mapped reads were subsequently converted to 270,293 (undifferentiated cultures) and 207,831 (differentiated cultures) cDNA counts for quantitative assessment of iCLIP targets as previously described (König et al., 2010). More than 97% of these cDNAs mapped to the sense strand indicative of high-quality iCLIP data. The five most enriched motifs surrounding the cross-linked position were variations of the Rbfox2 motif in both undifferentiated and differentiated cultures (Figure 3D). Rbfox2 binding was most enriched in intronic sequences (71% in undifferentiated cultures and 62% in differentiated cultures, Figure 3E). The distribution of Rbfox2-crosslinked sites within 500 nt upstream and downstream of all 375 cassette exons alternatively spliced in Rbfox2 knockdown (PSI ≥ 20%) showed significant binding of Rbfox2 downstream of Rbfox2-responsive exons compared to alternative exons not regulated by Rbfox2 (>3-fold) and constitutive exons (>5.5-fold) (Figure 3F). Using an analysis to associate two or more CLIP tags upstream or downstream of a cassette exon with Rbfox2-dependent skipping or inclusion, respectively, we identified Rbfox2-dependent splicing changes in 67 genes (Table S3) that were evaluated for potential roles in myoblast fusion.

**Rbfox2 Directly Regulates Muscle-Specific Alternative Splicing of Mef2d**

Mammals contain four paralogs of the Mef2 transcription factor (Mef2a, Mef2b, Mef2c, and Mef2d). Mef2a, Mef2c, and Mef2d are expressed in adult skeletal muscle and C2C12 cells, and all three genes contain mutually exclusive alpha exons and a cassette beta exon that undergo muscle-specific splicing (Morisaki et al., 1997; Potthoff and Olson, 2007). Our combined RNA-seq and iCLIP analyses identified the Mef2d beta exon as a target of Rbfox2 (Figure S3A; Table S3). This exon is included during differentiation and skipped in Rbfox2 knockdown, validating our approach to identify direct Rbfox2 targets (Figure 4A). Our RNA-seq data also indicated that transition of the Mef2d alpha exon required Rbfox2 (Figure 4A). We confirmed the Rbfox2-dependent transition of both exons during differentiation by RT-PCR (Figure 4B). Interestingly, the Mef2d protein isoforms produced from mutually exclusive splicing of the alpha exons have recently been shown to have opposite effect on skeletal muscle differentiation (Sebastian et al., 2013), supporting Mef2d as a critical splicing target for Rbfox2-dependent myoblast fusion. Rbfox2 knockdown also significantly reduced inclusion of the Mef2a beta exon and moderately affected differentiation-dependent splicing of the Mef2a and Mef2c alpha
exons (Figure S3B). Western blot analysis and quantitative RT-PCR analysis indicate modest but significant upregulation of Mef2a and Mef2d, indicating increased expression of undifferentiated isoforms in differentiated conditions (Figures 1A and S3C), raising the possibility of dominant-negative effects.

To test if the Mef2d alpha and beta exons were directly regulated by Rbfox2, we constructed minigenes containing genomic regions including the mutually exclusive alpha exons or cassette beta exon in a heterologous splicing reporter (Figures 4C and 4D) (Singh and Cooper, 2006). Both sets of exons contain Rbfox binding motifs within the vicinity of iCLIP crosslinks. Cotransfection of Mef2d alpha or beta exon minigenes with exogenously expressed Flag-tagged Rbfox2 in CosM6 cells promoted the differentiated splicing patterns from both minigenes (Figures 4C and 4D). Furthermore, mutation of the putative Rbfox binding site(s) in the minigenes abrogated the Rbfox2-dependent splicing effects in these cells, demonstrating that the Mef2d alpha and beta exons are direct splicing targets of Rbfox proteins. Separate mutation analysis demonstrated that binding of Rbfox2 downstream rather than upstream of the alpha exons is the dominant mechanism of Rbfox2-mediated regulation (data not shown).

**Rbfox2-Regulated Splicing of Mef2d and Rock2 Produces Muscle-Specific Activities**

To determine whether the muscle-specific isoform of Mef2d could rescue the fusion defect in Rbfox2 depleted cultures, we used...
lentivirus constructs for doxycycline-inducible expression of myc-tagged Mef2d isoforms in Rbfox2 knockdown cultures (Meerbrey et al., 2011). Immunofluorescence staining and western blot analyses confirmed depletion of endogenous Rbfox2 and comparable levels of expression of the myc-Mef2d isoforms that predominant either in myoblasts (Mef2d\(_{\alpha1}\)) or myotubes (Mef2d\(_{\alpha2}\)) (Figures 5A–5D). Expression of the muscle-specific Mef2d isoform (Mef2d\(_{\alpha2}\)) for myoblasts (Mef2d\(_{\alpha1}\)) produced a significant but partial rescue of myoblast fusion compared to uninduced cultures (Figures 5A–5C). We conclude that Rbfox2-mediated splicing of the muscle-specific Mef2d isoform is one of what is likely to be multiple Rbfox2-depend-
et al., 2001). Given that Rock kinases are regulated by autoinhibition via interaction of N-terminal kinase domains with C-terminal domains (Amano et al., 1999), we hypothesize that during differentiation, inclusion of one or both Rbfox2-dependent Rock2 alternative exons promotes intramolecular interactions, which inhibits the kinase activity. To test this hypothesis, we performed coimmunoprecipitation (coIP) experiments using a Flag-tagged N-terminal fragment of Rock2 with myc-tagged C-terminal fragments of Rock2 containing either no alternative exons (C0) or one (C1) or both (C2) alternative exons (Figure 6A). Our results demonstrated that Rock2 C termini containing one or both alternative exons interacted with the Rock2 N terminus. In contrast, the Rock2 C terminus lacking the alternative exons did not interact with the Rock2 N terminus (Figure 6A). Therefore, we conclude that Rbfox2-regulated splicing of Rock2 promotes intramolecular interaction and inactivation of its kinase activity.

Combinatorial Control of Mef2d and Rock2 Splicing Is Required for Myoblast Fusion

Because Rbfox2 coordinates splicing of Mef2d and Rock2 during differentiation, we wanted to determine whether there was a combined effect of Rbfox2-mediated isoforms on myoblast fusion. Therefore, we tested the effect of expressing the muscle-specific Mef2d isoform together with Rock2 depletion in cells lacking Rbfox2 (Figures 6B and 6C). Rock2-depleted cultures (Figure 6) or Rock2 inhibition (Figure S4) was used to reproduce the activity of the Rbfox2-dependent isoform that is hypothesized to be inactive and to block the activity of the active Rock2 isoform expressed in Rbfox2-depleted cells. The expression of myc-Mef2d and knockdowns of Rbfox2 and/or Rock2 was confirmed by western blot and immunofluorescence staining (Figures 6C and 6D). The rescue of myoblast fusion was significant upon induction of the Mef2d differentiation-specific isoform.
isoform in cells, with double knockdown of Rbfox2 and Rock2 (Figures 6B–6E). In addition, the rescue of myoblast fusion was confirmed independently by inhibition of Rock2 using Y27632 (Ishizaki et al., 2000; Uehata et al., 1997) (Figure S4).

We also observed a partial rescue of fusion in Rbfox2- and Rock2-depleted cultures, which is comparable to that produced by expression of the muscle-specific Mef2d isoform (Figures 6B–6E). The fact that rescue by Mef2d or Rock2 was partial is consistent with a requirement for multiple Rbfox2-dependent gene expression events for myoblast fusion. We conclude that Rbfox2-coordinated alternative splicing in Mef2d and Rock2 significantly contributes to control of myoblast fusion during myogenesis.

DISCUSSION

Transcription and cell signaling programs are required in distinct phases of myoblast differentiation (Braun and Gautel, 2011; Hinddi et al., 2013). Here we identified a role for alternative splicing during the dramatic transition from proliferative myoblasts to...
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differentiated multinucleated myotubes. Specifically we identified a role for Rbfox2 in myoblast fusion, a late step of differentiation. Furthermore, by the integration of RNA-seq, IPA, and iCLIP analyses in Rbfox2-depleted cultures, we identified Rbfox2-dependent splicing transitions in Mef2d and Rock2 that are required for myoblast fusion. Interestingly, the Rbfox2-mediated splicing transition leads to a gain of function of Mef2d and a loss of function for Rock2, illustrating an elegant utilization of coordinated splicing regulation during myogenesis.

Mef2 proteins are critical transcription regulators in embryonic and postnatal muscle (Potthoff et al., 2007a, 2007b; Potthoff and Olson, 2007). All four Mef2 genes are alternatively spliced; however, the specific functions of individual isoforms are not understood (Black and Olson, 1998; Martin et al., 1994; Ohkawa et al., 2006; Yu et al., 1992; Zhu et al., 2005). We showed that the transition to the muscle-specific splicing pattern for Mef2d during differentiation is directly regulated by Rbfox2 (Figures 4B–4D). Rbfox2 depletion in differentiated cultures not only fails to produce the differentiated Mef2a and Mef2d isoforms but also shows increased expression of the undifferentiated isoforms which could have an inhibitory effect on myoblast fusion, as over-expression of the undifferentiated Mef2d isoform has recently been shown to inhibit myoblast differentiation (Sebastian et al., 2013). Expression of the differentiated Mef2d isoform (Mef2d232) partially rescued the fusion defect caused by Rbfox2 depletion, demonstrating a role for this isoform in myoblast fusion.

An important finding of our study is that multiple splicing transitions coordinated by Rbfox2 contribute to myoblast fusion. In addition to Mef2d, we show that a change in Rock2 isoforms also contributes to myoblast fusion. Rock1 and Rock2 are primary effectors of RhoA that regulate the cytoskeleton during cell-cycle progression, cell migration, cell adhesion, and differentiation (Loirand et al., 2006; Riento and Ridley, 2003). RhoA is activated early in myogenesis; however, its activity must be downregulated for myoblast fusion to occur (Castellani et al., 2006; Charrassse et al., 2006; Hindi et al., 2013). Downregulation of Rock1 activity is mediated by its interaction with RhoE, a RhoA antagonist that does not interact with Rock2 (Fortier et al., 2008; Riento et al., 2003). Rock kinases are proposed to be regulated by autoinhibitory intramolecular interaction of their N-terminal kinase domains with their C-terminal domains (Amano et al., 1999). Based on results showing reduced autophosphorylation of the Rock2 isoform containing the upstream alternative exon (Pelosi et al., 2007; Singh et al., 2001) and our demonstration of increased interaction of C-terminal fragments containing the alternative exons with N terminus of Rock2 (Figure 5E), we hypothesize that Rbfox2 promotes myoblast fusion by including this exon, thus reducing kinase activity. We show by two independent methods, siRNA-mediated knockdown and chemical inhibition, that loss of Rock2 function partially rescues the fusion defect in Rbfox2-depleted cells. We conclude that Rbfox2-coordinated regulation of splicing of both Mef2d and Rock2 substantially contributes to the program regulating myoblast fusion. While the posttranslational mechanisms that modulate open (active) and closed (inactive) conformation of Rock kinases have been described (Loirand et al., 2006), our results provide a mechanism of modulation of Rock2 kinase activity by alternative splicing (Figure S4E).

We detected extensive transcriptome transitions associated with myoblast differentiation and Rbfox2 depletion. The changes in gene expression were relatively small in that 75% were less than a 2-fold effect. The majority of splicing transitions associated with differentiation are not affected by Rbfox2 loss of function (Figure 2C), indicating that there is not a general disruption of differentiation or alternative splicing regulation during differentiation. Of the events regulated during differentiation that were affected by loss of Rbfox2 (182 events, Figure 2C), the majority were affected exclusively in undifferentiated or differentiated cultures, with only 28 events that were commonly regulated in both undifferentiated and differentiated cultures (Figures 2C–2E, S2E, and S2F). Furthermore, the effects are consistent with a role for Rbfox2 in repressing the differentiated splicing pattern in undifferentiated cultures and activating the differentiated pattern during the conversion to myotubes. These results identify multiple roles for Rbfox2 with regard to the maintenance of exon inclusion in different cell states as separate from the regulated transition from one cell state to another. Rbfox2 affects subgroups of alternative splicing events differently, either directly or through secondary consequences. The mechanisms of these different effects undoubtedly require the action of other splicing regulators as well as changes in activities of the basal spliceosome.

EXPERIMENTAL PROCEDURES

Cells and Antibodies

C2C12, 293T, and COSM6 cells were purchased from ATCC. The Rbfox2 antibody was purchased from Bethyl Laboratories. We raised a mouse monoclonal antibody to mouse Rbfox1 using a unique internal peptide near the N terminus. Antibodies to myogenin, myosin heavy chain 3 or MHC, and fast skeletal troponin T were from Santa Cruz Biotech. The mouse monoclonal antibody to the Myh3 myosin heavy chain (MF20) antibody was from the Developmental Studies Hybridoma Bank, Iowa. Mef2d antibody was from BD Biosciences. Antibodies against Myo-tag, Mef2a, Mef2c, cyclin D, Rock2, Erk, and Phospho-Erk were from Cell Signaling Technologies.

Cell Culture, siRNA Transfection, and Immunofluorescence

C2C12, COSM6, and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 10% FBS with 2 mM L-glutamine, and 0.01% streptomycin (growth media). For differentiation, cultures at >90% confluency are placed in differentiation media (growth media with 2% horse serum replacing FBS). siRNAs (50–100 nM) (Supplemental Experimental Procedures) were delivered once either by electroporation using Neon transfection system or by RNAiMax transfection reagent (Life Technologies) according to the manufacturer’s protocol.

For immunostaining, C2C12 cells were differentiated in 12-well tissue culture plates and fixed using 4% paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were washed twice with PBS and permeabilized using 0.2% triton-X in PBS for 10 min followed by 1 hr blocking at room temperature in 5% BSA in PBS. Cells were incubated overnight with the indicated antibodies followed by 3× washing and incubation of Alexa Fluor-conjugated secondary antibody for 1 hr. DAPI stain was added for 5 min and washed twice in PBS. Photographs were taken using Nikon Eclipse TE2000 inverted microscope at 100× to include the maximum number of cells in each field for accurate calculations of fusion indices (percent of nuclei in myh3 +ve myofibers that has more than two nuclei). Two fields from each well and three biological replicates were used to calculate fusion index for each experiment.

Cloning, Lentivirus Production, and Rescue Experiment

The piNDUCER21 lentiviral construct (Meerbey et al., 2011) was used for doxycycline-inducible expression of siRNA-resistant Rbfox2 and Mef2d
cDNAs. The Rbfox2 cDNA was amplified from differentiated C2C12 cultures. The siRNA-resistant construct contained eight nucleotide substitutions in the region targeted by the Rbfox2 siRNA1 without changing the coding potential. The myc tag coding sequence was incorporated in the forward primer for amplified siRNA-resistant Rbfox2 cDNA cloning into pINDUCER21 using the Gateway system. The Met2d cDNA was amplified using a myc tag coding sequence in the reverse primer and cloned into pINDUCER21 using the Gateway system.

Lentivirus containing doxycycline-inducible cDNAs of interest was produced by cotransfection with pINDUCER21 containing cDNA plasmid with four packaging plasmids into 293T using TransIT-293 Transfection Reagent. Virus supernatant was collected 36 hr after transfection, filtered (0.45 um) and concentrated 30 x by centrifugation >20,000 × g for 3 hr at 4 °C. The pellet was resuspended in DMEM, aliquoted, and frozen at –80 °C. Undifferentiated C2C12 cells were transduced at 40%–60% confluency in a serum- and antibiotic-free medium containing 2 mM L-glutamine and polybrene (5 μg/ml) and iCLIP are included in Supplemental Experimental Procedures.

RNA was isolated using Trizol and Hi-Seq library preparation, and Illumina sequencing was performed by the Genomic and RNA Profiling Core (GARP) at Baylor College of Medicine (BCM). The details of RNA-seq data mapping and iCLIP are included in Supplemental Experimental Procedures.

Validation of RNA-seq Data
Alternative splicing detected by RNA-seq was validated by RT-PCR using primers annealing to the flanking constitutive exonic regions. PCR products were separated on a 5% acrylamide gel and the percent spliced (in PSI) during myogenic differentiation. Nucleic Acids Res. 38, W369–W373.

 Validation of RNA-seq Data

Alternating splicing detected by RNA-seq was validated by RT-PCR using primers annealing to the flanking constitutive exonic regions. PCR products were separated on a 5% acrylamide gel and the percent spliced (in PSI) (Wang et al., 2008) was calculated after ethidium bromide staining using Kodak El Logic 2200 imaging system. One set of RT-PCR validations was performed using the same RNA that was used for RNA-seq.

RNA-seq and iCLIP
RNA was isolated using Trizol and Hi-Seq library preparation, and Illumina sequencing was performed by the Genomic and RNA Profiling Core (GARP) at Baylor College of Medicine (BCM). The details of RNA-seq data mapping and iCLIP are included in Supplemental Experimental Procedures.

ACCESSION NUMBERS
All raw RNA-seq and iCLIP data files are available for download from NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE58928.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2014.06.035.

AUTHOR CONTRIBUTIONS
R.K.S. designed performed the experiments, analyzed the data, and wrote the manuscript. Z.K. and W.L. performed mapping and computation analysis for RNA-seq and iCLIP data. A.K. and C.S.B. performed iCLIP experiments and contributed to the manuscript. M.R. performed validation for RNA-seq data and calculation of fusion index. J.U. and T.C. sequenced the iCLIP library and analyzed the iCLIP data. T.A.C. supervised and designed research, analyzed the data, and wrote the manuscript.

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REFERENCES
Rbfox2 is required for both cerebellar development and mature motor function. Genes Dev. 26, 445–460.


