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Alternative Splicing of MEF2C Controls its Activity in Normal Myogenesis and Promotes Tumorigenicity in Rhabdomyosarcoma Cells

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*Running title: Muscle specific MEF2C isoform drives myogenesis

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Keywords: MEF2C, HDAC, SRPK3, myogenesis, rhabdomyosarcoma

Background: MEF2C is an important regulator of many developmental programs.
Results: Alternative splicing of the α exon of MEF2C regulates myogenesis. Loss of SRPK3 in rhabdomyosarcoma cells inhibits this splicing and blocks differentiation.
Conclusion: MEF2Cα2 promotes myogenesis and restoration of MEF2Cα2 in rhabdomyosarcoma cells inhibits growth.
Significance: Defining the function and deregulation of MEF2Cα2 enhances the understanding of normal myogenesis and RMS tumorigenesis.

ABSTRACT

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. Many cellular disruptions contribute to the progression of this pediatric cancer including aberrant alternative splicing. The MEF2 family of transcription factors regulates many developmental programs, including myogenesis. MEF2 gene transcripts are subject to alternate splicing to generate protein isoforms with divergent functions. We found that MEF2Cα1 was the ubiquitously expressed isoform which exhibited no myogenic activity and that MEF2Cα2, the muscle specific MEF2C isoform, was required for efficient differentiation. We showed that exon α in MEF2C was aberrantly alternatively spliced in RMS cells, with the ratio of α2/α1 highly downregulated in RMS cells compared with normal myoblasts. Compared with MEF2Cα2, MEF2Cα1 more strongly interacted with and recruited HDAC5 to myogenic gene promoters to repress muscle specific genes. Overexpression of the MEF2Cα2 isoform in RMS cells increased myogenic activity and promoted differentiation in RMS cells. We have also identified a serine protein kinase, SRPK3, which was downregulated in RMS cells and found that expression of SRPK3 promoted the splicing of the MEF2Cα2 isoform and induced differentiation. Restoration of either MEF2Cα2 or SPRK3 inhibited both proliferation and anchorage independent growth of RMS cells. Together, our findings indicate the alternative splicing of MEF2C plays an important role in normal myogenesis and RMS development. Improved understanding of alternative splicing events in RMS cells will potentially reveal novel therapeutic targets for RMS treatment.

Introduction

The myocyte enhancer factor 2 (MEF2) is a regulator of many developmental programs, including myogenesis (1). MEF2 is encoded by four vertebrate genes which encode MEF2A, MEF2B, MEF2C and MEF2D. The MEF2 family is expressed in distinct but overlapping temporal and spatial expression patterns in the embryo and adult (2). Both MEF2C and MEF2D are implicated in myogenesis (3,4), which is controlled by the concerted activity of the myogenic regulatory factors (MRFs), a group of four highly related bHLH transcription factors composed of Myf5, MyoD, Myf6, and myogenin (5). MEF2 factors alone do not possess myogenic activity, but work in combination with the MRFs to drive the myogenic differentiation program (6).

MEF2 proteins control differentiation, proliferation, survival and apoptosis in a wide range of cell types. The N-terminus of the MEF2 proteins contains a highly conserved MADS box and an immediately adjacent motif termed the MEF2 domain. Together, these motifs mediate dimerization, DNA binding and co-factor interactions (7). The C-terminus of the MEF2 proteins is highly divergent among the family members and functions as the transcriptional activation domain. MEF2 proteins function as endpoints for multiple signaling pathways and confer a signal-responsiveness to downstream target genes. MAP kinase pathways are known to converge on MEF2 (8,9), resulting in a phosphorylation of the transcriptional activation domain of MEF2 which augments its transcriptional activity. Calcium signaling pathways also modulate MEF2 activity through multiple mechanisms (10-13). The activity of MEF2 is tightly controlled by class II HDACs, which bind to the MADS domain and promote the formation of multiprotein repressive complexes on MEF2 dependent genes (14). Phosphorylation of class II HDACs is mediated by calcium regulated protein kinases, which promote the nuclear-cytoplasmic shuttling of the HDACs and subsequent activation of MEF2C (14,15).

Each of the MEF2 genes are subject to extensive alternative splicing. MEF2C contains three alternative exons: the mutually exclusive exons α1/α2, the skipping/inclusion exon β and the 3' splice site region γ. The α1 domain is expressed ubiquitously, while the α2 domain is strongly expressed in skeletal muscle (16). The function of the α domain is unknown, although it has been shown that isoforms entirely lacking the α domain have enhanced activity (17). Inclusion of the β exon has been described in neural cells (16,18) and the presence of the β-exon in MEF2C was found to strongly activate MEF2C responsive reporters (19). The γ domain, generated by alternative splice site acceptors, has an inhibitory effect on the activity of MEF2C and isoforms lacking this domain better synergize with MyoD (20). The use of alternative isoforms in skeletal muscle differentiation has been recently shown for MEF2D, which promotes late muscle differentiation through use of alternative isoforms which generates a muscle specific MEF2Dα2 isoform (21), which binds to the co-activator ASH2L and is resistant to phosphorylation by PKA and association with HDACs (22).

Rhabdomyosarcoma (RMS) is a highly malignant tumor that is the most common form of soft tissue tumors in children. It is thought to arise as a consequence of myogenic precursors failing to differentiate into normal muscle (23). There are two major histological categories of RMS, the embryonal (ERMS) and alveolar (ARMS) subtypes. The more common form of the disease is the ERMS subtype. ARMS, the more aggressive form of RMS, is characterized by chimeric transcripts that fuse the 5' DNA binding domain of PAX3 or PAX7, respectively, to the transactivation domain of a forkhead transcription factor, creating novel PAX3/7-FOXO1 fusion proteins (24,25).

Rhabdomyosarcoma tumors express the myogenic regulatory factors, but the MRFs are unable to promote differentiation (26). Indeed, MyoD and myogenin are used as diagnostic markers for RMS as they are expressed in almost every RMS tumor including both major histological subtypes, embryonal RMS (ERMS) and alveolar RMS (ARMS) (27). Many blocks to differentiation have been described and were the subject of a recent review (26). Exogenous expression of MEF2C (28) or MEF2D (29) can promote differentiation in RMS cells.

We have shown that the muscle specific MEF2C isoform (MEF2Cα2) was required for efficient differentiation of skeletal muscle cells and that this isoform was highly downregulated in

RMS cells. MEF2C isoforms containing the α2 exon have potent myogenic activity as assayed by muscle specific gene reporters, muscle specific gene expression and myotube formation, while isoforms containing α1 or lacking the α domain did not. Despite the robust expression of MEF2Cα1 in RMS cells, restoration of the MEF2Cα2 isoform promoted RMS differentiation and myotube formation. The MEF2Cα1 isoform had an enhanced association with HDAC5, which resulted in enhanced recruitment of class II HDACs to target promoters in the presence of MEF2Cα1. We found that the alterative splicing of the α1/α2 exon of MEF2C was controlled by the protein kinase SRPK3, which is specific for the SR (serine/arginine-rich domain) family of transcription factors, including the splicing factor ASF. We showed that SRPK3 was downregulated in RMS cells. Exogenous expression of SRPK3 in RMS promoted the splicing of the MEF2Cα2 isoform, induced expression of muscle specific genes and drove the formation of myotubes. Exogenous expression of MEF2Cα2 or SRPK3 inhibited the proliferation and anchorage independent growth of RMS cells.

Experimental procedures

Cell Culture

RD (ATCC), SJRH30 (RH30) cells (ATCC), C2C12 myoblasts (ATCC), 10T1/2 cells (ATCC) and HEK293 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) according to standard protocols. To induce differentiation of C2C12 myoblasts into myotubes, cells were grown to 70% confluence and the media switched to DMEM supplemented with 2% horse serum (Hyclone). C2C12 cells were grown in differentiation medium for the number of days indicated in each experiment.

Cloning

Murine *Mef2Cα2* (m*Mef2Cα2*) and *Srpk3* were PCR amplified from cDNA reverse transcribed from RNA isolated from C2C12 cells differentiated for four days. Human MEF2C isoforms (h*MEF2C*) were PCR amplified from cDNA generated from RNA isolated from human myoblasts (gift of Denis Guttridge, Ohio State University), RH30 cells or HEK293 cells. A common primer set, MEF2C TOPO F 5' ATGGGGAGAAAAAAGATTCAGA 3' and MEF2C TOPO R 5' TCATGTTGCCCATCCTTCA 3', was used to amplify both m*Mef2C* and hMEF2C. Each of the PCR amplified fragments were cloned into the pEF6/V5 His TOPO TA expression vector and the clones confirmed by sequencing.

Western Blot Analysis

Cell extracts were made by lysing PBS washed cell pellets in radio-immunoprecipitation assay buffer (RIPA) supplemented with protease inhibitors (Complete protease inhibitor, Roche Diagnostics). Following incubation on ice, clear lysates were obtained by centrifugation. Protein concentrations were determined by Bradford's assay (Bio-Rad). For each sample, 30 μg of protein was loaded on each gel. Proteins were transferred onto a PVDF membrane using a tank blotter (Bio-Rad). The membranes were then blocked with 5% milk in 1X Tris buffered saline plus Tween 20 (TBST) and incubated with primary antibody overnight at 4ºC. Membranes were then washed with 1X TBST before incubation with the corresponding secondary antibody. Membranes were washed again with 1X TBST, incubated with chemiluminescent substrate according to manufacturer's protocol (SuperSignal, Pierce) and visualized by autoradiography. The antibodies used include anti- MEF2C (D80C1, Cell Signaling), anti-HDAC5 (Cell Signaling), anti-V5 (Rockland), anti-MHC (MF-20, Developmental Studies Hybridoma Bank) and anti-GAPDH (Millipore).

Gene expression analysis

RNA was isolated from cells by Trizol extractions (Invitrogen). Following treatment with DNase (Promega), two micrograms of total RNA was reversed transcribed with MultiScribe™ MuLV reverse transcriptase (Applied Biosystems). cDNA equivalent to 40 ng was used for quantitative polymerase chain reaction (qPCR) amplification (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems). Samples in which no reverse transcriptase was added (no RT) were included for each RNA sample. The relative levels of expression of genes were normalized according to those of hypoxanthine guanine phosphoribosyl transferase (*HPRT*) qPCR data were calculated using the comparative Ct method (Applied Biosystems).

Standard deviations from the mean of the [Δ] Ct values were calculated from three independent RNA samples. Primers corresponding to the indicated genes were as described (30). Where possible, intron spanning primers were used. All quantitative PCR was performed in triplicate and three independent RNA samples were assayed for each time point. For measurements of relative gene expression (fold change), a fold change was calculated for each sample pair by dividing the mRNA expression values of each sample pair. Each experimental fold change was then normalized to the fold change observed at *HPRT*.

Chromatin immunoprecipitation

ChIP assays were performed and quantified as described previously (31) with the following modifications: $1x10^7$ cells were used for each immunoprecipitation and protein A agarose beads (Invitrogen) were used to immunoprecipitate the antibody:antigen complexes. The following antibodies were used: HDAC5 (Cell Signaling), HDAC4 (Cell Signaling) and rabbit IgG (Santa Cruz Biotechnology) as a non-specific control. Primers corresponding to the *LMOD2* and *CDKN1A* promoters were as described (32). The real time PCR was performed in triplicate. Values of [Δ] [Δ] Ct were calculated using the following formula based on the comparative Ct method: Ct, template (antibody) - Ct, template (IgG) = [Δ] Ct. Fold enrichments were determined using the formula : $2^{-[\Delta]\ Ct.}$ (experimental)/$2^{-[\Delta]\ Ct}$ (reference, CHR19). Standard error from the mean was calculated from replicate [Δ][Δ] Ct values obtained from at least three individual experiments.

Cell Transfections and Luciferase Assays

Cells were transfected with calcium phosphate according to standard protocols. The plasmids pEF6-mMef2Cα2, β^-, γ^+, pEF6-hMEF2Cα1, β^-, γ^+, pEF6-hMEF2Cα1, β^-, γ^-, pEF6-hMEF2Cα2, β^-, γ^- , pEF6-hMEF2Cα2, β^-, γ^+, and pEF6-hMEF2Cα$^-$, β^-, γ^+ were used for expressing different isoforms of mMef2C and hMEF2C. pEF6-SRPK3 was used to express SRPK3. The plasmid pEMCIIs (provided by Andrew Lassar, Harvard Medical School) was used for expressing MyoD. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). RH30 or RD cells were seeded at a density of $5x10^3$ cell per well in 96 well plates and transfected with 0.4 ug of DNA. Transfections were normalized to Renilla luciferase. Transfections were performed in triplicate and all data sets were repeated at least twice.

Stable Cell Lines

Stable C2C12, RD and RH30 cell lines overexpressing exogenous MEF2C or SRPK3 were constructed by transfecting cells with linearized pEF-V5 His vector (empty vector), linearized pEF-MEF2C or linearized pEF-SRPK3 and selecting for blasticidin (10 ug/ml) resistant colonies. Murine clones of *Mef2Cα1* and *Mef2Cα2* were used in murine cell lines and human clones were used in human cell lines. Individual clones were isolated and propagated.

Immunohistochemistry

Cells were grown on cover slips, fixed with paraformaldehyde, incubated with goat serum supplemented with 1.0 % NP-40 for one hour and washed with PBS. Primary antibodies against myosin heavy chain (1:100, MF20, Developmental Studies Hybridoma Bank) were incubated for 2 hours at room temperature, washed with PBS and detected by Alexa Fluor-488 goat anti-mouse antibody (1:500, Invitrogen). Cell nuclei were then stained by incubating with DAPI (1 μM, Invitrogen) for 5 min.

Proliferation

Cells were seeded in a six well plate at $6x10^4$ per well and harvested every two days for cell counts with a hemocytometer. All counts were performed in triplicate and individual experiments repeated three times.

Soft agar assay

Soft agar assays were carried out in 60 mm dishes in which 2 ml of 0.7% Noble agar (USB) in 1X DMEM with 10% FBS was overlaid with 2 ml of 0.35% agar in 1X DMEM with 10% FBS containing $3x10^5$ cells. RD and RH30 cells transected with pEF6 V5 His(vector), MEF2Cα2 and SRPK3 were grown to 70% confluence, trypsinized, and dispersed. Cells of each clone were plated in triplicate. 1 ml of culture medium was added to the top of each plate every 5 days and cells were grown at 37^{o}C for 30 days. The

plates were stained with 1 ml of 0.05% Crystal Violet (Fisher) for > 1 hour and colonies were counted using a dissecting microscope.

Statistics

qPCR data are presented as means ± standard deviation (SD). Statistical comparisons were performed using unpaired two-tailed Student's *t* tests, with a probability value of <0.05 taken to indicate significance.

Results

The muscle specific α2 exon of MEF2C is not expressed in RMS cells

To understand the blocks to differentiation in RMS cells, we undertook an analysis of the MEF2 family in RMS. During the course of this work, we found that both RD and RH30 cells highly expressed MEF2C (29), although MEF2C has also been reported to be downregulated in RD cells (28). MEF2C has been shown to play an important role in myogenesis and MEF2C is subject to alternative splicing by exclusion/inclusion of exon α1/2, exon β and exon γ (Figure 1A). The exon β has been reported to enhance MEF2C activity, while exon γ plays an inhibitory role. However, the function of the mutually exclusive exons α1/α2 has not yet been characterized. To characterize the function of the MEF2C isoforms, we cloned MEF2C from RH30 cells, human myoblasts, C2C12 cells and HEK293 cells. The isoforms recovered from each cell type are shown in Figure 1B. As has been previously observed (16), the muscle specific α2 exon was only found in mRNA from C2C12 cells and human myoblasts. The transcripts from C2C12 cells each contained the inhibitory γ domain, while human myoblast RNA produced transcripts with or without the γ domain. Both RD and RH30 cells contained the α1 exon with or without the gamma (γ) domain. HEK293 cells expressed isoforms either with the α1 domain or lacking the α domain entirely. The transcripts containing the α1 domain lacked the γ domain, and the transcripts without the α domain contained the γ domain. Consistent with the prior analyses which identified the β exon exclusively in neuronal tissue (16,18), we identified no transcripts which contained the β domain from any of the cell types in our study.

We sought to verify our results using reverse transcription PCR (RT-PCR) to detect the expression of exon α and exon β by exon specific primers in normal muscle and RMS cells. The location of the primers is shown in Figure 2A. Consistent with the results shown in Figure 1, we found that the MEF2Cα1 exon was ubiquitously expressed in both proliferating and differentiated C2C12 cells, human myoblasts and the RMS cell lines (Figure 2B). The MEF2Cα2 exon was only expressed in differentiated C2C12 cells and human myoblasts (Figure 2B). Expression of the β exon could not be detected in any of the samples tested here (Figure 2C). To verify detection of the β exon, we also assayed samples from the brain, induced pluripotent stem (iPS) cells and neural progenitor cells (NPC) derived from iPS cells (33). As anticipated, we found that brain and NPC cells expressed the β exon, while iPS cells did not (Figure 2C).

To further clarify our results, we used quantitative RT-PCR (qRT-PCR) to quantitate the expression pattern of MEF2Cα1/α2 isoforms during normal myogenesis and in RMS cells. Using primers specific to the α1 or α2 domain of MEF2C, we examined expression in C2C12 cells throughout a time course of differentiation. We found that the transcript for MEF2Cα1 was expressed in proliferating C2C12 cells (UD) and expression did not change significantly when cells were differentiated (Figure 2D). In RMS cells, expression of MEF2Cα1 was compared to the expression levels found in human myoblast RNA. We found that both RD and RH30 cells expressed very high transcript levels of MEF2Cα1 (Figure 2E). When the expression of MEFCα2 was examined, the expression was very low in proliferating C2C12 cells, but the ratio of α2 expression vs α1 expression sharply increased upon differentiation (Figure 2F). For RMS cells, very low expression of MEF2Cα2 was observed compared to the expression observed in human myoblast RNA and the ratio of α2/α1 expression did not increase significantly upon differentiation (Figure 2G).

MEF2Cα2 has myogenic activity while MEF2Cα1 does not.

We next compared the myogenic activity of the MEF2C isoforms on a muscle specific reporter. We chose a muscle specific reporter which contains the *Leiomodin2* (*Lmod2*) promoter

fused to luciferase, *Lmod2-luc*, which we have used previously to characterize the activity of the MRFs and MEF2D (29,34). The *Lmod2-luc* reporter shows very low activity in proliferating cells and is strongly upregulated upon differentiation. Transfection of MyoD or myogenin activate the reporter. Thus, we assayed for the activity of the *Lmod2-luc* reporter with MyoD alone, and in combination with each of the MEF2 isoforms in 10T1/2 cells, a fibroblast cell line considered poised for activation of muscle specific genes. We found that MEF2Cα1 did not enhance the activity of the reporter and in fact had a mild inhibitory effect (Figure 3A). The addition of the γ domain was also modestly inhibitory, as has been previously seen. The isoform lacking the α domain did not significantly inhibit or activate the *Lmod2-luc* reporter. The MEF2Cα2 isoform strongly activated the *Lmod2-luc* reporter. Addition of the γ domain again lead to a modest inhibition, but the γ domain containing isoform still robustly activated the *Lmod2-luc* reporter. The work indicates that transcripts including α2 are required for MEF2C myogenic enhancing activity, among which the isoform without γ domain is a modestly stronger than that with γ domain. Transcripts with the α1 exon appear to inhibit the myogenic activity of MyoD.

To confirm these results, we next assayed for the activity of the MEF2C isoforms on endogenous gene expression. 10T1/2 cells were tranfected with constructs expressing MyoD in combination with constructs expressing either MEF2Cα1 or MEF2Cα2. Gene expression analysis confirmed the expression of each MEF2C isoform (Figure 3B). We found that transfection of MEF2Cα2 with MyoD strongly induced muscle specific gene expression, including myosin light chain, phosphorylatable, fast (*Mylpf*), creatine kinase, muscle (*Ckm*) and troponin T, type 1 (*Tnnt1*), while MEF2Cα1 had no activity (Figure 3C).

Our data are consistent with previous findings which show that muscle expresses both MEF2Cα2 and MEF2Cα1 (16,18). To determine the effect of each isoform in muscle, MEF2Cα1 and MEF2Cα2 were individually ectopically expressed in C2C12 cells. Proliferating C2C12 cells were transfected with plasmids expressing MEF2Cα1 or MEF2Cα2 and then induced to differentiate. Expression of the individual isoforms was confirmed by qRT-PCR (Figure 4A). Expression of the exogenous epitope tagged MEF2C isoforms was also confirmed by western blot analysis (Figure 4B). When differentiation specific gene expression was examined, we found that exogenous expression of MEF2Cα2 stimulated the expression of actin (*Acta1*), troponin 1 type 2 (*Tnni2*) and leiomodin 2 (*Lmod2*), while the MEF2Cα1 isoform had a modest inhibitory effect (Figure 4C). The effect on differentiation was also assayed by immunostaining for expression of myosin heavy chain (MHC), which is commonly used as a marker for myogenesis. We found that ectopic expression of MEF2Cα2 significantly stimulated the formation of myosin heavy chain positive myotubes, while the expression of MEF2Cα1 was inhibitory for myotube formation (Figure 4D).

To determine if MEF2Cα2 could rescue the MRF dependent activation of muscle specific genes in RMS, we first asked if MEF2Cα2 could activate the *Lmod2* reporter in RD cells. We found that MEF2Cα2 robustly induced the *Lmod2* reporter, while the MEF2Cα1 isoform was not able to activate the reporter (Figure 5A). Consistent with our results in Figure 3, we found that MEF2Cα2 robustly stimulated the *Lmod-luc* reporter in RD cells. The isoform lacking an α domain did modestly activate the reporter (~2 fold), but not nearly to the degree as the MEF2Cα2 isoform. Next, we examined the effect on the expression of differentiation specific genes in RD cells and found that MEF2Cα2 stimulated the expression of *LMOD2*, *TNNI2* and *CDKN1A* (p21) (Figure 5B). The cell cycle regulator p21 is required for terminal differentiation (35) and is regulated in part by MyoD in muscle (36). To determine if the MEF2Cα2 isoform could promote differentiation in RMS cells, exogenous MEF2Cα1 and MEF2Cα2 were expressed in RD cells and myotube formation assayed by myosin heavy chain (MHC) immunohistochemistry. We found that MEF2Cα2 expression markedly induced differentiation in RD cells (Figure 5C).

MEF2Cα1 preferentially associates with HDAC5

To begin to address how the α2 exon of MEF2C promotes myogenesis while the α1 exon does not, we asked if the association with histone

deacetylases (HDACs) with each isoform was distinct. MEF2 is well known to interact with histone deacetylases (1), and the differential phosphorylation of the α1/α2 exon of MEF2D alters the association with HDACs (22). Thus, we asked if a difference in HDAC association could be observed for MEF2Cα1 vs MEF2Cα2. HEK293 cells, which express endogenous MEF2Cα1, were transfected with constructs expressing MEF2Cα1 or MEF2Cα2. MEF2C proteins were immunoprecipitated with antibodies against MEF2C and the immunoprecipitate probed for HDAC5. We found that the cells transfected with a plasmid expressing MEF2Cα2 immunoprecipitated HDAC5 less robustly than cells transfected with a plasmid expressing MEF2Cα1 (Figure 6A). The antibody used for the immunoprecipitation could immunoprecipitate both MEF2Cα1 and MEF2Cα2, so it is possible that the differential association of HDAC5 might be more significant than that indicated by our experiment as HEK293 cells have endogenous levels of MEF2Cα1. Selective immunoprecipitation of the isoforms using epitope tags on the constructs was attempted, but non-specific bands in the immunoprecipitate precluded analysis of HDAC association.

To understand if the differential association of HDAC5 observed would influence HDAC recruitment to target genes, we performed chromatin immunoprecipitation (ChIP) assays for HDAC5 in RD cells, which express MEF2Cα1, transfected with a vector control or with a construct expressing exogenous MEF2Cα2. We found that HDAC5 could be detected on muscle specific promoters in RD cells transfected with vector, but this association was decreased when MEF2Cα2 was expressed (Figure 6B). The decrease in HDAC recruitment was also observed at the *CDKN1A* (p21) promoter. We also examined the recruitment of HDAC4, an additional class II HDAC, by ChIP assays and found that HDAC4 association was also disrupted by MEF2Cα2 expression (Figure 6C). Our data indicate that MEF2Cα2 may promote muscle gene expression at least in part by reducing the recruitment of HDACs to target promoters and thus promoting gene activation.

SRPK3 is downregulated in RMS cells

As our data suggested that the lack of expression of MEF2Cα2 in RMS cells might contribute to the block to differentiation in these cells, we sought to understand why the MEF2Cα2 isoform was not expressed in RMS cells. To address this, we attempted to identify the splicing factors which controlled the α isoform selection. Two bioinformatic databases, Expasy (37) and Uniprot (38), were used to predict the splicing factors which might recognize the α exon splice sites in MEF2C. Both programs predicted the serine/arginine-rich splicing factor 1, SRSF1 (ASF), which is activated by phosphorylation. To initiate our analysis, we assayed for the expression of ASF in skeletal muscle and RMS cells. We found that the expression of ASF is modestly upregulated during myogenesis (Figure 7A), consistent with a role for promoting the MEF2Cα1 to MEF2Cα2 switch. However, when RMS cells were analyzed for expression of ASF, we found that ASF was highly upregulated compared to human myoblasts (Figure 7B). This result is consistent with many other studies which show that ASF is often highly upregulated in cancer (39).

We next looked for expression of upstream kinases required for activation of ASF. We choose SRPK3, a muscle specific protein kinase which is regulated by MEF2C in skeletal muscle (40). As has been previously shown (40), SRPK3 was strongly upregulated during normal myogenesis (Figure 7C). We also found that SRPK3 was downregulated in RMS cells (Figure 7D). To determine if SRPK3 was required for splicing of the MEF2Cα2 isoform, SRPK3 was depleted from C2C12 cells using shRNA constructs. Multiple shRNA constructs were used independently and the results of two individual constructs are shown. We found that the constructs depleted SRPK3 (Figure 7E) and inhibited splicing of the MEF2Cα2 isoform when assayed after two days of differentiation (Figure 7F).

SRPK3 activates the splicing of MEF2Cα2 and promotes differentiation in RMS cells

To determine if the downregulation of SRPK3 contributed to the isoform selection in MEF2C and the block to differentiation in RMS cells, we ectopically expressed SRPK3 in RD

cells. The expression of SRPK3 was confirmed by qRT-PCR (Figure 8A). The expression of the MEF2Cα1 and MEF2Cα2 isoforms was then analyzed and we found that expression of exogenous SRPK3 did not significantly alter the expression of the MEF2Cα1 isoform, but did strongly enhance expression of the MEF2Cα2 isoform (Figure 8B). To determine if the expression of SRPK3 could promote differentiation, we assayed these cells for differentiation specific gene expression including *LMOD2*, *ACTA1*, *TNNT1* and *CDKN1A*. We found that each of these genes were upregulated in cells expressing SRPK3 (Figure 8C), strongly suggesting the SRPK3 promotes differentiation specific splicing which allows expression of the appropriate transcripts required for differentiation. Differentiation was also assayed by immunostaining for MHC in RD cells transfected with expression constructs for vector, MEF2Cα2 or SRPK3. We found that MEF2Cα2 or SRPK3 promoted robust MHC signal and the appearance of myotubes (Figure 8D). To determine if SRPK3 and MEF2Cα2 could also promote differentiation in ARMS cells, the above experiment was repeated in RH30 cells and again, robust expression of MHC was observed (Figure 8D).

Finally, we sought to understand if SRPK3 or MEF2Cα2 could inhibit the proliferation and tumorigenic growth of RMS cells. RD cells expressing exogenous SRPK3 or MEF2Cα2 were assayed for proliferation and we found these cells had reduced proliferation rates when compared to the vector only controls (Figure 9A). To extend this result to the ARMS subtype of RMS, the proliferation assay was repeated in RH30 cells. We found that exogenous expression of SRPK3 or MEF2Cα2 also inhibited the proliferation of RH30 cells (Figure 9B). To determine if SRPK3 or MEF2Cα2 could inhibit anchorage independent growth of these cells, growth in soft agar media was assayed. We found that RD cells expressing exogenous MEF2Cα2 or SRPK3 formed fewer colonies in soft agar media (Figure 9C) and the colonies which did form were smaller in size than that observed for the vector only controls (Figure 9D). The data suggest that restoration of differentiation specific splicing may inhibit RMS tumor growth.

Discussion

We show here that the α2 exon of MEF2C confers myogenic activity on MEF2C and results in differential HDAC recruitment to target promoters. The expression of the MEF2Cα1 exon in RMS cells contributes to the lack of differentiation observed in those cells. The splicing of the α2 exon is promoted by SRPK3 and restoration of SRPK3 or MEF2Cα2 in RMS cells enhances differentiation and inhibits proliferation and tumorigenic growth. MEF2C has been previously shown to induce differentiation in RMS cells (28) and our results reveal that the deficiency in MEF2C activity is due to the lack of appropriate muscle specific splicing.

Defects in alternative splicing have been previously observed in RMS cells. The oncogenes Murine Double Minute 2 (MDM2) and MDM4 exhibit genotoxic-stress inducible splice forms in high risk metastatic disease represented by both ERMS and ARMS. Expression of these alterative isoforms promotes metastatic behavior of tumor cells (41). Multiple splicing isoforms of PAX3, PAX7 and the PAX-FOXO1 fusions have also been observed in RMS and differences in the PAX7 splicing pattern between murine skeletal muscle and RMS tumors has been observed (42). To our knowledge, our work is the first to implicate the deregulation of a splicing factor in RMS. We show here that SRPK3 is required for the isoform switch between MEF2Cα1 and MEF2Cα2, but likely controls the splicing of many other genes required for normal muscle differentiation.

A recent study has shown that the expression and alternative splicing of the MEF2 genes are deregulated in muscle from neuromuscular disorder (NMD) patients, including DM1 and DM2 (43). In DM, expression of a 224 bp isoform encompassing exons 4A and 4B (corresponding to MEF2Cα1) was found to expressed in muscle while normal muscle contained a 217 bp isoform encompassing exon 5a (corresponding to MEF2Cα2) (43). Our work suggests that expression of MEF2Cα1 in diseased muscle would prevent appropriate differentiation specific gene expression and contribute to the muscle dysfunction observed in the patients.

In a related study, MEF2C was found to be deregulated in cardiac tissue of DM1 patients (44). A screen of microRNAs revealed that

several miRNAs were differentially expressed in a mouse model of DM1, and many of these miRNAs were direct MEF2 transcriptional targets. A down regulation of MEF2C and MEF2A was observed in both the mouse models and in human DM1 cardiac tissues and restoration of MEF2C promoted expression of miRNA and mRNA targets in DM1. Cardiac tissue is thought to express the α1 isoform of MEF2C and it will be interesting to understand how alternative splicing of MEF2C contributes to the dysfunction of MEF2C observed in both cardiac and skeletal muscle tissue in DM1 patients.

It is intriguing that the MEF2Dα2 isoform has been recently shown activate differentiation specific transcription (22), while the ubiquitously expressed MEF2Dα1 form does not, similar to what we observed with MEF2C. In the case of MEF2D, the activity was shown to be due to differential phosphorylation of the α1 vs α2 exon mediated by PKA (22,45). Phosphorylation of the MEF2Dα1 isoform induces association with histone deacetylases (22). We also see that the α1 exon of MEF2C interacts preferentially with HDAC5 and induces the recruitment of HDAC5 and HDAC4 to target promoters. The basis of the differentiation interaction with HDAC5 is currently unclear for MEF2C, but it may also involve differential phosphorylation. The phosphorylation of MEF2C is unlikely to be mediated by PKA as MEF2C has been reported to be a poor substrate for PKA (45) and the α1 exon of MEF2C does not contain consensus sites for PKA phosphorylation.

Besides the modulation of MEF2C by HDACs, we cannot rule out the potential regulation of α1/α2 through differential interactions with other transcription factors and co-factors. Many factors have been shown to modulate the activity of MEF2C during myogenesis including the myogenic regulatory factors, MyoD and myogenin (46); the histone acetyltransferase, P300 (47); the steroid nuclear receptor coactivator NCOA2/GRIP-1 (48) and mastermind-like transcriptional coactivator (MAML) (49). The calcineurin inhibitor Cabin1 sequesters MEF2C in a transcriptionally inactive state which is released by an increase in intracellular calcium concentration (50). The differential interaction of MEF2Cα1 and MEF2Cα2 with any of these factors may contribute to the differences in myogenic activity we observe here. Intriguingly, the MEF2Cα1 domain has previously been shown to the target of the inhibitory effect of the Notch signaling pathway, which represses myogenesis (51). The SVGHSPESEDKY region, which is uniquely present in MEF2Cα1 and not in MEF2Cα2, MEF2A, MEF2B or MEF2D, was shown to be required for the Notch mediated repression. Activated Notch signaling is common in many cancers and activated Notch has also been observed in RMS cells (52). Thus, differential interactions of the MEF2C α1/α2 isoforms with the Notch signaling pathway may also contribute to the differential activity of the isoforms. The data shown here confirm that MEF2C constructs entirely lacking the α domain have higher activity than the MEF2Cα1 isoform found in RMS cells. Further understanding of how elevated Notch signaling and MEF2Cα1 expression in RMS cells may contribute to the pathology of RMS is an important future direction for these studies.

MEF2C is a direct transcriptional activator of many important developmental genes including c-jun (53) and matrix metalloproteinase 10 (MMP10) (54). MEF2C is also a direct transcriptional activator of several miRNAs including miR-1, miR-21, miR-29, miR-30, miR-133 (44). It will be important to understand which isoform of MEF2C directs transcription of these important targets in each system and how the differential expression and regulation of the isoforms contributes to appropriate expression of MEF2C target genes.

While the MEF2Cα2 isoform was known to be expressed in skeletal muscle, our results reveal the requirement for the α2 exon for myogenesis and show that the differentiation defect in RMS cells extends to the muscle specific splicing patterns required for differentiation. It will be important to further understand the deregulation of splicing factors such as SRPK3 in RMS as targeting these changes may offer novel therapeutic approaches for treating RMS. Defining the molecular basis for the myogenic activity of the α2 exon of MEF2C and the differential recruitment of HDAC5 will also be important in understanding normal skeletal muscle

differentiation. Understanding the function of the α exon of MEF2C and how the appropriate splicing is achieved also contributes to the understanding of muscle dysfunction in neuromuscular disease patients and may potentially offer new therapeutic approaches for this disease as well.

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Figure legends

Figure 1. MEF2C isoforms in muscle and RMS. A. A schematic of the MEF2C isoforms is shown. The sequences of the exons are indicated below. Murine and human sequences are indicated by m and h, respectively. Amino acids which differ among the species are indicated in red. B. MEF2C isoforms identified in indicated cell lines. The number beside each isoform indicates the number of individual isoform clones identified/total number of clones recovered.

Figure 2. Expression of the α and β exons of MEF2C in normal muscle and RMS. A. Schematic of the exon structure of MEF2C with the location of the primers used to detect the exons indicated. B. The α1 exon of MEF2C is expressed ubiquitously in skeletal muscle, but the α2 exon is strongly upregulated during differentiation. Exon expression was detected by RT-PCR on the indicated samples. Undifferentiated myoblasts are represented by UD and D represents the days of differentiation. Human myoblasts are represented by h.m. C. The β exon is not expressed in muscle or RMS cells. Exon expression was detected by RT-PCR on the samples indicated as in B. and from induced pluripotent stem (iPS) cells, neural progenitor cells (NPC) and brain. D. Expression of the α1 exon does not change during myoblast differentiation. Gene expression was assayed by qRT-PCR. Error bars, SD. ***P<0.001. E. The α1 exon is

highly expressed in RMS cells as assayed as in D. F. The α2 exon is upregulated during differentiation as assayed as in D. Data are shown as the ratio of α2 expression relative to the expression of α1. G. The α2 exon is highly down regulated in RMS and not induced by differentiation as assayed as in F.

Figure 3. MEF2Cα2 robustly enhances MRF activity, while MEF2Cα1 does not. A. MEF2Cα2 stimulates the activity of MyoD on a muscle specific luciferase reporter construct. 10T1/2 cells were transfected with the indicated constructs. Values are represented with respect to a luciferase vector with no promoter (pGL3 basic). pGL3 (+) represents a luciferase vector with the constitutive CMV promoter. Lmod2-luc represents a luciferase vector with a ~300 bp Leiomodin 2 (*Lmod2*) promoter. Error bars, SD. ***P<0.001, **P<0.01. B. Confirmation of the expression of MEF2Cα1 and MEF2Cα2. 10T1/2 cells were transfected with expression constructs for MyoD, MEF2Cα1 and MEF2Cα2 as indicated and gene expression was determined by qRT-PCR for the indicated genes. Vector represents a vector only transfection where the expression level was set to 1. Error bars, SD. ***P<0.001. C. MEF2Cα2 activates endogenous MRF target gene expression. 10T1/2 cells were transfected with expression constructs for MyoD, MEF2Cα1 and MEF2Cα2 and analyzed for the indicated genes as in B.

Figure 4. MEF2Cα2 promotes myogenesis in C2C12 cells. A. C2C12 cells were transfected with constructs expressing vector, MEF2Cα1 or MEF2Cα2. Expression of the isoforms was confirmed by qRT-PCR. Error bars, SD. ***P<0.001. B. Protein expression of the epitope tagged MEF2C isoforms was confirmed by western blot analysis. C. Differentiation specific gene expression is induced by overexpression of MEF2Cα2. Gene expression was assayed for the indicated genes by qRT-PCR. Error bars, SD. ***P<0.001. D. Over expression of MEF2Cα2 promotes myotube formation while MEF2Cα1 is inhibitory. Cell lines in A. were differentiated for 3 days and immunostained with antibodies against MHC and counterstained with DAPI. Images were taken at 200X magnification and scale bars represent 5 μms. The data are quantitated in the lower panel.

Figure 5. MEF2Cα2 promotes differentiation in RMS cells. A. MEF2Cα2 activates a muscle specific reporter in RMS cells. RD cells were transfected with the indicated constructs. Values are represented with respect to a luciferase vector with no promoter (pGL3 basic). pGL3 (+) represents a luciferase vector with the constitutive CMV promoter. *Lmod2-luc* represents a luciferase vector with a ~300 bp Leiomodin 2 (*Lmod2*) promoter. Error bars, SD. ***P<0.001. B. MEF2Cα2 promotes differentiation specific gene expression in RD cells. RD cells were transfected with constructs expressing vector, MEF2Cα1 and MEF2Cα2. Gene expression was assayed for the indicated genes by qRT-PCR. Error bars, SD. ***P<0.001. C. MEF2Cα2 promotes the expression of MHC in RD cells. RD cells expressing vector, MEF2Cα1 and MEF2Cα2 were immunostained for MHC and counterstained with DAPI. Images were taken at 100X magnification and scale bars represent 10 μms.

Figure 6. MEF2Cα1 recruits HDACs to target promoters. A. MEF2Cα1 interacts with HDAC5 more robustly than MEF2Cα2. HEK293 cells were transfected with expression constructs for MEF2Cα1 and MEF2Cα2, immunoprecipitated with an antibody against MEF2C and the blot probed with antibodies against HDAC5. B. MEF2Cα2 inhibits recruitment of HDAC5 to target

promoters. ChIP assays were performed on RD cells transfected with vector control or an expression construct for MEF2Cα2 with antibodies against HDAC5 and immunoprecipitated DNA probed with primers corresponding to the indicated promoters. Error bars, S.D. ***P<0.001. C. HDAC4 recruitment to target promoters is also inhibited by MEF2Cα2. ChIP assays were performed as in B. except with antibodies against HDAC4.

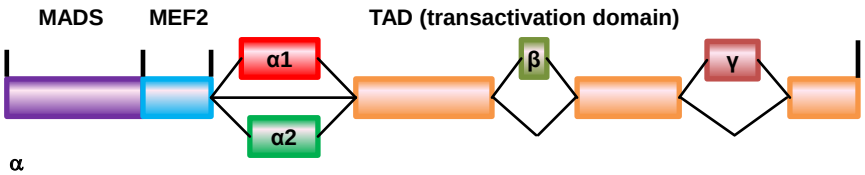
Figure 7. Expression of splicing factors in skeletal muscle and RMS. A. ASF is modestly upregulated upon differentiation in C2C12 cells. Gene expression was assayed by qRT-PCR and the days of differentiation are indicated. Error bars, S.D. B. ASF is highly expressed in RMS cells. Expression was assayed as in A. C. SRPK3 is robustly upregulated upon differentiation of C2C12 cells as assayed as in A. D. SRPK3 is highly downregulated in RMS cells as assayed as in A. ***P<0.001. E. Depletion of SRPK3. C2C12 cells were transfected individually with multiple shRNA contructs (shSRPK3) and stable transformants selected. The two constructs shown are represented by (1) and (2). Gene expression was assayed after two days of differentiation by qRT-PCR. Error bars, S.D. ***P<0.001. F. SRPK3 is required for MEF2Cα2 splicing. Gene expression was assayed on the shSRPK3 depletions as in E.

Figure 8. SRPK3 and MEF2Cα2 promote differentiation of RMS cells. A. SRPK3 overexpression in RD cells. RD cells were transfected with a vector control or an expression construct for SRPK3 and assayed for gene expression by qRT-PCR. B. SRPK3 induces the expression of MEF2Cα2. Gene expression was assayed by qRT-PCR. Error bars, S.D. ***P<0.001. C. SRPK3 induces differentiation specific gene expression in RD cells. Cells as in A. were assayed for gene expression by qRT-PCR for the indicated genes. Error bars, S.D. ***P<0.001. D. SRPK3 or MEF2Cα2 induce MHC expression in RMS cells. RD (left panel) and RH30 cells (right panel) were transfected with a vector control or expression constructs for SRPK3 or MEF2Cα2 and immunostained for MHC and counterstained with DAPI. Images were taken at 100X magnification and scale bars represent 10 μms.

Figure 9. SRPK3 and MEF2Cα2 inhibit growth of RMS cells. A. SRPK3 or MEF2Cα2 inhibit the proliferation of RD cells. RD expressing the indicated constructs were seeded at equivalent densities and harvested for cell counts every two days. Error bars, SD. B. SRPK3 or MEF2Cα2 inhibit the proliferation of RH30 cells. Proliferation was assayed as in A. C. SRPK3 or MEF2Cα2 inhibit the number of anchorage independent colonies formed. Error bars, S.D. ***P<0.001. D. SRPK3 or MEF2Cα2 inhibit the size of anchorage independent colonies formed. The largest colonies observed for each cell line are shown.

Figure 1

A



α
α1 (m/h) TLRKKGLNGCDSPDPDADDSVGHSPESEDKYRKINEDIDLMISRQRLC
α2 (m) ALNKKENKGSESPDPDSSYALTPRTEEKYKKINEEFDNMIKSHKIP
(h) ALNKKENKGCESPDPDSSYALTPRTEEKYKKINEEFDNMIKSHKIP
β
(m/h) SEDVDLLL
γ
(m) ACTSTHLSQSSNLSLPSTQSLSIKSEPVSPPR
(h) ACTSTHLSQSSNLSLPSTQSLNIKSEPVSPPR

B

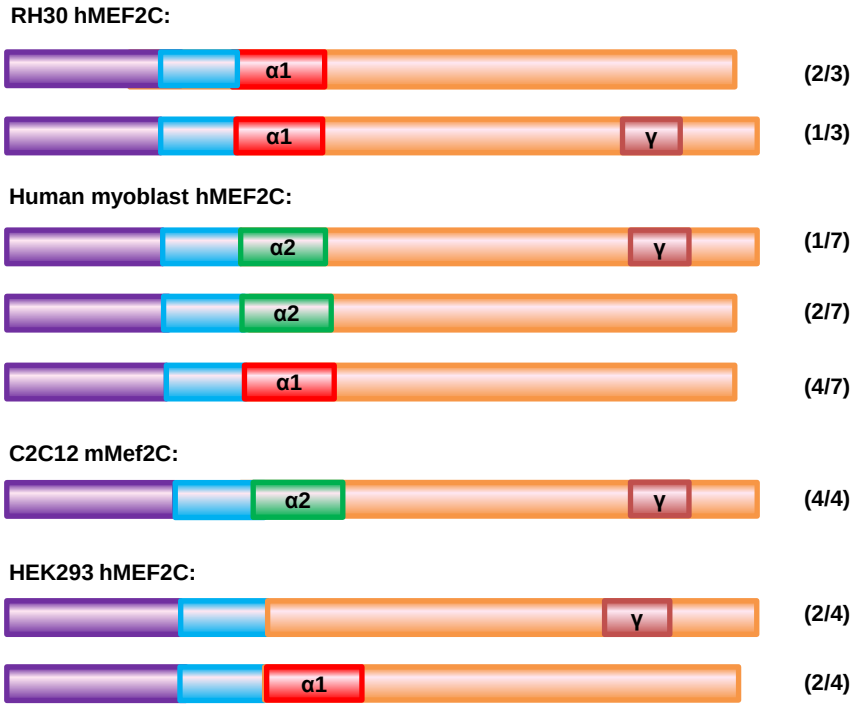


Figure 2

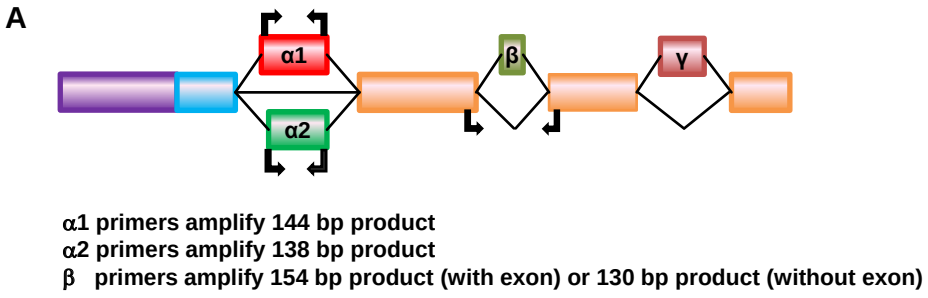

A
α1
α2
β
γ
α1 primers amplify 144 bp product
α2 primers amplify 138 bp product
β primers amplify 154 bp product (with exon) or 130 bp product (without exon)
B
C2C12
UD D2 D4 D6 h.m. RH30 RD
MEF2Cα1
MEF2Cα2
C
C2C12
UD D2 D4 D6 h.m. RH30 RD ESC nESC Brain
154 bp
130 bp
D
MEF2Cα1
Fold Change
1.4 1.2 1 0.8 0.6 0.4 0.2 0
C2C12: UD D2 D4
E
MEF2Cα1
Fold Change
1000 800 600 400 200 0
***
h.m. RD UD RD D2 RH30 UD RH30 D2
F
Ratio of α2/α1
250 200 150 100 50 0
***
***
C2C12: UD D2 D4
G
Ratio of α2/α1
1.2 1 0.8 0.6 0.4 0.2 0
***
h.m. RD UD RD D2 RH30 UD RH30 D2

Figure 3

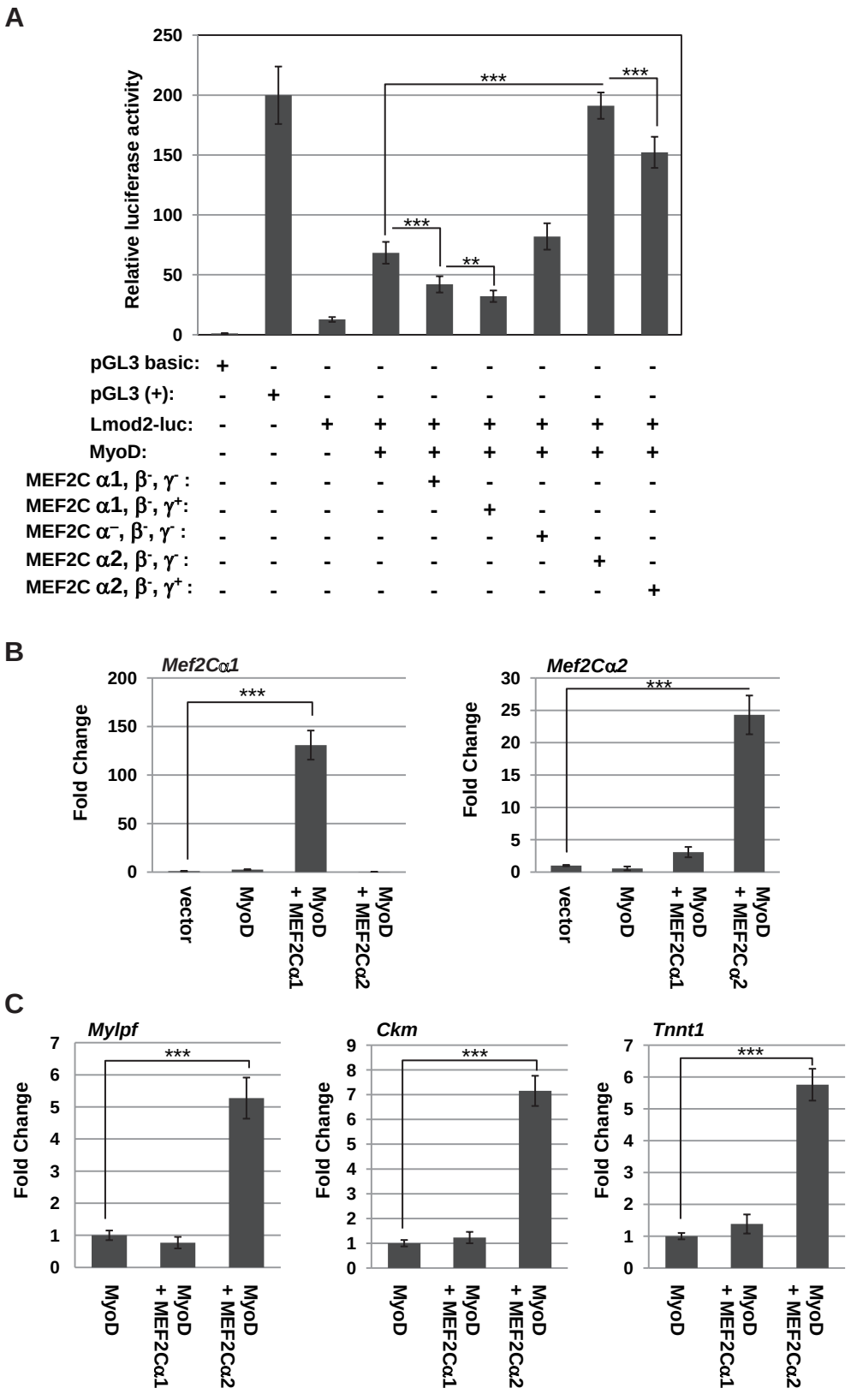

A
Relative luciferase activity
250
200
150
100
50
0
***
***
***
**
pGL3 basic: + - - - - - - - -
pGL3 (+): - + - - - - - - -
Lmod2-luc: - - + + + + + + +
MyoD: - - - + + + + + +
MEF2C α1, β-, γ- : - - - - + - - - -
MEF2C α1, β-, γ+: - - - - - + - - -
MEF2C α–, β-, γ- : - - - - - - + - -
MEF2C α2, β-, γ- : - - - - - - - + -
MEF2C α2, β-, γ+ : - - - - - - - - +
B
Mef2Cα1
Fold Change
200
150
100
50
0
***
vector
MyoD
MyoD + MEF2Cα1
MyoD + MEF2Cα2
Mef2Cα2
Fold Change
30
25
20
15
10
5
0
***
vector
MyoD
MyoD + MEF2Cα1
MyoD + MEF2Cα2
C
Mylpf
Fold Change
7
6
5
4
3
2
1
0
***
MyoD
MyoD + MEF2Cα1
MyoD + MEF2Cα2
Ckm
Fold Change
9
8
7
6
5
4
3
2
1
0
***
MyoD
MyoD + MEF2Cα1
MyoD + MEF2Cα2
Tnnt1
Fold Change
7
6
5
4
3
2
1
0
***
MyoD
MyoD + MEF2Cα1
MyoD + MEF2Cα2

Figure 4

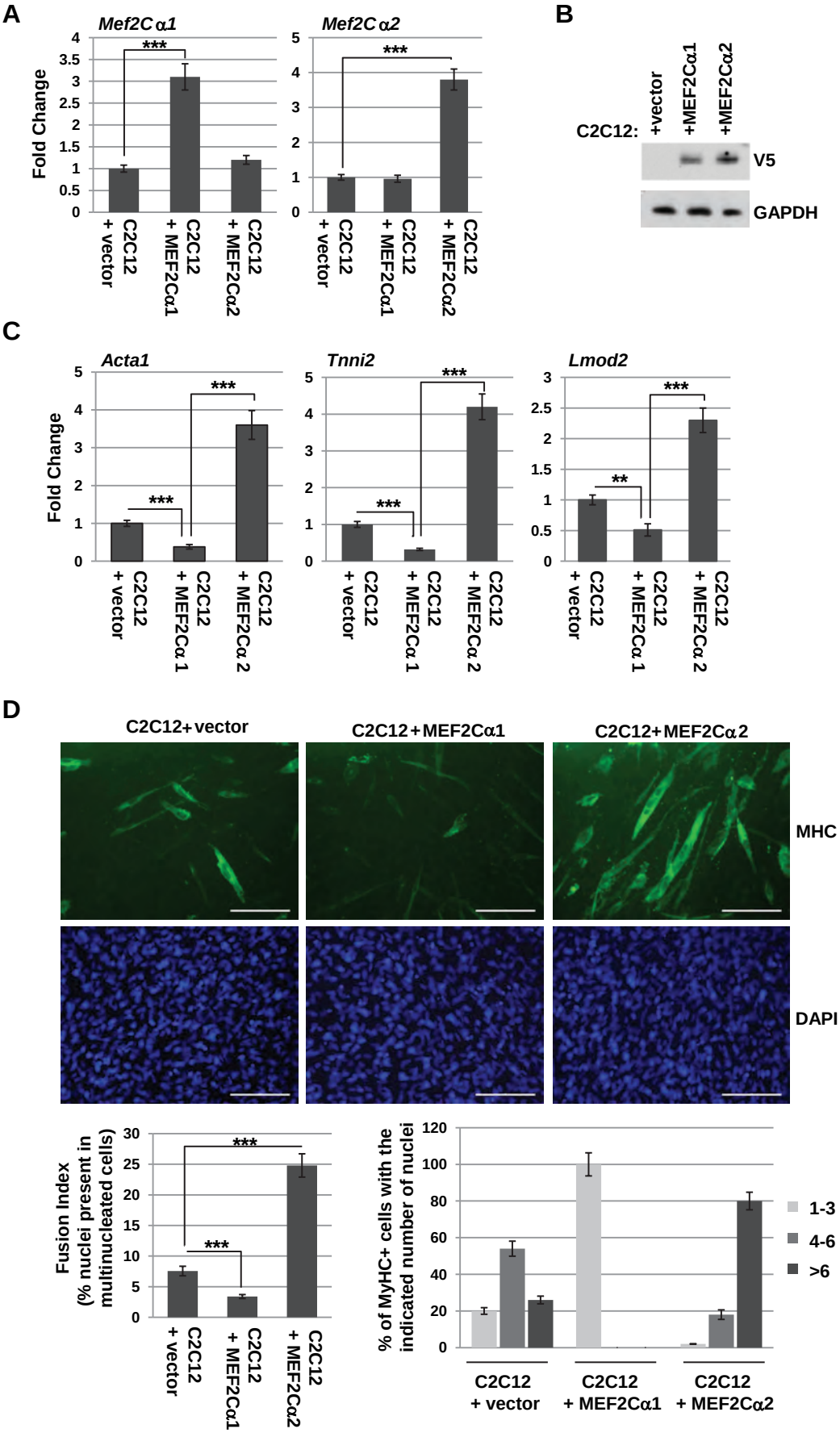

A
Mef2Cα1
Mef2Cα2
Fold Change
***
+ vector C2C12
+ MEF2Cα1 C2C12
+ MEF2Cα2 C2C12
B
C2C12: +vector +MEF2Cα1 +MEF2Cα2
V5
GAPDH
C
Acta1
Tnni2
Lmod2
**
D
C2C12+vector
C2C12+MEF2Cα1
C2C12+MEF2Cα2
MHC
DAPI
Fusion Index (% nuclei present in multinucleated cells)
% of MyHC+ cells with the indicated number of nuclei
1-3
4-6
>6
C2C12 + vector
C2C12 + MEF2Cα1
C2C12 + MEF2Cα2

Figure 5

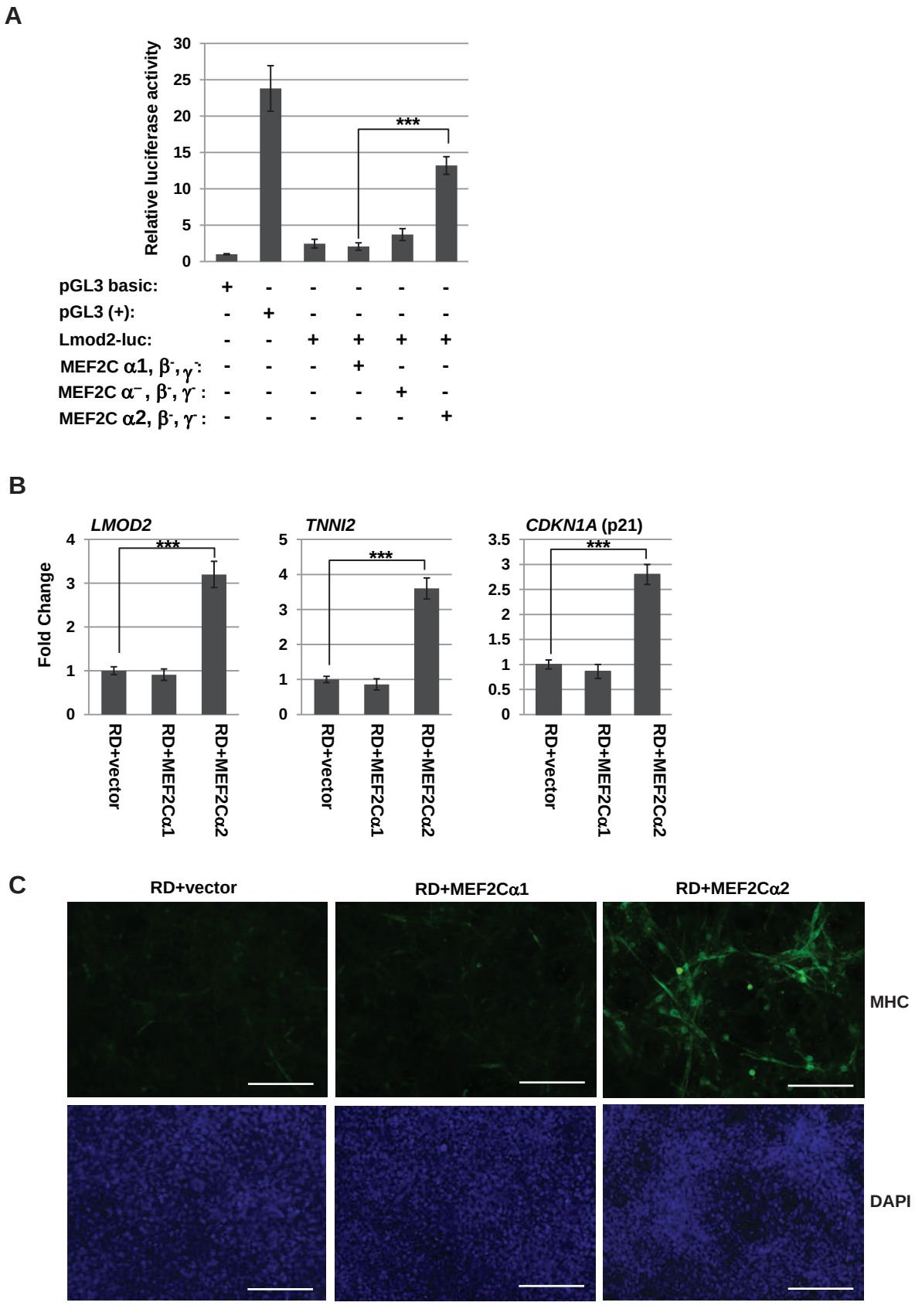

A
Relative luciferase activity
30
25
20
15
10
5
0
***
pGL3 basic: + - - - - -
pGL3 (+): - + - - - -
Lmod2-luc: - - + + + +
MEF2C α1, β-, γ-: - - - + - -
MEF2C α–, β-, γ- : - - - - + -
MEF2C α2, β-, γ- : - - - - - +
B
LMOD2
TNNI2
CDKN1A (p21)
Fold Change
***
RD+vector
RD+MEF2Cα1
RD+MEF2Cα2
C
RD+vector
RD+MEF2Cα1
RD+MEF2Cα2
MHC
DAPI

Figure 6

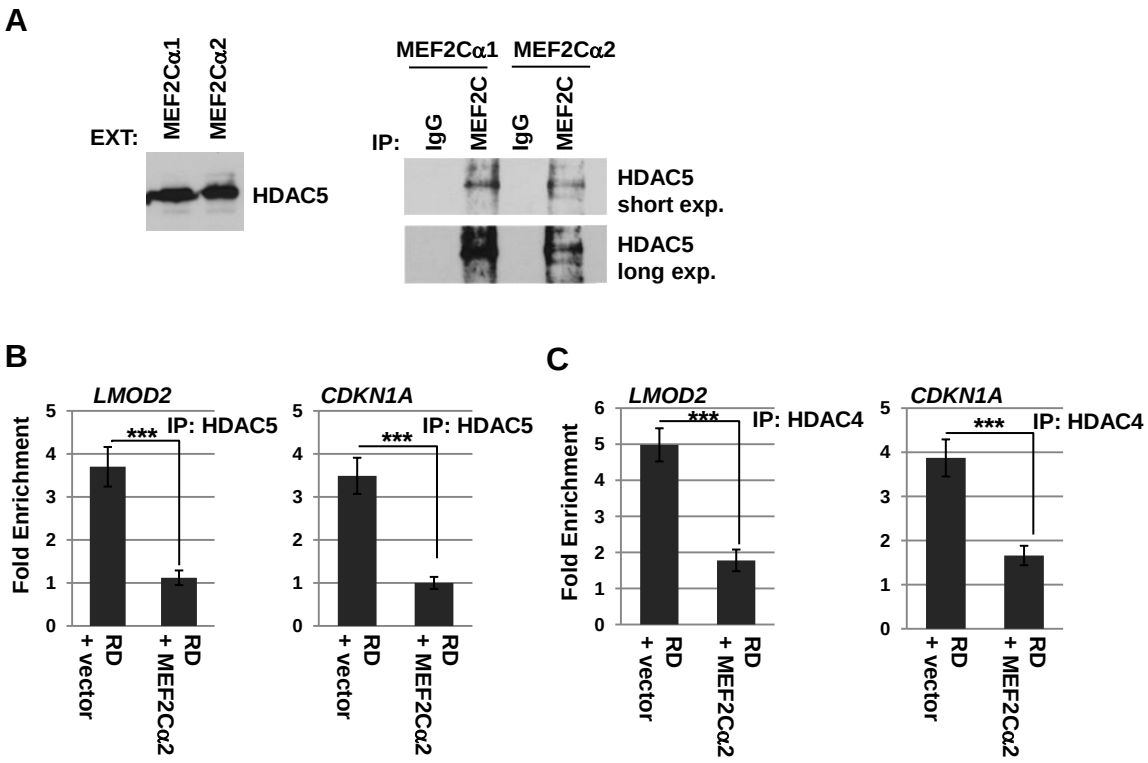

A
EXT:
MEF2Cα1
MEF2Cα2
HDAC5
IP:
MEF2Cα1
MEF2Cα2
IgG
MEF2C
IgG
MEF2C
HDAC5
short exp.
HDAC5
long exp.
B
LMOD2
IP: HDAC5
***
CDKN1A
IP: HDAC5
***
Fold Enrichment
0
1
2
3
4
5
RD
+ vector
RD
+ MEF2Cα2
C
LMOD2
IP: HDAC4
***
CDKN1A
IP: HDAC4
***
Fold Enrichment
0
1
2
3
4
5
6
RD
+ vector
RD
+ MEF2Cα2

Figure 7

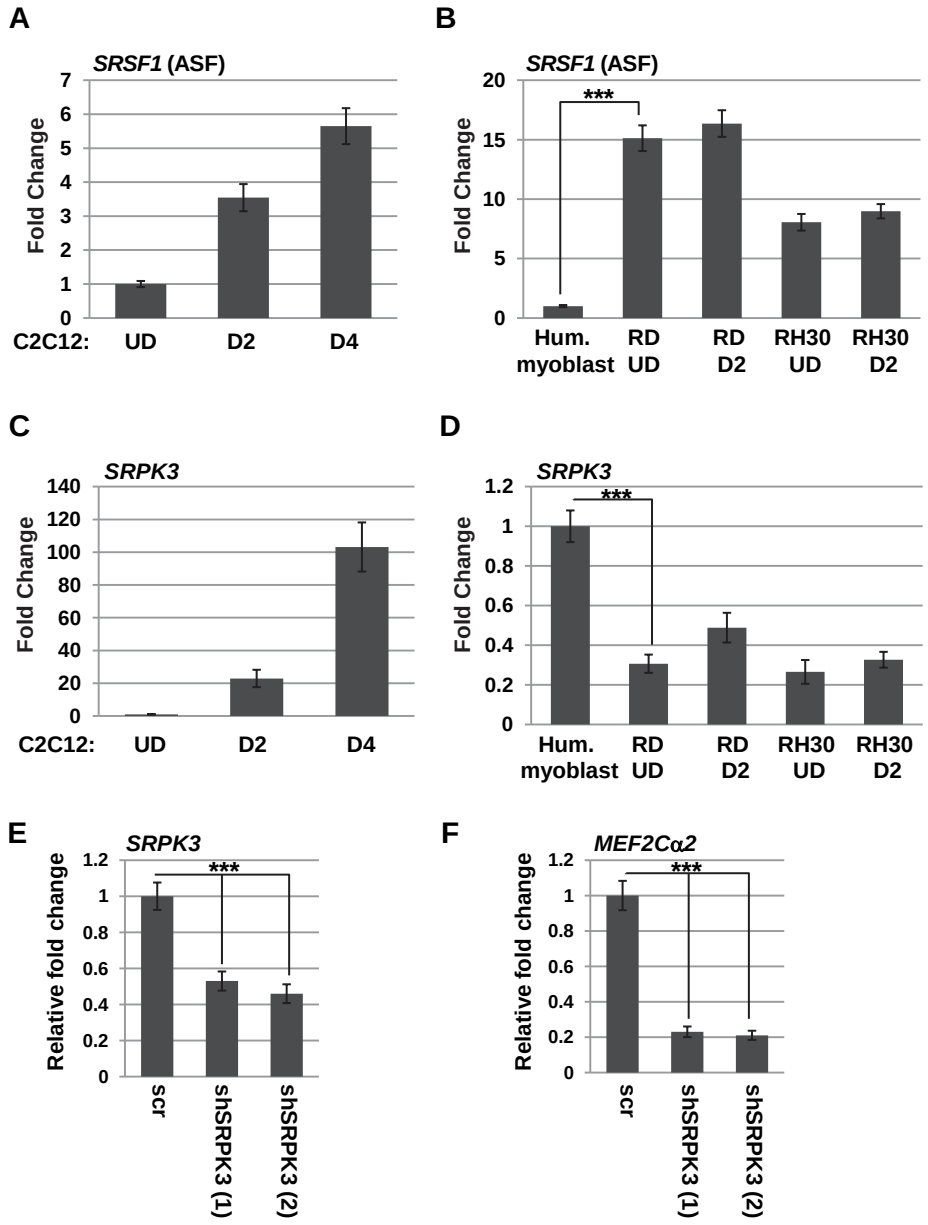
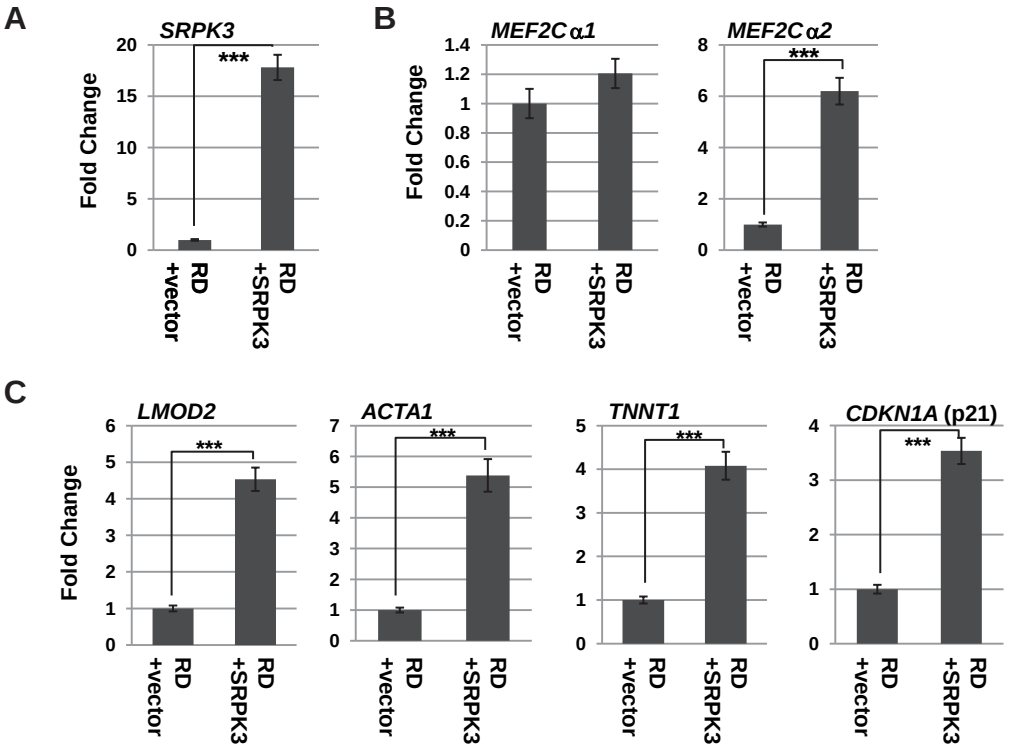

A
SRSF1 (ASF)
Fold Change
C2C12:
UD
D2
D4
B
SRSF1 (ASF)
***
Fold Change
Hum. myoblast
RD UD
RD D2
RH30 UD
RH30 D2
C
SRPK3
Fold Change
C2C12:
UD
D2
D4
D
SRPK3
***
Fold Change
Hum. myoblast
RD UD
RD D2
RH30 UD
RH30 D2
E
SRPK3
***
Relative fold change
scr
shSRPK3 (1)
shSRPK3 (2)
F
MEF2Cα2
***
Relative fold change
scr
shSRPK3 (1)
shSRPK3 (2)

Figure 8


A
SRPK3
Fold Change
20
15
10
5
0
***
+vector RD
+SRPK3 RD
B
MEF2Cα1
Fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
+vector RD
+SRPK3 RD
MEF2Cα2
8
6
4
2
0
***
+vector RD
+SRPK3 RD
C
LMOD2
Fold Change
6
5
4
3
2
1
0
***
+vector RD
+SRPK3 RD
ACTA1
7
6
5
4
3
2
1
0
***
+vector RD
+SRPK3 RD
TNNT1
5
4
3
2
1
0
***
+vector RD
+SRPK3 RD
CDKN1A (p21)
4
3
2
1
0
***
+vector RD
+SRPK3 RD

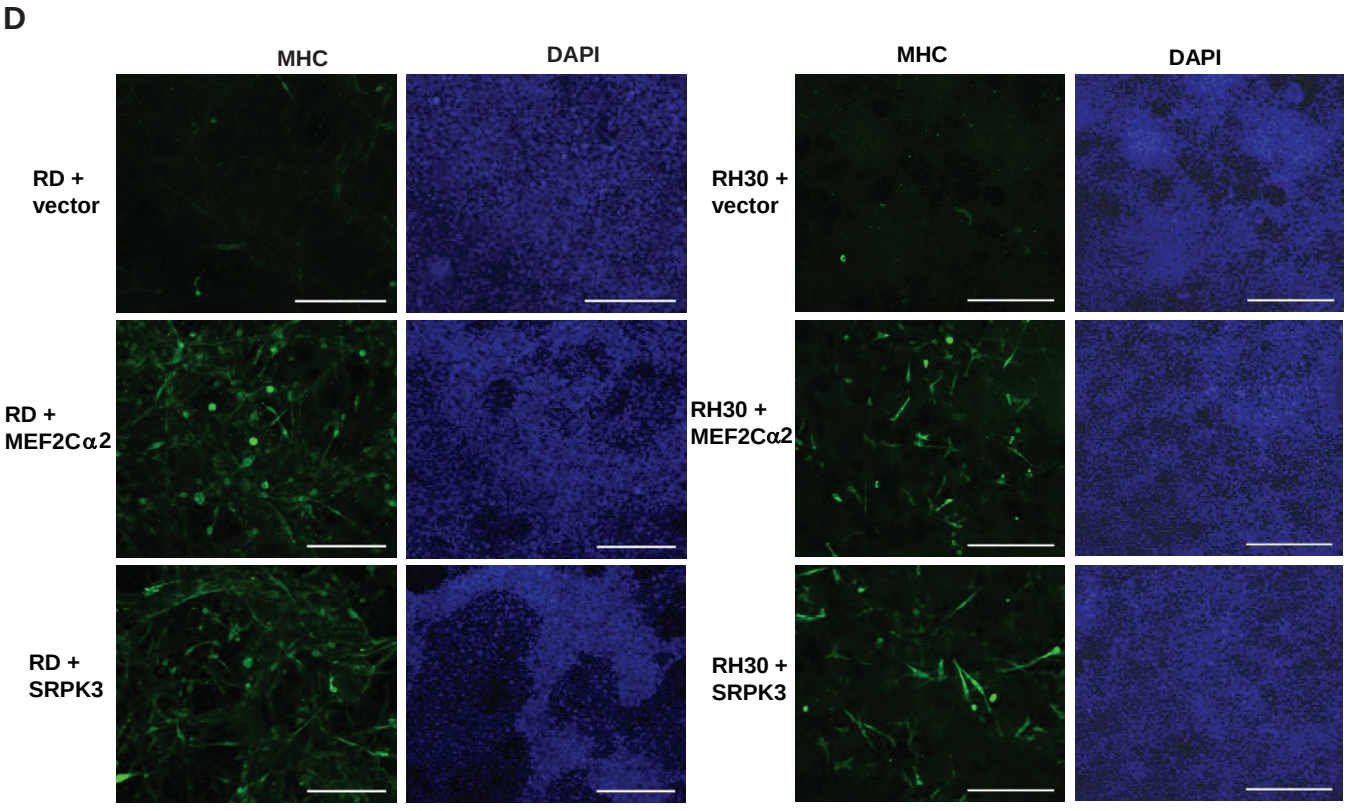
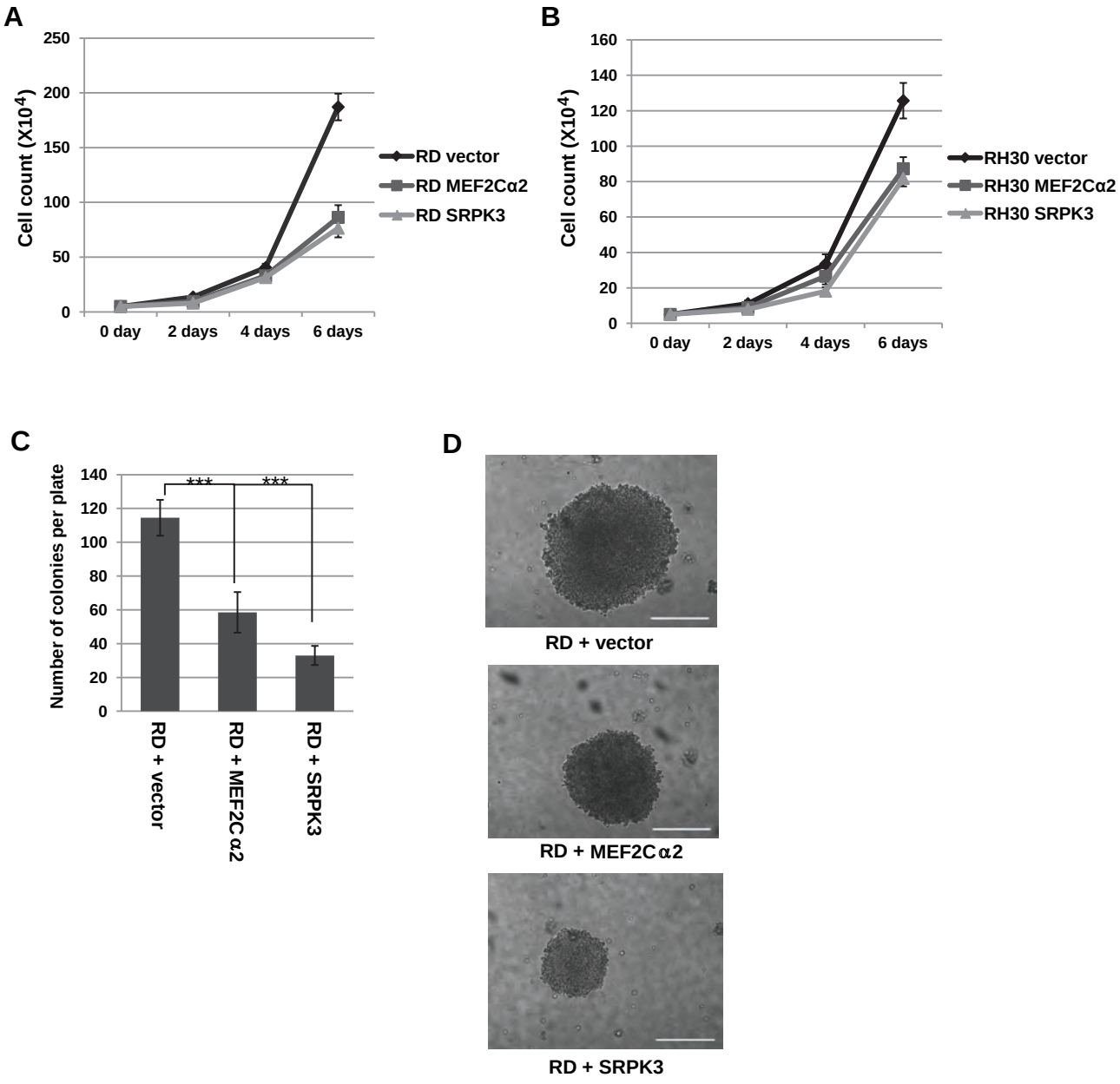

D
MHC
DAPI
MHC
DAPI
RD + vector
RH30 + vector
RD + MEF2Cα2
RH30 + MEF2Cα2
RD + SRPK3
RH30 + SRPK3

Figure 9


A
Cell count (X10^4)
250
200
150
100
50
0
0 day
2 days
4 days
6 days
RD vector
RD MEF2Cα2
RD SRPK3
B
Cell count (X10^4)
160
140
120
100
80
60
40
20
0
0 day
2 days
4 days
6 days
RH30 vector
RH30 MEF2Cα2
RH30 SRPK3
C
Number of colonies per plate
140
120
100
80
60
40
20
0
***
***
RD + vector
RD + MEF2Cα2
RD + SRPK3
D
RD + vector
RD + MEF2Cα2
RD + SRPK3