© 2013. Published by The Company of Biologists Ltd | Development (2013) 140, 4914-4925 doi:10.1242/dev.097428

RESEARCH ARTICLE

A new role for the calcineurin/NFAT pathway in neonatal myosin heavy chain expression via the NFATc2/MyoD complex during mouse myogenesis

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ABSTRACT

The calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway is involved in the modulation of the adult muscle fiber type, but its role in the establishment of the muscle phenotype remains elusive. Here, we show that the NFAT member NFATc2 cooperates with the basic helix-loop-helix transcription factor MyoD to induce the expression of a specific myosin heavy chain (MHC) isoform, the neonatal one, during embryogenesis. We found this cooperation to be crucial, as *Myod/Nfatc2* double-null mice die at birth, with a dramatic reduction of the major neonatal MHC isoform normally expressed at birth in skeletal muscles, such as limb and intercostal muscles, whereas its expression is unaffected in myofibers mutated for either factor alone. Using gel shift and chromatin immunoprecipitation assays, we identified NFATc2 bound to the neonatal Mhc gene, whereas NFATc1 and NFATc3 would preferentially bind the embryonic Mhc gene. We provide evidence that MyoD synergistically cooperates with NFATc2 at the neonatal Mhc promoter. Altogether, our findings demonstrate that the calcineurin/NFAT pathway plays a new role in establishing the early muscle fiber type in immature myofibers during embryogenesis.

KEY WORDS: MyoD, NFAT, Myogenesis, Calcineurin, Skeletal muscle, Myogenic regulatory factors, Myosin heavy chain

INTRODUCTION

Vertebrate skeletal muscles contain a heterogeneous population of muscle fibers. Depending on their contraction properties and the expression of activity-dependent genes, mammalian skeletal muscle fibers are classified into four types, including slow or type I and three subtypes of fast or type II fibers, type IIa, IIx and IIb (Brooke and Kaiser, 1970; DeNardi et al., 1993). Each fiber type is defined by the expression of specific isoforms of myosin heavy chains (MHC) (Schiaffino and Reggiani, 2011). The specification of mature fiber types is modulated by neural and hormonal influences but their early appearance and diversification takes place during the developmental and early postnatal life, independently of neural influences (Condon et al., 1990a). In contrast to the adult MHC isoforms expressed throughout adult life, the embryonic (emb-MHC) and neonatal (neo-MHC) MHC isoforms are expressed only in immature myofibers, during development, and in all muscles containing regenerating adult muscle fibers (Whalen et al., 1981; Lyons et al., 1990). In these immature myofibers, as well as in cultured myotubes, all MHC isoforms are expressed in a cell-autonomous manner, independently from the nerve activity. After birth and in regenerating muscle fibers, a switch from expression of embryonic and neonatal MHC to adult MHC occurs such as developmental MHC proteins (emb-MHC and neo-MHC) represent only 1% of the total MHC proteins 20 days after birth (Allen and Leinwand, 2001). As the expression pattern of each MHC isoform is different in time and in myofiber nature, the signaling pathways and transcriptional regulators of each MHC isoform are probably different.

One of the major modulators of the specification of the adult muscle fiber type is the calcineurin/NFAT signaling pathway. Calcineurin is a Ser/Thr phosphatase, the activity of which is regulated by the intracellular concentration of Ca^{2+}. Once activated, calcineurin dephosphorylates transcription factors, such as members of the NFAT (nuclear factors of activated T-cells) family, allowing NFAT to translocate to the nucleus where it cooperates with other transcription factors to induce transcription of target genes. Five NFAT genes have been identified: NFATc1-NFATc4 and NFAT5 (Rao et al., 1997). Only the NFATc1-c4 are regulated by calcineurin. All four NFATc proteins have been shown to be expressed in adult skeletal muscles at the RNA and protein levels (Calabria et al., 2009). Combination of these four NFAT family members determines the nerve activity-dependent specification of each adult fiber type by differentially regulating the expression of the MHC isoforms (Calabria et al., 2009): type I MHC is expressed as all four NFAT members are actively translocated; type IIb MHC is expressed in muscle cells with nuclear NFATc4 alone. These findings suggest that all four NFAT family members play a specific role in modulating the adult myofiber types in response to the nerve activity and that an increased activation of the calcineurin/NFAT pathway promotes the slow gene program, as has also been suggested by previous studies (Chin et al., 1998; Naya et al., 2000). Among the NFAT members, NFATc1 would play a crucial role in regulating this muscle slow phenotype, as NFATc1 would not only activate the expression of the slow MHC isoform (type I MHC), but also downregulate the type IIb MHC expression in adult myofibers (Schiaffino, 2010). Altogether, these results suggest a central role for the calcineurin/NFAT pathway in the activity-dependent specification of the adult muscle fiber type. However, its role in the establishment of the muscle phenotype remains elusive, particularly during embryogenesis.

Muscle fiber formation is under the control of muscle-specific transcription factors, the myogenic regulatory factors (MRF): MyoD (Myod1 – Mouse Genome Informatics), Myf5, MRF4 and

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Received 10 April 2013; Accepted 20 September 2013

Development (2013) doi:10.1242/dev.097428

myogenin. In concert with many co-factors, these basic helix-loop-helix (bHLH) transcription factors direct the expression of muscle-specific genes, via their DNA-binding elements – E-boxes (Buckingham, 1996). E-boxes are found in promoters of many muscle specific genes, including the MHC isoforms (Beylkin et al., 2006; Wheeler et al., 1999). Some *in vitro* studies suggest that MyoD would preferentially activate the emb-MHC isoform, whereas neo-MHC expression would better be regulated by the calcineurin/NFAT pathway (Beylkin et al., 2006).

We have previously demonstrated a new transcriptional paradigm where NFAT proteins could cooperate with MRF (Armand et al., 2008). One NFAT isoform, NFATc3, cooperates with MyoD to activate myogenin expression during somitogenesis, a specific role for NFATc3/MyoD, as a *Myod*/*Nfatc2* deletion does not induce any modification in myogenin expression in somites. In contrast to *Nfatc3*$^{-/-}$ mice, which are characterized by a decrease in the adult myofiber number (Kegley et al., 2001), the reduction of muscle mass in *Nfatc2*-null mice is probably due to a defect in skeletal muscle growth (Horsley et al., 2001). Indeed NFATc2 regulates the growth of nascent myotubes in primary cultures, by promoting the fusion of myoblasts to pre-existing myotubes (Horsley et al., 2001; Horsley et al., 2003), at a time corresponding to the establishment of the early specification of myofibers. As both MRF and NFAT transcription factors are involved in the regulation of the different MHC isoform expression and given that NFATc2 promotes the growth of myotubes, we hypothesize that cooperations between NFAT and MRF proteins could be one of the keys to acquiring the myofiber phenotype.

In this study, we provide a new regulatory network controlling the expression of one particular MHC isoform during embryogenesis. We show that the calcineurin/NFATc2 signaling pathway controls the neonatal MHC isoform expression *in vivo*, as hindlimb skeletal muscle myofibers of double-null *Myod/Nfatc2* E18.5 embryos are devoid of neonatal MHC, the major isoform expressed in muscle cells at birth, whereas neo-MHC expression is unaffected in muscles with single null mutations for either *Nfatc2* or *Myod*. The physical interaction between these two transcription factors is specific and both proteins are required on the promoter to activate synergistically the expression of neo-MHC. Overall, this study demonstrates that the calcineurin/NFATc2 pathway plays a crucial role in the establishment of the muscle cell fiber type during embryogenesis, by regulating specifically the neo-MHC isoform in cooperation with MyoD.

RESULTS

NFATc2 and MyoD cooperate to specifically induce the expression of the neonatal MHC isoform during embryonic myogenesis

In order to study a potential cooperation between NFATc2 and MyoD *in vivo*, we produced a mouse model combining defective alleles for both genes. Although *Myod*$^{-/-}$ and *Nfatc2*$^{-/-}$ mice were fertile, the genotypic distribution of the *Myod:Nfatc2*-null pups does not conform to Mendel's law: the prevalence of *Myod:Nfatc2* mutant pups is about 85% less than expected at birth (Table 1), suggesting embryonic or neonatal lethality. The collection of lungs from wild-type, *Nfatc2*$^{-/-}$, *Myod*$^{-/-}$ and double-null mutants at birth revealed that double mutants were not able to breath, as their lungs were the only ones sinking to the bottom of the tube, indicating that alveoli had never inflated (supplementary material Fig. S1A). The morphology of hearts dissected from newborn mice seems similar in each genotype (supplementary material Fig. S1B). These observations suggest that NFATc2 and MyoD could cooperate *in vivo* and that this cooperation is crucial for the embryo to live.

Table 1. Mendelian ratios of newborn mice from heterozygous crossings of double *myod/nfatc2* knockouts

	MyoD NFATc2 × MyoD NFATc2		
	+/− +/− × +/− +/−		
MyoD:NFATc2	Number	%	Expected %
+/+ +/+	4	4.2	6.3
+/− +/+	19	20.0	12.5
−/− +/+	2	2.1	6.3
+/+ +/−	15	15.8	12.5
+/+ −/−	4	4.2	6.3
+/− +/−	32	33.7	25
+/− −/−	12	12.6	12.5
−/− +/−	6	6.3	12.5
−/− −/−	1	1.1	6.3
Total:	95		

As MyoD expression is restricted to the skeletal muscle tissue, we decided to focus the analysis of the MyoD/NFATc2 cooperation during skeletal myogenesis. To describe the myogenic defects in *Myod*/*Nfatc2* mutants, immunochemistry experiments using desmin antibodies were performed on sections of hindlimbs from wild-type, *Myod*, *Nfatc2* and *Myod*/*Nfatc2* mutant embryos collected at different developmental time points [from the end of primary myogenesis until birth (E14.5 and E18.5)] and the morphology of muscle fibers was analyzed. Double-mutant muscle fibers displayed atrophy at E14.5 and E18.5 (Fig. 1). However, the atrophy observed is not specific to the double mutation, as the *Myod*/*Nfatc2*-null limb muscles were atrophied to the same extent as *Nfatc2*$^{-/-}$ muscles, whereas limb muscles from *Myod*$^{-/-}$ mice were not significantly different from wild-type muscles (Fig. 1A,B). These results showed that the reduction of size of *Myod*/*Nfatc2* mutant myofibers is not due to the combined absence of MyoD and NFATc2, but is only caused by the lack of NFATc2 protein, which is consistent with previous studies showing that the growth of multinucleated muscle cells is regulated by a NFATc2-dependent pathway (Horsley et al., 2001). Importantly, no apoptotic or necrotic myofibers have been identified using active caspase 3 immunohistochemistry or Alizarin Red staining, respectively (data not shown).

As it has been reported that the calcineurin/NFAT signaling pathway is involved in the acquisition of the adult myofiber phenotype (Calabria et al., 2009), the expression of several muscle specific genes was analyzed by qPCR (supplementary material Fig. S2). Among the genes analyzed, *neo-mhc* was specifically downregulated in *Myod/Nfatc2*-null E18.5 muscles. By western blot, a similar dramatic decrease of neo-MHC protein was observed in E18.5 muscles collected from *Myod*/*Nfatc2* mutants, whereas embryonic and type I MHC were similarly accumulated in E18.5 hindlimb muscles from mice of each genotype (Fig. 2A). This results in a decrease of the global amount of sarcomeric myosin protein detected by the MF20 antibody in *Myod/Nfatc2* mutant embryos compared with single mutant and wild-type mice. By immunohistochemistry on transverse sections of posterior limb muscles from wild-type, *Myod Nfatc2* and *Myod*/*Nfatc2* mutant embryos, the neonatal MHC protein was barely detected in limb and intercostal muscles of *Myod*$^{-/-}$*:Nfatc2*$^{-/-}$ embryos at E14.5 and E18.5 (Fig. 2B; supplementary material Fig. S3), whereas it was expressed in roughly 25 to 50% of the wild-type and *Myod*$^{-/-}$ myofibers and in 15 to 30% *Nfatc2*$^{-/-}$ myofibers from E14.5 and E18.5 embryos, respectively (Fig. 2C). At E18.5, the faint expression of neo-MHC detected in *Myod/Nfatc2*-null muscles is observed in few secondary myofibers, which do not express MHC-I (Condon et al., 1990b)

Development (2013) doi:10.1242/dev.097428

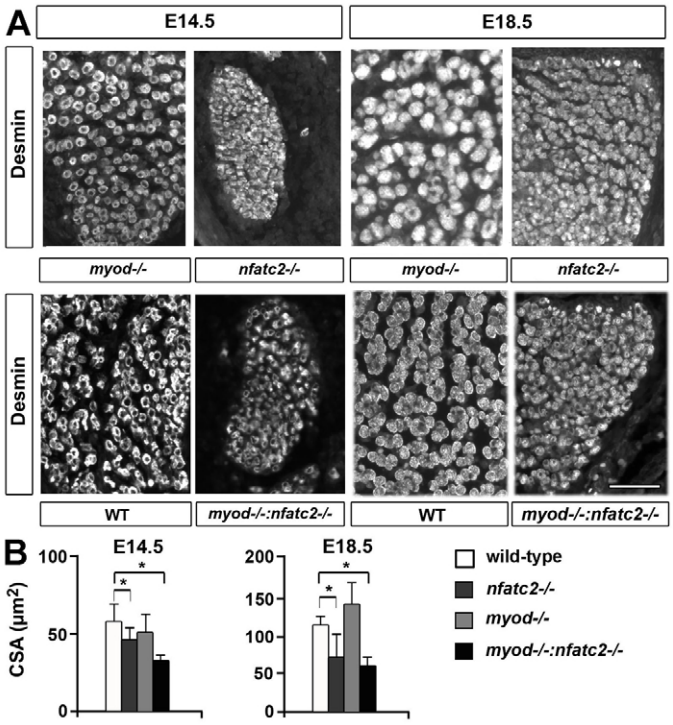

Fig. 1. Atrophy in double-null limb muscles. (A) Immunofluorescence analysis of desmin-positive myofibers on transverse sections of hindlimbs from wild-type, *Myod*$^{-/-}$, *Nfatc2*$^{-/-}$ and *Myod*$^{-/-}$:*Nfatc2*$^{-/-}$ E14.5 and E18.5 embryos. Scale bar: 60 µm. (B) Quantitation of fiber cross-sectional area (CSA) specifies the atrophy of myofibers in *Nfatc2*$^{-/-}$ and *Myod/Nfatc2*-null muscles compared with wild-type and *Myod*$^{-/-}$ muscles. **P*<0.05 versus wild type. Data represent mean ± s.e.m. Three to five embryos of each genotype were analyzed.

(supplementary material Fig. S4). In the diaphragm, the expression of neo-MHC is not modified by the absence of MyoD and NFATc2 expression (supplementary material Fig. S3). In contrast to neo-MHC, emb-MHC is expressed by almost all limb myofibers from embryos of each genotype (Fig. 2D). The percentage of fibers expressing the type I MHC isoform was similar in all wild-type and mutant muscles at E18.5, with a significant decrease in MHC I-expressing myofibers from E14.5 *Myod/Nfatc2* mutants, suggesting a delay in the expression of this MHC isoform (Fig. 2E). As only primary myofibers are present at E14.5 (Ontell et al., 1993), these results show that MyoD and NFATc2 cooperate during primary as well as secondary myogenesis to induce specifically the expression of the neonatal MHC isoform.

NFATc2 and MyoD are required for neonatal MHC expression in postnatal and cultured muscle cells

At birth, the neonatal isoform is the major MHC isoform expressed in hindlimb muscles (Allen and Leinwand, 2001): between 5 to 20 days after birth, its expression decreases to completely disappear in adult muscles. To verify whether MyoD and NFATc2 could regulate neo-MHC in muscle fibers after birth as during embryogenesis, we electroporated tibialis anterior (TA) of 5-day-old wild-type mice with either a scrambled siRNAs or siRNAs directed against MyoD, NFATc2 or a combination of both siRNAs. To verify the efficiency of electroporation, we electroporated siRNAs in combination with a GFP-expressing plasmid (Fig. 2G). Four days after electroporation, we observed by western blot and immunohistochemistry a decrease of neo-MHC expression in TA electroporated with both NFATc2 and MyoD siRNAs, compared with TA electroporated with either a scrambled siRNA or with NFATc2 or MyoD siRNA alone (Fig. 2F,I). The size of electroporated as well as non-electroporated myofibers was not significantly modified (Fig. 2H).

To verify the cooperative effect of MyoD and NFATc2 on neo-MHC expression in culture, we isolated myoblasts from E18.5 embryos. Plated at high density, these myoblasts were able to form proper, myogenin-positive myotubes, whatever the genotype is (Fig. 3A). The percentage of myotubes expressing neo-MHC was similar in all cell cultures after 3 days in differentiation medium (DM) (Fig. 3D). However, when cultured 6 days in DM, the proportion of neo-MHC positive myotubes from *Myod*/*Nfatc2* double mutant embryos was much smaller than in *Myod* or *Nfatc2* single mutant cultures (~5% versus 25 and 20%) and in wild-type cultures (more than 35%), whereas the percentage of myotubes expressing emb-MHC is similar in single and double mutant myotubes after both 3 and 6 days in DM (Fig. 3C). No significant difference of the fusion index was observed in any cell culture experiment (Fig. 3B), indicating that decrease of neo-MHC content in *Myod*/*Nfatc2* mutant cell cultures at 6 days of differentiation is not caused by an impaired myotube fusion. To test the differentiation state of myotubes, we determined the distribution of myotubes with equivalent numbers of nuclei (Fig. 3E). If wild-type myotubes were clearly more mature than single mutant myotubes, the *Myod*/*Nfatc2*-null myotubes were roughly at the same stage of differentiation as *Myod*$^{-/-}$ and *Nfatc2*$^{-/-}$ myotubes. Indeed, around 40% of the wild-type myotubes had two to five nuclei, 3 and 6 days after allowing myoblasts to differentiate, whereas myotubes with two to five nuclei represent more than 60% of *Nfatc2*-null myotubes and more than 75% of the *Myod*$^{-/-}$ and *Myod/Nfatc2*-null myotubes at both differentiation time points (Fig. 3E). Combined together, these results demonstrate the essential role for NFATc2 and MyoD in cooperatively regulating the neonatal MHC expression, in culture and *in vivo*, during embryogenesis and after birth.

NFATc2 cooperates only with MyoD to activate the promoter of neo-MHC

To better characterize the cooperation between NFATc2 and MyoD, we analyzed whether forced overexpression of calcineurin/NFAT and MyoD, Myf5 or myogenin could activate the promoter of the emb-MHC or the neo-MHC genes [*emb-mhc* and *neo-mhc* (*Myh8* and *Myh3* – Mouse Genome Informatics)]. Transient co-transfection assays were performed with luciferase reporters driven by the promoters of either *emb-mhc*, *neo-mhc* or *mhcI* (*Myh7* – Mouse Genome Informatics) (Fig. 4A). Combined tranfections of MyoD with either NFATc2 or NFATc3 were required to achieve the maximal transcriptional activation of *emb-mhc* and *mhcI* promoters, whereas transfection of MyoD with NFATc2, and not NFATc3, was the best combination to achieve the maximal induction of the neo-MHC luciferase activity compared with C2C12 cells transfected with the luciferase reporters alone (Fig. 4A). Myf5 or myogenin overexpression were overall much less efficient than MyoD at activating all three promoters in combination with any NFAT isoform, suggesting a specific regulation of neo-MHC expression by NFATc2 and MyoD.

We next tested whether endogenous NFATc2 and MyoD could regulate the endogenous expression of the different MHC isoforms. To do so, we first precisely analyzed their expression in differentiating C2C12 cells (Fig. 4B). The embryonic, type I, IIx and IIb isoforms of MHC were detected by qPCR soon after the cells

Development (2013) doi:10.1242/dev.097428

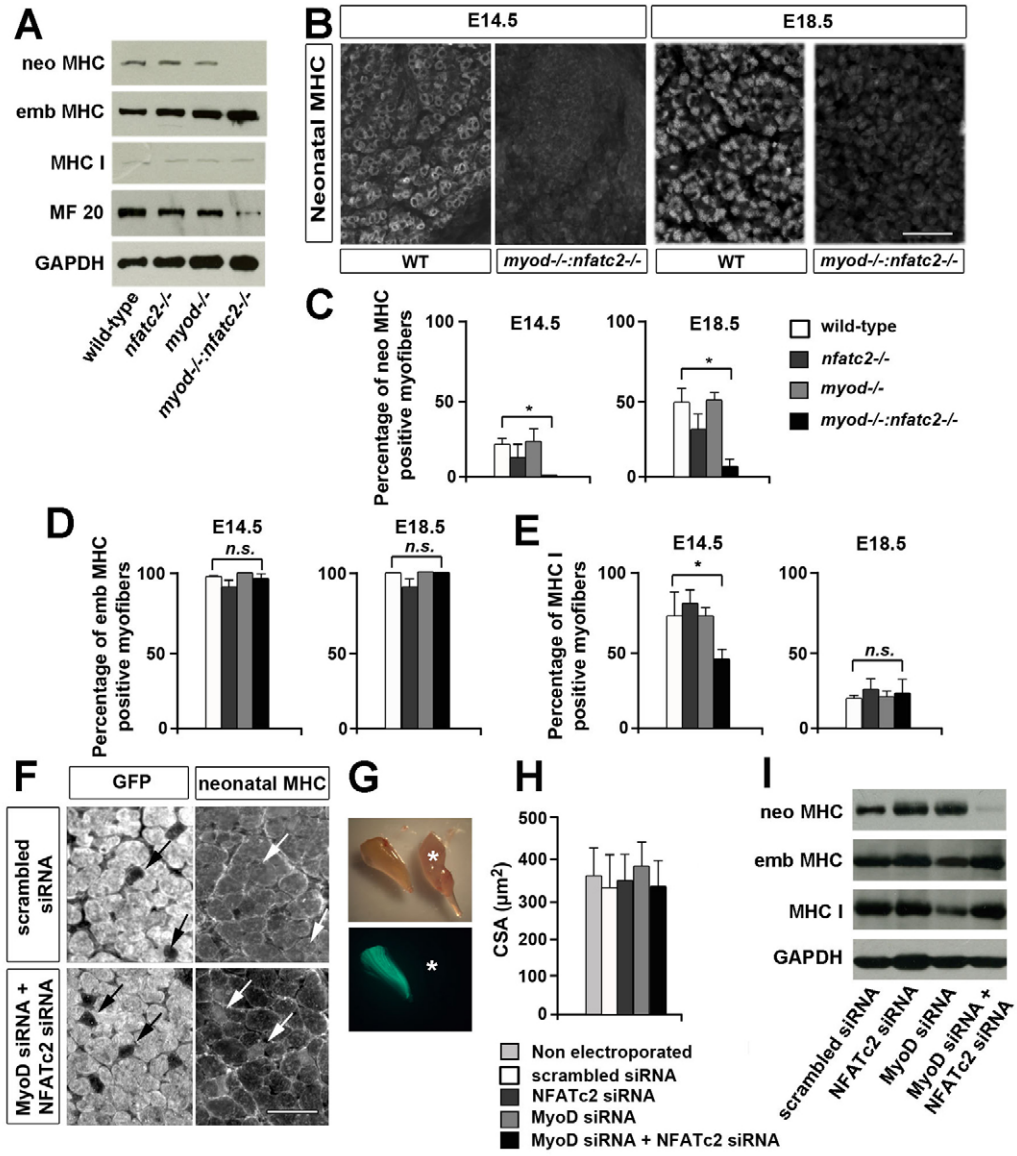

Fig. 2. NFATc2 and MyoD are required for neonatal MHC expression in mouse developing myofibers. (A) Western blot analyses of E18.5 hindlimb muscles collected from wild type, *Nfatc2*$^{-/-}$, *Myod*$^{-/-}$ and *Myod*$^{-/-}$:*Nfatc2*$^{-/-}$ indicate that double-null muscles are specifically deficient in one single MHC type: the neonatal. MF20 antibody recognizes all sarcomeric myosin. (B) Immunofluorescent analysis of neo-MHC-positive myofibers on transverse sections of hindlimbs from wild-type and *Myod*$^{-/-}$:*Nfatc2*$^{-/-}$ E14.5 and E18.5 embryos. Scale bar: 40 µm. Quantification of myofibers expressing neonatal (C), embryonic (D) and type I (E) MHC in wild type, *Myod*$^{-/-}$, *Nfatc2*$^{-/-}$and *Myod/Nfatc2*-null E14.5 and E18.5 hindlimb muscles confirms an important deficiency of neo-MHC-positive fibers in double-null embryos at both E14.5 and E18.5. Data represent mean ± s.e.m. Three to five animals of each genotype were analyzed. **P*<0.01 versus wild type. n.s., not significant. (F-I) MyoD and NFATc2 are necessary for the expression of neo-MHC in postnatal muscles. TA from 5-day-old mice were electroporated with scrambled siRNA or siRNA directed against MyoD, NFATc2 or both siRNAs, in combination with a GFP expression plasmid (*n*=3 per condition). Electroporation efficiency was controlled by visualizing TA using a light (up) or fluorescence (down) microscope (G). Asterisk indicates non-electroporated muscles. Muscles collected 4 days after gene delivery were analyzed by immunofluorescence on serial sections using antibodies against GFP or neo-MHC (F), or by western blot to detect neo, emb, type I MHC and GAPDH (I). The myofiber CSA was not modified under any experimental condition (H). Data represent mean ± s.e.m. Three to five electroporated wild-type neonates were analyzed. Arrows in F indicate non-electroporated myofibers. Scale bar: 50 µm.

were switched to DM. The neonatal and type IIa MHC isoforms were detected later in these cultured cells, after 4 days of differentiation. Transcripts of most of the MHC isoforms accumulated at high levels in C2C12 cells after 7 days of differentiation. To test the *in vitro* effect of either MyoD or NFATc2 on the expression of the MHC isoforms, differentiating C2C12 cells were transfected with siRNA directed against either MyoD, NFATc2 or both at day 4 of differentiation, and the MHC isoform transcripts were collected and analyzed 2 days later. These transfection assays resulted in the specific downregulation of neo-MHC in cells transfected with both MyoD and NFATc2 siRNA, confirming a regulation of this isoform by the cooperation of both transcription factors (Fig. 4C).

NFATc2 and MyoD directly activate the neonatal *mhc* promoter

To define the mechanisms behind the regulation of the neonatal and embryonic MHC expression by calcineurin/NFAT signaling, we

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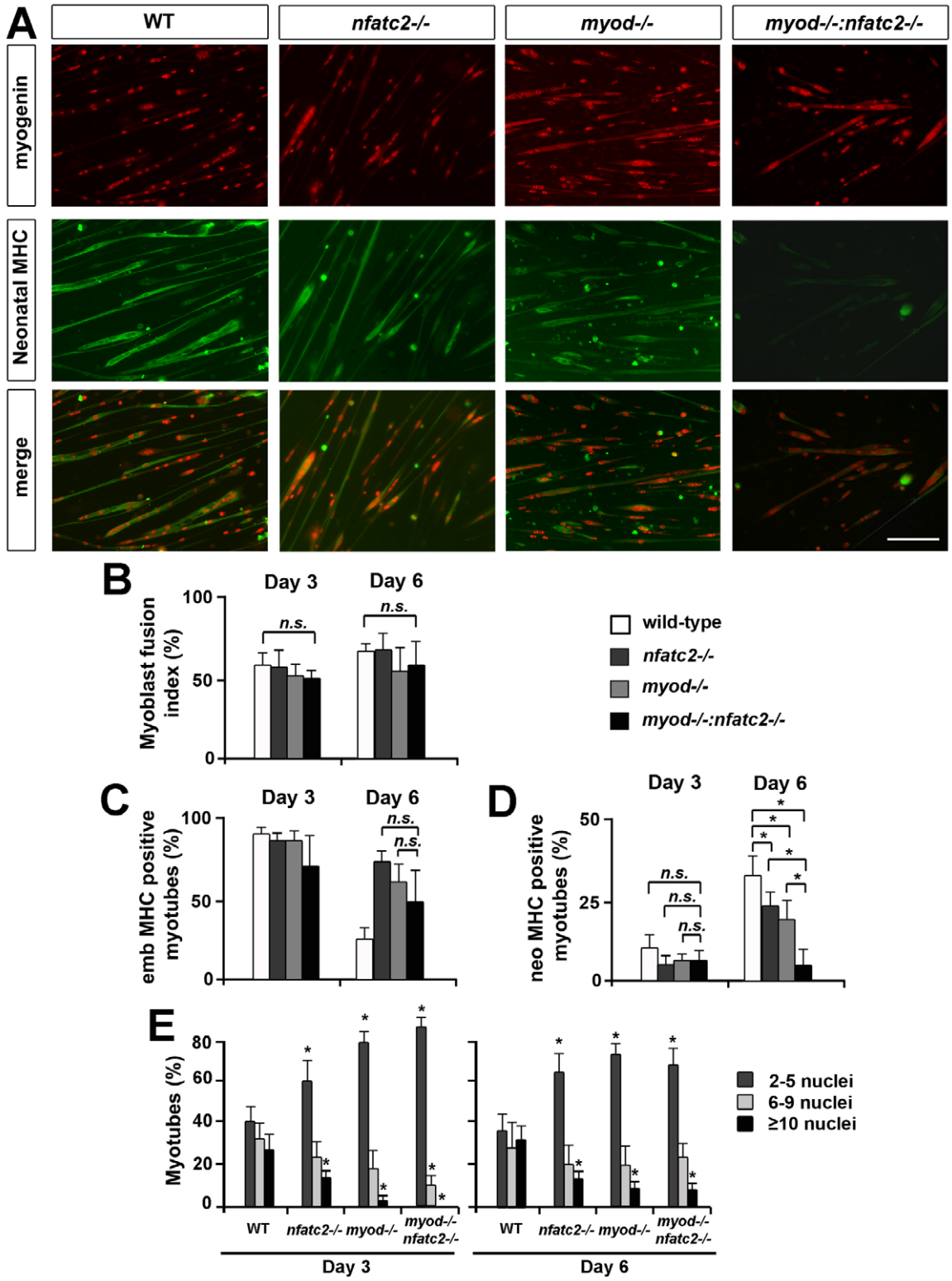

Fig. 3. NFATc2 and MyoD are wild-type required for neonatal MHC expression in primary cultured differentiating myotubes. (A) Differentiated WT, *Nfatc2*$^{-/-}$, *Myod*$^{-/-}$ and *Myod*$^{-/-}$:*Nfatc2*$^{-/-}$ myocytes plated at high density and grown for 6 days in DM were detected by immunostaining for myogenin (red) and neoMHC (green). Total nuclei were stained with bis-benzimide. Scale bar: 100 µm. (B) After 3 and 6 days in culture, the myoblast fusion index was similar in wild-type, single and double mutant primary cultures, suggesting that NFATc2 and MyoD are not involved in the early differentiation process. Quantification of myotubes expressing embryonic (C) or neonatal (D) MHC in wild type, *Myod*, *Nfatc2* and *Myod/Nfatc2*-null primary cultures confirms an important deficiency of neo-MHC-positive myotubes in double-null primary muscle cells cultured for 6 days in DM. **P*<0.05 versus wild type. At least 100 myotubes in three different cell culture experiments were analyzed. (E) After 3 and 6 days in DM, the distribution of myotubes with equivalent numbers of nuclei was similar in single and double mutant cell cultures. Primary myotubes were clearly more mature in wild-type cell cultures compared with single and double mutant cultures. At least 200 myotubes in three independent cell culture experiments were analyzed. n.s., not significant. **P*<0.05 versus wild type.

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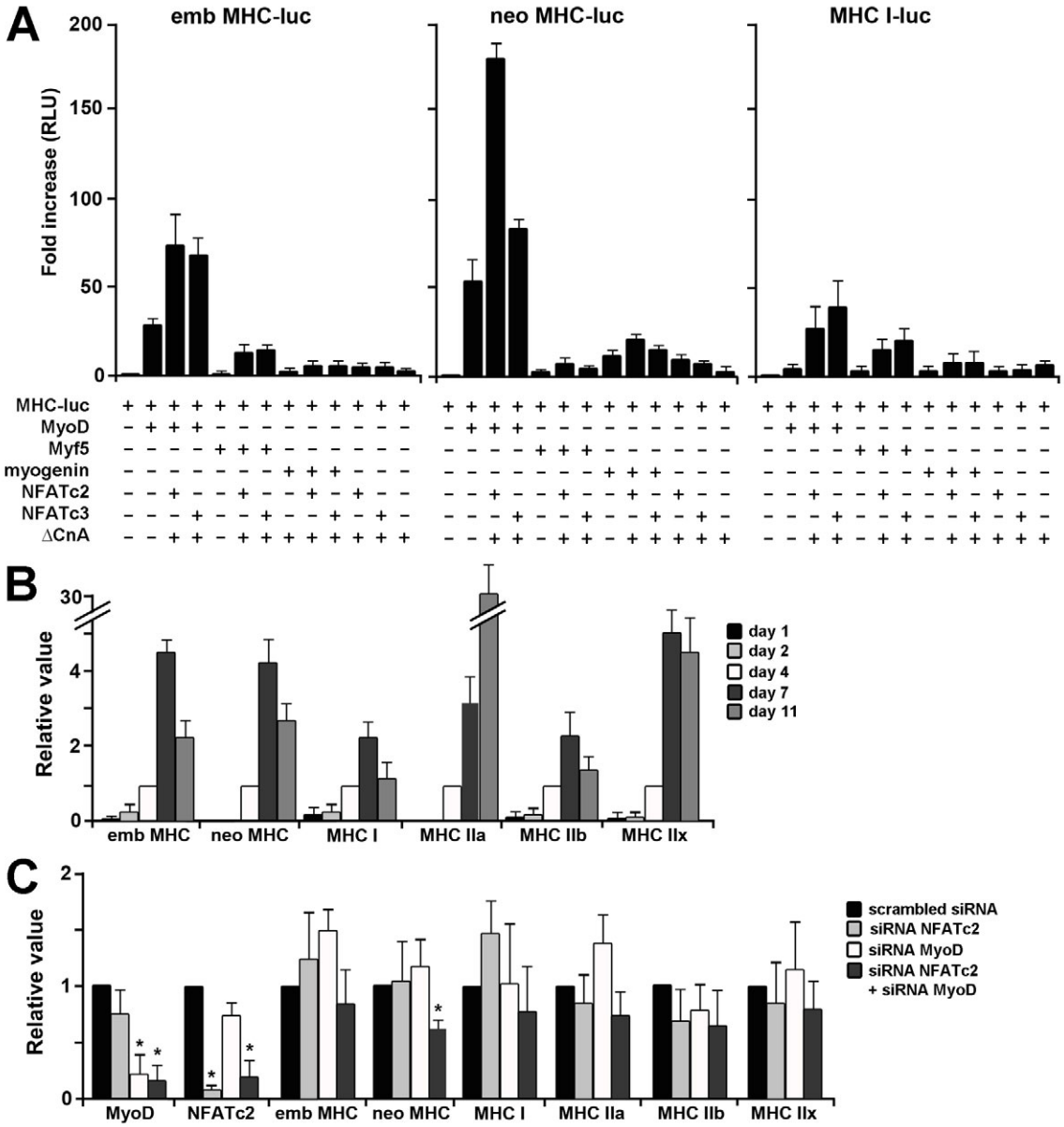

Fig. 4. NFAT transcriptional activity is involved in neonatal MHC expression. (A) Co-transfection assays in C2C12 cells cultured for 48 hours in PM using emb-MHC-luc, neo-MHC-luc or MHC I-luc vectors and the indicated expression vectors demonstrates a synergistic cooperation between MyoD and either NFATc2 or NFATc3. Data represent the mean ± s.e.m. of three independent experiments. (B) C2C12 myoblasts were cultured in DM for indicated time period and the different MHC isoforms were analyzed by real-time PCR. (C) C2C12 cells were cultured in DM and transfected, 4 days after the switch in DM, with scrambled siRNA or siRNA directed against NFATc2, MyoD or both siRNAs. Two days after transfection, MyoD, NFATc2 and MHC isoforms transcripts were analyzed by real-time PCR. *P<0.01 versus scrambled siRNA (n=3).

searched for enhancers that might regulate *in vivo* these two MHC isoforms. Using the rVista program, we identified several regions conserved between human and mouse that we tested by chromatin immunoprecipitation. Three putative NFAT consensus binding sites [(T/A)GGAAA] were identified in the most proximal 1.2 kb region of the *neo-mhc* promoter and designated N1, N2 and N3 (Fig. 5A). In the 0.7 kb proximal region and in the first intron of the *neo-mhc* gene, a series of conserved E-boxes were identified and named E1 to E11. In the 0.7 kb proximal region and in the first intron of the *emb-mhc* gene, three NFAT consensus binding sites and six E-boxes were identified and designated as N1, N2 and N3, and E1 to E6, respectively.

C2C12 myoblasts were either cultured in proliferating medium (PM) or were differentiating for 4 days, differentiation time point where both embryonic and neonatal MHC begins to be expressed (Fig. 4B). The resultant nuclear fractions were immunoprecipitated using specific antibodies to NFATc2 (Fig. 5B), NFATc1 (Fig. 5C), NFATc3 (Fig. 5D) or MyoD (Fig. 5E), and associated DNA was purified. Using specific primers to the different conserved regions in the *neonatal* and *embryonic mhc* gene by real-time PCR, we observed a very interesting difference in the occupancy of each NFAT isoform on each *emb* and *neo-mhc* gene, in proliferating and differentiating C2C12 cells. NFATc2 was barely detected on the *emb-mhc* gene in both proliferating and differentiating C2C12 cells

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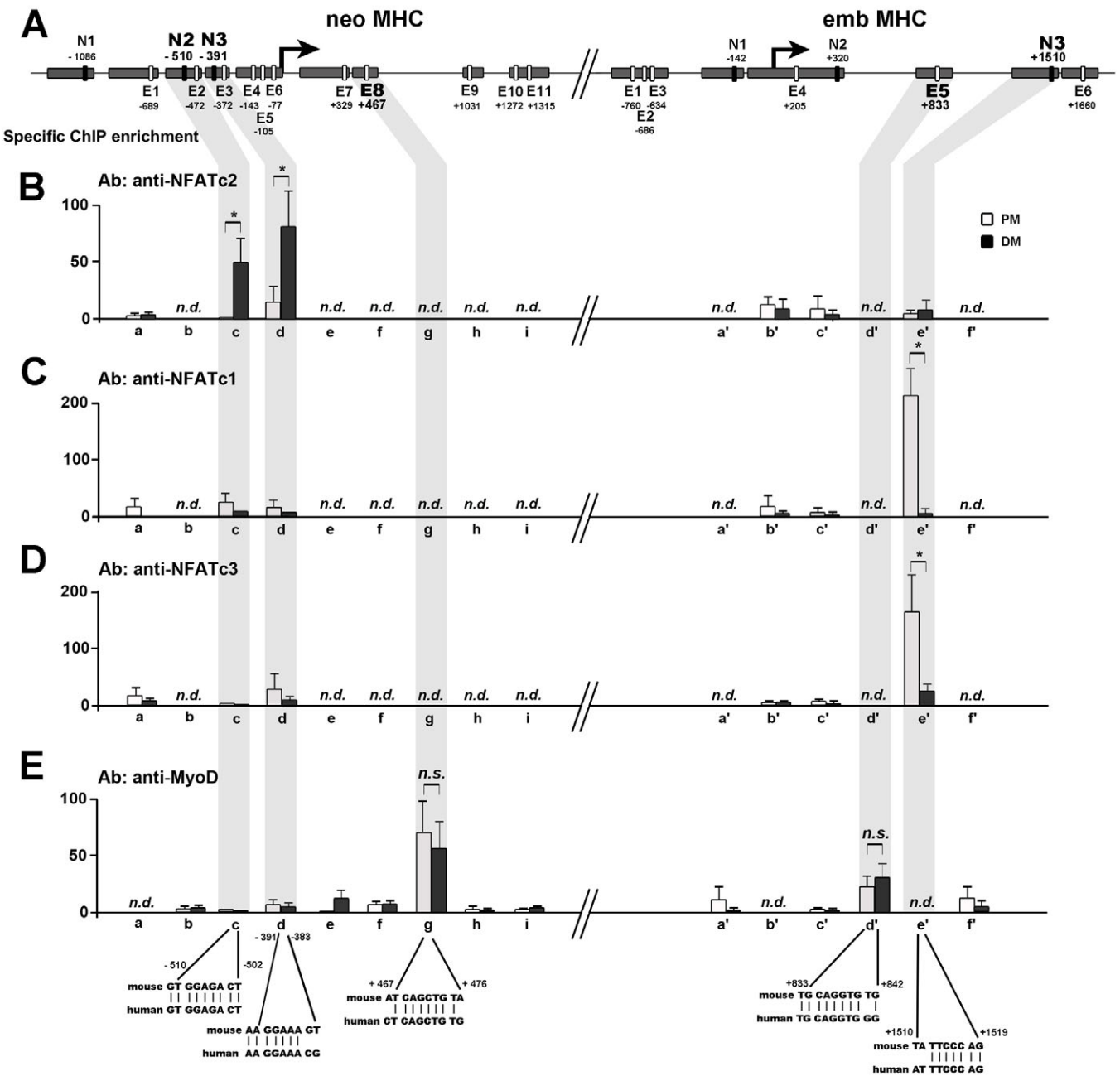

Fig. 5. Identification of NFAT consensus binding sites and E-boxes in neonatal and embryonic *mhc* promoters. (A) Schematic presentation of chromosome 11-specific regions in the mouse *Myh8* gene (*neo-mhc*) (−1.2 to +1.5 kb) and the mouse *Myh3* gene (*emb-mhc*) (−0.7 to +2 kb), and location of NFAT-binding sites (N1, N2, N3) and E-boxes (E1 to E11). Gray rectangles on the genes represent each ChIP amplicon amplified by PCR, designated as *a* to *i* on the *neo-mhc* gene and *a′* to *f′* on the *emb-mhc* gene. The specific mouse sequence of the NFAT-binding sites and E-boxes identified in the amplicons enriched by any ChIP is indicated above the figure with the aligned corresponding human sequence. (B-E) ChIP assays were performed on C2C12 cells grown in PM or DM with antibodies for NFATc2 (B), NFATc1 (C), NFATc3 (D) and MyoD (E). Bars represent fold enrichment of amplicons, normalized to input controls obtained with primers spanning a non-coding region 3′ of the myogenin gene. Three independent ChIP assays with three different antibodies directed against NFATc2 and two different against MyoD were performed. **P*<0.01. n.s., not significant; n.d., not determined.

(Fig. 5B). However, both c and d amplicons were observed to be significantly enriched in differentiating C2C12 cells, demonstrating the presence of endogenous NFATc2 in the most proximal region of the *neo-mhc* promoter. In the two regions covered by these amplicons, two putative NFAT binding sites, N2 and N3, were identified (Fig. 5A). Conversely, chromatin immunoprecipitation analyses carried out with antibodies against either NFATc1 (Fig. 5C) or NFATc3 (Fig. 5D) demonstrated that NFATc1 and NFATc3 were bound on the *emb-mhc* first intron (amplicon e′) in proliferating C2C12 myoblasts, but both proteins were absent from the *neo-mhc* promoter in proliferating and differentiating C2C12 cells, indicating a specific role for NFATc2 in the transcriptional control of the *neo-mhc* gene. One putative NFAT-binding site (N3) was identified in the DNA region, corresponding to amplicon e′ on the *emb-mhc* gene. ChIP experiments performed with an antibody against MyoD demonstrated the constitutive presence of MyoD in both *emb*

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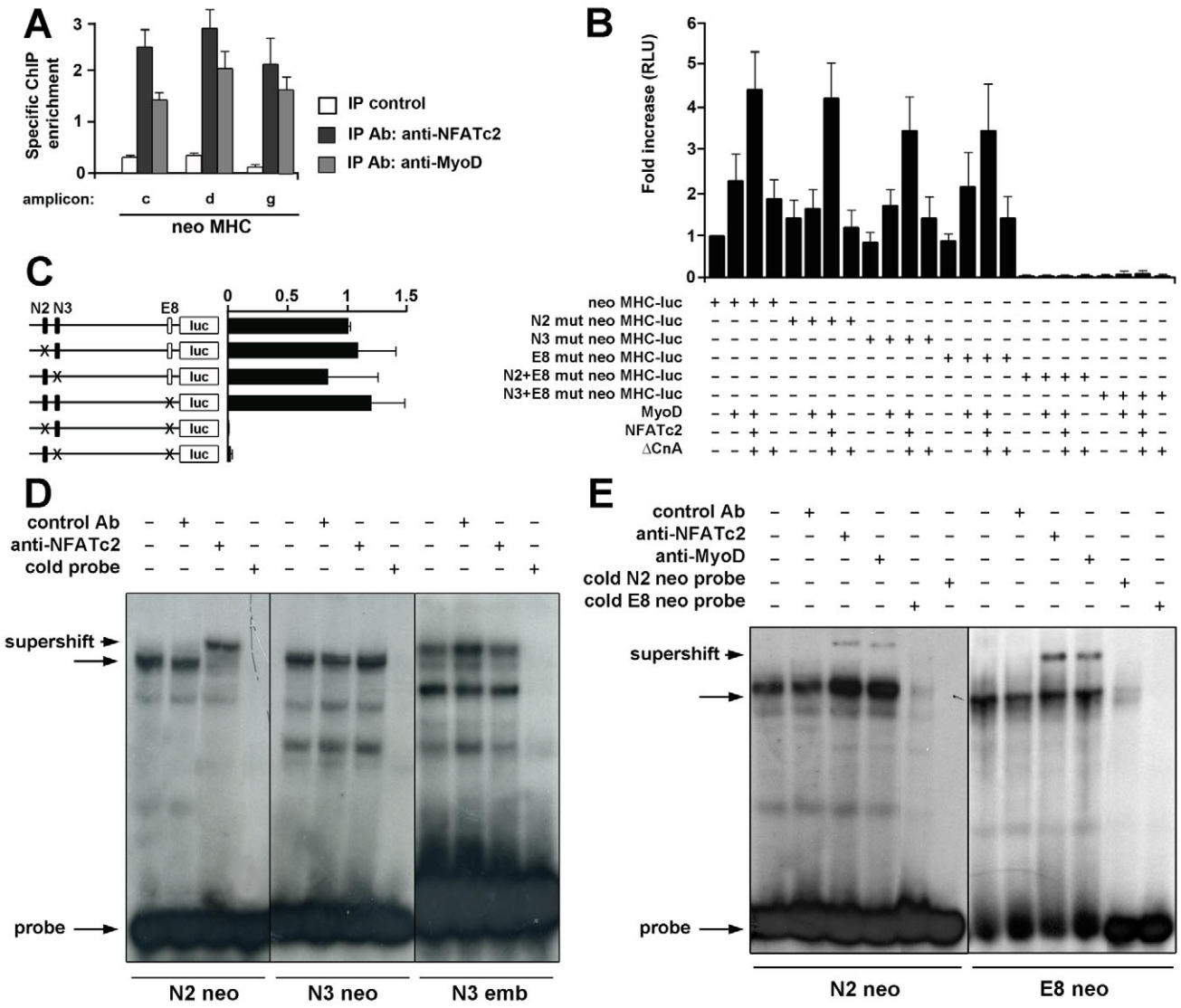

Fig. 6. Identification of the NFAT-binding sites and E-boxes bound by NFATc2 and MyoD on the *neo-mhc* gene. (A) *In vivo* ChIP assays using anti-MyoD, anti-NFATc2 or anti-laminin (control) antibodies revealed the presence of both MyoD and NFATc2 on the *neo-mhc* gene in developing limb muscles of P5 neonatal mice. Amplicons c, d and g are described in Fig. 5. Bars represent fold enrichment of amplicons, normalized to input controls obtained with primers spanning a non-coding region 3′ of the myogenin gene. (B) C2C12 cells were transiently transfected with the neo-MHC-luc vector and the same construct containing site-directed mutations for N2, N3 NFAT-binding sites and/or E8 E-box in combination with the indicated expression vectors. Data represent the mean ± s.e.m. of three independent experiments. RLU, relative light units. (C) P5 wild-type TA were electroporated with the different wild-type or mutated neo-MHC vectors with a GFP expression plasmid (n=3 per condition). TA muscles were microdissected 2 days later and luciferase activity was measured and normalized with GFP fluorescence intensity. (D,E) Nuclear extracts from C2C12 cells transfected with MyoD, NFATc2 and a constitutive activated form of calcineurin were analyzed by EMSA, using radiolabeled oligonucleotide probes of the NFAT-like sites N2 neo, N3 neo and the E8 E-box from *Myh8* promoter and of the NFAT-like site N3 emb inside the first intron of the *Myh3* gene in the presence or absence of 0.8 µg of the indicated antibodies. A 20-fold molar excess of the indicated unlabeled oligonucleotide was added to the binding reaction mixtures to determine the specific binding. Complexes are indicated by arrows, and the retarded complexes are indicated by arrowheads. The experiments presented in D and E were performed independently using the same protocol.

(amplicon d′) and *neo-mhc* (amplicon g) first introns in proliferating and differentiating C2C12 cells (Fig. 5E). *In vivo* ChIP experiments realized on P5 wild-type muscles indicated that endogenous MyoD and NFATc2 were both detected on *the neo-mhc* gene, on the same sites as above (amplicons c, d and g) (Fig. 6A). Furthermore, this result revealed that both proteins are detected on each amplicon, strongly suggesting that MyoD and NFATc2 are in the same complex linked to E-boxes and NFAT binding sites on the promoter and the 1st intron of the *neo-mhc* gene in developing muscles.

By transfection assays in C2C12 cells, the induction of luciferase activity by overexpression of both MyoD and NFATc2 was conserved when either N2, N3 or E8 were mutated on the neo-MHC-luc reporter (Fig. 6B). The combined mutation of both N2/N3 with E8 abrogates any cooperative induction by overexpressed MyoD and NFATc2 on the neo-MHC-luc reporter. Once electroporated in TA from P5 neonate mice, similar results were observed. Luciferase activity was barely detected when both N2/N3 and E8 were mutated, showing that MyoD and NFATc2

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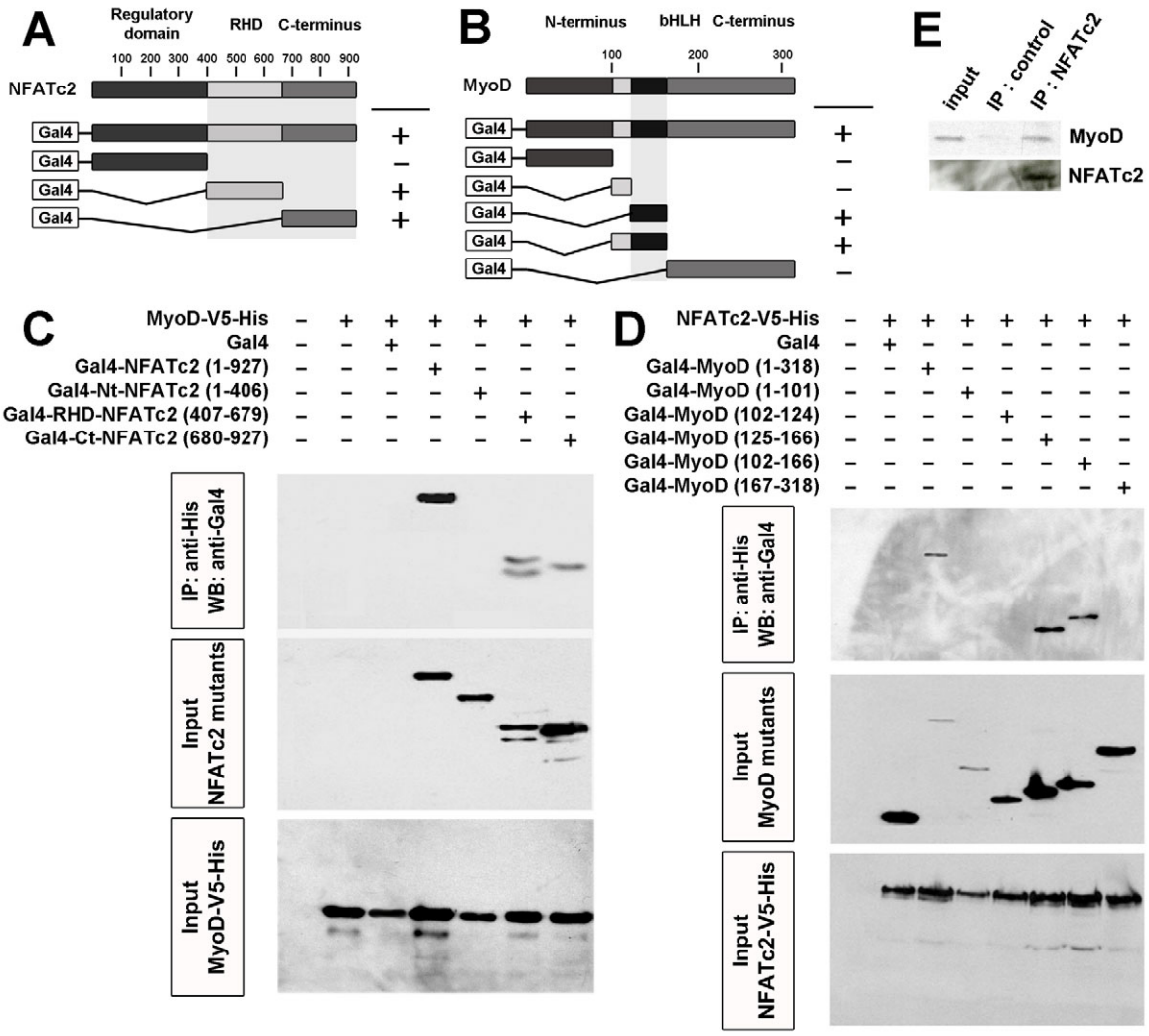

Fig. 7. Mapping of NFATc2 and MyoD interaction. (A,B) Schematic overview of Gal4-NFATc2 (A) and Gal4-MyoD (B) deletion constructs and their ability to bind MyoD or NFATc2, respectively. (C,D) Protein extracts from COS7 cells transfected with MyoD-V5-His, empty vector (Gal4) and Gal4-NFATc2 deletion constructs (C) or transfected with NFATc2-V5-His, empty vector (Gal4), and Gal4-MyoD deletion constructs (D) were immunoprecipitated (IP) with Ni-NTA beads and subjected to western blot (WB) using an anti-Gal4 antibody. (E). Co-immunoprecipitation of the endogenous proteins with anti-NFATc2 antibody and cell extracts from stimulated differentiating C2C12 cells were analyzed by immunoblotting with anti-MyoD and anti-NFATc2 antibodies.

cooperatively regulate neo-MHC expression, via N2 and/or N3 NFAT-binding sites and the E-box E8 (Fig. 6C).

To test whether NFATc2 could bind the sequences identified as NFAT-binding elements in the *neo-mhc* and *emb-mhc* genes, we performed electrophoretic mobility shift assays with nuclear extracts of C2C12 cells transfected with MyoD, NFATc2 and a constitutive activated form of calcineurin, and using radiolabeled DNA probes of N2 and N3 neo and N3 emb sites identified in amplicons c, d and e′, respectively (Fig. 6D). NFATc2 specifically bound the NFAT-like sequence N2 from the *neo-mhc* promoter, as a specific complex was supershifted by a specific anti-NFATc2 antibody. By contrast, NFATc2 failed to bind N3 neo and N3 emb, as no complex was supershifted with the anti-NFATc2 antibody. Further evidence on the specificity of NFATc2 binding was provided by competition experiments using a 20-fold molar excess of homologous oligonucleotide (Fig. 6D).

Remarkably, MyoD is also part of a complex that binds N2 neo, as such a complex was supershifted with a specific anti-MyoD antibody (Fig. 6E). The reverse is also observed on the E8 E-box sequence identified within amplicon g in the *neo-mhc* gene. Both MyoD and NFATc2 were supershifted by the anti-MyoD or anti-NFATc2 antibody, respectively. Furthermore, competitive experiments with cold E8 or cold N2 probes demonstrate that the MyoD/NFATc2 complex probably does not bind both sequences of the *neo-mhc* gene, but rather binds either N2 neo in the promoter or E8 neo in the 1st intron of *neo-mhc*.

Taken together, these results indicate that the MyoD/NFATc2 complex specifically regulates the *neo-mhc* gene by direct transcriptional activation and unambiguously show that endogenous NFATc2 and MyoD are present either on the proximal promoter or on the first intron of the *neo-mhc* gene *in vivo*. This supports the idea that NFATc2 and the bHLH transcription factor MyoD may cooperate to activate specifically neo-MHC transcription.

NFATc2 physically interacts with MyoD

Then, we tested by co-immunoprecipitation assays whether NFATc2 was able to interact with MyoD, as NFATc3 does (Armand et al., 2008). To map the MyoD binding site(s) on NFATc2, we generated a panel of NFATc2 deletion mutants (Fig. 7A). A C-terminal V5/His

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tagged full-length MyoD and Gal4-NFATc2 deletion constructs were co-expressed in COS7 cells and MyoD-immunoprecipitated with Ni-NTA beads, specific for the C-terminal His-tag on MyoD (Fig. 7C). The C-terminal transactivation domain (residues 680-927) and the DNA-binding Rel homology domain of NFATc2 (residues 407-679) are both sufficient to interact with MyoD (Fig. 7C). Similarly, a series of MyoD deletion mutants coupled to Gal4 were co-expressed with a C-terminal His/V5-tagged full-length NFATc2 and a constitutive activated mutant of calcineurin to ensure NFAT to be translocated into the nucleus (Fig. 7B). The co-immunoprecipitation of NFATc2-V5/His with Ni-NTA beads revealed that residues 125-166, corresponding to the HLH domain of MyoD, are required for this interaction (Fig. 7D). This interaction was corroborated by *in vivo* co-immunoprecipitation showing an endogenous MyoD/NFATc2 complex in differentiating C2C12 cells (Fig. 7E). Collectively, these data confirm that neo-MHC expression is induced *in vitro* and *in vivo*, in embryonic and in postnatal muscle fibers, by a cooperative interaction between MyoD and calcineurin/NFATc2 signaling.

DISCUSSION

Calcineurin/NFAT pathway regulates the acquiring of the myofiber phenotype during embryogenesis

In contrast to the implication of the calcineurin/NFAT pathway in the specification of the adult muscle fiber type that is no more debated, little is known about its involvement in the establishment of the muscle phenotype, particularly during the embryonic and perinatal periods. The calcineurin/NFAT pathway is considered as the best characterized signaling pathway mediating the neural activity in mature myofibers (Calabria et al., 2009). The MHC isoform expression would depend on the combination of translocated NFAT isoforms, type I MHC being regulated by the cooperation of all four NFAT members, whereas type IIb MHC is expressed in adult myofibers that accumulate in their nuclei only one NFAT isoform NFATc4. If it is clear that the calcineurin/NFAT pathway regulates the maintenance of the slow muscle phenotype in adult muscles, its involvement in the establishment of the muscle phenotype during embryogenesis is ambiguous. In transgenic mice overexpressing the calcineurin inhibitor MCIP1, hindlimb muscles have the same muscle phenotype as wild-type hindlimb muscles at birth (Oh et al., 2005). In these muscles, the expression of developmental (emb-MHC and neo-MHC) and adult (type I, IIa, IIb, IIx MHC) MHC is similar to wild-type muscles from E14.5 to 7 days after birth, suggesting that the calcineurin/NFAT pathway would not modulate or establish the muscle type specification in immature myofibers. An *in vitro* study suggested instead that promoters of both developmental *mhc* genes, *emb-mhc* and *neo-mhc*, could be under the control of MRFs and the calcineurin pathway (Beylkin et al., 2006). Despite contradictory results, the authors proposed a differential transcriptional mechanism, where MyoD would preferentially regulate emb-MHC expression, and the calcineurin/NFAT pathway, neo-MHC expression. Here, we provide the first evidence that the calcineurin/NFAT pathway plays a crucial role in establishing the muscle phenotype during embryogenesis. We demonstrate that the NFAT member NFATc2, in cooperation with MyoD, is required for neo-MHC expression during late stages of embryogenesis, from E14.5 to E18.5. We also show that this regulatory mechanism is conserved during early post-natal development, as neo-MHC expression was highly affected in myofibers electroporated 5 days after birth with a combination of siRNAs directed against MyoD and NFATc2 (Fig. 2F,I). Previous studies described NFATc2 as a major regulator of skeletal muscle growth during secondary myogenesis (Horsley et al., 2001). NFATc2 has been shown to promote the fusion of myoblasts with neighboring myotubes by directly activating its target gene, interleukin 4 (Horsley et al., 2003). We now show that NFATc2 plays an additional role in muscle development during secondary myogenesis, but also in primary myogenesis, by controlling the expression of the *neo-mhc* gene; this regulation requires its bHLH partner MyoD. This MHC isoform is an important one, the major MHC isoform at birth, as it represents 65% of the total MHC expressed at that time (Allen and Leinwand, 2001). As this MHC isoform is absent from *Myod/Nfatc2*-null hindlimb and intercostal muscles, it is possible that these muscles have difficulties in contracting due to a global deficit in myosin, as shown in Fig. 2A, and this could participate to the lethality of double-null mice at birth. We notice that some skeletal muscles as diaphragm were not affected by the absence of MyoD and NFATc2 expression that could be explained by the embryonic origin of this muscle; this should be further investigated. Overall, these combined observations point to the crucial role of the calcineurin/NFAT signaling in the establishment of the muscle fiber type in immature myofibers (this work), as well as in its maintenance in mature limb myofibers (Calabria et al., 2009).

Are specific NFAT/MRF complexes involved in fine regulations of MHC expression?

NFATc1 has been shown to maintain the slow muscle phenotype by activating type I MHC expression and downregulating type IIb MHC in mature myofibers (Schiaffino, 2010). Here, we show that NFATc2 is involved in the regulation of neo-MHC expression in immature myofibers. In line with these observations, we propose that the other NFATs could contribute to the maintenance and the establishment of the other embryonic and fast phenotypes. By chromatin immunoprecipitation, we show in this work that only NFATc2 and MyoD bind the *neo mhc* promoter in differentiating C2C12 cells (Fig. 5), as well as in neonatal limb muscles (Fig. 6). The other NFATc proteins, NFATc1 and NFATc3 were found on the promoter of the *emb-mhc* gene in proliferating C2C12 cells, whereas NFATc2 never binds this promoter in our experimental conditions. These results suggest that NFATc1 and NFATc3 would preferentially regulate the emb-MHC expression, whereas NFATc2 is specifically involved in the regulation of the neo-MHC isoform. They also suggest a differential activation of each MHC isoform by different combination of NFATc expression during muscle development.

Furthermore, NFATc proteins activate their target genes in cooperation with other transcription factors, such as MyoD (Armand et al., 2008), MEF2 (van Oort et al., 2006) and GATA4 (Molkentin et al., 1998). By cooperating with different partners, we could hypothesize that the calcineurin/NFAT pathway regulates different MHC isoforms: developmental and adult MHC; slow and fast isoforms. Among the NFAT partners, the myogenic factors would play an important role. We have previously shown that the NFATc3 isoform is able to interact with MyoD to regulate myogenin expression in somites, during early myogenesis (Armand et al., 2008). We now demonstrate that another NFAT isoform, NFATc2, is also able to interact with MyoD, but this complex has a completely different role as it specifically regulates the neo-MHC expression during myogenesis. Furthermore, each complex is unique, as the interacting domains involved in the interaction between NFATc2 and MyoD (this work) are different from those involved in the MyoD/NFATc3 interaction (Armand et al., 2008). MyoD is able to interact with NFATc3 via its basic domain, whereas its HLH domain is necessary for MyoD to interact with NFATc2. Similarly, the N-

terminal region of NFATc3 is required for NFATc3 to interact with MyoD and here, we show that NFATc2 interacts with MyoD via its C-terminal domain. Therefore, the MyoD/NFATc2 complex is as unique as MyoD/NFATc3, despite the homology in the sequence of both NFATc3 and NFATc2. It is then tempting to speculate that the other NFATc isoforms could interact with other myogenic factors, forming original MRF/NFATc complexes when both proteins are expressed and active in the nucleus. These original complexes would probably be involved in many different myogenic processes, as MRFs are differentially expressed and are involved in different specific myogenic steps. Moreover, we show in this work that Myf5 and myogenin are not able to cooperate efficiently with NFATc2 or NFATc3 to activate neo-MHC expression (Fig. 4A). The muscle phenotype of *Myod/Nfatc2*-null mice also suggests that no MRF/NFATc complex other than MyoD/NFATc2 is able to ensure the activation of neo-MHC expression in embryonic muscles. Therefore, the other MRF/NFATc complexes are likely to be involved in different other myogenic processes and we hypothesize that they could regulate different MHC isoforms in order to build a mosaic muscle.

MATERIALS AND METHODS

Animals

Myod- and *Nfatc2*-null mice were generously provided by Shahragim Tajbakhsh (Paris, France) and Laurie Glimcher (Boston, MA, USA) (Hodge et al., 1996; Rudnicki et al., 1993). *Myod/Nfatc2*-null mice were generated by cross-breeding single knockout mice. All protocols were performed according to institutional guidelines and approved by local Animal Care and Use Committees.

Cell culture and transfections

Cell culture and transfections of C2C12, COS7 cells and primary myoblasts have been described previously (Armand et al., 2008; Armand et al., 2011).

Cloning

NFATc2 constructs were inserted in frame into pBind (Promega) and pCDNA3.1/V5/His (Invitrogen) vectors. Expression vectors for an activated mutant of CnA or deletion fragments for MyoD have been described previously (Armand et al., 2008). MyoD, NFATc2, NFATc3 and MHC I-luc expression vectors were a kind gift from J. Molkentin, E. Olson and L. Leinwand. The emb-MHC-luc vector contains 1.1 kb of *emb-mhc* 1st intron (+661 to +1710). The neoMHC-luc plasmid contains 1.5kb of mouse *neo-mhc* gene (from –597 to +847). Both fragments were inserted upstream of a minimal TATA box into pGl4.23. Two NFAT-binding sites and one E-box were mutated by directed mutagenesis using primers described in supplementary material Table S1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described in detail previously (Armand et al., 2008), using proliferating or 3 days differentiating C2C12 cells or hindlimb muscles of wild-type P5 mice, homogenized with a tissue glass dounce after crosslinking. Precleared chromatin was immunoprecipitated with 5 µg of the indicated antibodies (supplementary material Table S2). Oligos used for the PCR amplifications were as described in supplementary material Table S1.

Nuclear extracts and electrophoretic mobility shift assay

C2C12 cells were transfected with expression vectors for MyoD, NFATc2 and a constitutive activated form of calcineurin, and cultured for 48 hours in proliferating medium (PM). Nuclear extracts and gel retardation assays were performed as described previously (Martínez-Martínez et al., 1997). The nucleotide sequences used as probes in EMSAs are described in supplementary material Table S1. Supershift assays were performed using the indicated antibodies (supplementary material Table S2).

Isolation and purification of total RNA and real-time PCR

Primers were designated to detect the different transcripts (see supplementary material Table S1) (Richard et al., 2011). Total RNA isolation and real-time PCR have been described previously (Armand et al., 2004).

Histological analysis and immunofluorescence

For paraffin sections, epitopes were demasked four times for 5 minutes at a time in boiling citric acid (pH 2) prior incubation with the primary antibody diluted in 1% BSA. For GFP and neo-MHC staining, cryosections were fixed, blocked in 4% goat serum/4% BSA/PBS/0.1% Tween and incubated with the appropriate concentration of antibody diluted in PBS/Triton X-100 0.5%.

Electroporation

Tibialis anterior (TA) of P5 wild-type mice were injected with 4 U hyaluronidase 2 hours prior the different injections combined with 10 µg of a GFP expression plasmid: a mixture of 10 µg of small interfering RNA (siRNA) in total or 1µg of non-mutated or mutated neo-MHC-luc vectors were injected. Six 65 V/cm pulses of 60 ms, with 100 ms interval were applied. Muscles were collected 2 (for luciferase activity) or 4 (for immunohistochemistry and western blot) days after gene delivery.

Immunocytochemistry

Myotubes were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 minutes and incubated with the indicated antibodies.

Co-immunoprecipitation assays

V5/His tagged MyoD or NFATc2 constructs were immunoprecipitated using Ni-NTA beads (Invitrogen), followed by western blotting procedures as described in detail previously (Armand et al., 2004). The MyoD/NFATc2 complex was immunopurified from C2C12 cells cultured 3 days in differentiation medium (DM) and stimulated for 4 hours with phorbol ester plus calcium ionophore (PMA + ionomycin). Cell extracts were incubated with anti-NFATc2 in 150 mM NaCl, 0.1% NP40 and purified using protein G-Sepharose.

Statistical analysis

The results are presented as means ± s.e.m. Statistical analyses were performed using INSTAT 3.0 software (GraphPad, San Diego) and Student's *t*-test or ANOVA followed by Tukey's post-test when appropriate. Statistical significance was accepted at a $P<0.05$.

Acknowledgements

We thank Marielle Saclier and Meryem Baghdadi for their early involvement in this project as M2 and rotation students. We also thank Laurie Glimcher and Sharagim Tajbakhsh for providing mouse models.

Competing interests

The authors declare no competing financial interests.

Author contributions

N.D., S. Lécolle., S. Lefebvre and A.-S.A. performed experiments. S. Lefebvre, F.C., A.S.A. and C.C. analyzed data. S. Lefebvre, A.-S.A. and C.C. designed research. B.d.G. provided reagents. C.C. and A.-S.A. wrote the manuscript. N.D. and S. Lécolle contributed equally as joint first authors.

Funding

This work was supported by grants from the Association Francaise contre les Myopathies [AFM13958].

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.097428/-/DC1

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Fig S1

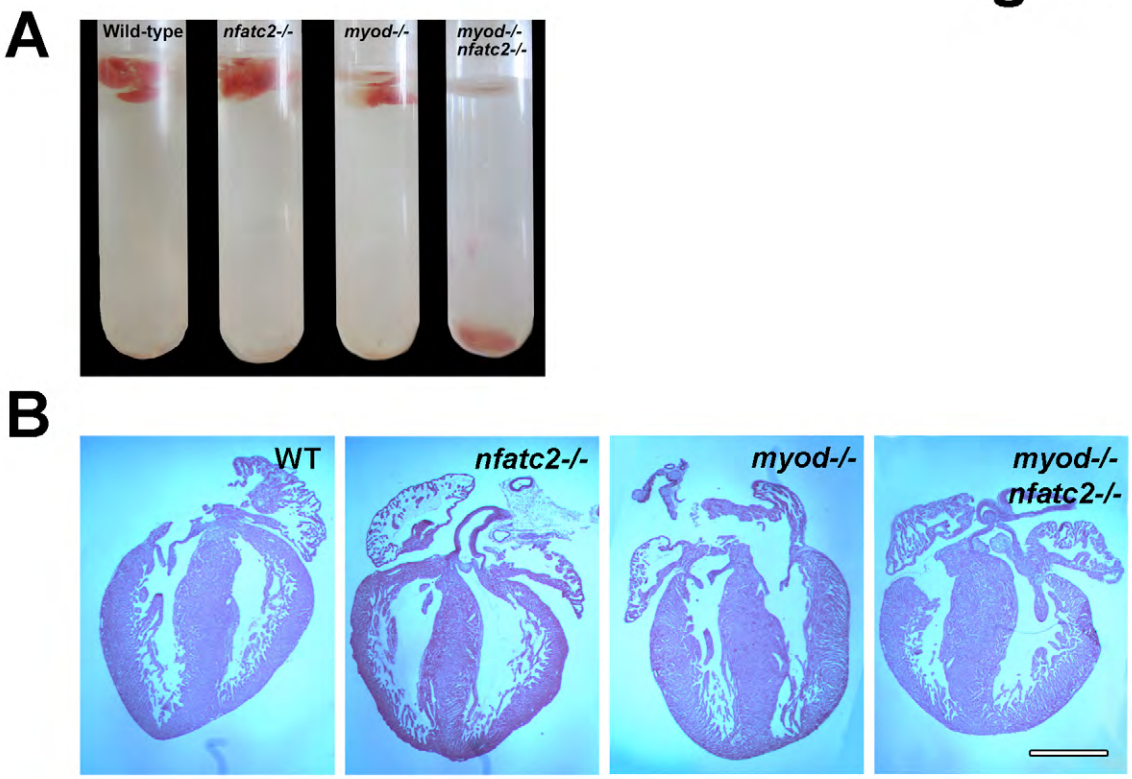

Fig. S1: *myod/nfatc2* double null mice die at birth with respiratory defects. (A) Lungs were dissected out from newborn mice and dropped in water. All lungs float except the *myod*−/−: *nfatc2*−/− lungs that sink to the bottom, indicating that they were not inflated with air. (B) Representative H&E stained four-chamber view of hearts dissected from newborn mice of indicated genotypes. No obvious cardiac phenotype was observed in any transgenic mice. Bar=750 μm.

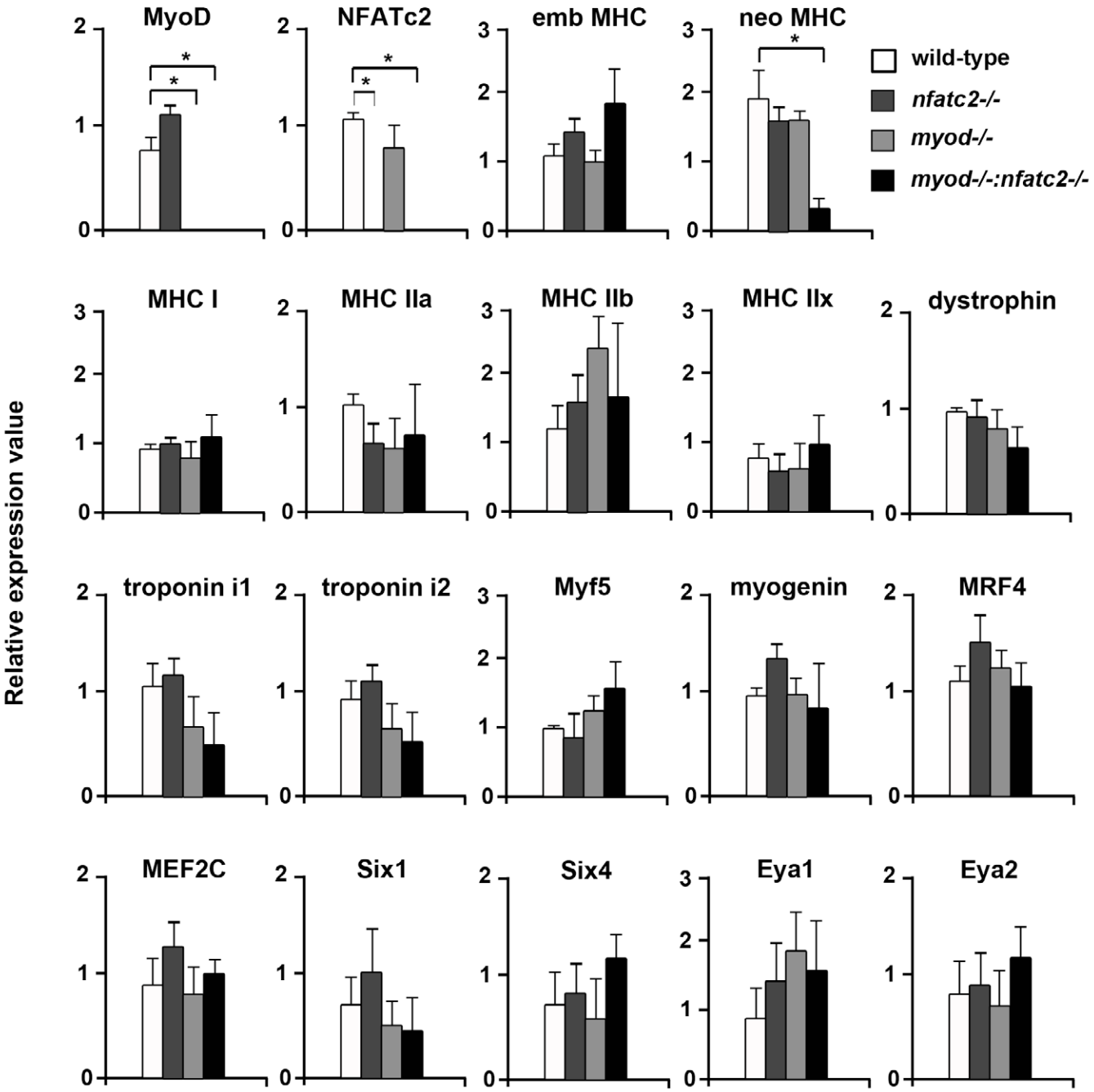

Fig. S2: The expression of the indicated muscle specific genes was analyzed by RT-qPCR from E18.5 limb muscles of WT, *nfatc2−/−, myod−/−, myod−/−:nfatc2−/−* mutant mice. Data are means±s.e.m. of three different experiments. Five to seven embryos of each genotype were analyzed. * indicates $P<0.01$ vs WT.

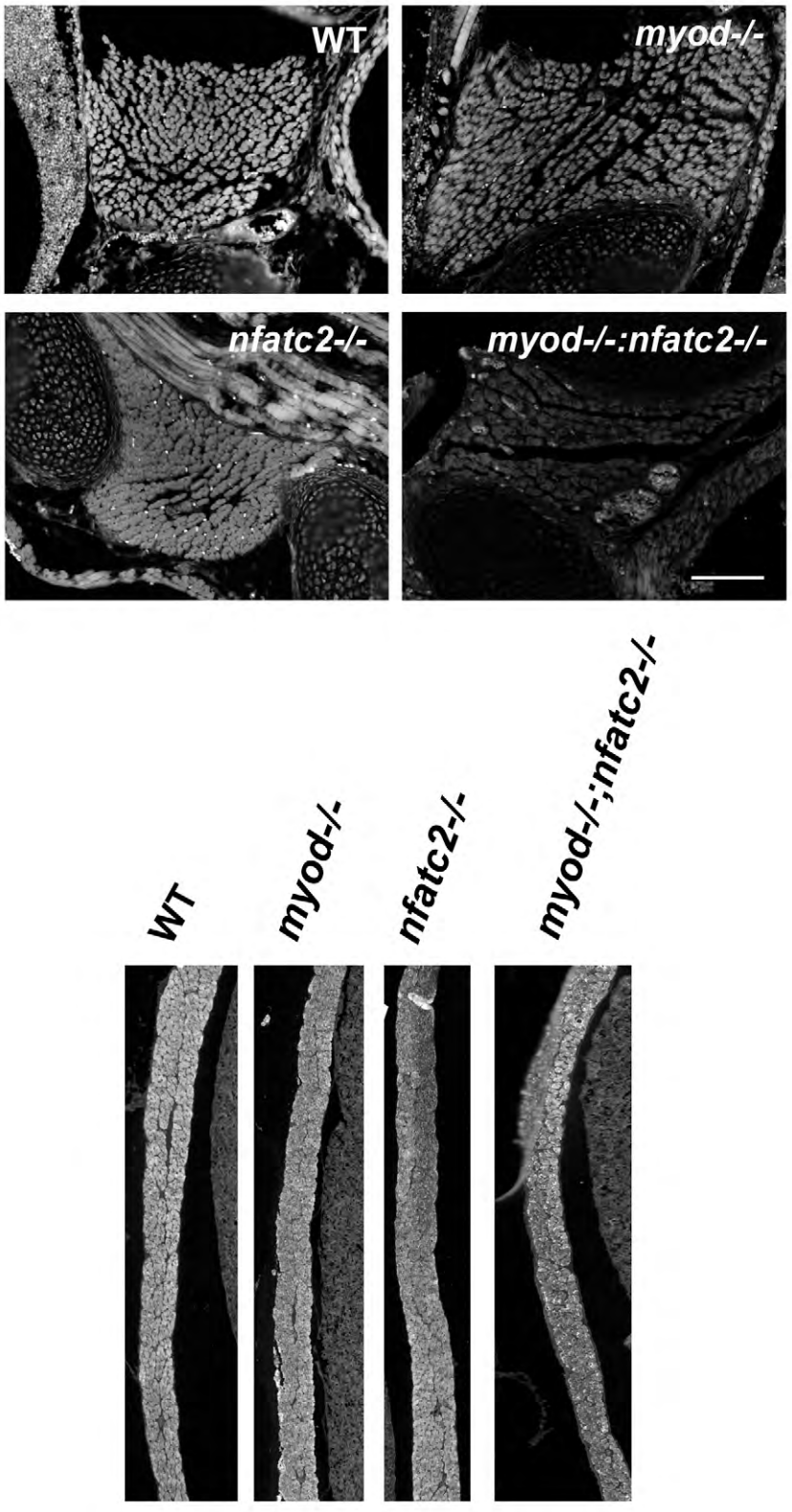

Fig. S3: MyoD and NFATc2 are required for neo-MHC expression in intercostal muscles at E18.5. Transverse cryosections of intercostal muscles (upper panels) and diaphragm (lower panel) were immunostained with neo-MHC antibodies, demonstrating that *myod/nfatc2* double null intercostal muscles but not diaphragm are deficient in neo-MHC proteins. Bar=40 μm.

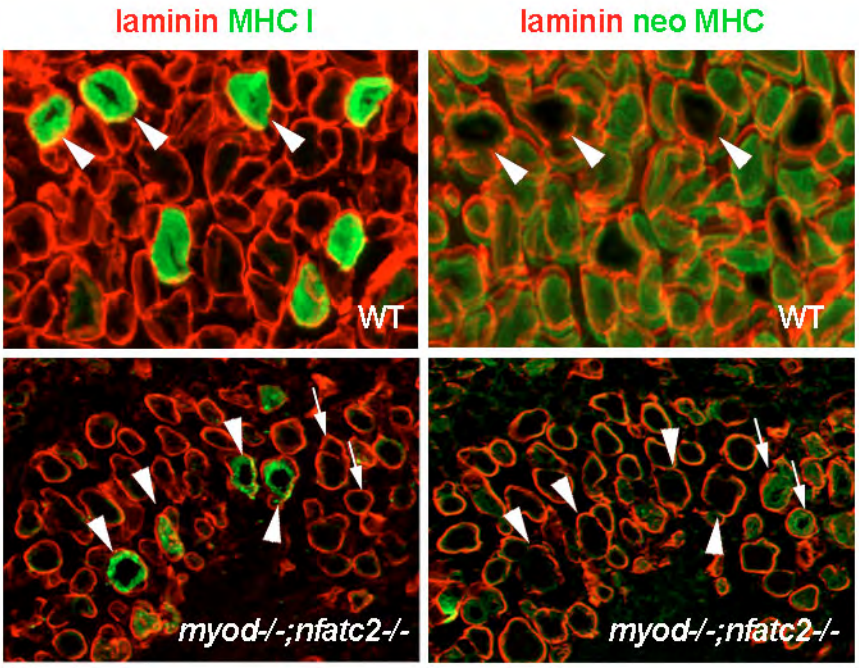

Fig. S4: Immunostaining of serial sections of E18.5 WT and *myod−/−;nfatc2−/−* limb muscles using anti-MHC I or anti-neo-MHC antibodies with the specific anti-laminin antibody. Primary myofibers are indicated by arrowheads. They express MHC I but not neo-MHC at this stage (Condon et al., 1990b). Secondary WT myofibers always express neo-MHC. In *myod/nfatc2* double null muscles, few secondary myofibers (pointed by arrows) express faint amount of neo-MHC.

Table S1. Primers

		Sequence (5'-3') Forward primer	Sequence (5'-3') Reverse primer
Chromatin IP	Amplicon a	AATGAGGGGATGGAAGATGA	CCAGACTTTGAGGGTGCATT
	Amplicon b	TACGACATCCCTGCCTTCAT	CGGAAAGAAAAGAGAAAATGC
	Amplicon c	ATCCCCCATGCTATCTCTCC	CTCATCCTTGGAGCCTGACT
	Amplicon d	AGTCAGGCTCCAAGGATGAG	CTGTGCTGCCAAAGATCAGA
	Amplicon e	TCTGATCTTTGGCAGCACAG	GCGGAGATAGGGTGTGAGG
	Amplicon f	GCTTGAGGGTAGGGGAACTT	CCAGGCTTCAGTGAGGGTAA
	Amplicon g	TTTTTGAGGGACGGCTTGTA	GGAGGCTTGGCATGAAATAG
	Amplicon h	GAACCAGGGCAGTGCTAAGT	CCATTGTTGCTCAAAACCAA
	Amplicon i	CAAAGCCATGTTTCCTTCCT	CTCGCCAGAATCTTGACTCC
	Amplicon a'	TGGACCAGGAAAGAGAGGAA	AGCAGGACCAACAAGCAAGT
	Amplicon b'	TGATTTCAGGGTGGGATGG	CGTATTTTTCGCCGTTGCC
	Amplicon c'	TCAAGGCGTTCTCAAGGAAT	GTCCCCTGTTCATTCTGTGG
	Amplicon d'	CCATACGTCCACAACTGCTG	AAGCCTGCACTTCTCCGTTA
	Amplicon e'	GTTTGCTCTACCCGTGC	GCAATGAATGGAAGCCTCTC
	Amplicon f'	GTGCTGAACCCTTGTGTGAA	TCCATCTCGGTGTCGCTACT
	Control amplicon	TCCTGGATTACTGTCAAGC	AACGAGTCTATGAGGGTAAG
qRT-PCR	NFATc2.2	ATCTACCCAGATCAGTATGG	TCAGGAGTATACCATTTCTC
	MyoD	GGCTCTCTCTGCTCCTTTGA	AGTAGGGAAGTGTGCGTGCT
	Emb MHC	GCAAAGACCCGTGACTTCACCTCTAG	GCATGTGGAAAAGTGATACGTGG
	neo MHC	GTCACGCAATGCAGAAGAGA	CAGGTCCTTCACCGTCTGTT
	MHC I	AGGGCGACCTCAACGAGAT	CAGCAGACTCTGGAGGCTCTT
	MHC IIa	CCAAGAAAGGTGCCAAGAAG	CGGGAGTCTTGGTTTCATTG
	MHC IIb	GCTTGAAAACGAGGTGGAAA	CCTCCTCAGCCTGTCTCTTG
	MHC IIx	CGGTGGTGGAAAGAAAGG	CAGGAGTCTTGGTTTCATT
	myogenin	TGACCCTACAGACGCCCACAATC	CACACCCAGCCTGACAGACAATC
	MRF4	TGCTAAGGAAGGAGGAGCAA	CCTGCTGGGTGAAGAATGTT
	Myf5	AGGAAAAGAAGCCCTGAAGC	GCAAAAAGAACAGGCAGAGG
	Six1	CTTTAAGGAGAAGTCTCGGG	TTCCAGAGGAGAGAGTTGAT
	Six4	TCACTCCACATCCCTCCTTC	CATCTGTTTCAAAGCGAGCA
	Eya1	ACCTCCCCGACTTTCTCATT	AGAAGTGCTGCTGACCCAGT
	Eya2	ACCCGTTACTCCCATTACCC	CCCCCTCTCCTACAACACAA
	Troponin i1	ATGCCGGAAGTTGAGAGGAAA	TCCGAGAGGTAACGCACCTT
	Troponin i2	AGAGTGTGATGCTCCAGATAGC	AGCCACGTCGATCTTCGCA
	Dystrophin	TCTCCTTTGCATTGGTTTCC	GAGTTTGGAGACGCTTTTGC
	MEF2C	TGCTGGTCTCACCTGGTAAC	ATCCTTTGATTCACTGATGGCAT
	26S	AGGAGAAACAACGGTCGTGCCAAAA	GCGCAAGCAGGTCTGAATCGTG
Mutagenesis	N2 neo	GGCACTGGTCCAATGGTCTCGAGTCAGCATAAAGGTTCTTCTCG	
	N3 neo	CCTGTTGGAACAAATACTCGAGGTGAGTTGCTGGGCATCTG	
	E8 neo	GAATTTTGTCAGTGAGTTATGGTACCTAACTCTAGTGGCAACC	
EMSA	N2 neo	gatcCCAATGGTGGAGACTCAGCATA	
	N3 neo	gatcACAAATAAGGAAAGTGAGTTGC	
	N3 emb	gatcGGATTATATTCCCAGTGCTGAA	
	E8 neo	gatcGAGTTATCAGCTGTAACTCTAGT	

Table S2. Antibodies

	Antibody		
Chromatin IP	NFATc3, monoclonal	Santa Cruz (sc-8405X)	5µg
	NFATc2, polyclonal	Santa Cruz (sc-13034X)	5 µg
	NFATc2, monoclonal	Santa Cruz (sc-7296X)	5 µg
	NFATc1, polyclonal	Santa Cruz (sc-1789)	5µg
	MyoD, monoclonal	Santa Cruz (sc-760)	5µg
	MyoD, polyclonal	Santa Cruz (sc-304X)	5µg
	NFATc2, polyclonal	Gift from JM Redondo, Madrid, Spain	5µg
	Laminin, polyclonal	Sigma	5µg
Immuno-detection	Sarcomeric MHC, monoclonal	DSHB (Iowa University) (MF20)	IHC: 1/100 WB: 1/20
	Embryonic MHC, monoclonal	DSHB (Iowa University) (F1.652)	IHC: 1/20 WB: 1/200
	Neonatal MHC, monoclonal	DSHB (Iowa University) (N3.36)	IHC: 1/20 WB: 1/200
	V5, monoclonal	Invitrogen	WB: 1/1000
	Gal4, monoclonal	Santa Cruz	WB: 1/2500
	Cleaved caspase-3, polyclonal	Millipore	IHC: 1/20
	GAPDH, monoclonal	Millipore	WB: 1/5000
	MHC I, monoclonal	Leica (NCL-MHCS)	IHC: 1/20 WB: 1/200
	Desmin, monoclonal	Dako	IHC: 1/200
	GFP, polyclonal	Abcam	IHC: 1/250
	Laminin, polyclonal	Sigma	IHC: 1/200
	Bis-benzimide	Sigma	IHC: 1/30 000
EMSA	Gal4, monoclonal	Santa Cruz	0.8µg
	NFATc2, monoclonal	Santa Cruz (sc-7296X)	O.8 µg
	MyoD, polyclonal	Santa Cruz (sc-759X)	0.8µg
IP	NFATc2, polyclonal	Santa Cruz (sc-13034)	2 µg