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Dynamic Phosphorylation of the Myocyte Enhancer Factor 2C α 1 Splice Variant Promotes Skeletal Muscle Regeneration and Hypertrophy

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ABSTRACT

The transcription factor MEF2C (Myocyte Enhancer Factor 2C) plays an established role in the early steps of myogenic differentiation. However, the involvement of MEF2C in adult myogenesis and in muscle regeneration has not yet been systematically investigated. Alternative splicing of mammalian MEF2C transcripts gives rise to two mutually exclusive protein variants: MEF2C α 2 which exerts a positive control of myogenic differentiation, and MEF2C α 1, in which the α 1 domain acts as trans-repressor of the MEF2C pro-differentiation activity itself. However, MEF2C α 1 variants are persistently expressed in differentiating cultured myocytes, suggesting a role in adult myogenesis. We found that overexpression of both MEF2C α 1/ α 2 proteins in a mouse model of muscle injury promotes muscle regeneration and hypertrophy, with each isoform promoting different stages of myogenesis. Besides the ability of MEF2C α 2 to increase differentiation, we found that overexpressed MEF2C α 1 enhances both proliferation and differentiation of primary myoblasts, and activates the AKT/mTOR/S6K anabolic signaling pathway in newly formed myofibers. The multiple activities of MEF2C α 1 are modulated by phosphorylation of Ser98 and Ser110, two amino acid residues located in the α 1 domain of MEF2C α 1. These specific phosphorylations allow the interaction of MEF2C α 1 with the peptidyl-prolyl isomerase PIN1, a regulator of MEF2C functions. Overall, in this study we established a novel regulatory mechanism in which the expression and the phosphorylation of MEF2C α 1 are critically required to sustain the adult myogenesis. The described molecular mechanism will represent a new potential target for the development of therapeutic strategies to treat muscle-wasting diseases. *STEM CELLS* 2017;35:725–738

SIGNIFICANCE STATEMENT

A deep understanding of the mechanisms that control adult muscle mass is crucial for developing strategies to counteract muscle wasting-associated disorders. Our work demonstrates that MEF2C transcription factor promotes a balanced muscle growth by acting at two cellular levels: by stimulating muscle differentiation of stem cell-derived muscle precursors and by activating the protein synthesis pathway in differentiated myofibers. We show that the timely coordination of these activities is ensured by a molecular mechanism involving the cross-talk of alternative splicing and protein phosphorylation. Our findings supply evidence that modulating MEF2C function might be a valuable therapeutic strategy for muscle wasting therapies.

INTRODUCTION

Skeletal muscle regeneration and hypertrophy are key adaptive responses to both pathological and physiological stimuli. Both processes are sustained by a population of resident self-renewing muscle stem cells, referred to as Satellite Cells (SC), located under the basal lamina [1–4]. SC are quiescent in the adult steady state, when triggered by signals resulting from

exercises or injuries, they become activated and give rise to a population of myogenic precursor cells (myoblasts) that proliferate, migrate and fuse with the host fibers or generate new myofibers leading to muscle growth/repair. SC self-renewal also ensures the maintenance of a stem cell pool [5, 6]. During the myogenic progression of SC, a controlled balance between proliferation, differentiation and self-renewal is required, to assure efficient

muscle repair and maintenance throughout life. The transcriptional control of these processes in vertebrates is mainly regulated by a handful of protein families, including the paired box transcription factors PAX3 and PAX7, the myogenic regulatory factors (MRFs), MYOD, MYOGENIN, MYF5, and MRF4, the Myocyte Enhancer Factor-2 (MEF2) transcription factors and the homeodomain transcription factors SIX [7–11]. In addition to their roles in the transcriptional activation of muscle-specific genes, these proteins are involved in controlling cell cycle progression. Importantly, they are the ultimate targets of multiple signaling pathways activated by external cues and, by integrating these various signals, they determine the balance between muscle precursors proliferation and cell cycle exit. There are four MEF2 proteins (MEF2A, -B, -C, and -D) in vertebrates, their structures are tailored for receiving and responding to multiple signaling pathways that control their functions at several levels, including alternative splicing (AS) of the encoding transcripts and post-translational modifications [12–16]. It has been shown that skeletal muscle-specific deletion of *Mef2* genes results in impaired muscle regeneration, however the involvement of MEF2 factors in the control of muscle growth and regeneration has not yet been systematically investigated [7, 8]. It has been reported that mutually exclusive AS of exons α 1 and α 2 in *Mef2c* and *Mef2d* transcripts is regulated during skeletal myogenesis and that inclusion of α 2 exons in *Mef2c* and *Mef2d* transcripts is important to guarantee efficient myogenic differentiation in cultured myoblasts and in vivo respectively, whereas the α 1 domains act as trans-repressors of MEF2 myogenic activity [15, 16]. However, the function associated with the α 1 domains might be more complex, as the α 1+ isoforms (MEF2C α 1 splice variants) are persistently expressed in differentiating cultured myocytes [15–17]. Likewise, we observed that inclusion of the α 1 exon in mouse *Mef2c* transcripts is upregulated in vivo in the early phases of muscle regeneration and is maintained during later stages of myogenesis, suggesting unexplored functions for this isoform during adult myogenesis. In addition to AS, covalent modifications such as phosphorylation, acetylation and sumoylation, are critical for modulating MEF2 function, however how these modifications are regulated during myogenesis in vivo is still unknown [18–22]. Finally, the function of MEF2C is widely regulated by its direct physical interaction with several coactivators and corepressors [23]. For instance, we had previously reported a regulatory mechanism that represses MEF2-dependent transcription in muscle precursors via physical interaction with the peptidyl-prolyl cis-trans isomerase PIN1 [24]. The PIN1/MEF2C interaction requires phosphorylation of two serine residues, Ser98 and Ser110, that are located in the α 1 domain, raising the question of how inclusion of the α 1 exon in *Mef2c* transcripts, phosphorylation of Ser98 and Ser110 in the encoded α 1 domain and interaction with PIN1 are coordinated for the regulation of MEF2C function in adult myogenesis in vivo. In order to elucidate the function of MEF2C α 1 in adult myogenesis, we have investigated the function of MEF2C protein variants in SC using both in vitro and in vivo approaches. In agreement with previous results [16], we observed that the MEF2C α 2 isoform exhibits its myogenic activity also in vivo, moreover, we provide the first evidence of pro-hypertrophic and pro-regenerative activities of MEF2C α 1 in skeletal muscle mediated by multiple sequential mechanisms, including

stimulation of SC expansion and subsequent terminal differentiation and activation of the PI3K/AKT-dependent protein synthesis pathway in adult myofibres. The switch between these multiple mechanisms is associated with phosphorylation of the Ser98 and Ser110 residues that regulates its interaction with PIN1. We thus conclude that, the timely inclusion of α 1 exon in *Mef2c* transcripts and phosphorylation of the corresponding MEF2C isoform determines its protein interactions: integration of these mechanisms leads to a coordinated modulation of MEF2C function during adult myogenesis.

MATERIALS AND METHODS

Cell Lines, Primary Cultures, and Single Myofibers

C2C12, COS1 and NIH 3T3 cells were maintained in DMEM/10% FBS. To induce C2C12 cells differentiation, confluent cells were maintained in DMEM/2% Horse Serum (HS). Primary cultures of SC were prepared from adult muscles of *Pax3*^{GFP/+} mice as described [25] and grown in SC plating medium (40% F12/40% DMEM/20% FBS, Ultraser). DMEM, F12 and FBS Gibco were provided by Life Technologies, ThermoFisher, <https://www.thermoFisher.com>, FBS by Gibco, HS Hyclone by GE Healthcare, <http://www.gelifesciences.com>, Ultraser Biosepra by Pall Corporation, <http://www.pall.com>. Adult (4 weeks old) C57BL6 mice were killed by cervical dislocation, and the extensor digitorum longus (EDL) muscles isolated and digested in collagenase as described [26]. Myofibres and associated SC were isolated and cultured in suspension or in adhesion on matrigel-coated plates (BD) in SC plating medium. To promote differentiation of primary SC-derived myoblasts, after 4–5 days of culture they were kept in differentiation medium with low serum [27].

Muscle Regeneration

Mouse studies were performed in accordance with the current version of the Italian Law on the Protection of Animals and approved by the local ethics committee. Tibialis anterior (TA) muscles of C57/BL6 mice (Charles River, <http://www.criver.com/>) were chemically injured using cardiotoxin (CTX) and after 2, 5 or 15 days of recovery, the injured and the contralateral control muscles were collected and used for protein and RNA extraction or embedded in OCT compound, frozen in isopentane cooled with liquid nitrogen and then sectioned (cross-section of 8 μ m thickness) for immunofluorescence staining. To induce protein overexpression during muscle regeneration, both TA muscles were injured and, after 48 h of recovery, lentiviruses (multiplicity of infection-MOI-25) encoding MEF2C isoforms were injected into one of the regenerating TA muscles, while the contralateral was injected with an empty lentivirus. Muscle samples were collected at day 5 and 15 postinjury. Changes in distribution of fiber size was assessed measuring the cross-sectional area (CSA) of myofibers. Counting was performed using NIH ImageJ and the distribution of muscle-fibers CSA was obtained by ranking the fibers by size and the mean \pm SEM, the number of muscle fibers of $n = 3$ animals was calculated for each size range.

Immunofluorescence and Bimolecular Fluorescence Complementation Assay

Isolated myofibers, muscle sections or plated cells were fixed with 4% paraformaldehyde, permeabilized (0.2% Triton X-100,

50 mM NH₄Cl in PBS) and then blocked for 1 hour with 2% HS, finally incubated overnight at 4°C with primary antibodies. Samples were stained with Hoechst (Sigma-Aldrich, Italy, Milan <http://www.sigmaaldrich.com>) and secondary antibody conjugated to a fluorochrome. After immunostaining coverslips were mounted in Mowiol mounting medium and observed under in a Zeiss Axioskop 40 fluorescence microscope equipped with an Axiocam HRC camera for image acquisition. For the bimolecular fluorescence complementation (BiFC) assay, isolated myofibers cultured in adhesion were transfected with the indicated plasmids using Lipofectamin LTX (Life Technologies), then treated as described [24].

Western Blot and Coimmunoprecipitation Assay

Western Blot assays were performed as described previously [21]. When shown, the results were quantified by densitometry using ImageJ software. CoIP assays were performed as described previously [24].

Transcription Reporter Assays

C2C12 and COS1 cells were cotransfected with pGL3(des-MEF2)3, pRSVβ-gal, and the MEF2C expression vectors, then analyzed as described [24].

Antibodies. Mouse monoclonal antibodies were directed against: PAX7 and MHC MF20 (Developmental Studies Hybridoma Bank, IA, Iowa City, Iowa, <http://dshb.biology.uiowa.edu/>), MYOD 5.8A (M3512 Dako, Milan, Italy, <http://www.dako.com>), MYOGENIN F5D (M3559, Dako), DESMIN (D33 Dako), VINCULIN (V4505 Sigma-Aldrich), DYSTROPHIN (ab 7164, Abcam, Milan, Italy, <http://www.abcam.com/>), FLAG M2 (F3165, Sigma), AKT1 (2967, Cell Signaling, www.cellsignal.com).

Rabbit Monoclonal anti-MEF2C (D80C1, Cell Signaling). Rabbit polyclonal antibodies were directed against: MYOD (C-20) (sc-304, Santa Cruz, <http://www.scbt.com/>), MYOGENIN M-225 (sc-576 Santa Cruz), CAVEOLIN-1 (a gift from A. Fanzani, Brescia), Ki67 (15580 Abcam), LAMININ (L9393 Sigma-Aldrich), PIN1 (PC270, Calbiochem, <http://www.abcam.com/>), MEF2C (SBS-002) (Sparrow Biosciences, <http://sparrowbiosciences.com/>), HA (H6908; Sigma-Aldrich), RFP (1R10367 Life Technologies), mTOR (2972, Cell Signaling), pAKT (Thr308) (4056 Cell Signaling), pAKT (Ser473) (9018 Cell Signaling), S6K (9202 Cell Signaling), pS6K (Thr389) (9205 Cell Signaling), P38 (9212 Cell Signaling). Anti-pSer98 MEF2C and anti-pSer110 MEF2C were generated in rabbit using synthetic phosphorylated MEF2C peptides.

Plasmids

pFLAG-MEF2Cα1 WT and 2SA are described in [21, 24], pFLAG-MEF2Cα2 was a gift from Tod Gulick (Orlando). Lentiviral vectors encoding MEF2Cα1 WT and 2SA were generated by cloning the respective cDNAs in the pLENTI-CMV-RFP-2A-PURO (Abm, www.abmgood.com). Vectors encoding for HA-tagged PIN1 protein and the plasmids used for BiFC assay were described in [24].

RNA Extraction and RT-PCR

Total RNA was extracted from muscles using TRIzol Plus RNA Purification System (Ambion, Milan, Italy, www.thermofisher.com)

or from SC using total RNA purification kit (Norgen Biotek, Thorold, ON, Canada, www.norgenbiotek.com). Reverse transcription was performed using SuperScript III (Life Technologies, Milan, Italy, www.thermofisher.com) and oligo dT (Life Technologies). The cDNA was amplified by semiquantitative PCR or quantified in qPCR. Primer sequences are available upon request. Real-Time PCRs were performed using SYBR green reagent (ThermoFisher, Milan, Italy, www.thermofisher.com) in the LightCycler Roche PCR machine.

Chromatin Immunoprecipitations

Chromatin immunoprecipitations were performed as previously described [21, 28, 29]. Five microgram of anti-MEF2 (C21 sc-313X, Santa Cruz) and anti-NFYB (GeneSpin, www.labome.com) antibodies were added to each IP and incubated overnight at 4°C on a rotating wheel. DNAs were resuspended in TE buffer and quantitative real-time PCR was performed using SYBR green reagent (ThermoFisher) in the LightCycler Roche PCR machine. The relative sample enrichment was calculated with the following formula: $2^{\Delta\Delta Ct} = 2^{\Delta Ct_{x} - \Delta Ct_{y}}$, where $\Delta Ct_{x} = Ct_{input} - Ct_{sample}$ and $\Delta Ct_{y} = Ct_{input} - Ct_{control}$. Data have been shown as means of three independent experiments.

RESULTS

Expression and Activity of MEF2Cα1 in Primary Myoblasts

To elucidate the role played by the α1 domain of MEF2C in primary myoblasts, we first determined the dynamics of inclusion of α1/α2 exons in *Mef2c* transcripts during the myogenic progression of murine SC by semiquantitative PCR analysis. For this purpose, we used exon-specific or common primers, whose location is shown in Figure 1A and Supporting Information Fig. 1A. Both strategies show that the α2 exon is ubiquitously expressed in quiescent, proliferating and differentiated SC-derived myoblasts. Inclusion of the α1 exon, almost undetectable in quiescent SC, is enhanced in activated SC-derived primary myoblasts, both α1 and α2 exons are highly expressed in differentiating cells (Fig. 1A and Supporting Information Fig. 1B). To monitor the dynamics of α exon at the protein level, we cultured primary SC for 0, 48 and 72 hours on floating myofibers from EDL muscles and we performed double immunolabeling for SC specific markers and MEF2C using a generic anti-MEF2C and two α1 isoform-specific antibodies developed and/or characterized in our laboratory (Supporting Information Fig. 1C-G). We show that, although all quiescent SC express MEF2C ($n = 9$ mice, >200 cells/mouse) (Fig. 1B day 0), only MYOD positive-dividing and MYOGENIN (MGN) positive-differentiating cells (Fig. 1C and 1D, days 2 and 3), express the α1 domain. We confirmed that 97% of SC on freshly isolated myofibers were quiescent, CAVEOLIN-1 positive (Supporting Information Fig. 1H) [30]. Immunoblot analysis confirmed the presence of MEF2Cα1 protein in proliferating and differentiated C2C12 cells (Supporting Information Fig. 1I). Next, we tested the function of MEF2Cα1 in SC myogenic progression by overexpression. As *Mef2c* transcripts have two additional alternatively spliced regions, β exon and γ domain [31–33] (Fig. 1A, left panel), we investigated their expression in SC and found exclusive expression of the exon β

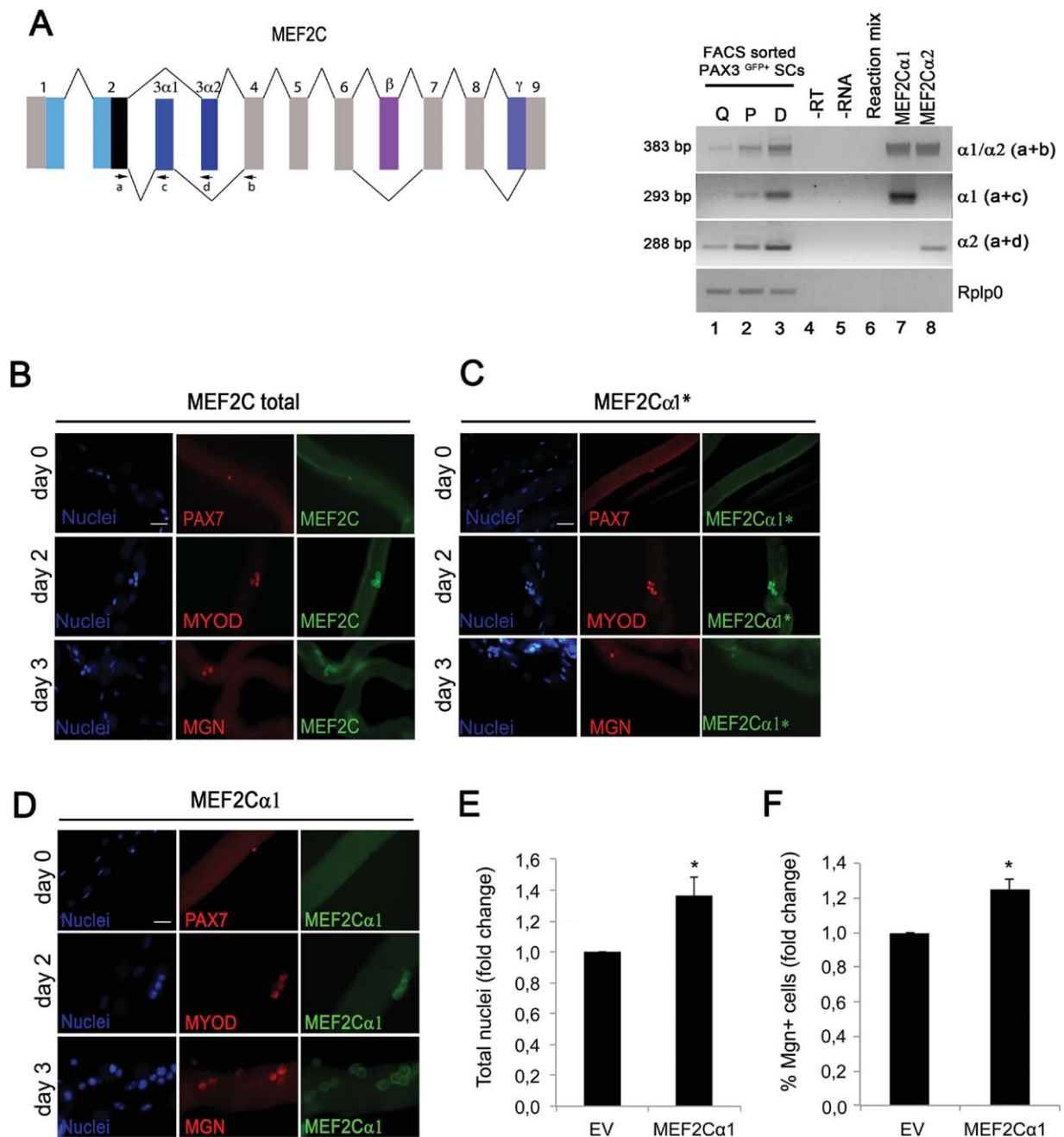


Figure 1. MEF2C α 1 promotes SC proliferation and differentiation. **(A):** Left: structure of the coding exons of the mouse *Mef2c* transcript. Arrows show the primers annealing sites used to evaluate the expression of α 1 and α 2 exons in quiescent (Q), proliferating (P) or differentiating (D) SC. Negative controls: -RT, -RNA, PCR reaction mix. Expression vectors of the MEF2C isoforms were used as control templates. *Rplp0* was used as a control of RNA quantities. Similar results were obtained from two sets of samples. **(B-D):** Single myofibers isolated from extensor digitorum longus muscles were cultured in floating conditions and immunostained for MEF2C, PAX7, MYOD or MYOGENIN. MEF2C was detected with a general antibody (total) (B), or two isoform-specific antibodies (MEF2C α 1* and MEF2C α 1) (C and D). Bars, 50 μ m. **(E, F):** Primary SC were transduced with either control EV or MEF2C α 1 coding lentiviruses. 48 hours later, nuclei were stained with DAPI (E), 72 hours later cells were immunostained for MYOGENIN (F) and counted. The number of control transduced cells was taken as one. Three independent experiments with duplicate samples were carried out for each measurement. Error bars represent SEM. *, $p < .05$. Abbreviations: EV, empty vector; MEF2C, myocyte enhancer factor 2C; *Rplp0*, Ribosomal protein large P0; -RT, nonreverse-transcribed RNA; -RNA, reverse transcription mixture; SC, satellite cells.

skipped isoform, whereas both γ + and γ - variants are equally expressed (Supporting Information Fig. 1L). Therefore, we decided to evaluate the activities of the α 1 exon in a α 1+ β - γ + context [24]. Freshly isolated SC were transduced with a lentiviral vector carrying RFP and mouse MEF2C α 1 cDNAs and

their proliferation and differentiation potential were compared to those of control cells (schematized in Supporting Information Fig. 2A, >80% of RFP+ infected cells, Supporting Information Fig. 2B). We observed a 38% increase in the capacity of the cells to proliferate, upon overexpression of

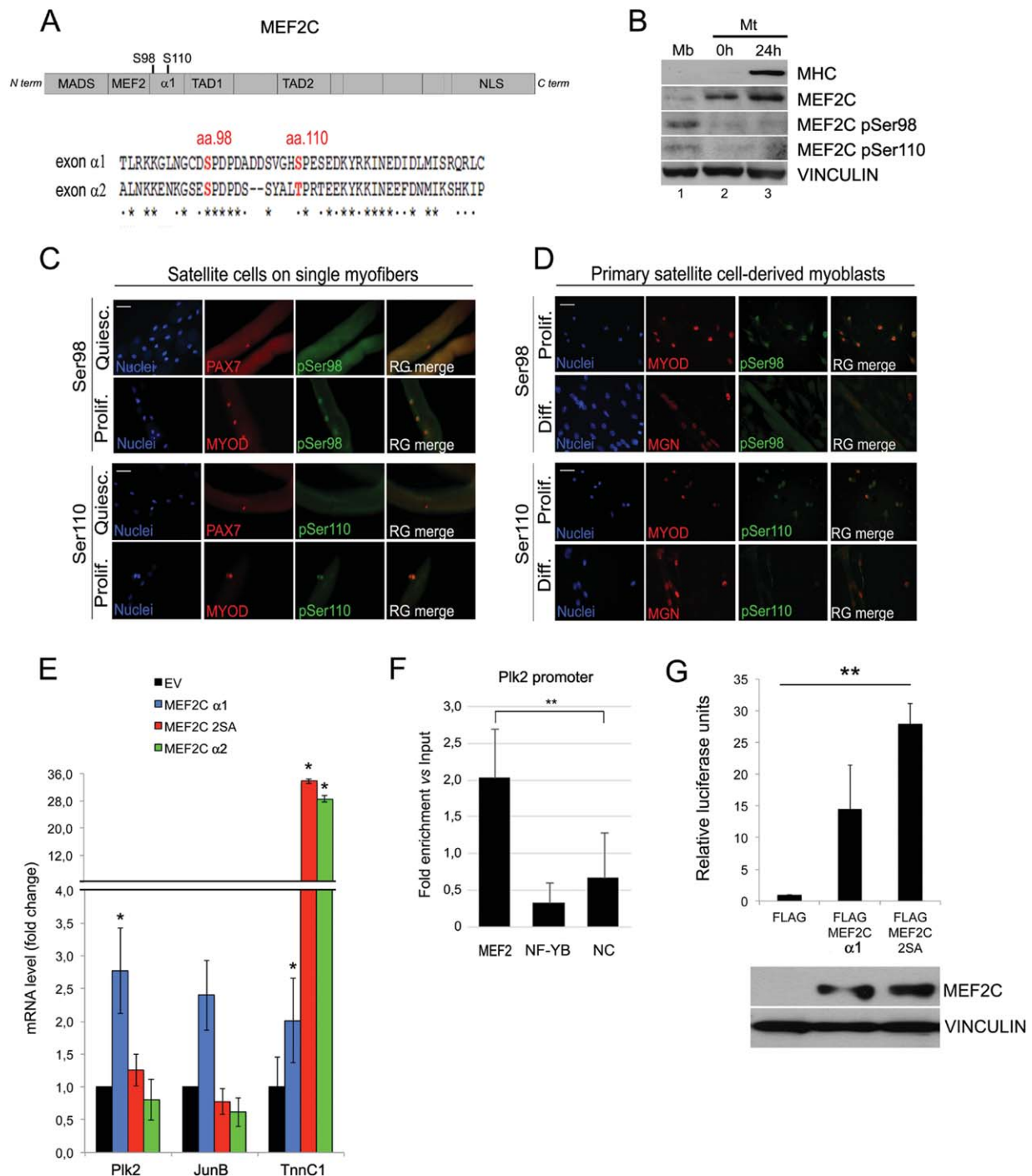


Figure 2. Dynamics of Ser98/110 Phosphorylation. **(A)** MEF2C protein structure and positioning of Ser98/110 residues. Sequences of the mouse MEF2C α exons were aligned with ClustalW software, similar and identical residues are marked with dots and asterisks. Ser98/110 residues in α 1 domain and the equivalent residues in α 2 are marked in red. **(B)** Extracts were prepared from Mb or cells differentiated at different times (Mt 0–24 hours) and immunoblotted for MHC, MEF2C, MEF2C phosphorylated on Ser98 and Ser110 (pSer98, pSer110). VINCULIN was used as loading control. **(C, D)** Single myofibers were immediately fixed (Quiescence), cultured for 48 hours (Proliferation) (C) or plated on gelatin-coated dishes and immunolocalized for pSer98/pSer110, and PAX7 or MYOD or MYOGENIN proteins at different times after isolation. Bars, 50 μ m (D). **(E)** C2C12 cells were transiently transfected with vectors encoding RFP and MEF2C α 1 wild type and mutated on Ser98/110 (2SA) or MEF2C α 2 vectors. RFP⁺ transfected cells were FACS-sorted, *Plk2*, *JunB*, and *TnnC1* expression levels were analyzed by qPCR. Data are expressed as fold induction relative to control cells (EV). **(F)** Chromatin Immunoprecipitation analysis showing MEF2 recruitment to the MRE in the regulatory region of *Plk2* gene in C2C12 myoblasts. Values are represented as % of Input DNA \pm s.e. Statistical analysis (unpaired *t* test) was performed to compare the enrichment of MEF2 versus anti-FLAG control antibody. **(G)** Upper panel: transactivation assays on C2C12 cells transfected with MEF2C α 1 or the 2SA mutant on a MEF2-dependent reporter. Luciferase activities were normalized for transfection efficiency by using β -Galactosidase. Lower panel: Western blot analysis of the expression levels of ectopically expressed FLAG-tagged MEF2C proteins in transiently transfected C2C12 cells using an anti-FLAG antibody. Vinculin was used as loading control. *, $p < .05$; **, $p < .01$. Abbreviations: Mb, myoblasts; MEF2C, myocyte enhancer factor 2C; MHC, myosin heavy chain.

MEF2C α 1, as measured by cell number 48 hours postinfection (Fig. 1E). The ability of MEF2C α 1 to promote cell proliferation was also observed in nonmuscle cells such as NIH 3T3 mouse fibroblasts (Supporting Information Fig. 2C) and colon cancer cells [17]. We used a similar approach to study the role of MEF2C α 1 overexpression on terminal differentiation. For that purpose, 3 days post-infection cells were fixed and immunostained for MYOGENIN. SC-derived myoblasts were kept subconfluent and in growth medium, to dissect the effect of MEF2C overexpression from that of density-dependent cell cycle exit. In these conditions we observed a 25% increase of MYOGENIN positive cells compared to controls (Fig. 1F), suggesting that MEF2C α 1 up-regulation promotes differentiation in addition to the pro-proliferative effect described above.

Phosphorylation of Ser98 and Ser110: A Regulatory Switch

To gain insight into the molecular mechanisms responsible for switching-on alternatively the pro-proliferative and the pro-differentiative activities of MEF2C α 1, we focused our attention on two phosphoacceptor sites, Ser98 and Ser110, located in α 1 domain, whose phosphorylation regulates MEF2C function in C2C12 myoblasts, allowing its association with PIN1, an inhibitor of muscle terminal differentiation (Fig. 2A) [17, 24]. As a first step, we characterized the level of phosphorylation of MEF2C α 1 during differentiation of C2C12 cells with phospho-specific antibodies, that specifically recognize MEF2C phosphorylated on Ser98 (anti-pSer98) or Ser110 (anti-pSer110) (Supporting Information Fig. 2D). We found that Ser98/110 are phosphorylated exclusively in myoblasts (Fig. 2B). Next, we performed the same analysis in SC retained in their niche on isolated myofibers. Again we observed Ser98/110 phosphorylation in proliferating MYOD+ primary myoblasts (Fig. 2C, proliferation). As concern differentiation, we plated single myofibers on Matrigel: Ser98 and Ser110 phosphorylation was detected in MYOD+ proliferating SC-derived myoblasts (Fig. 2D, proliferation), but not in differentiating MYOGENIN+ cells (Fig. 2D, differentiation). Overall our data indicate that MEF2C α 1 phosphorylation on Ser98/110 is restricted to proliferating myoblasts and absent in terminally differentiated cells. To test how the phosphorylation of PIN1 binding sites in MEF2C α 1 could affect proliferation and/or differentiation of SC, we investigated its transcriptional potential in promoting the expression of different target genes: two immediate early genes, polo like kinase 2 (*Plk2*) and *JunB* and troponin C1 (*TnnC1*), a myogenic target. We found that MEF2C α 1 induced the expression of both *Plk2* and *JunB* endogenous genes and that this activation depends on the presence of intact Ser98/110 phosphoacceptor sites, given that equivalent levels (Supporting Information Fig. 2E) of the non-phosphorylatable 2SA mutant had no activity (Fig. 2E). Conversely, the non-phosphorylatable 2SA mutant stimulated the expression of *TnnC1* more efficiently (32-fold) than the pro-myogenic MEF2C α 2 isoform (28-fold) (Fig. 2E). In order to investigate whether MEF2C α 1 directly regulates the transcription of *Plk2* and *JunB* genes, we looked at putative MEF2 recognition elements (MREs) around their transcription start sites by bioinformatics approach. We found putative MREs in the regulatory regions of both genes (Supporting Information Fig. 2F). Chromatin Immunoprecipitation (ChIP) experiments, with chromatin from proliferating myoblasts, showed a significant enrichment of MEF2 binding over the control antibody in

the *Plk2* amplicon containing the predicted MRE. The anti-NF-YB antibody, which is able to recognize the CCAAT-binding factor NF-Y, was used as negative control antibody (Fig. 2F). Oppositely, a significant percentage of input DNA was immunoprecipitated by the anti-NF-YB antibody, but not by the anti-MEF2, when qPCRs were performed with oligonucleotides specific for the *JunB* CCAAT-regulatory region (Supporting Information Fig. 2G). These results suggest a direct involvement of MEF2C α 1 in regulating the transcription of *Plk2* and an indirect role in *JunB* regulation, possibly through the regulation of other transcription factors. Overall our data indicate that phosphorylation of Ser98 and Ser110 inhibits the transcriptional potential of MEF2C α 1 toward muscle-specific genes, while boosting the activation, directly or indirectly, of cell-cycle related target genes. Coherently, the 2SA mutant is more transcriptionally active (two-fold) than the wild type MEF2C α 1 protein toward a myogenic luciferase reporter gene in muscle (Fig. 2G) and non-muscle cells (Supporting Information Fig. 2H). These findings indicate a critical role for phosphorylation of Ser98 and Ser110 in α 1-domain-mediated transcriptional activation of proliferation—versus differentiation—target genes, suggesting that this covalent modification is the molecular switch of the bi-modal activity of MEF2C in SC.

PIN1/MEF2C Interaction in Primary Myoblasts

Next we determined the role of the peptidyl-prolyl cys/trans isomerase PIN1 in modulating MEF2C function in SC. First, we observed that *Pin1* transcripts are upregulated in proliferating SC-derived myoblasts by RT-PCR (Fig. 3A) and that all MYOD-positive proliferating myoblasts express PIN1 by immunofluorescence on single myofibers (Fig. 3B). To demonstrate that the MEF2C α 1/PIN1 interaction takes place in adult myoblasts, we used the BiFC approach [34]. To this end, MEF2C α 1 and PIN1 were fused to the amino- or carboxyl-terminal fragment of YFP, respectively, and transfected into SC-derived myoblasts, the fusion proteins Jun-YN and Fos-YC were used as positive controls (Fig. 3C, panels ii, Fig. 3D, panels v). Coexpression of FLAG-tagged MEF2C α 1 and HA-tagged PIN1-YC in plated SC resulted in complementation of the YFP in the nucleus of MYOD positive proliferating SC (Fig. 3C, panels iii, Fig. 3D, panels vi). Given that adult myoblasts also express *Mef2c* transcripts including the α 2 exon, we next investigated whether PIN1 distinguishes between the two isoforms by coimmunoprecipitation experiments in transfected COS cells. As shown in Figure 3E, we found that only MEF2C α 1 interacts with PIN1, unlike the muscle-specific MEF2C α 2 isoform, devoid of the Ser110 residue (Fig. 2A). Altogether our data indicate that the MEF2C α 1/PIN1 interaction might play a role in regulating the proliferative potential of adult myoblasts. To investigate this, equal numbers of freshly isolated adult SC were transduced with combinations of lentiviral vectors carrying PIN1 and/or MEF2C α 1 cDNAs, and their proliferation potential was assayed by evaluating the number of cells after 3 days in culture. We show that PIN1 and MEF2C α 1 synergize to promote proliferation, with an average 60% increase of cell number compared to control cells and about 15% increase versus MEF2C α 1 overexpressing cells (Fig. 3F). These data indicate that association with PIN1 increases the pro-proliferative activity of MEF2C α 1 in primary myoblasts. To reinforce these observations, we used lentiviral vectors encoding MEF2C α 1 mutated in the PIN1 binding sites located in the

$\alpha 1$ exon (Ser98/110) and in the C-terminus (Ser254 and Ser388) [24] (SA mutant) and we evaluated the proliferation and differentiation potential of transduced SC. We found that the SA mutant is not able to efficiently stimulate myoblasts

expansion (Fig. 3G) but retains the ability to promote their differentiation, expressed as in increase of the percentage of MGN+ (50%) and TROPONIN T+ (TNT+) (60%) cells (Fig. 3G). Thus, phosphorylation of the PIN1 binding sites

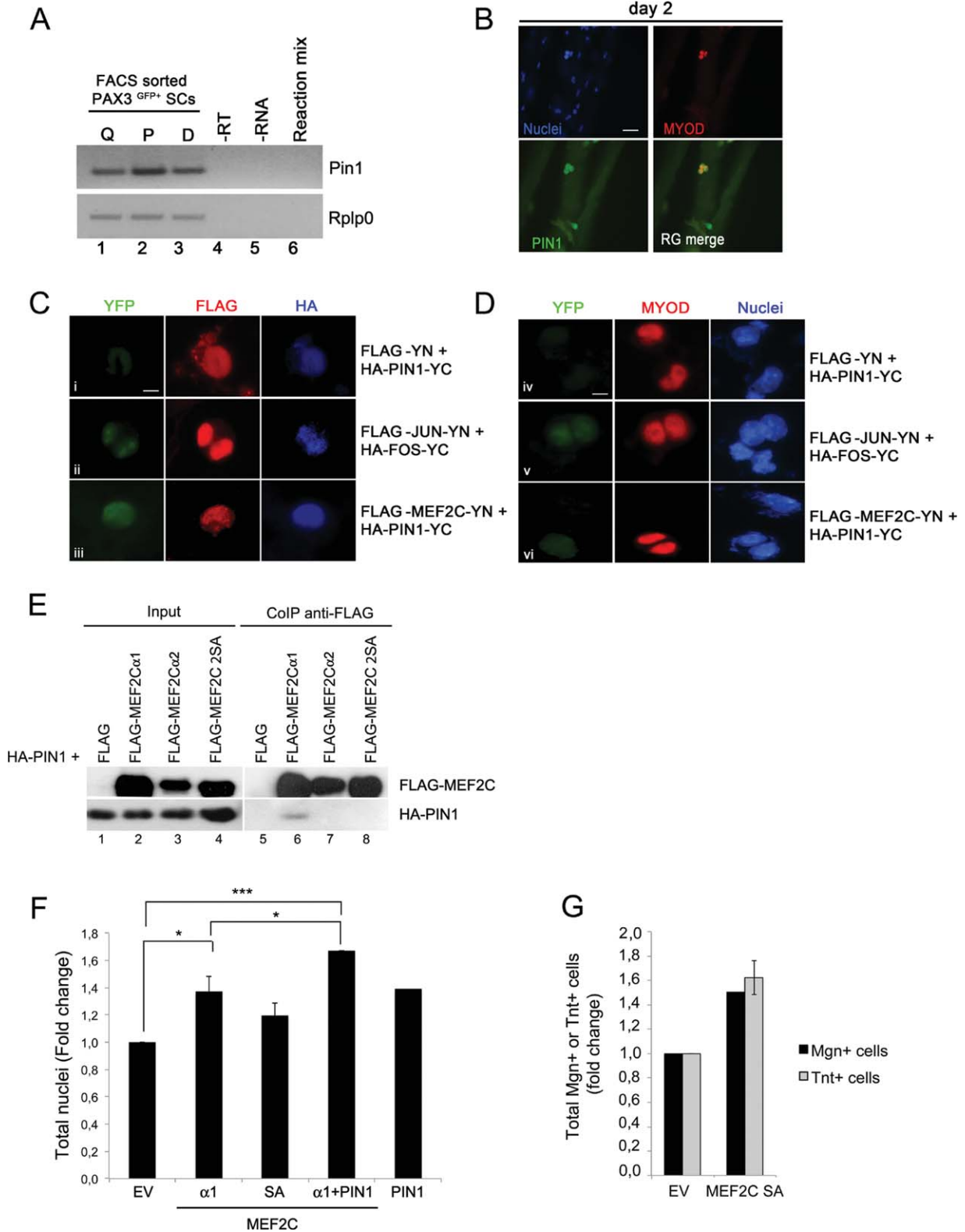


Figure 3.

represents a switch to turn-on and turn-off alternatively the pro-proliferative or the pro-differentiative activities of MEF2C α 1 in cultured adult myoblasts.

Phosphorylation of the α 1 Domain in Muscle Regeneration

We aimed to confirm the relevance of MEF2C α 1 phosphorylation in SC function *in vivo* during muscle regeneration. We first validated that this regulatory mechanism is active during skeletal muscle regeneration upon cardiotoxin (CTX)-induced injury. Western blot analysis showed that the expression level of the α 1 domain increased in regenerating muscle at 2 days post-injury (p.i.) and, although it gradually decreased, it remained expressed throughout the course of regeneration (Fig. 4A, left and middle panels), together with a remarkable level of phosphorylation at Ser98/110 at 2 and 5 days p.i., a time frame concomitant with proliferation of activated SC (Fig. 4A, right panel). Next we investigated the cellular source of MEF2C protein by immunofluorescence analysis on cryosections of regenerating TA muscles at 5 days p.i.: MEF2C protein is expressed in 100% of centrally located nuclei of newly formed myofibers and in 30% of mononucleated interstitial cells (Fig. 4B). In summary our results indicate that the α 1 domain is expressed throughout muscle regeneration and is subjected to a dynamic phosphorylation on Ser98/110, suggesting multiple roles played by MEF2C α 1 *in vivo*.

Effects of MEF2C Splice Variants Overexpression in Adult Muscle

To investigate the role of MEF2C α 1/ α 2 splice variants and of MEF2C α 1 phosphorylation in SC function *in vivo*, we performed a muscle regeneration assay. Due to the high functional redundancy of MEF2 proteins [7], we decided to adopt a gain of function strategy. TA muscles were subjected to a single CTX injury and 2 days later they were injected with lentiviruses encoding the MEF2C α splice variants and the mutant MEF2C α 1 protein or the RFP cassette alone. Muscles were analyzed 5 or 15 days later (as schematized in Supporting Information Fig. 3A). Infection efficiency was >60%–70% for all vectors (Supporting Information Fig. 3B) and qRT-PCR analysis confirmed the ectopic expression of transcripts encoding RFP and MEF2C (Supporting Information Fig. 3C). Immunofluorescence analysis confirmed that MEF2C protein is expressed and phosphorylated on Ser98 in activated, DESMIN-positive SC (Supporting Information Fig. 3D). Examination of serial sections of muscles overexpressing MEF2C proteins revealed an increase in the overall size

throughout the body of the muscle that is particularly pronounced with the 2SA mutant at 15 days p.i. (Fig. 5A). Evaluation of the cross-sectional areas (CSA) of regenerated muscles showed the presence of a number of hypertrophic myofibers, in muscles that overexpress all MEF2C proteins, in comparison to control injected muscles (Fig. 5B). However, the hypertrophic effect is particularly pronounced upon ectopic expression of the non-phosphorylatable 2SA mutant, already at an early stage of regeneration (5 days p.i.). The upper panels of Figures 5B show the frequency distribution of myofiber CSA in the transduced regenerating muscles. Table C shows the mode and median values inferred from the histogram and the calculated shifts of CSA versus the EV control injected muscles. The modal values at 15 days reflect the left-shift (880 μm^2) with MEF2C α 1 wild type and the right shift (1440 μm^2) with MEF2C α 2 and the non-phosphorylatable 2SA mutant, in comparison to control regenerating muscle, where the majority of fibers were 1280 μm^2 in size. Similarly, we found that the median cross-sectional area of myofibers in muscles overexpressing MEF2C α 2 and 2SA were respectively 1536 μm^2 and 1684,53 μm^2 versus 1348 μm^2 for the control muscles and 1314,81 μm^2 for muscles that overexpress the wild type protein. The hypertrophic effect of the 2SA mutant was already evident at the very early stages of muscle regeneration as indicated by the observation that the median CSA of myofibers was higher (660 μm^2) than those of the other samples, with a shift of +202,88 μm^2 , the shift is more evident at later stages of regeneration (+336,51 μm^2). At the 5-day time point regenerating muscle that overexpress MEF2C α 2 is instead characterized by a high proportion of smaller newly regenerating myofibers, with a shift of $-80 \mu\text{m}^2$ and $-97,64 \mu\text{m}^2$ of the mode and median CSA, probably due to the ability of this splice variant to promote myotube formation [16].

Exclusively in the case of muscles overexpressing MEF2C α 1, at 15 days p.i., we observed the coexistence of hypertrophic fibers (>2400 μm^2 , 9% of totally counted myofibers) with small, regenerating ones (<200 μm^2) suggesting that, in addition to the hypertrophic effect, a regenerative process persists at a stage in which this process has normally ended. This observation suggest that MEF2C α 1 might stimulate SC proliferation also *in vivo*. To address this issue, we quantified the number of mononucleated interstitial cells expressing *Ki67*, a marker of proliferation, in transduced adult muscles 5 days p.i. In agreement with the *in vitro* data, we found that the percentage of *Ki67*⁺ nuclei was increased in MEF2C α 1 (6.5%) compared to MEF2C α 2 overexpressing (5.38%) and control muscles (4.8%), whereas ectopic expression of the 2SA mutant

Figure 3. PIN1/MEF2C α 1 interaction in primary myoblasts. **(A):** Expression level of *Pin1* in quiescent (Q), proliferating (P) or differentiating (D) SC. Negative controls: $-RT$, $-RNA$, PCR reaction mix. *Rplp0* was used as a control of RNA quantities. Similar results were obtained from two sets of samples. **(B):** Single myofibers were cultured for 48 hours and immunolocalized for PIN1 and MYOD. Bar, 50 μm . **(C, D):** Bimolecular Fluorescence Complementation (BiFC) assay. Myoblasts were transfected with FLAG-YN and HA-PIN1-YC negative controls (panels i and iv), FLAG-JUN-YN and HA-FOS-YC positive controls (panels ii and v) and FLAG-MEF2C α 1-YN and HA-PIN1-YC (panels iii and vi). Cells were stained with anti-FLAG (red), anti-HA (blue) or anti-MYOD antibodies and DAPI. The fluorescence of the YFP (green stain) results from the bimolecular complementation assay. Scale bar, 50 μm . **(E):** COS-1 cells were cotransfected with HA-tagged PIN1 and FLAG-tagged MEF2C vectors. CoIP was performed with anti-FLAG antibody and immunoblotted for FLAG or HA tags. As shown in the representative immunoblot, HA-PIN1 is coimmunoprecipitated exclusively with the wild type MEF2C α 1 isoform. These findings were replicated over three independent experiments. **(F):** Myoblasts were transduced with the indicated combinations of lentiviruses. 48 hours later, DAPI-stained nuclei were counted. Three independent experiments were carried out for each measurement. Error bars = standard deviations. ***, $p < .001$, *, $p < .05$. **(G):** Myoblasts were transduced with lentiviral vectors encoding MEF2C α 1 mutated on the PIN1 binding sites (MEF2C SA), 72 hours later cells were immunostained for MYOGENIN or TROPONIN T (TnT). Three independent experiments were carried out for each measurement. Error bars represent SEM. Abbreviations: CoIP, coimmunoprecipitation; MEF2C, myocyte enhancer factor 2C; $-RT$, nonreverse-transcribed RNA; $-RNA$, reverse transcription mixture; SC, satellite cells TnT, troponin T.

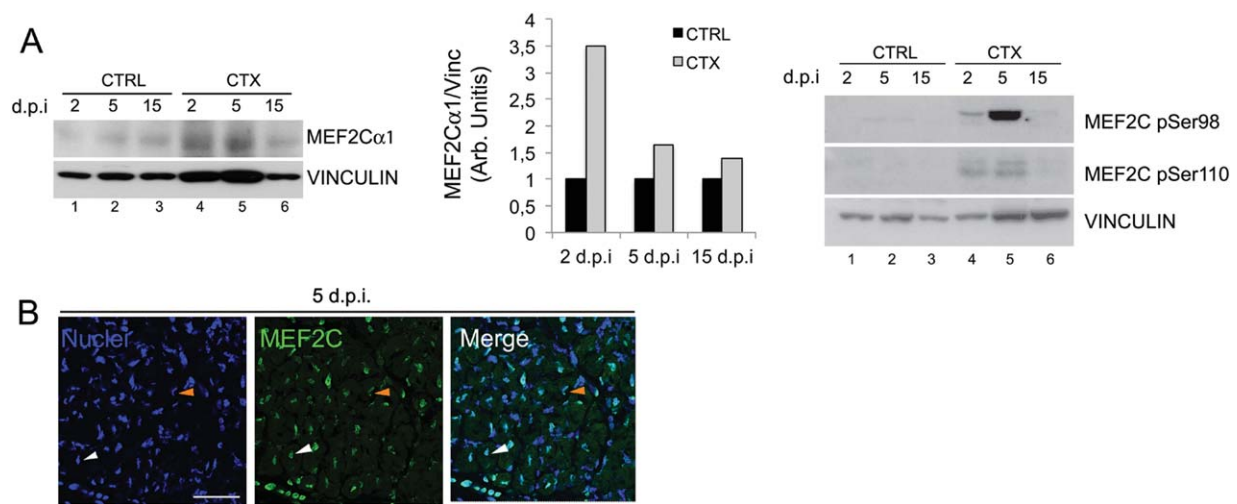


Figure 4. MEF2C α 1 is expressed during muscle regeneration and is dynamically phosphorylated on Ser98 and Ser110. **(A):** Left: Western blot analysis of protein lysates from murine injured (CTX) TA muscles analyzed at 2, 5, and 15 days postinjury with MEF2C α 1. The controlateral TA muscles were used as control (CTRL). Middle: densitometric analysis of the immunoblot experiment for MEF2C α 1 expression. Each time point, after normalization versus VINCULIN, was compared with the correspondent baseline level in the controlateral leg considered as 1. Right: representative Western Blot analysis of protein lysates from injured TA muscles at 2, 5, and 15 days postinjury with pSer98 and pSer110 antibodies. The controlateral TA muscles were used as control tissues. For all analysis, VINCULIN was used as control of the quantity of proteins. **(B):** Cryosections of regenerating TA muscle 5 d.p.i.; nuclei were stained with DAPI, MEF2C protein is revealed by anti-MEF2C antibody and localizes in central nuclei (white arrowhead) and in mononucleated interstitial cells (red arrowhead). Scale bars are 50 μ m. Abbreviations: CTX, cardiotoxin; CTRL, control tissues; MEF2C, myocyte enhancer factor 2C.

correlates with an earlier reduction of proliferating interstitial cells (2.5%) (Fig. 5D). Overall our findings indicate that both MEF2C α 1 and MEF2C α 2 splice variants promote muscle regeneration and hypertrophy in adult muscles. Furthermore, we present evidence indicating a bimodal activity of MEF2C α 1 for which the level of phosphorylation of the PIN1 binding sites tips the balance toward its ability to promote cell proliferation, differentiation and muscle growth.

Targets of Overexpressed MEF2C Splice Variants in Adult Muscle

As MEF2C modulates alternatively muscle- and proliferation-related genes in a splice variant- and phospho-dependent manner in cultured myoblasts, we tested whether these regulatory mechanisms operate also *in vivo*. Indeed, qPCR analysis of RNA extracted from regenerated muscles (15 d.p.i.) showed that MEF2C α 1 overexpression promotes the expression of the immediate early gene *Plk2* in adult muscle more efficiently than the 2SA mutant or the MEF2C α 2 splice variant, which in contrast are stronger activators of the muscle-specific gene *TnnC1* (Fig. 6A). In addition, we observed that both the MEF2C α 2 isoform and the 2SA mutant are powerful activators of the expression of insulin like growth factor 1 and 2 (*Igf1* and *Igf2*), two stimulators of myogenesis by activating progenitor cell proliferation, terminal differentiation and muscle growth (Fig. 6B). In line with these results, we observed an increase in the levels of MYOD and of P38, an inducer of terminal differentiation and of MHC, a myogenic marker, in muscles that overexpress the non-phosphorylatable MEF2C α 1 mutant and MEF2C α 2 (Fig. 6C). These results suggest that, analogously to what observed in cultured SC, MEF2C α 1 controls distinct sets of target genes associated with early (myoblast proliferation) or late (terminal differentiation) stages of muscle regeneration *in vivo* through a Ser98/110-

phosphorylation switch. Furthermore, we found that solely MEF2C α 2 is able to potently activate the expression of the cyclin-dependent protein kinases (CDK) inhibitor *Cdkn1a* (*p21*) gene, a MEF2 target gene required for cell cycle withdrawal of skeletal myoblasts, in accordance with its predominant myogenic role [16, 35–37].

Next we investigated the pathway underlying the observed MEF2C-dependent muscle hypertrophy, focusing on the IGF-1 signaling cascade, a major regulator of skeletal muscle mass through the PI3K/AKT pathway [38, 39]. We, therefore, checked the activation of AKT/mTOR signaling in regenerating adult muscles overexpressing MEF2C proteins and we found that total protein levels of AKT1, mTOR and the ribosomal protein p70S6K S6 kinase (S6K) as well as the phosphorylation level of AKT were upregulated upon overexpression of MEF2C α 2 and the 2SA mutant compared to the wild type MEF2C α 1 protein and the control samples (Fig. 6D). Noticeably, we observed that only the non-phosphorylatable 2SA mutant has the ability to increase the levels of phosphorylated S6K, a key step for the activation of protein synthesis [40]. We also evaluated the myonuclear number in the same samples by determining the number of nuclei within the dystrophin-stained sarcolemma and we observed an increase in myonuclear number in muscles overexpressing MEF2C α 2 (50%) and the 2SA mutant protein (60%) (Fig. 6E). These data indicate that MEF2C α 1-dependent muscle hypertrophy is the result of both protein synthesis and myonuclear fusion and that phosphorylation of Ser98/110 negatively controls these functions.

DISCUSSION

Our major finding is that MEF2C α 1, a MEF2C splice variant, is a strong stimulator of skeletal muscle hypertrophic growth, an effect that depends on two mechanisms, including the activation

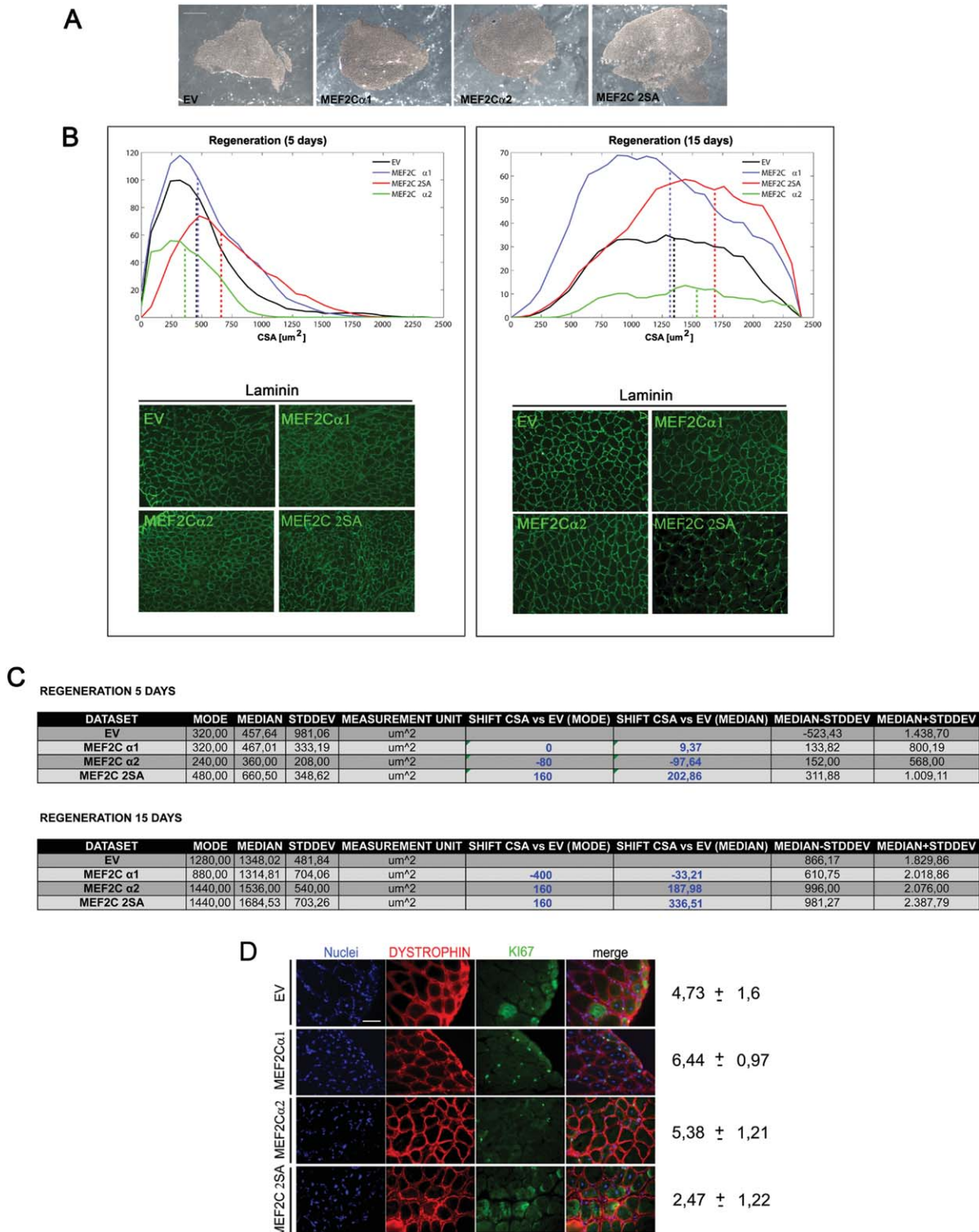


Figure 5. Overexpression of both MEF2C α 1 and MEF2C α 2 splice variants stimulates muscle regeneration and hypertrophy. **(A):** Representative anatomical sections of TA muscles transduced with lentiviral vectors encoding the indicated proteins removed from mice at 15 days after cardiotoxin injection. Bar: 50 μ m. **(B):** Top: histograms showing the frequency distributions of myofiber cross-sectional areas in regenerating TA muscles transduced with EV or lentiviruses bearing the coding sequences of MEF2C α 1 and MEF2C α 2 or MEF2C α 1 mutated on Ser98 and Ser110 (2SA) 5 or 15 days p.i. MEF2C α 2- and 2SA-dependent hypertrophy are indicated by the displacement of the mode from 1280 μ m² in the control group to 1440 μ m². Bottom: representative images of regenerating TA muscle sections immunostained for LAMININ (green). **(C):** Tables reporting Mode, Median and shifts obtained from the histograms in B and C. **(D):** Double immunostaining for KI67 (green) and DYSTROPHIN (red) and nuclear staining with DAPI (blue) in muscle sections from TA regenerating muscles transduced with EV or MEF2C α 1, MEF2C α 2 or the 2SA mutant at 5 days p.i. Scale bar, 50 μ m. On the right are reported the relative percentages of total KI67-positive nuclei in regenerating TA muscles. Data are mean \pm SEM. Abbreviations: EV, empty lentivirus; MEF2C, myocyte enhancer factor 2C.

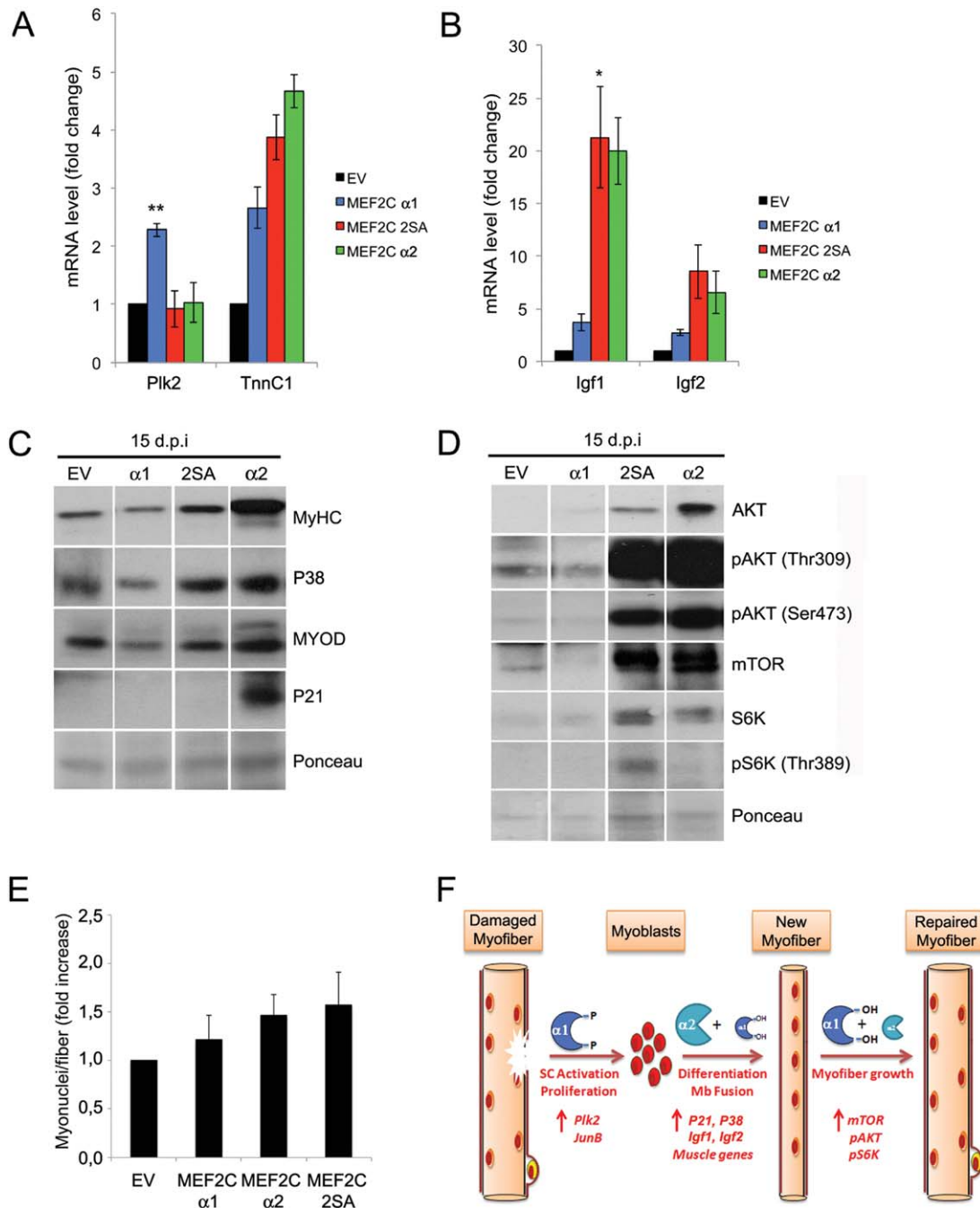


Figure 6. MEF2C-dependent expression of proliferation-, differentiation- and growth- associated genes is regulated by alternative inclusion of $\alpha1/\alpha2$ exons in *Mef2c* transcripts and by Ser98/110 phosphorylation. The mRNA and protein lysates were obtained from 15 d.p.i. regenerating TA muscles transduced with the indicated lentiviruses and analyzed as described below. **(A, B):** *Plk2*, *TnnC1*, *Igf1*, *Igf2* mRNA expression levels were analyzed by qPCR. Data are expressed as fold induction relative to control muscle transduced with the EV. At least three independent experiments with duplicate samples were carried out for each measurement. Error bars represent SEM. *, $p < .05$, **, $p < .01$. **(C):** Representative Western blot analysis of protein lysates from transduced regenerating muscles with MyHC, P38, MYOD, and P21 antibodies. **(D):** Representative Western blot analysis of protein lysates from transduced regenerating muscles with, AKT1 and 70S6K protein, pAKT on Thr308 and Ser473, mTOR, S6K, and pS6K antibodies. Red Ponceau staining was used as a loading control in C and D. **(E):** The number of DAPI-stained nuclei within dystrophin-positive sarcolemma were counted in transduced regenerating muscles, they are expressed per 100 myofibers. Data are mean \pm SEM. **(F):** A model depicting the potential roles played by MEF2C proteins in adult myogenesis. Phosphorylated MEF2C $\alpha1$ promotes satellite cells-derived myoblasts (red cells) proliferation via upregulating, directly or not, the immediate early genes *Plk2* and *JunB*. Subsequent increase of MEF2C $\alpha2$ level drives cell cycle exit, terminal differentiation and myoblast fusion to give newly formed myofibers, by inducing the expression of the CDK inhibitor *P21* as well as *P38* and *MyoD*. Dephosphorylated MEF2C $\alpha1$ contributes to the activation of muscle genes in nascent myofibers and it promotes myofiber growth by activating the anabolic AKT/mTOR/S6K pathway. Abbreviations: EV, empty lentivirus; MEF2C, myocyte enhancer factor 2C; MyHC, myosin heavy chain; pAKT, phospho-AKT; S6K, ribosomal S6Kinase; pS6K, S6K phosphorylated on Thr389.

of the anabolic AKT/mTOR pathway and its downstream target pS6K and the addition of new myonuclei via the proliferation and further fusion of SC-derived myoblasts to the adult myofiber. These different activities are alternatively switched-on by a dynamic phosphorylation of Ser98 and Ser110, two phosphoacceptor sites encoded by the alternative α 1 exon, their phosphorylation is paramount in the control of the pro-proliferative function, given that substitution of these two phosphoacceptor sites with non-phosphorylatable Alanine residues represses the pro-proliferative activity and at the same time enhances the pro-myogenic and hypertrophic abilities of MEF2C. Previous studies have implicated MEF2 proteins in the control of adult skeletal muscle regeneration, where they activate the differentiation gene expression program [7, 8]. In this study we present evidence to suggest that MEF2C, in addition to the pro-differentiating function, mainly due to the MEF2C α 2 isoform, also promotes expansion of primary myoblasts, an activity exhibited by the MEF2C α 1 protein in a phosphorylation-dependent manner. On the basis of our results, we hypothesize that the pro-proliferative activity of MEF2C α 1 on SC is related to its ability to direct the expression of Immediate Early Genes, including, *JunB*, *Plk2*, which are to be added to other cycle-related genes that are regulated by MEF2 proteins in muscle [17, 41] and nonmuscle cells [42, 43].

The observed MEF2C α 1-dependent upregulation of *JunB* is in line with the results of ChIP analyses performed by the Encyclopedia of DNA Elements (ENCODE) consortium in human cells, demonstrating that MEF2 binds to regions upstream of the *JunB* gene, suggesting a direct role of MEF2C in regulating this gene. We identified a putative MRE in the promoter region of the *JunB* gene, near one conserved CCAAT elements, however, in our experimental conditions, we have not detected a recruitment of MEF2 to the identified MRE in front of an efficient binding of the NFY transcription factor to its binding site localized in the same region. This data may then indicate an indirect role of MEF2C α 1 in regulating the expression of *JunB*, potentially through other transcription factors. *Plk2* transcripts present a broad tissue distribution and have been also detected in mouse SC [44]. Our findings also show that MEF2 proteins directly bind to the putative MRE located in the upstream region from the transcription sites of *Plk2* suggesting that MEF2C α 1 might actively regulate *Plk2* transcription by binding to an active enhancer.

One process contributing to the pro-proliferative function of MEF2C in primary myoblasts is the phospho-dependent interaction with PIN1. In this study we show that PIN1 and MEF2C physically interact in primary myoblasts and we found not only that PIN1 inhibits myogenic differentiation [24] but also that this interaction synergistically stimulates their expansion. The use of phospho-specific antibodies allowed us to follow the in vivo kinetics of phosphorylation of the PIN1 binding sites in the α 1 domain: we observed a strong phosphorylation at Ser98/110 within five days after injury, when the regeneration response is at its highest. Later on, during remodeling of regenerated muscle we found maintenance of MEF2C α 1 expression but we did not detect phosphorylation at the PIN1 binding sites, suggesting an important role for the dephosphorylated protein in late phases of adult myogenesis. Our in vivo studies revealed that ectopic expression of the non-phosphorylatable MEF2C α 1 mutant protein induces the expression of myogenic markers and of P38, whose activity is important for early phases of myoblast differentiation [45–49] and myocyte fusion [50]. However, the pro-differentiative activity of the 2SA mutant is less pronounced than that exerted by MEF2C α 2, which instead

potently stimulates the expression of the CDK inhibitor *p21* gene, a key event for cell cycle exit and myogenic terminal differentiation. Conversely we observed that the 2SA mutant robustly promotes a notable increase of the cross-sectional area of myofibers that correlates with the activation of the AKT/mTOR anabolic pathway and the phosphorylation of the downstream target S6K [40, 51, 52] and the concomitant increase in the number of myonuclei. Altogether our data suggest a model whereby MEF2C protein variants promote different stages of myogenesis, ranging from SC proliferation, terminal differentiation and myofiber growth. MEF2C α 1 stimulates muscle growth through two mechanisms, by controlling first SC proliferation when phosphorylated on the PIN1 binding sites, subsequently, upon dephosphorylation it stimulates myonuclear accretion and protein synthesis through activation of the mTOR/S6K pathway (Fig. 6F). In support for this model, fully regenerated muscles overexpressing MEF2C α 1 are characterized by the coexistence of hypertrophic as well as small newly formed myofibers and an increase of *Ki67*-positive cells and *Plk2* expression, reflecting the pleiotropic function played by this protein. Given our data, dynamic phosphorylation of Ser98/110 plays a key role in adult myogenesis. Both Ser residues are predicted to be dynamically phosphorylated by proline-directed protein kinases, including CDKs and mitogen-activated protein kinases (MAPKs), a prediction supported by our finding that CDK6/Cyclin D1 phosphorylates MEF2C at Ser110 in vitro [17] and treatment of cultured cells with CDK6 inhibitors reduces MEF2C phosphorylation. Experiments directed toward the identification of these kinases are the matter of ongoing work. Although MEF2C has been already implicated in activation of genes that promote cardiac hypertrophy in response to IGF1 signaling, this is the first report of its implication in skeletal muscle hypertrophy [53–55]. We hypothesize that MEF2C indirectly activates the AKT/mTOR pathway by inducing an up-regulation of *Igf1* and *Igf2* gene expression. ChIP analyses performed by the ENCODE consortium demonstrate that MEF2 is bound to MEF2 sites located in the promoter as well in an upstream region of the *Igf1* gene, suggesting a direct role of MEF2C in promoting the expression of this gene. Interestingly previous studies indicate that forced expression of IGF-I can induce *Mef2c* gene expression in muscle cells [56]. Our observations of a MEF2C-dependent induction of *Igf1* gene expression suggest a positive-feedback loop underlying muscle growth [56].

CONCLUSION

In general, our data demonstrate that MEF2C promotes muscle regeneration and growth at two cellular levels: by means of activating SC proliferation and differentiation to provide myonuclei to growing myofibers and through promotion of the IGF1/AKT anabolic signaling pathway in differentiated myofibers to increase muscle mass. The timely coordination of these activities in adult myogenesis is ensured by a molecular mechanism in which alternative splicing and phosphorylation finely regulate the activity of MEF2C. Our data supply preliminary evidence that modulating MEF2C α 1 function might be a valuable therapeutic strategy for muscle wasting therapies.

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AUTHOR CONTRIBUTIONS

F.B.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript; D.M.: Conception and design, collection and/or assembly of data, data analysis and interpretation; C.N.: Collection and assembly of data; L.D.F.: collection and/or assembly of data; S.B., M.G., and R.B.: Data analysis and interpretation; V.B.: Collection and/or assembly of data, data analysis and interpretation; C.I.: Conception and design, data analysis and interpretation; A.M.: Conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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