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ORIGINAL ARTICLE

CREB, NF-Y and MEIS1 conserved binding sites are essential to balance *Myostatin* promoter/enhancer activity during early myogenesis

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Abstract Myostatin (MSTN) is a strong inhibitor of skeletal muscle growth in human and other vertebrates. Its transcription is controlled by a proximal promoter/enhancer (*Mstn* P/E) containing a TATA box besides CREB, NF-Y, MEIS1 and FXR transcription factor binding sites (TFBSs), which are conserved throughout evolution. The aim of this work was to investigate the role of these TFBSs on *Mstn* P/E activity and evaluate the potential of their putative ligands as *Mstn* trans regulators. *Mstn* P/E mutant constructs were used to establish the role of conserved TFBSs using dual-luciferase assays. Expression analyses were performed by RT-PCR and in situ hybridization in C2C12 myoblasts and E10.5 mouse embryos, respectively. Our results revealed that CREB, NF-Y and MEIS1 sites are required to balance *Mstn* P/E activity, keeping *Mstn* transcription within basal levels during myoblast proliferation. Furthermore, our data showed that NF-Y site is essential, although not sufficient, to mediate *Mstn* P/E transcriptional activity. In turn, CREB and MEIS1 binding sites seem to depend on the presence of NF-Y site to induce *Mstn* P/E. FXR appears not to confer any effect on *Mstn* P/E activity, except in the absence of all other conserved TFBS. Accordingly, expression studies pointed to CREB, NF-Y and MEIS1 but not to FXR factors as possible regulators of *Mstn* transcription in the myogenic context. Altogether, our findings indicated that CREB, NF-Y and MEIS1 conserved sites are essential to control basal *Mstn* transcription during early myogenesis, possibly by interacting with these or other related factors.

Keywords Myostatin · Myogenesis · Promoter · C2C12 · CREB · NF-Y · MEIS1 · FXR

Introduction

The embryo has the challenging task of unfolding different cellular phenotypes from a common repertoire of genes shared by all cells of its developing body. Success in this enterprise requires gene regulation, which enables different genes to be turned on/off as development proceeds. Transcriptional control of gene expression involves regulatory DNA sequences such as promoters, enhancers, silencers and insulators that cooperate to establish the spatial/temporal expression pattern of specific genes. RNA polymerase II, pre-initiation complex and tissue specific transcription factors bind at specific sites on these regulatory elements to drive gene expression [1]. A finely orchestrated interaction among DNA elements and regulatory proteins is therefore essential for the generation of different tissues during development.

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Myostatin (MSTN) is a signaling molecule that plays an essential role in regulating development, homeostasis and repair of skeletal muscles [2]. This protein belongs to the transforming growth factor-β (TGF-β) superfamily and presents highly conserved structure and function. *Mstn* knockout in mice was shown to cause a dramatic increase in skeletal muscle mass due to both hyperplasia and hypertrophy [2]. Accordingly, natural mutations in this molecule have been associated to a hyper-muscular phenotype in many species [2–5]. In contrast, increased levels of *Mstn* expression or circulating protein were described to reduce muscle size and lead to muscle atrophy [6, 7] while inhibition of MSTN improves skeletal muscle regeneration in mouse models for muscle dystrophies [8]. In accordance to its role, *Mstn* is expressed in the myotome of developing somites, in fetal and adult muscles as well as in satellite cells [2, 9, 10]. However, variations in expression patterns are known to occur among different species of vertebrates (reviewed by Rodgers and Garikipati [11]). For instance, in mouse embryos *Mstn* expression seems to be limited to differentiating myoblasts of somites and limbs, while in chicken several other embryonic tissues express *Mstn*, such as testis and ovaries, suggesting yet unknown developmental functions [2, 12]. In mammals, several adult tissues express *Mstn*, such as heart, mammary gland, adipocytes, olfactory neurons and brain [11, 13–17]. Furthermore, *Mstn* expression changes under different nutritional states [18], aging [19], practice of physical exercises [20] and diseases associated to muscle wasting, such as the acquired immunodeficiency syndrome—AIDS [21], cirrhosis [22, 23], chronic obstructive pulmonary disease [24] and cachexia, a degenerative syndrome associated to several kinds of cancer [25, 26].

Its dynamic expression suggests that *Mstn* transcription is finely regulated, making the study of its promoter and other regulatory elements of particular interest. In a previous work, we have characterized a proximal promoter/enhancer (P/E) for *Mstn* using a phylogenetic approach and functional assays [27]. This proximal element is 260-bp-long and is conserved from human to fish. As about 10% of gene promoters, *Mstn* P/E has a canonical TATA box, which possibly recruits the TATA-binding protein (TBP) subunit of the TFIID complex, as a first step to form the RNA polymerase II preinitiation complex [28, 29].

Besides TATA-box, four other conserved transcription factor binding sites (TFBS) were found within *Mstn* P/E. These sites possibly interact with the factors CREB, NF-Y, MEIS1 and FXR, or related proteins [27]. The first TFBS corresponds to a cAMP response element binding (CREB) site, a cAMP responsive element (CRE) potentially bound by members of the CREB family of Basic Leucine Zipper transcription factors, which also includes activating transcription factor (ATF) and cAMP response element modulator (CREM) [30]. These factors bind to DNA as homo or heterodimers to activate transcription upon phosphorylation by protein kinase A (PKA) [31]. The second TFBS is for nuclear transcription factor Y (NF-Y), which is a widely expressed heteromeric protein composed by the subunits NF-YA, B and C. This complex binds to CCAAT boxes, which are ubiquitous elements found in several gene promoters, especially in those which also possess a CRE site [32, 33], as is the case of *Mstn* P/E. The third binding site is for the homeobox protein myeloid ecotropic viral integration site 1 (MEIS1), which belongs to the three aminoacid loop extension (TALE) subclass of transcription factors [34]. MEIS1 is known to heterodimerize with pre-B-cell leukemia homeobox 1 (PBX1), another TALE protein, to induce or repress transcription in several cellular contexts [35]. Finally, the fourth TFBS is likely bound by farnesoid X receptor (FXR), a member of the nuclear receptor superfamily of transcription factors. FXR is activated by ligands such as farnesol metabolites or bile acids, and is capable of mediating transcriptional activation or repression through a large variety of FXR response elements (FXREs), either as a monomer or as a FXR/RXR heterodimer [36].

Considering that the conserved TFBSs compose an essential part of vertebrate *Mstn* P/E architecture, in this work we aimed at establishing the transcriptional role of these sequences and evaluating the potential of CREB, NF-Y, MEIS1 and FXR factors as regulators of *Mstn* expression during myogenesis.

Experimental procedures

Cell culture

C2C12 mouse myoblast cell line was cultivated in growth medium composed by Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum containing the antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were maintained in 6-wells plates at 37 °C in humidified atmosphere containing 5% CO_2. Once achieving ~60% confluence, myoblasts were trypsinized and used to perform the RT-PCR assays.

RT-PCRs

Total RNA from proliferating C2C12 myoblasts was obtained using Trizol reagent (Invitrogen) and cDNA was synthesized with RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). Gene-specific primers, annealing temperatures and predicted PCR product sizes are detailed in Online Resource Table 1. PCRs were performed in 20 µL reactions, using 1 U of GOTaq® Polymerase (Promega), 300–450 ng of template cDNA, 0.3 mM dNTP mix, 0.5 mM of each primer, 1× Green GOTaq® reaction buffer with the

addition of $MgCl_2$ to 1.9 mM final concentration. Cycling conditions started with an initial denaturation at 94 °C for 1 min, followed by 35–40 cycles of denaturation at 95 °C for 1 min, annealing at 55–57 °C for 30 s, extension at 68 °C for 1 min. A final extension was performed at 72 °C for 10 min. Transcripts identity was confirmed by sequencing.

Site-directed mutagenesis

The tE4 construct, which contains the mouse *Mstn* P/E driving the expression of luciferase reporter gene (as previously described elsewhere [27]) was used as template to mutate specific TFBSs. Site-directed mutagenesis assays were performed using QuikChange II Site-Directed Mutagenesis kit (Agilent). Primers used to introduce single or multiple mutations in TFBS (Online Resource Table 2) were designed with QuikChange Primer design program. Details about the site-directed mutagenesis strategy are available in Fig. 1 of the Online Resource. All mutant constructs were sequenced to confirm the mutagenesis success.

Transfections and dual luciferase assay

Mutant constructs were transfected into C2C12 mouse myoblasts in order to measure differences in luciferase activity. On the day before transfection, C2C12 cells cultured in proliferating conditions were trypsinized and plated in semi-confluency (~60%). Transfections were performed using 10 µg PEI (*polyethyleneimine*) for 10 µg of test DNA (wild-type and each of the eleven mutant constructs of *Mstn* P/E). In all assays, 5 µg of the control vector pRL-TK (Promega) DNA was co-transfected to normalize the transfection efficiency. Myoblasts were harvested 18 h after transfection for reporter gene luciferase assay. Transfections were always performed in duplicate and each experiment was repeated four times. The Dual-Luciferase Reporter Assay System kit (Promega) was used to measure Firefly and Renilla luciferase enzymatic activities.

Statistical analysis

After normalization with Renilla luciferase, the activity of Firefly luciferase of each tested construct was calculated as fold change in comparison to the wild-type *Mstn* P/E. Data were presented as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software.

Synthesis of antisense RNA probes

Templates to generate antisense RNA probes were prepared by RT-PCR. Primers were initially used to amplify gene specific products using E10.5 mouse embryos cDNA. A second PCR was performed to add a T7 RNA polymerase promoter tail in synthesized amplicons. All primers used to produce probe templates are listed in Online Resource Table 1. T7 RNA polymerase (Invitrogen) and Digoxigenin RNA labeling mix (Roche) were used to synthesize probes, which were purified with SigmaSpin™ Post-Reaction Purification Clean-up kit (Sigma).

In situ hybridizations

Animal care was carried out in accordance with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). The protocol was approved by the Committee for Ethics in Animal Research of the State University of Campinas (CEUA/UNICAMP), under the permit number 3366-1.

E10.5 mouse embryos were fixed in 4% paraformaldehyde/PBS and dehydrated in methanol. Specimens were permeabilized by treatment with 10 µg/mL proteinase K/PBS at 37 °C during 20 min, followed by proteinase K inactivation in 2 µg/mL glycine/PBS for 10 min and post-fixation. Hybridizations with gene specific digoxigenin labelled probes were conducted at 70 °C overnight. Post-hybridization washes, antibody incubation and signal revelation were conducted as described by Wilkinson [37]. To detail gene expression patterns, some embryos were embedded in 20% gelatin and sectioned in vibratome (50 µm).

Results

Creb, Nf-Y, Meis1 and *Fxr* are co-expressed with *Mstn* during C2C12 myoblast proliferation

We started this work by investigating whether *Creb*, *Nf-Y*, *Meis1* and *Fxr* gene expression profiles are related to that of *Mstn* during in vitro myogenesis. For that, RT-PCR was carried out in proliferating C2C12 myoblasts, a well-established model to study myogenesis [38]. Besides *Mstn* and its putative regulators, expression of *MyoD* and *Myogenin* (myogenic controls) as well as *16S* rRNA (endogenous control) was analyzed (Fig. 1a). Our data showed that all tested molecules were expressed in C2C12 myoblasts. Therefore, *Mstn* transcription occurs in proliferating C2C12 myoblasts concomitantly with *Creb, Nf-Y, Meis1* and *Fxr* mRNAs, indicating that proteins encoded by these genes are available to interact with their respective binding sites in the *Mstn* P/E.

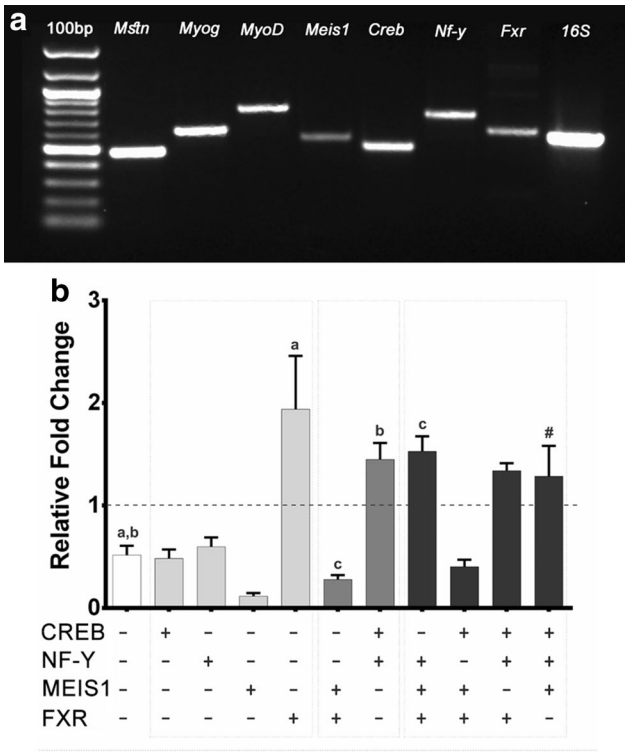

Fig. 1 Conserved TFBSs affect *Mstn* P/E basal levels of expression during C2C12 myoblast proliferation. **a** *Mstn* and its putative regulators are co-expressed in proliferating C2C12 myoblasts; myogenic (*MyoD* and *Myogenin*) and endogenous (*16S* rRNA) controls were also detected. **b** In vitro activity of *Mstn* P/E-Luc constructs containing different mutations. Mutant constructs were transfected into C2C12 cells for measurement of luciferase reporter gene activity, which was normalized to the wild-type control. Wild-type *Mstn* P/E-Luc activity is indicated by the dashed line. Bars indicate means ± SEM of the independent experiments. (a) $p < 0.05$ for FXR+ versus Δ*Mstn*P/E-Luc; (b) $p < 0.05$ for CREB+/NF-Y+ versus Δ*Mstn*P/E-Luc; (c) $p < 0.05$ for NF-Y+/MEIS1+/FXR+ versus MEIS1+/FXR+; (#) CREB+/NF-Y+/MEIS1+ is the only construct with equivalent activity to the wild-type control

Conserved TFBSs for CREB, NF-Y and MEIS1 cooperate to drive *Mstn* P/E basal activity

Having established the expression profiles of *Mstn* potential regulators during C2C12 myoblast proliferation, we next worked to elucidate the role of CREB, NF-Y, MEIS1 and FXR sites. For that, we investigated the effects of single or multiple mutations in the conserved TFBSs of *Mstn* P/E by measuring luciferase activity after transfection of proliferating C2C12 myoblasts with different constructs (Fig. 1b). For each construct, the relative values of activity were calculated as fold change in comparison to the wild-type *Mstn* P/E. It is worth noting that TATA-box was preserved in all constructs in order to allow transcription.

Taken as a whole, the site-directed mutagenesis assays showed that the individual or combined disruption of any of the conserved TFBS can cause changes in the basal activity of *Mstn* P/E in proliferating C2C12 myoblasts. In the more drastic scenario, disruption of all four TFBS (Δ*Mstn*P/E-Luc) caused a 50% reduction in reporter expression relative to wild-type P/E. This result was used as reference for comparisons with the effects in *Mstn* P/E caused by four combinations of triple mutations, in order to mimic the restoration of each one of the TFBS to Δ*Mstn*P/E-Luc (Fig. 1b; Online Resource Fig. 2).

The single recovery of CREB, NF-Y or MEIS1 sites did not change P/E activity in relation to Δ*Mstn*P/E-Luc (Fig. 1b). In contrast, restoration of FXR site caused a significant increase in reporter activity (a, $P < 0.05$), which was 1.94-fold higher than that observed for the wild-type P/E (Fig. 1b). Therefore, in a background lacking other conserved sites, most TFBSs were unable to modulate transcription by themselves, except for FXR, which mediated a positive effect.

Subsequently, analysis of double mutations showed that when only MEIS1 and FXR sites were intact, P/E activity was maintained equivalent to that observed for Δ*Mstn*P/E-Luc and for the construct containing only MEIS1 site intact (Fig. 1b; Online Resource Fig. 2). This result also revealed an antagonistic interaction between MEIS1 and FXR sites, since in the presence of MEIS1, FXR site failed to induce P/E activity. In contrast, the presence of CREB and NF-Y binding sites significantly raised reporter expression in comparison to Δ*Mstn*P/E-Luc (b, $P < 0.05$), reaching levels 1.45-fold higher than wild-type *Mstn*P/E-Luc (Fig. 1b). Since the effect of CREB and NF-Y sites in *Mstn* P/E transcription changed from repression, when alone, to upregulation, when acting together (Online Resource Fig. 2), these sites probably interact synergistically.

Finally, *Mstn*P/E-Luc constructs containing three intact conserved TFBS (or, in other words, constructs with single TFBS disrupted) were evaluated. In this set of assays, we initially analyzed the restoration of CREB or NF-Y in the MEIS1+/FXR+ construct, and then, conversely, the restitution of MEIS1 or FXR in the CREB+/NF-Y+ construct (Fig. 1b). After rescuing NF-Y in the MEIS1+/FXR+ background, there was an induction instead of repression (c, $P < 0.05$), leading to transcription levels 1.53-fold higher than those of wild-type *Mstn*P/E construct (Fig. 1b). In contrast, CREB restoration had no effect in comparison to the transcription levels of MEIS1+/FXR+ construct (Fig. 1b). Taken together, these results show that MEIS1 and CREB sites cannot change the downregulation status of *Mstn* P/E without the NF-Y site (Online Resource Fig. 2). Meanwhile, only the restitution of MEIS1 in the CREB+/NF-Y+ background was able to reestablish wild-type expression levels, indicating that the CREB, NF-Y and MEIS1 sites are sufficient to control *Mstn* P/E activity during myoblast proliferation.

In short, our data revealed that in the context of myoblast proliferation, NF-Y site is essential, although not sufficient, to mediate *Mstn* P/E activity. In turn, CREB and MEIS1 binding sites seem to depend on the presence of NF-Y site to induce *Mstn* P/E activity. FXR appears not to confer any effect on the transcriptional activity of *Mstn* P/E, except in the absence of all other conserved TFBS. Taken together, our results showed that cooperation among CREB, NF-Y and MEIS1 TFBSs is crucial to balance *Mstn* P/E basal activity during myoblast proliferation.

Expression patterns indicate that CREB, NF-Y and MEIS1 are good candidates to regulate *Mstn* expression during in vivo myogenesis

After identifying the cooperation among the conserved TFBS in the regulation of *Mstn* P/E in vitro, we decided to investigate whether *Creb*, *Nf-Y*, *Meis1* and *Fxr* gene expression patterns could also be temporally and spatially related to *Mstn* during in vivo myogenesis. For that, we evaluated the expression of *Mstn* and that of its putative regulators in E10.5 mouse embryos (Fig. 2), a stage in which *Mstn* is known to be strongly expressed [2]. *MyoD* expression was also used as a marker for skeletal myogenesis. Our results showed that *Mstn* transcripts (Fig. 2a) are located in the myotome of developing somites, overlapping with sites of *MyoD* expression (Fig. 2f). *Creb* (Fig. 2b) and *Nf-Y* (Fig. 2c) mRNAs are diffuse and distributed in a dorsal to ventral gradient with expression levels declining from dermomyotome towards myotome. *Meis1* expression (Fig. 2d) is highly upregulated in the central dermomyotome, avoiding the myotome, *Mstn* main domain of expression. In turn, *Fxr* transcripts (Fig. 2e) were not associated to myogenic sites. Since *Creb, Nf-Y* and *Meis1* are expressed in myogenic compartments of developing somites, the proteins encoded by these genes are possibly being synthesized in vivo in proper position and time to regulate *Mstn* expression.

Discussion

In the current study we carried out expression analysis and functional assays to evaluate the potential of CREB, NF-Y, MEIS1 and FXR as regulators of *Mstn* transcription, since TFBSs for these proteins have been conserved in *Mstn* P/E throughout vertebrate evolution [27]. Considering that *Mstn* is expressed mainly in skeletal muscles and its progenitors during development, and given its crucial function in determining muscle fiber number and size [2, 9], we focused our studies in the myogenic context, both in vitro and in vivo.

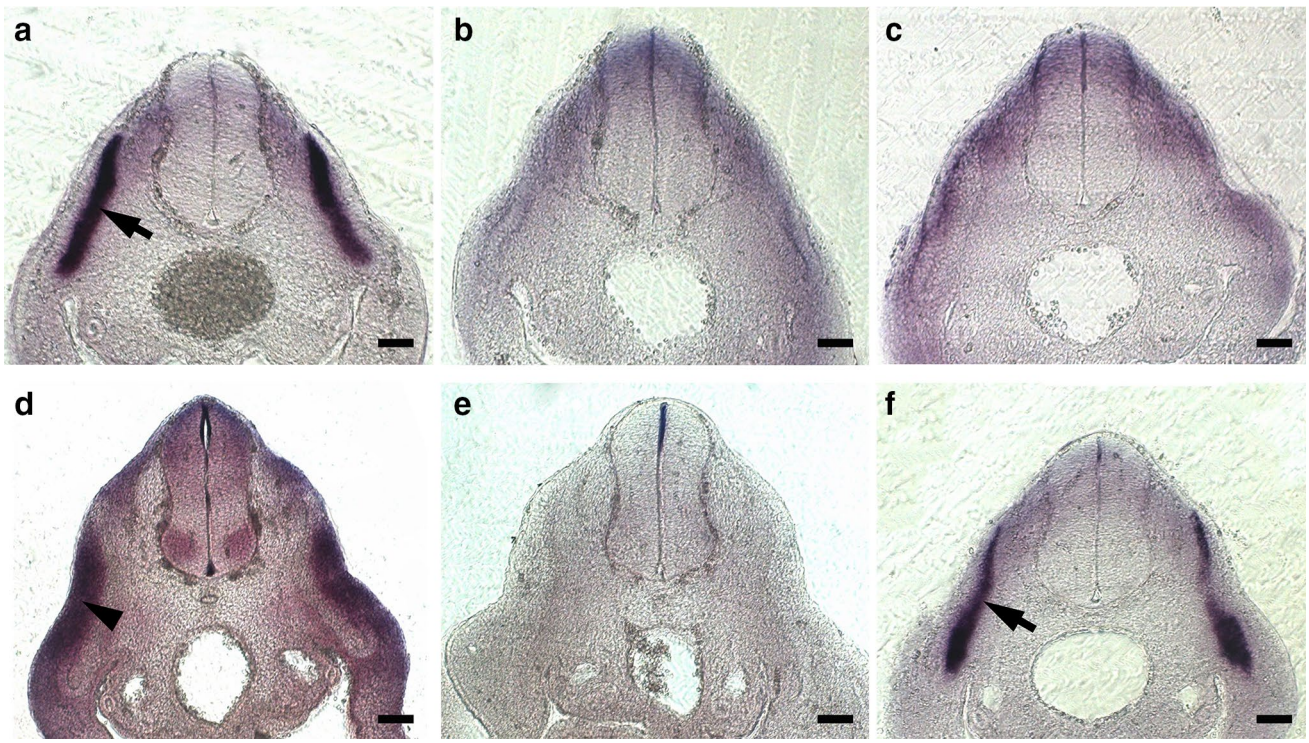

Fig. 2 Expression patterns indicate that CREB, NF-Y and MEIS1 are good candidates to regulate *Mstn* expression during in vivo myogenesis. Transverse sections through somites of E10.5 mouse embryos at interlimb levels show prominent expression of **a** *Mstn* and **f** *MyoD* in the myotome (*arrows*). **b** *Creb* and **c** *Nf-Y* present similar diffuse expression patterns that comprise the developing somites. **d** *Meis1* transcripts are strongly upregulated in the dermomyotome (arrowhead). **e** *Fxr* is not expressed in myogenic regions. Scale bar = 200 μm

Using C2C12 as an in vitro model, we identified basal levels of *Mstn* expression in proliferative myoblasts. Expression data in C2C12 myoblasts are conflicting, as some works report no expression [38, 39] while others describe basal levels of *Mstn* expression [40, 41]. This heterogeneity probably reflects variations in the sensitivity of the techniques used to evaluate *Mstn* expression. Even so, it is consensus that *Mstn* expression in vitro is reduced during proliferation in comparison to differentiation [38, 40]. Thus, the *Mstn* expression detected in our work correlates to this scenario where *Mstn* is strictly downregulated to allow C2C12 myoblasts to undergo several rounds of cell division before differentiation begins. Along with *Mstn*, we found *Creb, Nf-Y, Meis1* and *Fxr* expression, indicating that *Mstn* P/E may be regulated by the proteins encoded by these genes.

In this context, we further evaluated the role of *Mstn* potential regulators performing a systematic study of *Mstn* P/E-Luc constructs containing different sets of mutations on conserved TFBSs. Our data revealed that disruption of these binding sites interferes with *Mstn* P/E activity. It is relevant to mention that the conserved TATA box was left intact in all *Mstn* P/E-Luc constructs. TATA boxes have been commonly associated with highly regulated and strong tissue-specific promoters [28, 42]. This characteristic, however, is not due to TATA box itself, but most likely conferred by adjacent TFBSs. In fact, without any of the conserved TFBSs neighboring the TATA box in *Mstn* P/E, expression levels decrease substantially.

As an essential part of *Mstn* P/E, the conserved TFBSs seem to act in concert to balance *Mstn* expression. NF-Y site was shown to be necessary, although not sufficient, to promote *Mstn* P/E activity. In fact, recent works revealed that NF-Y causes epigenetic modifications that change chromatin conformation to facilitate the binding of other transcription factors to DNA [43, 44]. Moreover, CCAAT boxes are typically flanked by at least one functionally important promoter element, and the interaction between them is essential for the function of the regulatory unit [32]. In the *Mstn* P/E, this essential DNA element seems to be the adjacent CREB binding site (CRE site), which increases *Mstn* P/E activity whenever in the presence of NF-Y site. Interestingly, a similar promoter organization was also described for *Hexokinase II* (*HKII*), a gene that encodes an important enzyme for glucose metabolism in skeletal muscle [45, 46]. Therefore, NF-Y and CREB sites compose an important regulatory module of *Mstn* P/E.

It is important to highlight that CREB functional status changes after phosphorylation by PKA, as in the transition from proliferation to differentiation of C2C12 myoblasts [47]. In this context, phosphorylated CREB (P-CREB) was shown to interact with p300 to recruit MYOD on *Retinoblastoma* promoter, an important gene involved in cell cycle arrest [47]. P-CREB was also shown to modulate *Mstn* activity in differentiating C2C12 cells treated with IGF-1 factor [48]. In contrast, in the absence of phosphorylation by PKA, CREB is capable of inhibiting promoters of several genes [49–52]. Taken together, these data indicate that cAMP/PKA signaling pathway may be relevant in the regulation of *Mstn* transcriptional activity.

Another factor related to cAMP signaling is MEIS1, which also presents a transcriptional activation domain responsive to PKA [53]. Our data revealed that MEIS1 site contributes to *Mstn* P/E function in proliferating C2C12 myoblasts and, as CREB, requires NF-Y to mediate its transcriptional effects. In fact, MEIS1 interacts with PBX1, another HOX–TALE transcription factor, to recruit the heterodimer MyoD/E12 to a noncanonical E-box located in *Myogenin* promoter, activating its transcription [35]. Since MyoD participates in *Mstn* regulation [54], it may be speculated that MEIS1 could mediate non canonical binding of this factor to *Mstn* P/E.

Differently from the other TFBSs, FXR presented a neutral role on *Mstn* P/E activity except when it is the only intact TFBS. Although *Fxr* transcripts were detected in proliferating C2C12 cells, they were not found in mouse embryos somites where myogenic precursor cells are located. Furthermore, FXR is not expressed in adult skeletal muscle, but it is detected in liver, intestine and adipose tissue [55, 56]. As there has been growing evidence of roles for *Mstn* in the above mentioned sites of *Fxr* expression [57], it is possible that FXR has a more active post-natal role on the regulation of *Mstn* in a tissue-specific manner, acting in other locations such as liver and adipose tissue, where *Mstn* is also expressed [58].

Except for FXR, *Mstn* regulators studied in vitro are also expressed in myogenic territories of developing somites in E10.5 mouse embryos. At this stage, most somites have two compartments committed to skeletal muscle formation: the medially/laterally located dermomyotome, which contains proliferating myoblasts and, beneath it, the myotome, bearing differentiating cells [59]. Our in situ hybridization assays unraveled a complementary nature of *Mstn* expression, restricted to the myotome, in comparison to *Meis1* expression, strongly upregulated in the dermomyotome. This fact suggests a negative effect of MEIS1 on *Mstn* transcription in vivo. In turn, *Creb* and *Nf-Y* present identical patterns with diffuse expression encompassing both the myotome and dermomyotome. In this scenario, it can be speculated that the proteins encoded by these genes may interact in vivo to regulate *Mstn* activity in both compartments. In the future, it would be interesting to investigate the role of CREB, NF-Y and MEIS1 on *Mstn* regulation in vivo to elucidate how these factors participate in the *Mstn* transcription when integrated to its complete regulatory network.

In conclusion, in the current paper we showed that CREB, NF-Y and MEIS1 conserved TFBSs mediate *Mstn* P/E

transcriptional activity in the myogenic context. Future studies are needed to confirm the physical interaction between the conserved sites of *Mstn* P/E with the transcription factors CREB, NF-Y and MEIS1. It would be interesting to test whether overexpression or knockdown of these transcription factors in vivo or in vitro modulate *Mstn* expression. Besides, as both CREB and MEIS1 are affected by cAMP signaling it would be important to evaluate the effects of this pathway on *Mstn* transcription. It is also important to consider that there may be other non-conserved TFBSs influencing *Mstn* P/E activity and also working to keep an appropriate balance of *Mstn* expression. A good candidate to be investigated in the future is MYOD, a myogenic factor that has already been related to *Mstn* regulation. Finally, it is important to remember that probably besides the promoter there is a complex network of cis-regulatory elements working to determine when, where and at what levels *Mstn* transcription occurs during development and at different postnatal physiological contexts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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