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Nfix Regulates Fetal-Specific Transcription in Developing Skeletal Muscle

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SUMMARY

Skeletal myogenesis, like hematopoiesis, occurs in successive developmental stages that involve different cell populations and expression of different genes. We show here that the transcription factor nuclear factor one X (Nfix), whose expression is activated by Pax7 in fetal muscle, in turn activates the transcription of fetal specific genes such as MCK and β-enolase while repressing embryonic genes such as slow myosin. In the case of the MCK promoter, Nfix forms a complex with PKC theta that binds, phosphorylates, and activates MEF2A. Premature expression of Nfix activates fetal and suppresses embryonic genes in embryonic muscle, whereas muscle-specific ablation of Nfix prevents fetal and maintains embryonic gene expression in the fetus. Therefore, Nfix acts as a transcriptional switch from embryonic to fetal myogenesis.

INTRODUCTION

During skeletal myogenesis, stage-specific transcriptional changes occur in muscle fibers derived from differentiation of different precursors: embryonic, fetal myoblasts and satellite cells (Biressi et al., 2007a; Stockdale, 1992). "Embryonic" or primary fibers appear at E11 in the mouse and establish the basic muscle pattern. A second wave of myogenesis, termed fetal or secondary, takes place between E14.5 and E17.5 and involves the fusion of fetal myoblasts either with each other to form secondary fibers (initially smaller and surrounding primary fibers), or with primary fibers. At E16, satellite cells appear as mononucleated cells underneath the newly formed basal lamina of each individual fiber: they are responsible for muscle postnatal growth and regeneration. Previous work identified specific features of embryonic, fetal myoblasts and satellite cells and of the myotubes they give rise to (Biressi et al., 2007a). In particular, primary fibers express both slow and embryonic fast MyHCs and ubiquitous isoforms of metabolic enzymes. Conversely, secondary fibers express fast but not slow MyHC and muscle-specific enzymes such as MCK, β-enolase and PKCθ (Biressi et al., 2007a).

A genome-wide expression analysis on purified embryonic and fetal myoblasts (Biressi et al., 2007b) revealed that the transcription factor nuclear factor I X (*Nfix*) is robustly expressed in the fetus but absent in the embryo. Nuclear factor one (Nfi) proteins act as transcriptional activators and/or repressors of cellular and viral genes. In amniotes, the *Nfi* gene family consists of four closely related genes, named *Nfia*, *Nfib*, *Nfic*, and *Nfix* (Gronostajski, 2000). They encode for proteins with a conserved N-terminal DNA-binding and dimerization domain and a C-terminal transactivation/repression domain, which exhibits a high variability due to extensive alternative splicing. Nfi family members act as homo- and heterodimers and bind with high affinity to the palindromic consensus sequence 5′-PyTGGCA-N3-TGCCAPu-3′. Nfi binding motifs were detected in promoters of genes expressed in different organs, including brain, lung, liver, intestine, muscle, connective tissue, and skeletal elements (Gronostajski, 2000); recently *Nfia* was shown to regulate fate choice between erythrocytes and granulocytes (Starnes et al., 2009).

Gene ablation studies revealed that *Nfi* genes have essential and distinct roles in different organ systems including brain (*Nfia*) (das Neves et al., 1999); lung and brain (*Nfib*) (Steele-Perkins

et al., 2005); tooth (*Nfic*) (Steele-Perkins et al., 2003); and brain, intestine, and skeleton (*Nfix*) (Driller et al., 2007). *Nf1*-binding sites are present on the *Myogenin* promoter (Johanson et al., 1999) and Nfi can form a complex with Myogenin, thus increasing its affinity for a number of muscle-specific genes (Funk and Wright, 1992). This suggested a role for Nfi as co-factor in muscle differentiation but its precise function was not subsequently studied. We investigated the function of Nfix during mammalian myogenesis and report here that Nfix represses the expression of embryonic genes while activating fetal genes in developing skeletal muscle; thus it acts as a main regulator of the embryonic to fetal transcriptional switch, a role that has evolutionary significance in amniotes.

RESULTS

Nfix2 Represses Embryonic and Activates Fetal Muscle Genes

Although the pattern of embryonic, fetal, and adult gene expression in skeletal muscle has been described (Gunning and Hardeman, 1991), the underlying molecular control remains elusive. A genome-wide screen in fetal and embryonic myoblasts purified from Myf5$^{GFP-P/+}$ mice (Biressi et al., 2007b) identified the transcription factor nuclear factor one among the differentially expressed genes; in particular *Nfix* appeared as the most differentially expressed (>8-fold change) being abundant in the fetus and virtually absent in the embryo (Figures S1A–S1C available online). Of the various splicing isoforms, we focused on the best characterized, Nfix2, which was shown to transactivate expression of a variety of promoters in fibroblasts, glial cells, and other cell types (Chaudhry et al., 1998) and is strongly expressed in fetal skeletal muscle (Figure S1D). To investigate the possible role of Nfix in fetal myogenesis, we initially performed loss-of-function experiments (dominant negative Nfi-engrailed) in fetal myoblasts and gain of function experiments (expression of Nfix2 isoform) in embryonic myoblasts. As a model of fetal myogenesis, we initially used the myogenic cell line C2C12, which allows more in-depth biochemical analysis than in purified fetal myoblasts, which are limited in amount. Although derived from postnatal muscle, C2C12 cells share many features with fetal myoblasts, including the expression of high levels of Nfix (Biressi et al., 2007b) (Figure S1A). C2C12 cells were transduced with a lentiviral vector expressing the dominant-negative Nfi-engrailed (NfiEngr), containing the DNA binding and dimerization domain of Nfia fused together with the *Drosophila* Engr transcriptional repression domain that inhibitis transactivation activity of all Nfi proteins (Bachurski et al., 2003). A lentivirus expressing only the engrailed domain was used as control (Engr). As shown in Figure S1E, Nfi inhibition negatively affects myogenesis, likely because of reduced Myogenin expression and consequent reduced expression of Myosin Heavy Chains and MCK (Figure S1F). These data are consistent with previous studies (Funk and Wright, 1992; Johanson et al., 1999). BrdU incorporation indicated that in C2C12 cells the NfiEngr block of terminal differentiation was not accompanied by enhanced proliferation rate (data not shown). To test whether a possible modulation of embryonic and fetal gene expression might exist independently of a generic inhibition of terminal differentiation in the whole culture, we analyzed embryonic/fetal-specific gene expression in the few differentiated C2C12NfiEngr myotubes. We used the short trypsinization method (Kitzmann et al., 1998), which causes detachment of myotubes from substrate at a low dose of trypsin. C2C12Engr and C2C12NFIEngr differentiated myotubes (DM mt) were collected and analyzed by western blot in comparison with the whole differentiated culture (DM). As shown in Figure S1G, the C2C12NFIEngr myotubes express total sarcomeric MyHC at levels comparable to Engr controls, but show a strongly reduced expression of fetal markers such as MCK and β-enolase. Conversely, slow MyHC, normally repressed during fetal myogenesis and almost absent in control C2C12 is expressed at high levels. We repeated these experiments on Myf5$^{GFP-P/+}$-purified fetal myoblasts, which were infected by LentiNfiEngr or Engr, as a control. NfiEngr causes a less dramatic inhibition of differentiation (e.g., MyHC expression) in fetal myoblasts than in the C2C12 cells (Figures S1E and S1H). Nevertheless, NfiEngr-expressing fetal myoblasts acquire the typical aspect of their embryonic counterparts, characterized by smaller myotubes and a lower number of nuclei/myotube (data not shown); in addition, western blot analysis confirmed that the two fetal markers analyzed, MCK and β-enolase, were strongly downregulated, whereas slow MyHC was robustly induced in fetal myotubes (Figure S1I). Since NfiEngr blocks the function of all the members of the Nfi family, we investigated whether the effect on fetal myogenesis was specific to Nfix. Fetal myoblasts were isolated by cell sorting from the *Nfix* null//Myf5$^{GFP-P/+}$ mouse embryos (described below). In addition, small interfering RNA (siRNA) silencing of Nfix and Nfia was also performed. Myf5$^{GFP-P/+}$-purified fetal myoblasts were transduced with lentivectors expressing siNfix, siNfia, or the nontargeting control siRNA (CTL). As Figure 1D shows, the inhibition of both Nfix and Nfia does not modulate the expression of the other members of the family, suggesting the absence of reciprocal regulation among the Nfi proteins. Most importantly, the selective inhibition of Nfix, both in the Nfix null myoblasts and in the Nfix silenced cells, dramatically impairs fetal myoblast differentiation and fusion (Figures 1A and 1E) and abolishes the expression of MCK and β-enolase (Figure 1C); despite reduced differentiation, slow MyHC was activated in the *Nfix* null and siNfix myotubes. Silencing of Nfia did not inhibit myogenesis and did not modify the profile of gene expression. As a reciprocal experiment, purified embryonic myoblasts were isolated and infected by a lentiviral vector expressing the Nfix2 and Nfia1.1 isoforms (or an empty vector, as a control). After 3 days in culture, the Nfix2-embryonic myoblasts (Nfix2) formed larger myotubes with an increased fusion index (Figure 1E) in comparison with control cells (CTL) (Figure 1B). In contrast, induction of Nfia1.1 reduced myoblast fusion (Figures 1B and 1E). RT-PCR analysis showed that Nfix2- but not Nfia1.1-differentiated myotubes expressed the fetal marker β-enolase and downregulated expression of the slow MyHC; unexpectedly, MCK, the other fetal marker analyzed, was not expressed (Figure 1C), suggesting the requirement of a cofactor(s) for Nfix2-mediated induction of MCK. In approaches of both loss and gain of function, myogenin was not dramatically affected (data not shown), suggesting that Nfix modulates fetal/embryonic gene expression independently from its role of

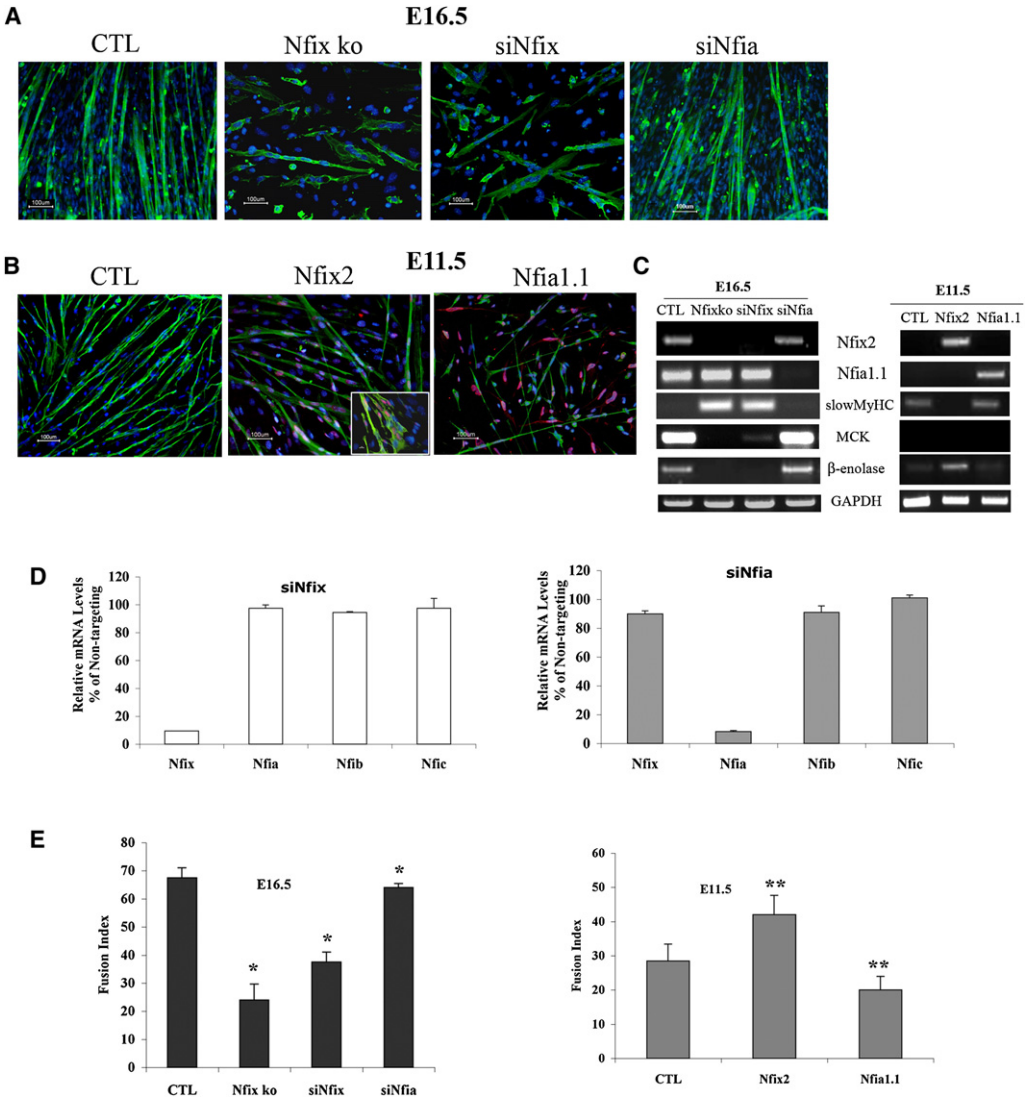

Figure 1. Nfix2 Modulates Embryonic and Fetal Muscle Genes

(A) Analysis by immunofluorescence of muscle differentiation of the Nfix ko//Myf5$^{GFP\text{-}P/+}$- and Myf5$^{GFP\text{-}P/+}$-purified fetal myoblasts (E16.5), the latter infected by Lentivector expressing the siRNA for Nfix (siNfix) or Nfia (siNfia), compared to nontargeting control (CTL). Cells are stained with anti-MyHC, antibody (green) and DAPI (blue). The scale bar represents 100 μm.

(B) Immunofluorescence on Myf5$^{GFP\text{-}P/+}$-purified embryonic myoblasts (E11.5) infected by lentiviruses expressing the HA-Nfix2 (Nfix2), the HA-Nfia1.1, or the empty vector (CTL), as control, stained with anti-MyHC (green), HA (red) antibody, and DAPI (blue). The insert in the Nfix2-infected cells highlights the increased fusion index (arrow). The scale bar represents 100 μm.

(C) RT-PCR on differentiated fetal (CTL, Nfix ko, siNfix, and siNfia) and embryonic myoblasts (CTL, Nfix2, and Nfia1.1) analyzing the expression of embryonic and fetal markers following Nfix2 or Nfia1.1 modulation.

(D) qRT-PCR on fetal myoblasts infected by Lenti-siNfix or Lenti-siNfia analyzing the relative Nfis mRNA levels after Nfix or Nfia silencing.

(E) Fusion index of the differentiated fetal (CTL, Nfix ko, siNfix, and siNfia) and embryonic myoblasts (CTL, Nfix2, and Nfia).

The scale bars represent means of three experiments ± s.d. * ($p < 0.05$) and ** ($p < 0.01$) indicate significantly different from control (CTL). See also Figure S1 and Table S1.

coactivator of myogenin. To identify other genes potentially modulated by Nfix2, we performed a quantitative real-time PCR for 95 muscle characteristic genes on the transduced myoblast populations (Engr and NfiEngr fetal myotubes, CTL and Nfix2-embryonic myotubes). The results showed that the induction of Nfix2 activated many genes in embryonic myotubes, while NfiEngr inhibited a more restricted number of genes in fetal myotubes (respectively, 68 and 43 genes present a fold change >2). Genes known to be specific of fetal muscle (e.g., MCK, integrin-alpha7, β-enolase, and PKCθ) were downregulated in NfiEngr fetal myotubes; in addition, other genes such as M-cadherin, FGFR4, decorin, α-sarcoglycan, dystroglycan-1, and desmin were also downregulated. Most of these genes were induced in embryonic myotubes expressing Nfix2. Interestingly,

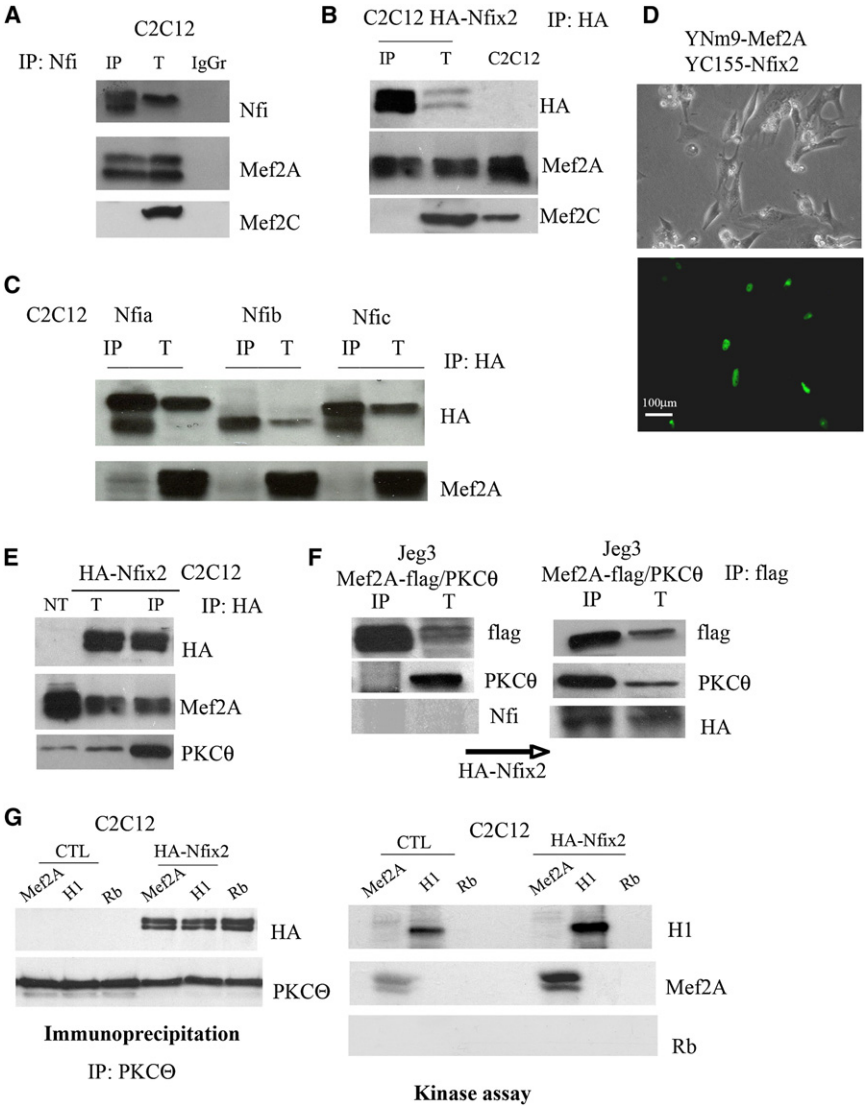

Figure 2. Nfix2 Forms a Complex with Mef2A and PKCθ

(A) Nfi was precipitated (IP) in extracts of C2C12 myotubes with anti-Nfi rabbit polyclonal antibody. The immune precipitate was separated by SDS-PAGE and reacted with anti-Mef2A and Mef2C antibodies. Immunoprecipitation with nonrelated rabbit IgG (IgGr) was performed to show the specificity of the assay.

(B) C2C12 cells were transfected with a plasmid vector expressing HA-Nfix2 plasmid, immunoprecipitated (IP) with anti-HA antibody, separated by SDS-PAGE, and probed with anti-Mef2A and anti-Mef2C antibodies.

(C) C2C12 cells were transfected with plasmid vectors expressing HA-Nfia1.1, HA-Nfib2, or HA-Nfic and the different Nfis were immunoprecipitated (IP) with anti-HA antibody, separated by SDS-PAGE, and probed with anti-Mef2A and anti-Mef2C antibodies.

(D) BiFC assay. C2C12 cells were transfected with vectors expressing YNm9-Mef2A and YC155-Nfix2. Thirty-six hours after transfection, YFP fluorescence accumulated in the nucleus, demonstrating Mef2A and Nfix2 binding. The scale bar represents 100 μm.

(E) C2C12 cells were transfected with plasmid vector expressing HA-Nfix2 and immunoprecipitated (IP) with anti-HA antibody. The binding among Nfix, Mef2A, and PKCθ was tested with specific anti-Mef2A and anti-PKCθ antibodies.

(F) The JEG3 cell line was cotransfected with both Mef2A-flag and PKCθ plasmids. Mef2A was immunoprecipitated with anti-flag antibody, and binding with PKCθ was tested. The absence of binding between PKCθ and Mef2A was rescued after cotransfection of the JEG3 cells with HA-Nfix2 plasmid (see arrow).

(G) PKCθ kinase assay on C2C12 myoblasts. PKCθ was immunoprecipitated (IP) in C2C12 cells transfected with the empty vector (CTL) or transfected with HA-Nfix2 plasmid. The amount of PKCθ and Nfix (by anti-HA antibody) in the immunoprecipitates was assessed by western blot (left). The activity was assayed by phosphorylation of GST-Mef2A fusion protein, histone H1 (H1, as positive control), and GST-Rb fusion protein (Rb, as negative control).

IP, immunoprecipitated; T, total lysate; CTL, control.

proteins responsible for sarcomere contraction (i.e., the troponin/tropomyosin complex) were strongly upregulated in these myotubes. Details are presented in Table S1. These data indicate that Nfix2 is a key regulator of the transcriptional switch of many embryonic/fetal muscle genes.

Fetal MCK Expression Depends upon Cooperation between Nfix2, Mef2A, and PKCθ

We reported previously that Mef2 transcription factors are expressed both in embryonic and fetal muscle but bind the MCK proximal enhancer region only in the fetus as a consequence of stage-specific Mef2 phosphorylation (Ferrari et al., 1997). We had also shown that the θ isoform of PKC is expressed in fetal muscle (Zappelli et al., 1996), but whether this isoform is responsible for the direct phosphorylation of Mef2 had not been evaluated. Thus, we initially tested whether Nfi was able to bind Mef2, and in particular the forms predominantly expressed in muscle, Mef2A and Mef2C, by coimmunoprecipitating the endogenous Nfi in C2C12 cells. As Figure 2A shows, Nfi is able to specifically bind Mef2A but not Mef2C. As the antibody we used did not discriminate the different members of the Nfi family, we transfected C2C12 myoblasts with the HA-tagged Nfix2 expression vector and immunoprecipitated Nfix2 containing complexes with an anti-HA antibody (Figure 2B). The same approach was followed with vectors expressing the HA-Nfia1.1, HA-Nfib2, or HA-Nfic2 isoforms (Figure 2C). The coimmunoprecipitations

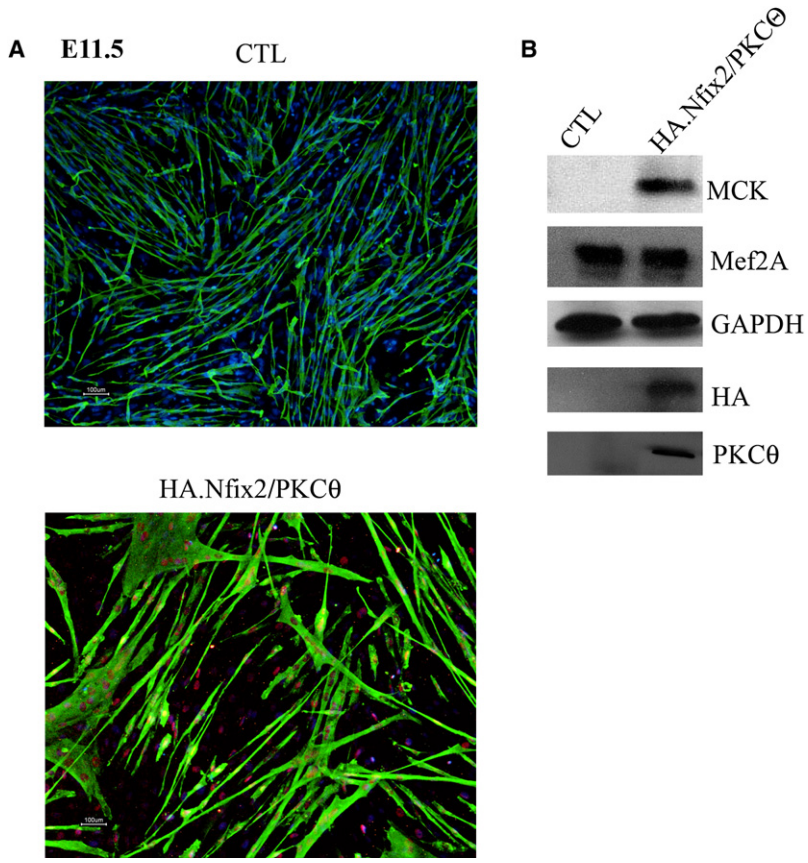

Figure 3. Nfix2, PKCθ, and Mef2A Cooperate in MCK Transactivation

(A) Immunofluorescence on Myf5$^{GFP-P/+}$-purified embryonic myoblasts (E11.5) cotransfected with HA-Nfix2 and PKCθ plasmids (HA-Nfix2/ PKCθ) or the empty vector as control (CTL) showing MyHC (green), HA (red), and DAPI staining (blue). The scale bar represents 100 μm.

(B) Western blot on the HA-Nfix2/ PKCθ or CTL embryonic differentiated myoblasts. GAPDH was chosen to normalize the amount of proteins loaded.

See also Figure S2.

clearly revealed that Nfix2 is the only member of the Nfi family able to bind Mef2A. To further confirm this result, we used the bimolecular fluorescence complementation (BiFC) approach (Saka et al., 2008), which allows detection of protein-protein interactions in living cells. To this end, Nfix2 and Mef2A were fused to the amino(N)- or carboxy(C)-terminal of the yellow fluorescent protein (YFP), respectively, and transfected into C2C12 myoblasts. Coexpression of YNm9-Mef2A and YC155-Nfix2 in C2C12 cells resulted in complementation of the YFP, indicating in living cells that Nfix2 directly interact with Mef2A in the nucleus of living cells (Figure 2D), while no complementation occurred when the YN fragment alone was expressed in association with Mef2A-YC (data not shown).

We then tested whether PKCθ could be immunoprecipitated in HA-Nfix2-expressing C2C12 myoblasts, and the possible binding to Nfix2 and/or Mef2A was evaluated. As shown in Figure 2E, Nfix2 was able to bind not only Mef2A but also PKCθ. This result prompted us to determine whether Mef2A was bound directly to PKCθ or whether this binding was Nfix2 mediated. To address this point, we used the JEG3 cell line, which expresses essentially no Nfi proteins. JEG3 cells were cotransfected with both Mef2A-flag and PKCθ, and Mef2A was immunoprecipitated with an anti-flag antibody. It appeared that Mef2A and PKCθ were not able to bind directly to each other, while binding was rescued in the presence of Nfix2 (Figure 2F), suggesting that Nfix acts as a bridge between Mef2A and PKCθ, likely to allow Mef2A phosphorylation. To investigate this point, we performed a PKCθ kinase assay on control and HA-Nfix2-expressing C2C12 cells. The kinase activity of the immunoprecipitated PKCθ was tested using as possible substrates Mef2A, histone H1 (as a positive control), or retinoblastoma protein (pRB, as a negative control). As shown in Figure 2G, PKCθ was able to weakly phosphorylate Mef2A, but this ability was strongly enhanced in the presence of Nfix2. To test whether the ternary complex among Mef2A, Nfix2, and PKCθ was responsible for MCK transactivation in fetal myotubes, we cotransfected purified-embryonic myoblasts with both Nfix2 and PKCθ and allowed them to differentiate in vitro. The resulting myotubes showed a typical fetal morphology, even more pronounced than that after induction of Nfix2 alone (compare Figure 3A with Figure 1B). Most importantly, Nfix2- and PKCθ-coexpressing myotubes expressed MCK (Figure 3B). A chromatin immunoprecipitation (ChIP) assay was then performed on embryonic and fetal myotubes. In the case of *MCK*, we tested two different putative Nfix binding regions (according the Genomatix MatInspector database) (Cartharius et al., 2005), MCK-2500/-2400 and MCK-1200/-1048, close to the Mef2 box domains. For *β-enolase*, we used the well-characterized region located at +504/+637 from the transcription starting site (Feo et al., 1995). As shown in Figures S2A and S2B, neither Nfi nor Mef2A were able to bind to *MCK* and *β-enolase* enhancer/promoters in nuclear extracts of embryonic (E11.5) myotubes, while this binding was detected in extracts of fetal (E16.5) myotubes. Moreover, ChIP assay performed on HA-Nfix2-expressing fetal myotubes (E16.5 HA-Nfix2) confirmed the direct binding of Nfix on MCK and β-enolase enhancer/promoter (Figure S2C), while the PKCθ seems only to participate in Mef2A phosphorylation not being detectable in the complex on MCK and β-enolase regulatory regions (Figure S2D).

Together, these results demonstrate the existence of a Mef2A/Nfix2/PKCθ complex responsible for MCK transactivation and that Nfix acts as a regulator of fetal myogenesis by different molecular mechanisms.

Fetal Skeletal Muscle Deficient for *Nfix* Displays Defects in Myogenesis and a Delayed Sarcomerogenesis

To confirm the results obtained in in vitro and ex vivo experiments, we studied fetal myogenesis in the *Nfix*-deficient mouse.

Since Nfix is expressed in different tissues and the *Nfix* knockout mouse shows brain and other malformations (Driller et al., 2007), we conditionally ablated *Nfix* in skeletal muscle. To this aim, we crossed the floxed $Nfix^{c/+}$ mouse (Campbell et al., 2008) with the *MyoD*-Cre transgenic mice (F3/-2,5cre) (Chen et al., 2005); in this mouse, the expression of the Cre recombinase is under the control of the regulatory sequences of the muscle specific gene MyoD. While muscle-specific *Nfix*-deficient embryos (E11.5) were indistinguishable from wild-type (WT) littermates (Figure S3), at E16.5 muscle-specific *Nfix* null fetuses were smaller than the WT even though their gross morphology was apparently normal (Figure 4A). Hematoxylin and eosin (H&E) staining on hind-limb sections revealed dramatic disorganization of muscle fibers in *Nfix* null fetuses (Figure 4B). Transmission electron microscopy (TEM) on hind-limb sections from WT and *Nfix* null fetuses showed poorly assembled sarcomeres in the *Nfix*-deficient versus WT fetuses (Figure 4C), consistent with H&E staining (Figure 4B), likely due to a delay in sarcomere organization since at P3 the sarcomeres appeared to be indistinguishable from WT (Figure 4D). Western blot analysis on the different *Nfix* null and WT fetuses confirmed the in vitro data: *Nfix*-deficient fetuses showed downregulation of β-enolase and MCK but persistent expression of slow MyHC (Figure 4E). Interestingly, *Myogenin* expression was not decreased in *Nfix* null fetuses. Immunofluorescence on muscle sections with antibodies directed against all sarcomeric or slow MyHC confirmed the data from western blots (Figure 4I). To explain the different phenotype of the *Nfix* null fetus, we tested whether changes in size may be due to altered proliferation (Ki67 staining), apoptosis (ApoTag detection), or fiber number, but no significant differences were noticed (data not shown). However, a reduced amount of MyHC/fetus was detected in the *Nfix*-deficient fetuses in comparison with their WT siblings (Figure 4G), which corresponded to reduced cross-sectional area of the MyHC positive fibers (Figures 4F and 4H), whose number was unchanged (data not shown).

These data show that besides its role in regulating the transition from the embryonic to the fetal program, Nfix plays an important role in sarcomere organization depending, at least in part, upon the expression of the mature isoforms of contractile proteins.

Nfix2 Is Required In Vivo to Drive the Developmental Shift from Embryonic to Fetal Myogenesis

We then analyzed the gain of Nfix function in transgenic mice overexpressing Nfix during embryonic myogenesis (E12.5) under the transcriptional control of the myosin light chain 1F promoter/enhancer (Jiang et al., 2002) (Figure S4A). Different founders originated independent lines indistinguishable from each other (Figure S4B). Transgenic mice showed no overt differences in terms of fertility, behavior, and life span with respect to the littermate controls. We tested the expression of Nfix2 in transgenic embryos (Nfix2) at E12.5 by RT-PCR using primers specific for Nfix2 and for the other members of the Nfi family (Figure S4C). As expected, the expression of Nfix2 but not of the other family members was much greater in the transgenic embryos than in WT littermates. At E12.5, Tg:Mlc1f-*Nfix2* embryos were larger in size than the WT littermates (Figure 5B, a and b). H&E staining of muscle fibers did not reveal obvious differences between WT and transgenic embryos (data not shown), while TEM hind-limb sections showed an almost completely organized sarcomerogenesis (Figure 5B, c and d, see Z bands) in transgenic embryos, with an apparent precocious maturation in comparison with WT embryos. The amount of MyHC accumulation together with the increased fiber size (as shown in the forelimb transverse sections of the Tg:Mlc1f-*Nfix2* embryos, Figures 5D and 5E) may in part explain the increased size of transgenic mice. Interestingly, transgenic embryos have much increased levels of Igf-1 (Figure 5F), which is known to affect prenatal growth (Liu et al., 1993). No differences in proliferation or apoptosis were noticed (data not shown). In Figure 5A, western blot analysis of WT and Tg:Mlc1f-Nfix2 embryos revealed premature expression of β-enolase and downregulation of slow MyHC (the latter confirmed by immunofluorescence on a limb muscle section, Figure 5C). In accordance with in vitro observations, premature Nfix2 expression was not sufficient to promote MCK expression. To test in vivo for an Nfix/Mef2A requirement for PKCθ in inducing MCK expression, we generated a Tg:Mlc1f-*PKCθ* transgenic mouse by using the same strategy designed for the Tg:Mlc1f-*Nfix2* mouse (Figure S4D). Different founders (Figure S4E) were crossed with the Tg:Mlc1f-*Nfix2* with the aim of examining MCK expression in the double-transgenic Tg:Mlc1f-*Nfix2*//Tg:Mlc1f-*PKCθ* embryos. Unexpectedly, no embryos of the appropriate genotype were obtained from these crosses (although different founders were tested); the reason for this observation remains unclear. To overcome this problem, we established cocultures of embryonic myoblasts from Tg:Mlc1f-*Nfix2* and Tg:Mlc1f-*PKCθ* embryos, reasoning that fusion of the two different transgenic myoblasts would lead to coexpression of Nfix and PKCθ in the same cell. After 3 days in culture, the mixed population differentiated properly (Figure 5G), and MCK was expressed only in the cocultured Tg:Mlc1f-*Nfix2* and Tg:Mlc1f-*PKCθ* myotubes (Figure 5H), formally demonstrating the cooperation of Nfix2 and PKCθ in the induction of MCK.

Nfix2 Regulates Slow MyHC Expression through NFATc4

Beside its role of transcriptional activator, Nfix is also able to negatively modulate the expression of embryonic genes, such as slow MyHC. We thus tested whether Nfix directly binds to the promoter of slow MyHC. The 5 kb region upstream of the transcription starting site of murine, rat, and human slow MyHC were analyzed with Genomatix, but no conserved putative binding sites were identified, and a ChIP assay for Nfix2 failed to detect direct binding (Figure 6C), suggesting the existence of an indirect mechanism through which Nfix inhibits slow myosin.

It was recently shown that transcription of slow MyHC in adult muscle depends on the NFAT family members NFATc1–4 (Calabria et al., 2009). The NFATc4 isoform is more expressed in embryonic than in fetal myoblasts (Biressi et al., 2007b), and its expression dramatically changed after Nfix modulation in myoblasts (Table S1); we thus tested whether Nfix could repress slow MyHC expression by modulating the transcription of NFATc4. $Myf5^{GFP\text{-}P/+}$-purified fetal myoblasts were transfected with a NFATc4-expressing vector, and the amount of slow MyHC versus the expression of all sarcomeric myosins was

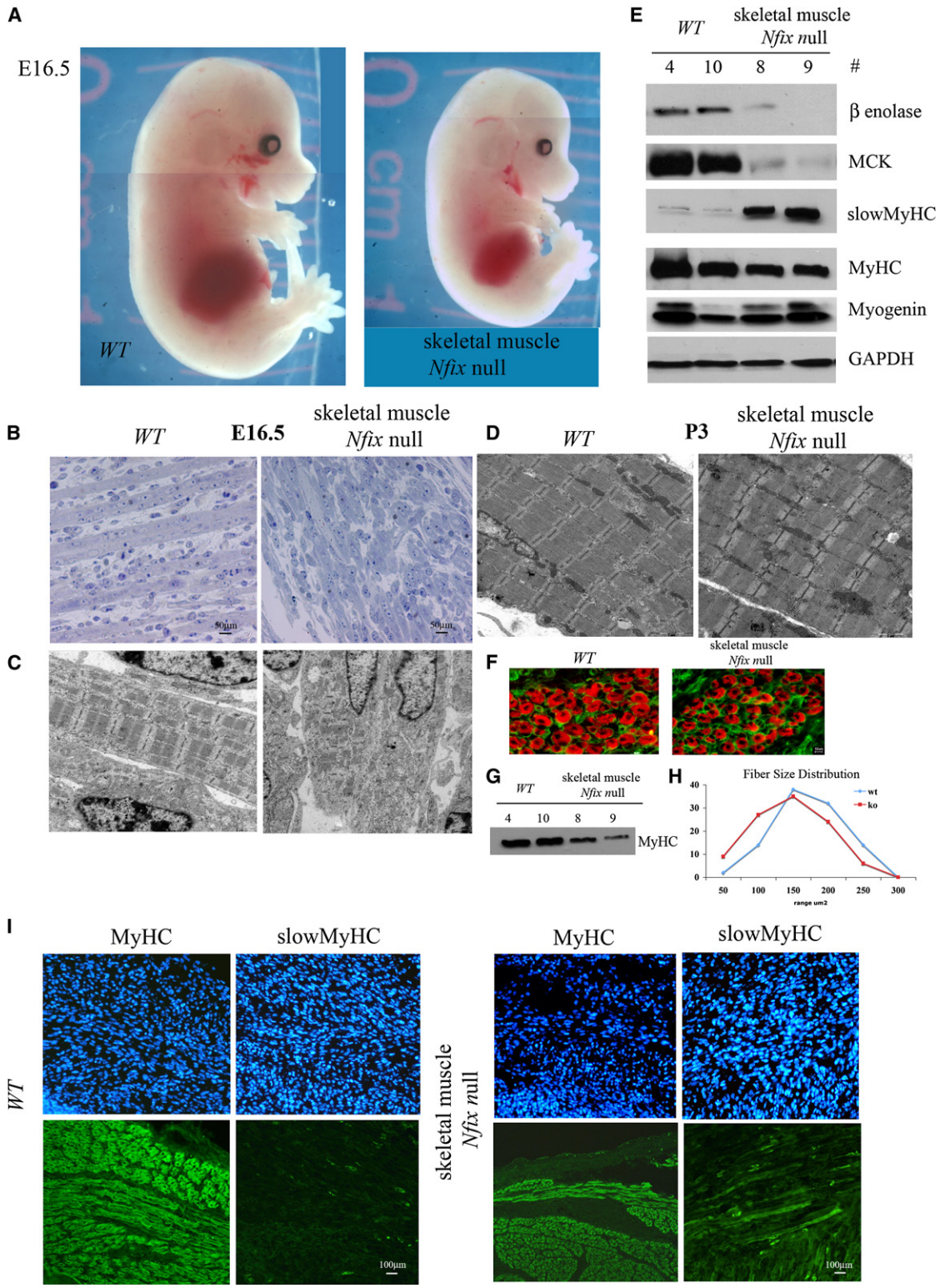

Figure 4. Phenotype, Muscle Morphology, and Gene Expression of the Skeletal Muscle-Specific *Nfix* -Deficient Fetuses

(A) Morphology of the skeletal muscle-specific *Nfix*-deficient and WT fetus at E16.5

(B) H&E staining on serial transverse sections of hind limbs of skeletal muscle-specific *Nfix*-deficient and WT fetus at E16.5. The scale bar represents 50 μm.

(C and D) Transmission electron microscopy (TEM) on serial transverse sections of hind limbs of skeletal muscle-specific *Nfix*-deficient and WT fetus at E16.5 (C) and at P3 (D). The scale bar represents 2 μm.

evaluated. As Figures 6A and 6B show, NFATc4-expressing cells differentiated similarly to CTL cells, but robustly expressed slow MyHC. A ChIP assay performed on HA-Nfix2-expressing fetal myotubes (E16.5 HA-Nfix2) demonstrated direct binding of Nfix on the promoter of NFATc4 (Figure 6C). These data suggest that Nfix negatively modulate the expression of NFATc4, which in turns is responsible for the normal expression of slow MyHC.

Pax7 Is Sufficient but Not Required for the Induction of Nfix

To date, the regulation of Nfix gene expression is completely unknown. Since Pax7 expression levels are higher in Myf5$^{GFP\text{-}P/+}$-purified fetal myoblasts than in their embryonic counterpart (Biressi et al., 2007b) and Pax7+ cells give rise to and are required for fetal myogenesis (Hutcheson et al., 2009), we investigated a possible role of Pax7 in regulating the fetal-specific expression of Nfix. To this aim, E11.5 Myf5$^{GFP\text{-}P/+}$-purified embryonic myoblasts were transduced with pLentiPax7 lentivector after 2 days in culture, they robustly expressed Nfix (Figures 7A and 7B). Moreover, a ChIP assay on unpurified HA-Pax7-expressing fetal myotubes (E16.5 HA-Pax7) revealed that Pax7 directly binds, with different affinity, to four putative Pax7 binding sites identified by Genomatix MatInspector analysis on the promoter of Nfix (Figure 7C). On the other hand, the expression of Nfix in limbs of E16.5 Pax7$^{-/-}$ mice was only modestly decreased, and it was normal in postnatal (P14) Pax7 null muscle (Figure 7D), suggesting that other genes besides Pax7 may activate Nfix during fetal and perinatal development.

DISCUSSION

Progressive changes in size and shape characterize the development of most vertebrate organs and tissues. These changes often reflect transcriptional modulation of stage (and tissue)-specific genes, according to a complex developmental program, evolved to express genes that best fit the changing needs of the developing organism. These genes are often developmentally regulated isoforms, usually evolved in vertebrates from a common ancestral gene. For example hemoglobin expression switches from an embryonic, to a fetal, to an adult form (Peterson, 2003) to optimize gas exchange with embryonic, fetal, and adult tissues, respectively. The transcriptional regulation of this switch is very complex and involves several proximal and distant regulatory elements (Palstra et al., 2008). Several other gene families, such as myosins and troponins (Gillis et al., 2007), are similarly regulated in development, but little is known about the relative molecular regulation.

In this work, we identify a major molecular mechanism that controls the switch from embryonic to fetal gene transcription in mammalian skeletal muscle. We show that the transcription factor Nfix, expressed in fetal but not in embryonic muscle, activates transcription of fetal-specific genes while repressing expression of the embryonic specific genes.

Pax7 is sufficient to activate Nfix (and thus the fetal program) in embryonic myoblasts but is not strictly necessary because in fetal and neonatal limbs of *Pax7* null mice Nfix is modestly reduced or normal. The only partially overlapping patterns of expression shown by Pax7 and Nfix during organogenesis (Chaudhry et al., 1997; Jostes et al., 1990) further corroborate the idea that additional genes can also be responsible for Nfix expression.

Nfix is required and sufficient to modulate expression of a number of genes, such as slow myosin, troponins, M-cadherin, α7-integrin, LDH, FGFR4, and MCK. Interestingly, modulation of Nfix levels in purified-embryonic and fetal myoblasts produced modest alterations in the differentiation potential per se, but induced a fine-tuning of the fetal program of muscle gene expression, causing the observed morphological changes: fetuses lacking Nfix in skeletal muscle are smaller and have fibers with reduced diameter, whereas embryos prematurely expressing Nfix are larger, likely reflecting more robust transcription in fetal muscle. Interestingly, expression of Igf-1, a major regulator of embryonic growth (Liu et al., 1993), is strongly induced in Tg:Mlc1f-*Nfix2* embryos.

Nfix-deficient fetuses also showed disorganized sarcomerogenesis, likely due to a delayed assembly of sarcomeres as postnatal sarcomeres of the mutant mouse are normal. It is possible that this phenomenon may depend on the persistence of cardiac troponin C and absence of skeletal isoforms, since perturbed expression of troponins has been shown to induce sarcomere abnormalities (Siedner et al., 2003).

Like other transcription factors, Nfix acts as a transcriptional activator or repressor, likely depending upon interacting proteins. It is possible that Nfix directly bind its consensus on the DNA of some embryonic/fetal genes to directly activate or repress their transcription with its C-terminal domain; for other genes, such as MCK, it may recruit other proteins (PKCθ/Mef2A) on embryonic and fetal muscle promoters. It is likely that Nfix may act with a similar mechanism on additional genes that were not overtly modulated in our real-time PCR screening because of the lack of coactivation by either Mef2A/PKCθ or other partners yet to be identified. The role of Nfix as a transcriptional repressor remains to be elucidated. We have shown that it does not bind directly the slow MyHC promoter but rather

(E) Western blot analysis of extracts from two different skeletal muscle-specific *Nfix* null (#8, 9) and two WT (#4, 10) fetuses at E16.5. GAPDH was used to normalize the amount of proteins loaded.
(F) Immunofluorescence analysis on hind-limb transverse sections of *Nfix*-deficient and WT fetuses at E16.5 stained with antibodies anti-MyHC (red) and laminin (green). The scale bar represents 100 μm.
(G) Quantification by western blot of MyHC content per fetus in the skeletal muscle-specific *Nfix*-deficient and WT fetuses. Each lane has been loaded with the same amount of each fetus extracted in the same volume of lysis buffer.
(H) Frequency histogram showing the CSA distribution of myofibers in WT (n = 867 myofibers) and *Nfix*-deficient (n = 870 myofibers) fetuses at E16.5.
(I) Immunofluorescence analysis on hind-limb sections of skeletal muscle-specific *Nfix*-deficient and WT fetuses at E16.5, stained with antibodies anti-MyHC or slow MyHC (green) and with DAPI (blue). The scale bar represents 100 μm.
See also Figure S3.

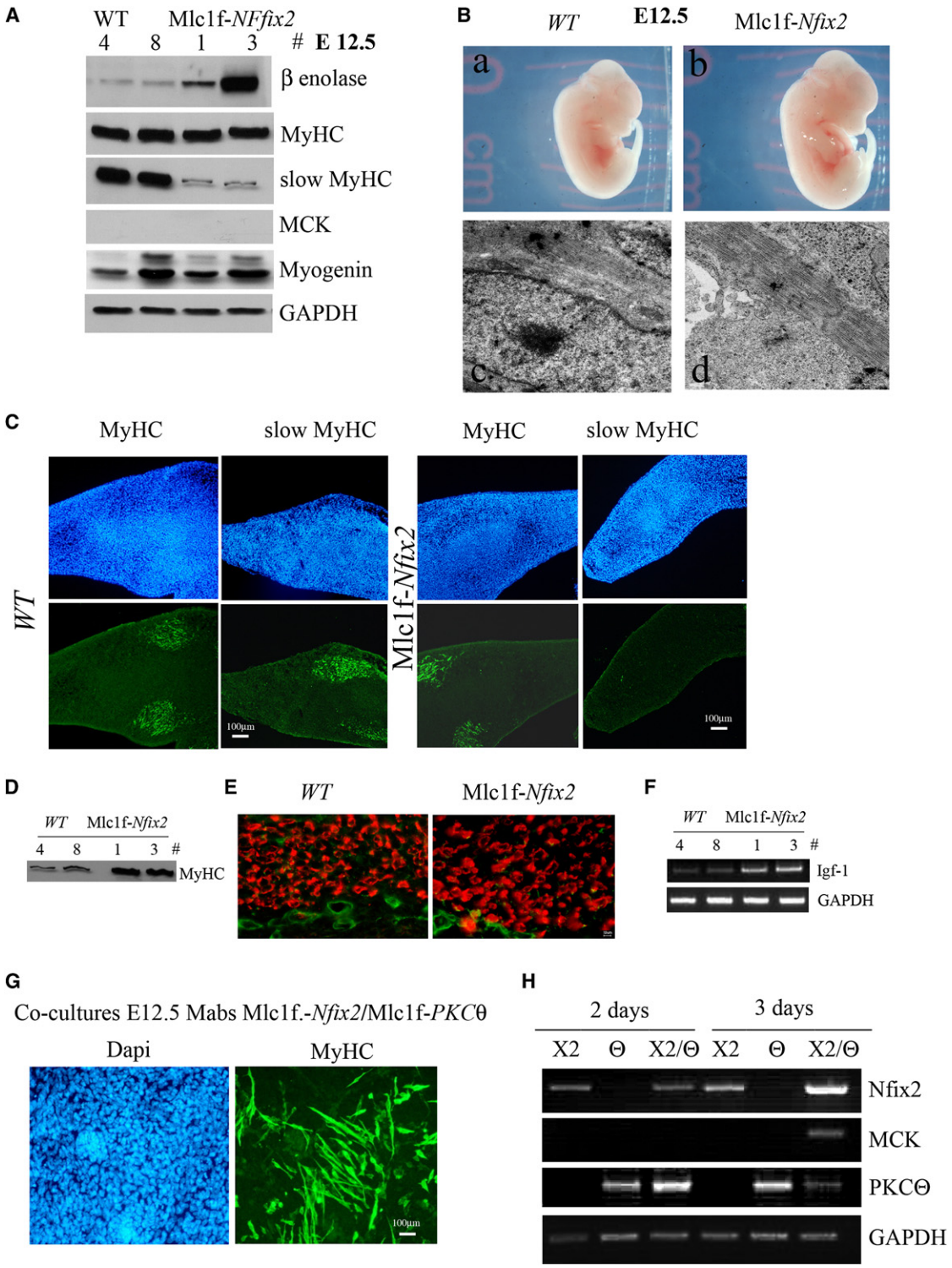

Figure 5. Phenotype, Muscle Morphology, and Gene Expression of Tg:Mlc1f-*Nfix2* Embryos at E12.5 of Development

(A) Western blot analysis of extracts from Tg:Mlc1f-*Nfix2* and WT embryos at E12.5. GAPDH was used to normalize the amount of proteins loaded.

(B) Tg:Mlc1f-*Nfix2* (b) and WT (a) embryos at E12.5. Transmission electron microscopy (TEM) on serial transverse sections of forelimbs of Tg:Mlc1f-*Nfix2* (d) and WT (c) embryos at E12.5. The scale bar represents 2 μm.

(C) Immunofluorescence on forelimb sections of Tg:Mlc1f-*Nfix2* and WT embryos at E12.5, using antibodies anti-MyHC or anti-slow MyHC (green) and DAPI (blue) to stain the nuclei. The scale bar represents 100 μm.

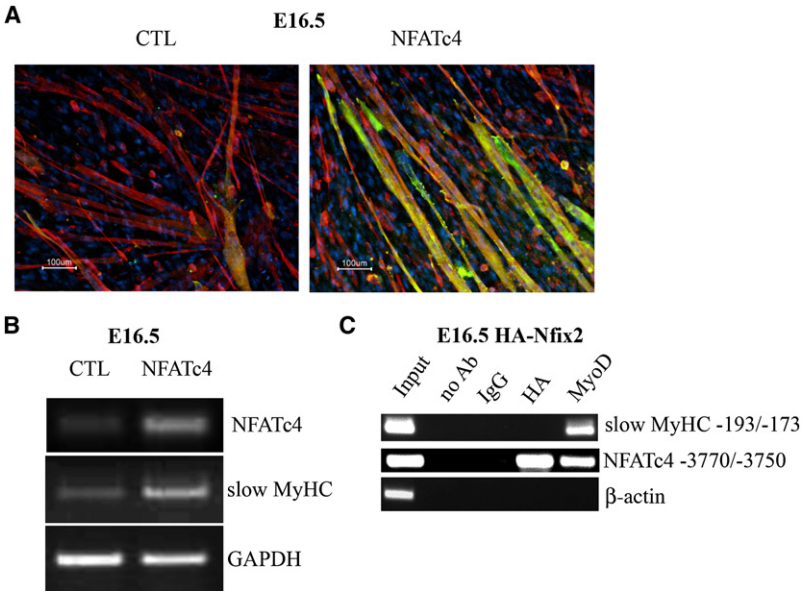

Figure 6. Slow MyHC Regulation through Nfix2-Dependent NFATc4 Expression

(A) Immunofluorescence analysis of slow MyHC expression in Myf5$^{GFP-P/+}$-purified fetal myoblasts (E16.5) transfected with NFATc4-expressing vector (NFATc4) compared to the empty vector (CTL). Cells are stained with anti-slow MyHC (green), anti-all sarcomeric MyHCs (red) and DAPI (blue). The scale bar represents 100 μm.

(B) Slow MyHC expression analyzed by RT-PCR analysis of in the Myf5$^{GFP-P/+}$-purified fetal myoblasts expressing NFTAc4 (NFATc4).

(C) ChIP assay on unpurified fetal myoblasts transduced by HA-Nfix2 lentivirus to test Nfix2 binding (by using the anti-HA antibody) on slow MyHC or NFATc4 promoters. β-actin has been used as a negative control and IgG as an unrelated antibody. I, input; no Ab, not antibody. ChIP with anti-MyoD antibodies was used as positive control.

represses transcription of NFATc4 a protein responsible for activation of this gene. A schematic model of the possible mechanisms through which Nfix acts is shown in Figure 7E.

Although no skeletal muscle defects were reported (Driller et al., 2007; Campbell et al., 2008), the *Nfix*-deficient mice are significantly smaller than their WT counterparts and the cause of their premature lethality is still not completely understood. The conditional ablation of *Nfix* in skeletal muscle allowed us to highlight specific defects in fetal myogenesis. Interestingly, skeletal muscle-specific *Nfix*-deficient mice are also smaller than their WT littermates.

From the data presented here, it appears that Nfix is essential for the expression of fetal markers such as MCK, β-enolase, PKCθ, or the troponin/tropomyosin complex, thus ensuring the appropriate enzymatic and contractile apparatus normally required for fetal and postnatal muscle. Moreover, the Nfix-dependent expression of α7-integrin, already known as a fetal marker (George-Weinstein et al., 1993; Biressi et al., 2007b), and of M-cadherin, important for myoblast fusion (Kaufmann et al., 1999), may modulate the selective fusion of myoblasts with either primary or secondary fibers, necessary to form the adult myofiber pattern.

Our observations, as well as those from previous studies, point to a dual role for Nfix in myogenesis. It cooperates with myogenin in the transcriptional activation of muscle genes, as originally described by Funk and Wright (1992). This function is shared by many different genes that directly or indirectly promote myogenesis. On the other hand, Nfix is the only gene so far identified that specifically represses expression of embryonic genes and activates expression of fetal genes in fetal muscle. In embryonic mammalian muscle, slow myosin heavy chains and ubiquitous isoforms of metabolic enzymes contribute to a slow twitching, low performant contractile activity; in the fetus, fast contractile proteins together with muscle-specific isoforms of metabolic enzymes, required to produce high level of ATP, result in fast twitching contraction.

Nfi transcription factors are well-conserved proteins across different species. In particular, there are four *NFI* genes in mammals and birds (*NFIA*, *NFIB*, *NFIC*, and *NFIX*) and a single NFI gene in *Drosophila*, *C. elegans*, *Anopheles*, sea urchin, and other invertebrates (Kruse et al., 1991; Gronostajski, 2000). No *NFI* genes have been found in plants, bacteria, or single-cell eukaryotes. Our preliminary data indicate that the Nfix and Mck (cmka) genes in the developing zebrafish are expressed at the onset of myogenesis, when fast MyHC is promptly expressed in the large majority of skeletal muscles. This has evolutionary relevance. A progressive maturation of skeletal muscle is probably useful for tetrapods, which first develop muscle anlagens with a complex tridimensional pattern, then increase the muscle mass by formation of secondary (fetal) fibers

(D) Quantification by western blot of MyHC content per fetus in the Tg:Mlc1f-*Nfix2* and WT embryos. Each lane has been loaded with the same amount of each fetus extracted in the same volume of lysis buffer.

(E) Immunofluorescence analysis on hind-limb transverse sections of the Tg:Mlc1f-*Nfix2* and WT embryos, stained with antibodies anti-MyHC (red) and laminin (green). The scale bar represents 100 μm.

(F) Igf-1 expression analyzed by RT-PCR of RNA extracts from Tg:Mlc1f-*Nfix2* (#1, 3) and WT (#4, 8) embryos at E12.5.

(G) Immunofluorescence analysis of cocultures of Mlc1f-*Nfix2* and Tg:Mlc1f-*PKCθ* embryonic myoblasts (E12.5) after 3 days in culture. The staining was done using the antibody against the MyHC (green) and DAPI (blue) to stain the nuclei. The scale bar represents 100 μm.

(H) MCK is induced in Tg:Mlc1f-Nfix2/Tg:Mlcif-PKCθ embryonic myotubes as assayed by RT-PCR on unpurified single (X2 and θ) and cocultured Mlc1f-*Nfix2* and Tg:Mlc1f-*PKCθ* (X2/θ) embryonic myoblasts (E12.5) after 2 and 3 days.

Mabs, myoblasts. See also Figure S4.

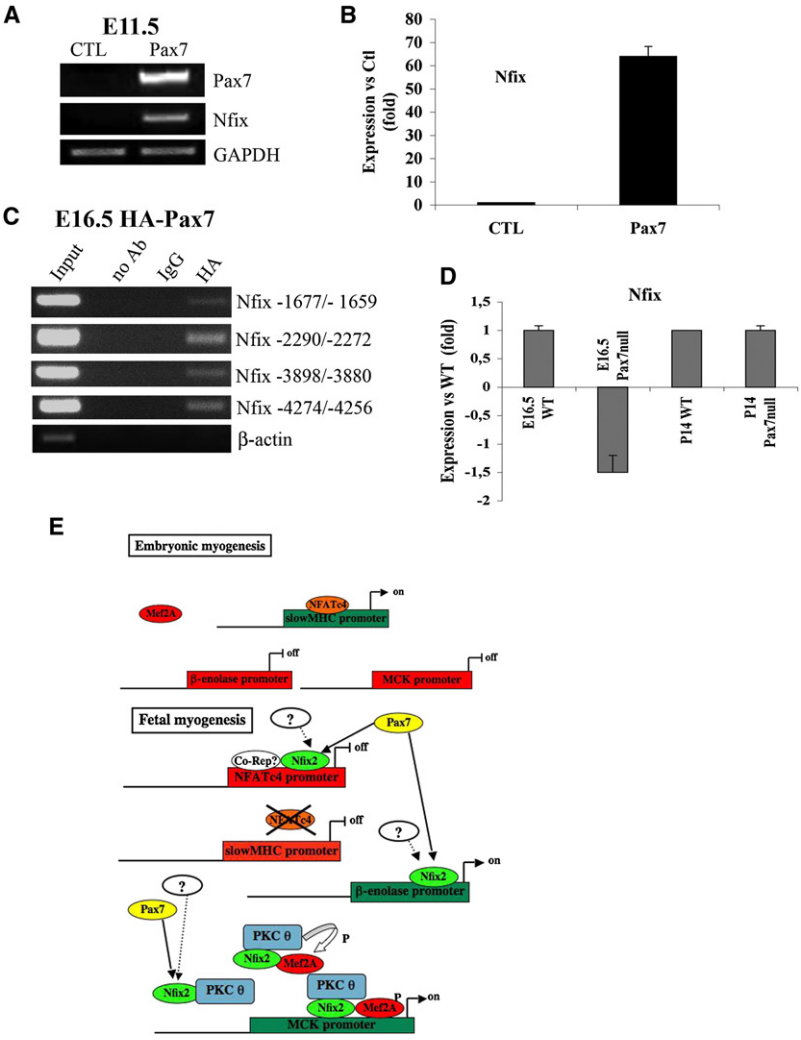

Figure 7. Nfix Regulation by Pax7 and Proposed Model for Nfix Action

(A and B) Nfix induction analyzed by semiquantitative (A) and real-time (B) RT-PCR on Myf5$^{GFP-P/+}$-purified embryonic myoblasts (E11.5) infected with lentivector expressing Pax7. CTL, control. Overexpression of Pax7 was verified with RT-PCR (A).

(C) ChIP assay on unpurified fetal myoblasts transfected with pCMV.HAPax7 (HA-Pax7) to test Pax7 binding (by using the anti-HA antibody) on four different regions on the Nfix promoter. β-actin has been used as a negative control and IgG as an unrelated antibody. I, input; no Ab, not antibody.

(D) Real-time RT-PCR showing the Nfix mRNA levels on E16.5 WT and *Pax7* null, P14 WT and *Pax7* null RNA extracts. Error bars represent means of three experiments ± standard deviation.

(E) Model of the possible mechanisms through which Nfix acts in modulating embryonic and fetal genes.

and finally increase progressively the fiber size by fusion of subsequent generations of myoblasts. Mammals and birds develop in a protected environment and show an initially slow muscle phenotype. In contrast, zebrafish embryos acquire motility when they are still in the chorion membrane (22–24 hours postfertilization [hpf]) and begin to swim rapidly (48 hpf). In this species, developing muscle fibers are predominantly fast twitching, and muscle-specific isoforms of metabolic enzymes such as MCK are expressed at the onset of myogenesis, as is its regulator, Nfix. Therefore, a direct causal correlation between Nfix expression and a fetal/fast myogenic program seems to be conserved from zebrafish to mouse while the timing of Nfix differs, in apparent correlation with their particular lifestyles. Although further work will be required to clarify the developmental role of Nfix in vertebrate evolution, the in vitro and in vivo data presented here conclusively demonstrate that in the mouse, Nfix acts as a positive regulator of the fetal (fast) myogenic program. The function of Nfix in postnatal growth and regeneration, as well as the identification of genes that contribute (together with Pax7) to the activation of Nfix in the late embryonic muscle, remain open questions for future studies.

EXPERIMENTAL PROCEDURES

Cell Cultures/Cell Isolation

C2C12 myoblasts and JEG3 chorion carcinoma cell line were grown as detailed in the Extended Experimental Procedures. Embryonic and fetal myoblasts were isolated from Myf5$^{GFP-P/+}$ mice (Kassar-Duchossoy et al., 2004) as previously described (Biressi et al., 2007b). Unpurified embryonic and fetal myoblasts were obtained from CD-1, Tg:Mlc1f-*Nfix2*, or Tg:Mlc1f-*PKCθ* embryos/fetuses via the same procedure/enzymatic digestion utilized for the Myf5$^{GFP-P/+}$-sorted myoblasts. In the coculture experiment, unpurified embryonic myoblasts from the Tg:Mlc1f-*Nfix2* or Tg:Mlc1f-*PKCθ* E11.5 embryos were obtained and mixed at a ratio 1:1. The short trypsinization assay was performed as described in Kitzmann et al. (1998).

Mutant Animals and Genotyping

The Myf5$^{GFP-P/+}$ mice (Kassar-Duchossoy et al., 2004), MyoDCre mice (Chen et al., 2005), *Nfix*$^{c/+}$ mice (Campbell et al., 2008) and Pax7$^{-/-}$ mutant mice (Seale et al., 2000) and their genotyping strategies have been published.

Transgenic Tg:Mlc1f-*Nfix2* and Tg:Mlc1f-*PKCθ* mice were generated as detailed in the Extended Experimental Procedures. Expression of the transgene in the various tissues was evaluated by RT-PCR. All experiments were performed under internal regulations for animal care and handlings (IACUC 355).

Plasmids

The plasmids used were as follows: pMef2AFlag and pMef2CFlag (kindly provided by S.Molinari, Modena), pNfiEngrailed and pEngrailed (kindly provided by C. Bachurski), pPKCθ (kindly provided by Marina Bouchè), pcDNA3NFATc4 (kindly provided by S. Schiaffino), pLKO.1siNfix and pLKO.1siCONTROL (Darmacon, Open Biosystems), and pLentisiNfia (kindly provided by Clara Nervi). The pCMVHA-Pax7 was obtained by cloning of the Pax7 complementary DNA (cDNA) into the pCMV-HA plasmid (Clontech).

pLentiHA-Nfix2, pLentiHA-NfiEngr, pLentiEngr, and the empty pLentiVector have been produced by subcloning of the HA-Nfix2, the NfiEngrailed and Engrailed cDNAs in the pLentiV5/TOPO (Invitrogen, Carlsbad, CA). pLenti4TO/mPax7 (Pax7) was engineered with Gateway technology (Invitrogen). Pax7 cDNA was amplified with a 5′-CACC flanking sequence and ligated into the pENTR/D TOPO vector. Entry vector was used for the final step of recombination into the pLenti4TO destination vector. A HA-tagged

cDNA encoding murine Nfia1.1 was inserted into the lentiviral vector pRRL.cPPT.hCMV.hPGK.GFP.WPRE under control of the CMV promoter. The pYNm9-Mef2A and pYC155-Nfix2 plasmids were obtained as detailed in the Extended Experimental Procedures.

Cell Transfection and Transduction

Purified embryonic and fetal myoblasts were transfected with the Lipofectamine LTX transfection Reagent (Invitrogen) and allowed to differentiate. C2C12 myoblasts and the JEG3 cell line were transfected using Lipofectamine with Plus reagent (Invitrogen). Thirty-six hours after the transfection, cells were processed for the coimmunoprecipitation assays. For the BiFC assay, C2C12 cells were transfected with the YNm9-Mef2A and YC155-Nfix2 plasmids, and 36 hr after transfection, they were incubated 30 min at 30°C to enhance YFP's fluorophore maturation before the analysis.

The lentiviral particles were produced as detailed in the Extended Experimental Procedures. Transduction of C2C12, embryonic, and fetal myoblasts was performed by addition of the viral preparation. Polybrene was added for the C2C12 cells (8 μg/ml, Sigma Aldrich). After overnight incubation, the medium was changed. These preparations were used to infect C2C12 myoblasts at a multiplicity of infection (MOI) of 100 and purified embryonic/fetal myoblasts at an MOI of 10.

Immunofluorescence on Cultured Cell and Tissue Sections

Cell cultures and 8 mm cryostat sections were fixed in 4% paraformaldehyde for 10 min at 4°C. When necessary, adjacent sections were stained with H&E.

Immunofluorescent staining was carried out with the following antibodies: rabbit polyclonal (Biressi et al., 2007b) and mouse monoclonal (MF20, DHSB) antibody to all sarcomeric MyHCs; mouse monoclonal slow MyHC (BA-D5, DSMZ); mouse monoclonal HA.11 (Covance, Princeton, NJ); and rabbit polyclonal laminin (Sigma). Secondary antibodies used were Alexa Fluor 488 or 594-conjugated donkey anti-mouse. DAPI was used to stain the nuclei.

Fiber Size Distribution

Morphometric analyses were performed on sections collected from similar regions of embryonic or fetal limbs (on WT, null, or transgenic mice).The images were captured from each section, and Image 1.63 (Scion Corporation) was used to determine the cross sectional area (CSA) of 700–1000 myofibers per section.

Transmission Electron Microscopy

The samples were fixed for 2 hr at 4°C with 4% paraformaldehyde and 2.5% glutaraldehyde in 125 mM phosphate buffer. Samples were then postfixed (1 hr) with OsO4 in 125 mM phosphate buffer, was washed and embedded in Epon. Conventional thin sections were collected on uncoated grids, stained with uranyl acetate and lead citrate, and examined with a Leo912 electron microscope.

Western Blot, Coimmunoprecipitation, and Kinase Assay

The western blot, the coimmunoprecipitation and kinase assay were performed as described previously (Messina et al., 2005).

For the coimmunoprecipitation, the antibodies used were rabbit anti-HA (Santa Cruz), mouse anti-PKCθ (BD Bioscience), mouse anti-flag (Sigma), and rabbit anti-Nfi (Santa Cruz); normal rabbit IgG (Santa Cruz) was used as control.

Western blot analysis of immunoprecipitates and total extracts was carried out with the following antibodies: mouse anti-MyHC (MF20), mouse anti-slow-MyHC (BA-D5, DSMZ), mouse anti-MCK (Abnova), mouse anti-β-enolase (BD Bioscience), mouse anti-PKCθ (BD Bioscience), mouse anti-Myogenin (F5D), rabbit anti-Mef2C (Santa Cruz), rabbit anti-Mef2A (Santa Cruz), mouse anti-HA (Covance, Princeton, NJ, USA), rabbit anti-Nfi (Santa Cruz), mouse anti-βtubulin (Covance, Princeton, NJ, USA), and mouse anti-GAPDH (Biogenesis).

PKCθ-associated kinase activity is detailed in the Extended Experimental Procedures.

ChIP Assay

The ChIP protocol was performed on unpurified embryonic (E11.5) and fetal (E16.5) myoblasts as described (Molinari et al., 2004). The following antibodies were used: rabbit anti-MEF2 (C21 sc-313X, 200 mg/0.1 ml, Santa Cruz), rabbit anti-Nfi (Santa Cruz), anti-PKCθ (BD Bioscience), mouse anti-MyoD (Dako), mouse anti-HA.11 (Covance, Princeton, NJ, USA), and, as a control, normal rabbit IgGr (Santa Cruz). Immunoprecipitated DNA was subjected to PCR as detailed in the Extended Experimental Procedures.

RT-PCR

The RNA from mouse embryos was isolated from CD-1 mice at different developmental stages. One microgram of RNA, collected from cells or tissues with the RNeasy mini (or micro) kit (QIAGEN) or TRIZOL (Invitrogen) for tissues, was converted into double-stranded cDNA with the cDNA synthesis kit ThermoScript RT-PCR system (Invitrogen), according to the manufacturer's instructions. The primers used are described in the Extended Experimental Procedures.

Quantitative Real-Time PCR

RNA was extracted and quantified as described above. Each tested RNA sample was obtained by pooling of at least five independent RNAs extracted from different experiments. cDNAs were reverse transcribed from total RNA samples (100 ng per sample) with the High Capacity cDNA Archive Kit (Applied Biosystems) as described in the manufacturer's protocol. Details of the procedure are described in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.01.027.

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